HHS Public Access

Author manuscript

Semin Perinatol. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

Semin Perinatol. 2017 February ; 41(1): 15–28. doi:10.1053/j.semperi.2016.09.014.

Pathogenesis of NEC: Role of the Innate and Adaptive Immune Response

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Abstract

Necrotizing enterocolitis (NEC) is a devastating disease in premature infants with high case fatality and significant morbidity among survivors. Immaturity of intestinal host defenses predisposes the premature infant gut to injury. An abnormal bacterial colonization pattern with a deficiency of commensal bacteria may lead to a further breakdown of these host defense mechanisms, predisposing the infant to NEC. Here, we review the role of the innate and adaptive immune system in the pathophysiology of NEC.

Introduction

Necrotizing enterocolitis (NEC) remains a leading cause of morbidity and mortality in the neonatal intensive care unit (NICU)[1-4]. Because epidemiologic studies demonstrate that NEC incidence is inversely proportional to gestational age at birth[5,6], Immature intestinal host defenses are thought to play a major role in its pathogenesis. These key immature defenses include intestinal barrier function, intestinal regulation of microbial colonization, regulation of intestinal circulation, and intestinal innate and adaptive immunity.

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Interestingly, NEC onset has also been associated with a developmental window of susceptibility (30-32 weeks postmenstrual age)[7,8]. Changes in microbial colonization patterns during postnatal development may explain this apparent window of susceptibility. Recent clinical studies implicate the importance of the intestinal microbial community in regulating health and disease in the premature infant. First, increased NEC incidence has been associated with increased early empiric antibiotic use[9-12]. Second, administration of probiotic bacteria has been associated with decreased risk of NEC[13]. Finally, longitudinal stool colonization studies using molecular techniques have implicated specific changes in microbial patterns prior to NEC onset[14-22].

An imbalance in the maturation of intestinal innate and adaptive immune defense mechanisms may also explain the apparent developmental window of NEC susceptibility. Adaptive immunity is often thought to regulate the innate immune system which can cause disease when allowed to respond unchecked. Neonates, especially preterm infants, are born with underdeveloped adaptive immunity. Adaptive immune defenses transferred from mom (through breast milk and placental transfer of maternal IgG) are meant to protect the newborn infant until their own adaptive immunity develops[23]. Maternal transfer of these adaptive immune defenses are significantly reduced in preterm infants (especially formula fed infants)[24], thus placing them at greater risk for inflammatory disorders such as NEC.

In this review, we will summarize the current evidence regarding the role of the innate and adaptive immune response in the pathophysiology of NEC. Specifically, we will discuss the relative contributions of passive immunity, physical barriers protecting the gastrointestinal (GI) tract, innate immune cells, and cytokines in NEC pathogenesis.

Passive Immunity in NEC

Passive antibody transfer

The two main mechanisms of passive immunity which may act to protect the preterm infant from NEC are passive transfer of maternal antibodies in the form if IgG from the placenta or secretory IgA (sIgA) from breast milk (Table 1). Neonates are known to be born with deficiencies in both cellular and humoral immunity and this passive immunity received from the mother is meant to protect the infant from disease until its own immune system can mature[25,26]. Placental transfer of IgG is mediated by the FcRN receptor in the syncytiotrophoblast and maternal antibodies have been shown to protect the infant in the first 6 months of life[25]. Successful placental transfer of IgG is dependent upon maternal IgG levels and gestational age of the infant[27]. Antibody transfer begins as early as 13 weeks gestation but the greatest amount of antibody transfer occurs in the last 4 weeks of pregnancy. Preterm infants at less than 22 weeks gestation have antibody levels at < 10% maternal levels, which raises to 50% by 28-32 weeks, and continues to raise to 20-30% above maternal levels by term[27]. In contrast, breast milk from mothers of preterm infants have been found to have higher levels of sIgA compared to term mothers' milk[28-30]. Based on relative deficiency of IgG and IgA in preterm infants, several clinical trials have evaluated the effect of oral immunoglobulin administration in preterm infants[31]. However, the results of these trials have found no effect of oral immunoglobulin administration on risk of NEC. Of note, intestinal epithelial expression of the FcRN receptor has been demonstrated in

fetuses and may play a role in additional passive immunity in the preterm infant[32]. FcRN expression and function in humans is reduced compared to rodents which may explain partly why rodents are relatively resistant to NEC-like injury in animal models[33,34].

Breast milk nutrients

Breast milk contains multiple additional components that help to protect the newborn infant from infectious and inflammatory diseases in the first 6-12 months of life[35,36]. These include antimicrobial and anti-inflammatory factors and components that also promote maturation of intestinal host defenses[37]. First, breast milk contains sugars, proteins, and fats that confer dual roles in nutrition and promoting intestinal homeostasis. Oligosaccharides, nondigestible sugars which promote the growth of commensal bacteria in the GI tract[38-40]. Oligosaccharide supplementation may reduce NEC risk in human[41,42] and animal studies[43]. Caseins in breast milk are highly glycosylated proteins that are also thought to promote intestinal defenses by stimulating increased numbers of goblet and Paneth cells and also by promoting increased MUC2 gene expression (see more detailed discussion of importance of goblet and Paneth cells below)[44]. One casein subunit, κ-Casein, has also been shown to prevent attachment of bacteria to intestinal mucosal epithelia[45]. Triglycerides in human milk have also been shown to provide antiviral, antibacterial, and antiprotozoal activity[26,46,47].

Breast milk bioactive proteins

Second, breast milk contains bioactive proteins, lactoferrin and lysozyme, with antipathogenic activity. Lysozyme can act synergistically with lactoferrin to kill gram negative bacteria[48], but can independently have antibacterial effects as well[49]. Lactoferrin has been shown to have antibacterial, antifungal, and antiviral activity[50-52], can reduce microbial activity by limiting iron availability, and be converted by gastric pepsin to lactoferricin, which disrupts gram-negative cell walls[53]. A recent meta-analysis reports that lactoferrin supplementation with or without probiotics may reduce the incidence of NEC and late-onset sepsis in preterm infants[54]. Breastmilk has also been shown to contain platelet activating factor acetylhydrolase (PAF-AH), which is thought to prevent NEC by inactivating the key pathogenic mediator PAF (see below for a more detailed discussion on PAF)[55,56].

Breast milk immunoregulatory cytokines

Third, breast milk contains immunoregulatory cytokines, such as IL-10 and TGF-β[57,58]. Monocytes obtained from preterm infants also seem to have lower ability to produce IL-10[59] and TGF-β[60], potentially putting them at greater risk for inflammatory diseases. IL-10 is believed to be an anti-inflammatory cytokine critical for intestinal homeostasis[61,62]. Multiple animal and human studies implicate the importance of IL-10 in protecting the preterm infant from developing NEC. Both IL-10 deficient mice[63,64] and human infants with IL-10 receptor genetic defects[65] are at increased risk for colitis. Animal models of NEC also show that maternal milk can reduce NEC severity while increasing intestinal IL-10[66]. Human infants whose mothers have low levels of IL-10 are at increased risk for NEC[67] and probiotics may regulate IL-10 signaling in the immature gut[68]. TGF-β is also thought to regulate the inflammatory response[69] and promote wound healing[70,71]. TGF-β can also initiate local production of IgA in the gut, providing additional protection[72]. Levels of TGF-

β in human milk may predict feeding intolerance in growth restricted infants[73]. Both IL-10 and TGF-β have been shown to reduce inflammatory signaling by fetal human enterocytes in vitro[74].

Breast milk growth factors

Finally, breast milk contains growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF). IGF family members include IGF-1 and IGF-2 and have been thought to help intestinal homeostasis by promoting IEC proliferation and preventing IEC apoptosis[36]. Low serum IGF-1 levels in preterm infants may correlate with risk for NEC[75] and IGF-1 supplementation has been shown to reduce NEC in animal models[76,77]. Increased IGF-1 receptors have also been detected in NEC tissue[78]. The EGF family members most studied are EGF and heparin-binding EGF (HB-EGF). Both are thought to be important for intestinal homeostasis and protective against NEC[79]. EGF is secreted by multiple cells throughout the GI tract. EGF promotes IEC proliferation and differentiation[80], IEC restitution after injury[81], and reduces IEC autophagy[82]. EGF may also act by increasing production of helpful mucus by increasing goblet cells and their production of MUC2 (for more detailed discussion on the importance of intestinal mucus layers, see below); by improving intestinal barrier function through increased tight junction protein expression (occludin and claudin)[79,83]; by reducing TLR-4 signaling[84]; and by promoting anti-inflammatory macrophages and reducing pro-inflammatory macrophages[85] (for more detailed discussion of the role of TLR-4 and macrophages in NEC, see below). EGF is supplied by amniotic fluid throughout pregnancy and by colostrum in human milk. Extremely preterm human milk contains 50-80% more EGF when compared to milk from mother's with full-term infants,[86] which may help to protect against NEC, but decreases over time[79]. Salivary EGF levels increase in preterm infants postnatally[87,88] and low EGF levels in cord blood[89] and preterm saliva and serum has been associated with increased risk of NEC[90]. EGF and HB-EGF supplementation in animal NEC models reduces NEC incidence[91-103] and EGF supplementation in human neonates has been shown to have trophic effects on intestinal mucosa[104].

Physical Barriers Protecting the GI Tract

The physical barriers protecting the GI tract include gastric acid, the mucus layer present throughout the GI tract, the intestinal epithelial barrier, and antimicrobial peptides (Table 2). The intestine is lined by a single layer of highly polarized epithelial cells. Four different types of cells make up the intestinal epithelial layer: hormone-secreting enteroendocrine cells, mucus-secreting goblet cells, enterocytes with absorptive and secretory functions, and antimicrobial-secreting Paneth cells (specialized secretory enterocytes located at the base of small intestinal crypts). Below, we summarize how these cells contribute the physical barriers protecting the GI tract.

Gastric acid

Gastric acid protects the GI tract by decreasing the number of viable pathogens that can pass into the distal intestine. Enteroendocrine cells and the autonomic nervous system coordinate the secretion of hydrochloric acid by parietal cells located in gastric glands within the

epithelial lining. This, in turn, creates the acidic and bactericidal gastric environment. Mature gastric acid secretion seems to be present by 24 weeks gestation[105-107]. The importance of this acidic gastric environment to host defense is demonstrated by multiple observational studies linking the use of acid suppression by H2 antagonists to both NEC and late-onset sepsis[108-111].

Mucus layer

The mucus layer lining the GI tract protects by lubricating and minimizing contact between the epithelium and commensal bacteria. The major proteins of mucus in the intestine are highly glycosylated proteins called mucins, secreted by goblet cells[112,113]. Goblet cells also produce secretory Immunoglobulin A (sIgA), which contribute to the function of mucus. Mucins secreted by salivary glands coat food and assist with esophageal transit[114]. The mucus layer in the stomach plays a role in protecting the epithelium from the harsh acidic environment[114]. MUC2 is the most predominant mucin in both the small and large intestine[115]. The single unattached layer of mucus in the small intestine works with antibacterial proteins to limit the ability of bacteria to reach the epithelium[116]. The mucus also moves along the small intestine with peristaltic waves, thus making it even more difficult for bacteria to approach the epithelial layer. Attached to the apical side of enterocytes in the small intestine is a separate, thin layer of mucus made up of transmembrane mucins. This layer is commonly referred to as the glycocalyx and affords protection to the intestinal epithelial cells (IECs) by means of a physical barrier and plays a role in cellular signaling[117]. The goblet cells in the large intestine contribute to an inner and an outer layer of mucus. The inner mucus layer is inpenetrable to larger entities, such as bacteria. The outer layer is the area where the commensal bacteria of the large intestine reside. In this way, the commensal bacteria can help in digestion of the glycans found on the mucins.[118] Mucins also bind and stabilize key trophic and reparative factors (intestinal trefoil factor and epidermal growth factor, EGF) at the epithelial surface, which may aid epithelial repair[119,120].

Human infants with NEC have fewer mucin-containing goblet cells[121,122]. The premature infant's impaired ability to secrete mucus in response to an infection, coupled with a poorly developed mucus system may contribute to the increased risk of NEC[123]. Reduced number of MUC2 and trefoil factor 3 goblet cells have been found in both human[121,122] and rodent[83] NEC, and mice with genetically aberrant MUC2 develop more severe disease[124]. Trefoil factor 3 supplementation may reduce NEC in animal models[125].

Growth restriction may also impair intestinal barrier defenses. In rats with intrauterine growth retardation (IUGR), colonic barrier function was impaired[126]. This is of interest due to the possible association between NEC and IUGR[127-130]. The decreased function was a product of decreased colonic length, fewer goblet cells per crypt, and disruption of the normal gene expression and amount of mucin throughout the large intestine[126]. Decreased Paneth cell number has also been reported in murine models of IUGR and human IUGR intestinal tissue[131]. The combination of these differences in intestinal integrity of premature and IUGR infants compared to term infants may play a role in the possible association with NEC.

Epithelial barrier

The intestinal epithelial barrier is composed of a single layer of highly polarized intestinal epithelial cells (IECs), which creates a physical barrier regulated by the apical junction complex (AJC), consisting of tight junctions (TJ) and adherens junctions[132]. Tight junctions (TJs) regulate paracellular permeability and maintain separation of tissue compartments by sealing the intercellular space[133,134]. Three types of proteins make up TJs: occludins, claudins, and junctional adhesion molecules. The AJC starts to form as early as 10 weeks human gestation when intercellular tight junctions can be detected. However, full secretory and absorptive capabilities of the intestinal epithelia continues to occur *in utero* due to amniotic fluid growth and trophic factors, which induces mucosal maturation from 26 weeks to term[135]. Ongoing postnatal intestinal epithelial barrier maturation can also be induced by multiple factors including diet[136-138], epidermal growth factor[80], endogenous glucocorticoids[139], and commensal bacteria[140,141].

Premature infants have impaired epithelial barrier function compared to term infants[142,143], which is thought to contribute to the pathogenesis of NEC[2,3,144-147]. The role of TJ proteins in the pathogenesis of NEC has been extensively studied in human[148,149] and animal[83,148,150-152] studies. Many studies have also demonstrated how cytokines induced during intestinal inflammation can further weaken intestinal barrier function[153-158], leading to a vicious cycle of increased intestinal inflammation and injury. In addition, two promising biomarkers in early detection of NEC, I-FABP and claudin-3, are measures that indicate gut barrier disruption[159].

IECs are also responsible for sampling intraluminal contents which instigates transcellular signaling and transcription of genes resulting in a defense response via the release of cytokines and chemokines and subsequent attraction of leukocytes. This function is mediated by multiple pattern recognition receptors (PRRs) critical for the identification of both foreign elements such as peptidoglycan, lipoproteins, viral DNA and commensal microflora. The remarkable ability of these receptors to distinguish between harmful and helpful bacteria with subsequent appropriate signaling is critical to intestinal homeostasis[141]. Toll-like receptors (TLRs) are the predominant type of PRR found on the apical side of IECs. Another group of PRRs that cooperate with TLRs are the intracellular Nod-like receptors (NLRs). Nod1 is expressed by IECs, and Nod2 is found in monocytes, dendritic cells, and Paneth cells[117]. Multiple TLRs (TLR-2, TLR-4) as well as NOD2 have been implicated in the pathogenesis of NEC in human[160-164] and animal studies[165-175]. TLR-2 primarily senses peptidoglycan (a component of gram positive bacteria cell wall); TLR-4 primarily senses lipopolysaccharide (LPS, a component of gram negative bacteria cell wall); and TLR-9 primarily senses bacterial or viral DNA (CpG dinucleotides)[176]. In particular, exaggerated TLR-4 signaling and LPS are thought to play a major role in the inflammatory signaling in NEC[177,178]. Of note, platelet activating factor (PAF) is also an important acute mediator in the pathogenesis of NEC, which is not only a chemokine that induces inflammatory signaling but also can increase expression of TLR-4[179-182]. TLR-9 may play a protective role[183-186].

Antimicrobial peptides

Antimicrobial peptides can be secreted into the lumen of the gut by IECs, Paneth cells, and recruited neutrophils. Antimicrobial peptides are thought to promote intestinal homeostasis by regulating the microbial population[187]. Traditional antimicrobial peptides are directly microbicidal (defensins (α and β), cathelicidins); other peptides regulate microbes by sequestering nutrients (e.g. iron, zinc, manganese) necessary for growth (calprotectin, REG3γ)[188]. For the purposes of this review, we will limit our discussion to the first group. Initially discovered in human neutrophils, defensins are small cationic peptides that kill microbes in an oxygen-independent manner[189]. Defensins and cathelicidins function by inserting into the membranes of a broad range of prokaryotic cells, including gram-positive and gram-negative bacteria, fungi, protozoa, spirochetes, and enveloped viruses[187,190]. Once inside the microbial cell membrane, they form pores allowing the passage of anions through the membrane, thus depolarizing and killing the organism[191].

IECs primarily secrete β-defensins (hBD1, 2, and 3) with specific tissue distribution varying along the intestinal axis for each member of the β-defensin family[191]. Paneth cells secrete lysozyme, phospholipase A2, and antimicrobial peptides (defensins (α and β) and cathelicidins[189,192]). Paneth cells secrete α-defensins (human defensin, HD5 and HD6) in response to microbial or cholinergic stimuli, contributing to the relatively sterile and protected environment within intestinal crypts.

In vitro studies suggest that antimicrobial peptides may also contribute to host defense indirectly, by inducing host responses[193]. Cathelicidins and defensins may have proinflammatory properties by activating chemokine release resulting in immune cell chemotaxis and differentiation. α-defensins released into the intestinal crypt may stimulate chloride secretion from nearby enterocytes in order to flush pathogens and toxins away from sensitive stem cells[194]. β-defensins may promote homeostasis by promoting IEC migration, barrier function, and reducing pro-inflammatory cytokine expression[195]. Stool hBD2 expression has been reported as high in neonates with NEC[196] and may increase in response to changes in microbiota composition associated with NEC so has been proposed as a possible biomarker for early detection[197]. Intestinal expression of hBD2 is high in resected NEC tissue but low in more severe cases[198].

Ontogeny studies have demonstrated that Paneth cells can be detected by 12 weeks gestation and begin to produce antimicrobial defensins at 13 weeks and lysozyme at 20 weeks[199,200]. Significant HD5 expression can be detected at above 29 weeks[201]. Premature infants have been shown to have fewer Paneth cells with decreased function[191,199,201,202]. Multiple animal studies implicate the importance of Paneth cells in NEC pathogenesis[203,204]. Preterm infants with NEC have been shown to have normal[4] to reduced[122,205,206] numbers of Paneth cells or poorly functioning Paneth cells[202,206], but infants recovering from NEC have been shown to demonstrate Paneth cell hyperplasia[207].

Contribution of Innate and Adaptive Immune Cells

In addition to the physical and chemical barriers that limit unrestricted translocation of intestinal microbiota, numerous innate immune cells coordinately regulate responses that

contribute to barrier fortification and host defense[208,209]. In the process of attempting to protect the host from real or perceived threats, however, innate immune cells can elaborate serious bystander effects that are associated with the pathogenesis of NEC including excessive intestinal damage and impaired repair process. Below, we briefly outline the current state of knowledge with regards to complex role of innate and adaptive immune cells and cytokines in regulating NEC.

Intraepithelial lymphocytes

Positioned directly between intestinal epithelial cells in both the small and large intestine are intra-epithelial lymphocytes (IEL). The two main subsets of IEL can be distinguished by expression of either αβ or γδ T cell receptors and can be further categorized into specific subsets using CD4, CD8α, and CD8β co-receptors[210]. γδ IEL are the pioneer T cells that colonize the intestinal epithelium during embryogenesis and the very early postnatal period when conventional αβ T cell responses are not yet fully established[211,212]. Given their "front-line" positioning at epithelial surfaces and expression of NK receptors, γδ IEL are poised to contribute to barrier protection and mucosal defense in response to infection and stress[213]. Thus, at the earliest stages of ontogeny γδ IEL are among the first intestinal-resident immune cells contributing to the maintenance of epithelial integrity.

Given the putative beneficial role for γδ IEL in the intestine early in life, these cells may fundamentally contribute to barrier defense in the preterm infant. Consistent with this hypothesis, γδ IEL were observed to be preferentially reduced in the ileum of surgical NEC patients when compared to non-NEC controls. Additionally, TCR8-deficient mice, which lack γδ IEL altogether, were more susceptible to experimental NEC-like intestinal injury[149]. These complementary observations from both human and experimental NEC further provided a link between loss of γδ IEL and reduction of IL-17 and RORC, the master transcription factor involved in the differentiation of IL-17 producing T cells (Th17)[214]. IL-17A was originally viewed as a pro-inflammatory cytokine involved in driving systemic and intestinal inflammation. However, more recent data suggests that IL-17A is involved in maintaining barrier function via regulation of tight junction proteins[215-217]. In addition to IL-17A, γδ IEL can afford barrier protection and repair of damaged mucosa by secretion of other factors such as epithelial growth factor[218]. Collectively, during the precarious developmental window in preterm infants when the intestinal epithelial barrier is functionally immature, γδ IEL appear to provide important early immune-mediated barrier protection[219].

Natural killer (NK) cells and innate lymphoid cells (ILCs)

The function of natural killer (NK) cells in anti-tumor and anti-viral mediated immunity is well established[220]. More recently, accumulating evidence suggests a fundamental contribution of NK cells in intestinal barrier protection and regulation of inflammation. Using the DSS model of acute intestinal damage, depletion of NK cells was reported to significantly augment colonic damage, neutrophil infiltration, and proinflammatory cytokine production[221]. The mechanism of NK cell-mediated protection from acute barrier damage in this study was linked to expression of the NK cell inhibitory receptor NKG2A. NK cells have also been implicated in protection from chronic T cell-dependent intestinal

inflammation. In the CD45RBhi model of colitis, loss of NK cells results in dramatically accelerated Th1-driven disease[222]. Consistent with these data, flow cytometric analyses of immune cell subsets in preterm infants established a link between a decrease in NK cells and the development of NEC[223]. Thus, NK cells and perhaps NK-like innate lymphoid cells[224] may protect from intestinal barrier damage, promote barrier repair and decrease the risk of NEC.

Neutrophils

Despite being one of the most well studied innate immune cell populations, the role of neutrophils in NEC has remained enigmatic. Neutrophils are the most abundant= innate immune cell population among white blood cells and are normally absent from healthy peripheral tissues including the intestine. In response to intestinal damage or danger signals. However, neutrophils rapidly exit the circulation, enter affected tissues, and elaborate numerous “pro-inflammatory” effector functions including phagocytosis, production of reactive oxygen and nitrogen intermediates and ultimately killing of microbes[225]. While the function of neutrophils is aimed at host protection, localized tissue damage can be an unfortunate complication of neutrophil effector responses. Interestingly, neutrophils isolated from blood of preterm infants have been reported to exhibit defective phagocytic and microbicidal activities as well as impaired chemotaxis and adhesion, which could increase the risk of developing NEC[226].

In the preterm infant intestine, which is developmentally immature, neutrophils may provide transient barrier protection in response to threats from potentially pathogenic bacteria or tissue damage/injury. In support of this concept, early-onset neutropenia in small-for-gestational-age infants has been shown to correlate with increased odds for developing NEC[227]. Additionally, depletion of neutrophils and macrophages in an experimental model of NEC induced by treating newborn mice with the virulent gram-negative pathogen *Cronobacter sakazakii* resulted in exacerbated disease[228]. Somewhat paradoxically, neutrophils are increased in intestinal tissue obtained from NEC patients[229]. However, whether these cells are simply “guilty-by-association” as they attempt to provide critical barrier fortification remains unclear. Consistent with these findings, inducing acute intestinal damage in mice using dextran sodium sulfate (DSS) results in neutrophil accumulation in the colon as damage to the mucosa increases in severity. This accumulation coincides with barrier repair and resolution of damage and depletion of neutrophils impairs this process[230]. Interestingly, one of the key mediators of neutrophil-dependent barrier repair following DSS-induced acute intestinal damage is the IL-10 family cytokine IL-22. IL-22 is a potent inducer of intestinal epithelial proliferation and mucosal healing and also leads to enhanced production of antimicrobial peptide expression by IECs[231-233]. Thus, it is tempting to speculate that neutrophil recruitment and neutrophil-dependent IL-22 production may help to protect the premature intestine during development.

Macrophages and dendritic cells

Like neutrophils, macrophages play important roles in host defense at barrier surfaces such as the intestine. An important dichotomy between neutrophils and macrophages, however, is that the latter reside in peripheral tissues in the steady state even very early in life.

Interestingly, macrophages are already present in the fetal intestine where they are believed to contribute to maintenance of tissue homeostasis and tolerance[234,235]. At this stage of development, intestinal macrophages are hypo-responsive to stimulation with lipopolysaccharide (LPS). So-called “endotoxin tolerance” of intestinal macrophages is likely beneficial in establishing and maintaining a mutualistic relationship with the intestinal microbiota[234].

With intestine resident macrophages being in a state of hypo-responsiveness, blood monocytes can be rapidly mobilized into the intestine in response to microbial threats and or tissue damage/stress. During the intestinal damage that accompanies NEC, blood monocytes are recruited to the damaged intestine and an acute drop in blood monocyte counts can be observed and may be useful as a biomarker for NEC in VLBW infants[236]. After entering the damaged intestine, blood monocytes rapidly differentiate into pro-inflammatory M1-type macrophages. These M1 macrophages isolated from human and experimental NEC exhibit high-level expression of Smad7 making them refractory to TGF-β signaling, while promoting NF-κB-mediated signaling and secretion of pro-inflammatory cytokines including IL-1β, IL-6, IL-12, and TNFα[237]. Macrophage products such as the proinflammatory chemokines IL-6, IL-8, and TNF-α have been found to be greatly elevated in infants with surgical NEC as compared to other preterm intestinal injury[238]. Activated M1 macrophages can further potentiate intestinal damage during NEC by augmenting intestinal epithelial cell apoptosis[85]. Thus, inhibiting the differentiation and/or effector functions of M1 macrophages has been considered as an approach to limit the dysregulated inflammatory response in the NEC intestine. A recent report has provided evidence that this may be a feasible approach by showing that heparin-binding epidermal growth factor-like growth factor (HB-EGF) can protect from experimental NEC by preventing M1 and promoting M2 polarization of macrophages[85].

In addition to macrophages, dendritic cells (DCs) are another population of antigen-presenting cells that are capable of regulating intestinal immune responses[239]. Intestinal DCs are positioned in the muscularis mucosae and lamina propria where they can access bacterial antigens and initiate innate and adaptive immune responses[240]. In the steady-state, intestinal DCs promote the induction of regulatory T cells and tolerance in adults[241,242], but the functions in the preterm infant intestine remain unclear. Current evidence from *C. sakazakii*-induced experimental NEC suggests that DC influx into the intestine during disease contributes to pathological inflammation. Using this model of NEC, the authors found that depletion of DCs in mice protected against *C. sakazakii*-induced intestinal damage and conversely that adoptive transfer of DCs promoted epithelial barrier disruption and the onset of NEC[243]. Altogether, these data suggest that controlling the activation and differentiation of intestinal macrophages and dendritic cells may hold potential for NEC therapy.

CD4+ T lymphocytes

Compared to the role of innate immune cells in NEC as described above, there exists relatively little evidence examining the role of lymphocytes in this disease process. Interestingly, a recent study showed that the intestine in mouse and human NEC contains an abundance of CD4+ T cells that are recruited in response to TLR4-mediated induction of the

CCR9/CCL25 axis[244]. CD4+ T cells in the NEC intestine were enriched in Th17 cells, while Foxp3-expressing regulatory T cells (Tregs) were diminished, consistent with previously published data[245]. Th17 cells in the NEC tissue appeared to contribute to intestinal damage as blocking of IL-17 receptor or STAT3 was capable of ameliorating disease. Additionally, oral delivery of retinoic acid was sufficient to skew the polarization of CD4+ T cells away from the Th17 lineage and towards Tregs, which resulted in diminished NEC severity. These results provide an exciting new avenue for exploring the contribution of effector and regulatory T cells in the pathogenesis of NEC.

Conclusion

While it is clear that immune responses are associated with NEC, the precise role of specific innate and adaptive cells and factors in mediating protection versus contributing to pathogenesis continues to emerge. Rapid innate immune cell recruitment and cytokine production in response to barrier threats is a highly evolutionarily conserved process that is critical for host protection. However, if the threat is not efficiently and effectively neutralized, uncontrolled intestinal damage may ensue. From this standpoint, NEC may be a disease initiated, in part, due to suboptimal innate immune responses in response to dysbiotic microbiota in the preterm intestine. Following initial tissue damage, activated innate and adaptive immune cells may then accumulate in the intestine where they are associated with further tissue damage while attempting to contain invading bacteria.

Acknowledgments

Supported in part by National Institutes of Health Award R01 DK097256 (TLD), K23 HL128942 (RMP), Children's Healthcare of Atlanta Friends Grant (AFB, PLD), and Emory and Children's Center for Infections and Vaccines Pilot Grant (PLD).

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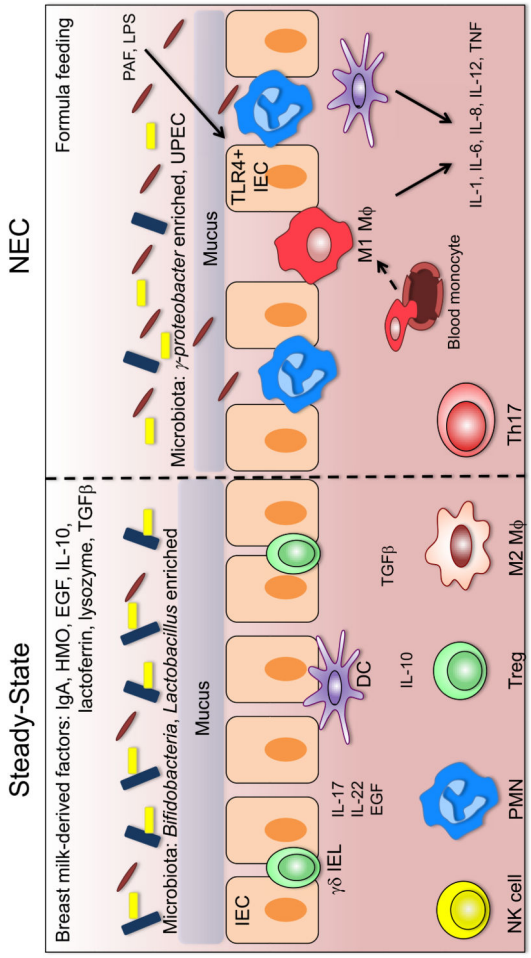

figure 1. Premature infant gut in the steady state and during NEC

In the steady state, homeostasis is promoted by beneficial bacteria *(Bifidobacteria, Lactobacillus)* and breast milk components (IgA, HMO, EGF, IL-10, lactoferrin, lysozyme, TGF-β). In the preterm gut, γδ IEL are among the first intestinal-resident immune cells contributing to the maintenance of epithelial integrity via IL-17A and EGF. Natural killer (NK) cells also protect against and repair barrier damage. Neutrophils (PMN) may be important during initial colonization in the neonatal gut, providing transient barrier protection in response to threats from potentially pathogenic bacteria, via IL-22 production. Resident macrophages (Mϕ) and dendritic cells (DCs) maintain tolerance toward the intestinal microbiota via the production of IL-10, which, in combination with transforming growth factor TGF-β, induce regulatory T cells (Treg) cells. During NEC, lack of breast milk protective components and dysbiotic flora (e.g. *Gammaproteobacter*) may allow barrier breakdown and bacterial translocation. This leads to innate signaling via TLR-4 (in response to PAF and LPS), which in turn causes recruitment of neutrophils and monocytes into the intestine, where they, along with resident DCs drive proinflammatory cytokine production, including IL-1β, tumor necrosis factor (TNF), IL-8, and IL-12, which can promote pathogenic Th1 and Th17 responses.

Table 1A
Passive Immunity Protecting the GI Tract in the Preterm Infant

	Time of Maturation	Role in NEC
Placental transfer of IgG	Starts at 13 weeks Mature by term Preterm infants with reduced IgG transfer	Deficiency may predispose to NEC
Breast milk transfer of sIgA	Preterm human milk with higher levels	Unclear benefit of oral IgA administration in decreasing risk of NEC

B. Additional Breast Milk Components Protecting the GI Tract in the Preterm Infant

	Mechanism of Protection	Role in NEC
Nutrients:		
Oligosaccharides	Promote growth of commensal bacteria	Oligosaccharide supplementation may reduce NEC risk
Caseins	Stimulate increased Paneth cell and goblet cell number and possibly function May also reduce bacterial adherence to intestinal epithelia	
Triglycerides	Stimulate increased Paneth cell and goblet cell number and possibly function May also reduce bacterial adherence to intestinal epithelia	
Bioactive proteins:		
Lysozyme	Antibacterial, synergistic with lactoferrin	
Lactoferrin	Antibacterial, antifungal, antiviral Reduces bioavailability of iron to pathogens	Lactoferrin supplementation (+/- probiotics) may reduce NEC risk
PAF-AH	Inactivates PAF (key mediator of NEC)	
Immunoregulatory cytokines: IL-10	Anti-inflammatory cytokine important for intestinal homeostasis Genetic defects in IL-10R cause colitis	IL-10 supplementation in animal models is protective Increased IL-10 in human milk associated with a decreased risk of NEC
TGF-β	Involved in regulating inflammation and wound healing	Low levels in human milk may predict feeding intolerance in growth restricted infants
Growth factors:		
IGF family	Promotes IEC proliferation; reduces IEC apoptosis	IGF supplementation reduces NEC in animal models
EGF family	Promotes IEC proliferation/differentiation restitution and TJ expression Reduces IEC autophagy Increases mucin production Inhibits TLR-4 signaling Promotes anti-inflammatory macrophages	Decreased EGF associated with increased NEC risk EGF supplementation reduces NEC in animal models EGF supplementation in humans promotes intestinal mucosa trophic effects

Table 2
Physical Barriers Protecting the GI Tract in the Preterm Infant

Physical Barrier Component	Time of Maturation	Role in NEC
Gastric Acid	Mature secretion by 24 weeks	Acid suppression associated with an increase risk of NEC
Mucus layer (Goblet Cells)	Term Premature infants with immature mucus layer	Deficiency may predispose to NEC NEC causes reduced number & reduced production of mucins and trefoil factor
Epithelial barrier (AJC)	Mature structure of AJC at 12 wks gestation (in utero) Premature infants with increased intestinal permeability Mature function at term	Immature barrier function may increase NEC risk Breast milk and probiotics may reduce NEC risk by improving epithelial barrier function
Antimicrobial peptides	Paneth cells detectable at 12 wks gestation with secretory capability at 13-20 wks Premature infants with decreased Paneth cell number and secretory capability	Deficiency of Paneth cell number and function may predispose to NEC NEC causes upregulated Paneth cell numbers but these cells are dysfunctional