Pathogenesis of Necrotizing Enterocolitis

Modeling the Innate Immune Response

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Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants. The pathophysiology is likely secondary to innate immune responses to intestinal microbiota by the premature infant’s intestinal tract, leading to inflammation and injury. This review provides an updated summary of the components of the innate immune system involved in NEC pathogenesis. In addition, we evaluate the animal models that have been used to study NEC with regard to the involvement of innate immune factors and histopathological changes as compared to those seen in infants with NEC. Finally, we discuss new approaches to studying NEC, including mathematical models of intestinal injury and the use of humanized mice. (Am J Pathol 2015, 185: e16; http://dx.doi.org/10.1016/j.ajpath.2014.08.028)

Necrotizing enterocolitis (NEC) is a disorder characterized by intestinal necrosis in premature infants that results in significant morbidity and mortality.1 Approximately 7% of infants with a birth weight between 500 and 1500 g develop NEC.1 The pathogenesis is characterized by intestinal inflammation that can progress to systemic infection/inflammation, multiorgan failure, and death. The bowel is distended and hemorrhagic on gross inspection. On microscopic examination, signs of inflammation, mucosal edema, epithelial regeneration, bacterial overgrowth, submucosal gas bubbles, and ischemic transmural necrosis are seen (Figure 1, A–E).2

Currently the pathogenesis of NEC is believed to have multifactorial causes, including intestinal immaturity and microbial dysbiosis. Intestinal immaturity leads to a compromised intestinal epithelial barrier, an underdeveloped immune defense, and altered vascular development and tone. The compromised epithelial barrier and underdeveloped immune system, when exposed to luminal microbiota that have been shaped by formula feedings, antibiotic exposure, and Cesarean delivery, can lead to intestinal inflammation and sepsis. Despite therapeutic success in animal model systems, there are relatively few therapeutic strategies that have allowed for significantly improved outcomes in infants with NEC. Two hurdles that persist are our incomplete understanding of the developing immune system in premature infants and our inability to adequately replicate these complex factors in animal models.3,4 This review summarizes the complex intestinal immune system in premature infants and details what is known about the involvement of innate immune factors in NEC, both in animal models and in human disease.

The Neonatal Intestinal Ecosystem

The neonatal intestinal ecosystem is extremely fragile. At birth, the newborn is exposed to the external environment for...
the first time, and the immune response must begin to distinguish between self and nonself. In particular, the recognition of food antigens and commensal microbiota must be distinguished from that of potential pathogens. This process is even more challenging in premature infants, as they are typically placed on broad-spectrum antibiotics that disrupt both the timing and the diversity of the initial bacterial colonization of the intestinal tract. In addition, the immature epithelial barrier appears to be more sensitive to the detection of bacteria and more susceptible to bacterial translocation, allowing for both an unwarranted inflammatory response to commensals and the translocation of pathogens, both of which may contribute to intestinal damage.

The innate immune system is the first line of defense against infections. Innate immune cells respond in a nonspecific manner and do not confer long-lasting immunity to the host. The major components are cells (including macrophages, neutrophils, dendritic cells (DCs), natural killer cells, B1 B cells, innate lymphoid cells, and γδ T cells) and anatomical barriers (such as the intestinal epithelium and the gastrointestinal mucus layer). In addition, the presence of commensal microbiota can serve as a part of the innate immune system by preventing the colonization by pathogenic bacteria (colonization resistance).

Mucosal Innate Immunity

Several major components of the human mucosal immune system are in place before birth. These components include IgM B cells; γδ T cells found in the intraepithelial lymphocyte compartment, which are seen as early as 12 to 15 weeks of embryonic age; and Peyer’s patches, which can
be seen by about 30 weeks of gestation. This development contrasts with that of the murine mucosal system, in which the same B cells and intraepithelial lymphocytes are seen only about 1 day before birth and macroscopic Peyer’s patches are not seen until at least 1 week after birth. These differences in intestinal immune system development imply that a newborn mouse may have intestinal immaturity very similar to that of a premature infant—one reason that murine models are widely used to study the immune mechanisms that predispose to NEC development.

Anatomical Barriers

Separation of the intestinal lumen from the rest of the organism is accomplished through a physical barrier established by the intestinal epithelial cells. All intestinal epithelial cells, including enterocytes, Paneth cells, and goblet cells, play an important role in maintaining barrier integrity. The barrier is maintained by the presence of tight junctions between the epithelial cells. In humans, tight junctions are formed by 10 weeks of gestation and are composed of claudins, junctional adhesion molecules, and zonula occludins.

Goblet cells, positioned throughout the intestine, are involved in the secretion of mucin glycoproteins, which generate the mucus layer of the intestine. Goblet cells can be found as early as 9 to 10 weeks of gestation, and mucins reach adult levels by 27 weeks of gestation. Animal model studies suggest that mucus gene expression is influenced by bacterial colonization, that mucus protein glycosylation patterns are developmentally regulated, and that glycosylation alters interactions with bacterial pathogens; however, equivalent studies in humans have not been reported. Mucus forms in two distinct layers; the outer, thicker layer prevents intestinal bacteria from reaching the epithelial layer, whereas the inner layer is vital for cell signaling if a disruption occurs. In addition to preventing bacterial interaction with the epithelium, the mucus layer also provides scaffolding for antimicrobial peptides (AMPs) and secretory IgA (SIgA), which are discussed in the following section.

Microbial Sensors and AMPs

Should pathogenic or commensal bacteria penetrate the mucus layer and reach the epithelial cells, pattern-recognition receptors (PRRs) are able to sense the presence of the bacteria and initiate an appropriate response. The most common PRRs are Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains (NODs), which are expressed by both epithelial cells and immune cells, particularly macrophages and DCS, throughout the intestinal compartment. The PRRs detect common bacterial structures, such as lipopolysaccharide (LPS), flagella, and CpG-rich DNA. Current dogma indicates that abnormal TLR4 signaling in the premature intestinal epithelium is associated with NEC, perhaps by increasing platelet-activating factor (PAF) levels. However, on detection of microbial components, protective PRRs can initiate signaling pathways leading to the production of AMPs, IgA, and epithelial healing factors.

Paneth cells—found at the base of the intestinal crypts—are yet another secretory cell type that provides protection to the host. Paneth cells are present at around 13 weeks of gestation and produce AMPs, which function to disable or kill microorganisms that have entered the digestive tract. Enterocytes, macrophages, and human neutrophils can also produce AMPs, including α-defensins, β-defensins, lysozyme, and type IIA secretory phospholipase A2, with α-defensins and lysozyme being the most common in the neonatal intestine. Type IIA secretory phospholipase A2 hydrolyzes the phospholipids in bacterial cell walls, which results in the production of arachidonic acid and lyso-PAF. As lyso-PAF is converted to the highly active inflammatory mediator PAF, type IIA secretory phospholipase A2 provides a mechanism that is at the intersection of bactericidal and inflammatory host defenses.

Luminal and Environmental Factors

As the newborn immune system begins its development, SIgA is transferred via maternal breast milk. Additionally, growth factors, cytokines, and other immune modulators are components of human milk, including IL-6, tumor necrosis factor α, IL-10, transforming growth factor (TGF)-β, and lysozyme. Newborns acquire IgA through passive transfer via the ingestion of human milk after birth. The levels of IgA in human milk remain high throughout the breastfeeding period and help to provide protection and aid in the colonization of commensal bacteria. Although neonatal IgA production begins at approximately 2 weeks after birth, it does not reach adult values until 2 to 6 years of age. Maternal IgA in milk is thought to react with the microbiota present in the maternal gastrointestinal tract, and the passive transfer of these antibodies not only is thought to prevent the adherence of bacteria to mucosal surfaces and bacterial penetration into the mucosa but also is vital for the formation and composition of the newborn’s intestinal microbiota. Maternal milk has been shown to promote colonization with bifidobacteria. As NEC cases are more highly associated with formula feeding, lower numbers of bifidobacteria and bacteroidetes, and higher proportions of proteobacteria and actinobacteria, it would appear that the shaping of the intestinal microbiota by the maternal IgA is one of the crucial factors in preventing NEC. Discussion of the multiple studies that have investigated the dysbiosis seen in NEC are outside of the scope of the review, but those studies were recently reviewed by Torrazza and Neu. IL-10 and TGF-β are anti-inflammatory cytokines involved in regulating T-cell responses and in down-regulating proinflammatory secretions from macrophages and neutrophils. Neonatal monocyte and T cell–derived IL-10 levels are significantly lower than are adult levels; however, IL-10 has been detected in human milk, suggesting an important role for milk-derived IL-10 in neonatal immune development. A recent study has indicated that a low blood TGF-β level is associated with a higher risk for NEC.
Modeling NEC

Numerous animal models are currently used for studying NEC disease pathogenesis. Although each model has specific advantages in mimicking certain aspects of the human disease, it has limitations, including the fact that laboratory animal models lack genetic diversity, are subject to static environmental conditions, and do not have innate immune and intestinal development identical to that in humans. However, human tissue is also not optimal, as surgical specimens are often necrotic and may not reflect the mechanisms that lead to the development of NEC. An ideal model of NEC would: i) be based on the mediators thought to contribute to and induce human disease, ii) take into account the developmental differences specific to the neonatal period, iii) have impaired intestinal restitution and development of necrosis, iv) include factors shown to play a role in the human disease (prematurity, hypoxic stress, formula feeding, dysbiosis), and v) have increased severity in the ileum. As this review will demonstrate, there is no model that perfectly replicates NEC; however, each of the following models is a useful tool for evaluating the specific mechanisms of the disease (Table 1): gavage/hypoxia (G/H) model, ischemia/reperfusion (I/R) model, PAF administration, Paneth cell depletion, xenograph model, infection model, and chemical injury models.

G/H Model

Perhaps the most commonly cited animal model of NEC is the G/H model, which has been described in mice, rats, and piglets. This model links the altered vascular development and tone that are thought to be components of intestinal immaturity with early exposure to cold stress and a nonmilk diet. Rats are popular for use in these studies because they are easier to gavage than are mouse pups and are easier and less expensive to maintain than are piglets. The G/H models are characterized by sloughing epithelial cells, severe edema in the submucus and muscle layers, villous damage and destruction, separation of the mucosa and lamina propria, and, ultimately, complete destruction and necrosis of all epithelial structures, representing a presentation similar to that of human NEC (Figure 1, F–J). This model has been adapted and more fully understood over the years, and it is now known that hypoxia, formula gavage, and intestinal bacteria are required for the development of NEC-like pathophysiology and histological factors. The mouse G/H model was originally described with pups delivered by Cesarean section (E20-21) but has recently been simplified to use newborn pups gavaged with adult commensal bacteria. This simplified model will encourage the use of the plethora of available knockout and transgenic mice to further our understanding of NEC. In addition, the G/H model has also been used for linking several components of the innate immune system to the pathogenesis of NEC. Mucin production, SIGA, TLR4 signaling, AMP expression, and epithelial junctional proteins have all been shown to impact the degree of intestinal injury in animals that are subjected to the G/H model (details in Proposed Innate Immune Mechanisms Leading to NEC); however, the role of innate immune cells in the pathogenesis of NEC has not been investigated to date.

Challenges associated with the G/H model include significant variability across research groups regarding the specific age at which the protocol is started, the type of formula administered, whether the formula is supplemented with bacteria and/or LPS, and the modality of hypoxia treatment. These challenges make it difficult to reproduce the model between laboratories and to unify the knowledge gained in the multitude of studies. Although the piglet model is not widely used because of costs and a lack of reagents, the piglet gastrointestinal system offers the resemblance perhaps closest to that in humans, particularly in the neonatal and newborn stages. In pigs, the hypoxia and gavage are not always paired because one insult (either hypoxia/hypothermia or formula feedings) can result in NEC-like illness.

I/R Model

The I/R model of intestinal injury is caused by occluding the superior mesenteric artery, thus obstructing blood flow

<table>
<thead>
<tr>
<th>Components of an optimal animal model</th>
<th>G/H</th>
<th>I/R</th>
<th>PAF administration</th>
<th>Paneth cell depletion</th>
<th>Xenograft</th>
<th>Infection and chemical injury</th>
</tr>
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<tbody>
<tr>
<td>Based on predicted mediators of NEC pathogenesis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Reflects developmental differences specific to the neonatal period/prematurity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
<td>Impaired intestinal restitution</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Development of necrosis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Hypoxic stress</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Temporal relationship to feeding/formula feeding</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Dysbiosis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>More severe disease in the ileum</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The Institutional Care and Use Committee and the institutional review board of the University of Alabama at Birmingham approved all experiments.

G/H, gavage/hypoxia; I/R, Ischemia/reperfusion; NEC, necrotizing enterocolitis; PAF, platelet-activating factor.
to the small bowel for a short time, followed by a period of reperfusion. In the intestine, the initial ischemia results in damage to the epithelial cells, whereas the subsequent reperfusion injury causes significant damage to the rest of the mucus layer. Histologically, this model is typically evaluated based on the extent of necrosis in the intestine, ranging from necrosis of the villus tip to transmural necrosis penetrating the muscle layer (Figure 1, K–O). This model attempts to replicate the ischemia that is thought to occur before NEC; however, it is more accurately classified as a model of intestinal injury rather than as a true model of NEC. The I/R model is particularly useful for investigating the role of free-radical damage in intestinal injury, as high levels of oxidative damage are induced after reperfusion (mimicking the potential reperfusion after an ischemic event in newborn humans). The primary strength of the I/R model is that it leads to defined and reproducible mucosal injury and barrier dysfunction. Both TLR4 and junctional protein expression have been shown to impact intestinal injury in the I/R model.

**PAF Administration**

The administration of PAF, combined with bacterial LPS, has been reported to initiate NEC-like pathologies in both mice and rats. PAF has been identified as a proinflammatory phospholipid, causing vascular permeability, enhanced leukocyte degranulation, adhesion, and chemotaxis, as well as the production of tumor necrosis factor α, IL-6, and IL-8. PAF is primarily produced by innate immune cells, such as neutrophils, basophils, and monocytes, as well as endothelial cells and platelets. To induce NEC-like necrosis, PAF and LPS are injected directly into the mesenteric vascular circulation. In rats, the hemorrhagic lesions of necrosis observed in the jejunum and ileum are similar to those observed in human NEC. On microscopic examination, severe cases demonstrate transmural necrosis, with loss of mucosal architecture and sloughing of epithelial cells.

**Paneth Cell Depletion**

Dithizone, when administered i.v. to rats, leads to cell death in 25% to 30% of Paneth cells. This technique was used in conjunction with the administration of enteric pathogens (Escherichia coli, Klebsiella pneumonia) to induce NEC-like pathology in one study. That study revealed gross necrotic lesions as well as damage to the intestinal tissue, including mucosal edema, loss of villous integrity, areas of transmural necrosis, and intramural air, all of which are observed in human patients. It is interesting to note that when this treatment was performed in mice before mature Paneth cell development, no disease was observed. The Paneth cell depletion model is useful as it is simpler to administer dithizone and an enteric pathogen than it is to perform feeding gavages or ischemia-reperfusion operations in young mice. However, it implies a central role of Paneth cells in disease initiation, a hypothesis that has recently been proposed but not proven. In adults, it is clear that Paneth cell dysfunction, secondary to genetic risk factors, results in dysbiosis and plays a role in intestinal inflammation; therefore, the evaluation of NEC patients for similar genetic defects might yield support for the role of Paneth cells in NEC.

**Xenograft Model of Human Intestinal Development**

The xenograft model provides a means of recapitulating the developmental changes in innate immunity and intestinal development, both of which may have a role in NEC pathophysiology. Human fetal intestine is implanted s.c. into an immunodeficient mouse host, the host is allowed to heal, and the fetal intestinal tissue matures for a designated period. The xenograft model allows for the assessment of ontogenic changes at points in human intestinal development that correspond to the second and third trimesters of pregnancy. Although this is a potentially accurate model of human intestinal development, it does not mirror NEC pathogenesis as other models do.

**Infection and Chemical Injury Models**

A mouse model of NEC based on Cronobacter sakazakii infection was recently reported. The justification of using this specific pathogen is the relatively common contamination of various formula preparations by this bacteria. This model involves the recruitment of innate immune cells (DCs, macrophages, and neutrophils) and has been used for showing that the depletion of DCs in newborn mice protects against C. sakazakii—induced NEC, whereas the depletion of neutrophils and macrophages exacerbates inflammation. Macrophages also may have a role in a chemically induced model of NEC. This model is based on the clinical observation of numerous macrophages and few neutrophils in tissue samples from NEC patients. MohanKumar et al hypothesized that this observation was a reflection of the underdeveloped innate mucosal immune system, and they designed a model that used trinitrobenzene sulfonic acid—induced inflammation for comparing mucosal injury in newborn versus adult mice. The neonatal mouse has a macrophage-predominate infiltrate similar to that in human NEC. These two new models may serve as tools useful for the study of innate immune cells in NEC, which to date have not been adequately investigated.

**Proposed Innate Immune Mechanisms Leading to NEC**

**Dysbiosis and Barrier Dysfunction**

A recent study demonstrated a lack of diversity in fecal bacteria from patients who developed NEC compared to
that from unaffected patients. A further study described an increase in proteobacteria and a decrease in firmicutes in NEC patients relative to healthy neonatal controls. These studies clearly demonstrate a difference in intestinal microbiota in NEC patients. Because these alterations were detected at the initial colonization phase in the patients, it is possible that this dysbiosis may contribute to the development of NEC and is not just a reflection of the disease state. Several factors may result in a bacterial dysbiosis after birth, including Cesarean instead of vaginal delivery, feeding of breast milk instead of formula, and broad-spectrum antibiotic exposure.

It is becoming increasing clear that dysbiosis can drastically impact intestinal immune defenses by altering apoptotic and inflammatory signaling, barrier function, and bacterial detection mechanisms in the intestine. Increased intestinal permeability and epithelial damage, coupled with deficient epithelial healing, have been identified in NEC, although it is unclear whether these aspects precede or result from NEC. Alterations in the tight junctions and the mucus layer would provide a mechanism for dysbiotic bacteria to reach and cross the epithelial barrier and initiate altered immune responses. Conversely, full development of a functional mucosal immune system requires the normal bacterial colonization that occurs after birth, as newborn pups raised by dams on broad-spectrum antibiotics have increased bacterial translocation, decreased numbers of CD4+ T cells in mesenteric lymph nodes, and altered cytokine production (a pattern that is similar to that in infants with NEC).

Several G/H studies have demonstrated a loss of mucin 2, which might allow for increased bacterial translocation from the lumen (Table 2). In addition, rat pups subjected to G/H and subsequently treated with epidermal growth factor (EGF) showed an amelioration of experimental NEC that was correlated with increases in mucin 2, occludin, and claudin-3 and decreased epithelial cell apoptosis. These studies demonstrate the importance of the epithelial barrier and imply its role in preventing bacterial translocation that may be crucial for the prevention of NEC.

Also controlling microbial colonization in the intestines of neonates are AMPs, several of which are produced by Paneth cells. Studies have demonstrated that AMP secretion is altered in infants with NEC; specifically, lysozyme is decreased, whereas the α human defensins 5 and 6 are increased. Human NEC samples have been reported to have both an increase and a decrease in Paneth cell numbers relative to control samples. Because Paneth cell numbers increase during gestation and are regulated by inflammatory mediators, these discrepancies may be attributed to differences in gestational age at birth or they might reflect the heterogeneity of samples. As the dithizone model certainly mimics some of these observations, it should be useful for further studies on the importance of dysbiosis and the innate immune components that shape the microbiota in NEC.

**TLRs**

One potential initiating factor of NEC is the response of epithelial and immune cells to the microbiota that colonize infants after birth. The expression of TLR4, a sensor of the Gram-negative bacterial component LPS, is increased in human NEC as well as fetal intestine, and this increase appears to be dependent on microbial colonization (Table 2). PAF has also been shown to induce TLR4 expression in intestinal epithelial cells, and PAF levels are elevated in newborns with advanced NEC, whereas levels of acetylhydrolase (the PAF-inactivation enzyme) are decreased in NEC patients. Mouse G/H studies have demonstrated that the changes in TLR4 expression precede histological injury and that epithelial expression of TLR4 is required for NEC development. Also, treatment with polyunsaturated fats to reduce TLR4 expression or with amniotic fluid to inhibit TLR4 signaling reduces the severity of G/H-induced NEC, and the absence of TLR4 protects from disease. TLR4 signaling during bacterial colonization results in inhibited proliferation, migration, and survival and increased endoplasmic reticulum stress and apoptosis of enterocytes in the intestine. These findings suggest that TLR4 activation leads to impaired enterocyte repair and migration, therefore inhibiting the effective repair of the intestinal epithelium and subsequently resulting in increased bacterial translocation, inflammation, and NEC.

Recently, Yazji et al. described a novel role for TLR4 on endothelial cells in the regulation of intestinal perfusion. Intestinal ischemia has been thought to be a potential trigger of NEC; this study shows the importance of innate immune recognition outside the intestinal mucosa in the development of NEC. The role of TLR4 in NEC has also recently been linked to other PRRs, including TLR9 and NOD2. The administration of bacterial CpG DNA (the ligand of TLR9) or the activation of NOD2 inhibited TLR4 signaling and greatly reduced the incidence of murine NEC. The dysbiosis seen in infants predisposed to NEC may contribute to the disease through differential ligation of TLR4 versus TLR9/NOD2.

Using the xenograft model of intestinal development, studies indicate that immature xenograft and NEC intestinal epithelial cells have increased mRNA expression of TLR2, TLR4, myeloid differentiation primary response 88 (MyD88), NF-κB, and IL-8 and decreased expression of the TLR and NF-κB negative regulators, toll-interacting protein, and single Ig IL-1-related receptor compared with that in mature controls. Additionally, reports describe that the treatment of immature xenografts with media containing probiotic factors led to the down-regulation of TLR2 and TLR4 as well as the up-regulation of toll-interacting protein and single Ig IL-1-related receptor comparable to those in mature...
These data suggest that differences in the maturation of the TLR-induced NF-κB signaling pathway may be a part of the mechanism of NEC development, and that these pathways may be controlled by interaction with probiotic microorganisms.

Together, these data highlight the importance of proper bacterial detection by the intestinal epithelial barrier (particularly through TLR4 and TLR9) and their subsequent signaling cascades to induce a proper immune response. When this sensing is disrupted through antibiotic treatment,
or occurs prematurely in neonatal infants, the physical barrier is breached and bacteria are exposed to the infant’s immune system. In contrast to the increased epithelial expression of TLR4 in premature infants, it has been reported that infants born at <28 weeks of gestation have very low levels of innate immune receptors (CD14, TLR2, TLR4) on their core blood leukocytes and have severely impaired leukocyte inflammatory responses to bacteria. This impaired innate immune function likely leads to an inability to control infection due to the impaired barrier, and subsequently aberrant immune activation results, leading to NEC. However, further studies are needed to specifically investigate the contributions of PRRs on leukocytes in the development of NEC.

**EGF**

Studies in both animal models and human patients have identified EGF as a potential biomarker for NEC development and as a future treatment of NEC. EGF is found in the amniotic fluid and is produced by the salivary glands, Brunner’s glands in the duodenum, Paneth cells, and maphrophages. EGF is important for cell survival (reduced autophagy), division, and migration in the intestine. Human studies have revealed that decreased cord blood EGF levels are predictive of NEC in very-low-birth-weight infants and that heparin-binding EGF (HB-EGF) treatment in rat pups subjected to the G/H NEC model protects the pups from injury (Table 2). In addition, in the G/H model, the administration of mesenchymal stem cells along with HB-EGF results in a synergism that reduces injury and improves survival. These studies demonstrate that impaired epithelial healing secondary to reduced EGF/HB-EGF may be a major component in the pathogenesis of NEC.

**External Modulators**

Full-term infants acquire several immunomodulatory components from the breast milk of their mothers, including PAF acetylhydrolase; growth factors such as EGF, HB-EGF, insulin-like growth factor (IGF), and TGF-β; and immune factors such as SIgA and IL-10 (Table 2). The immaturity of a preterm infant’s immune system may make the presence of these molecules even more vital for preventing intestinal damage during the first weeks of life. Due to the nearly absent levels of IgA in newborn infants and the importance of IgA in regulating the commensal microbiota, it has been suggested that a lack of IgA may be involved in NEC, particularly in formula-fed infants. To address this issue, several human studies have attempted to supplement IgA levels, as well as other immunoglobulins, in newborns; however, there has not been a striking improvement in outcomes. In interpreting these data, it should be noted that in all studies in which formula was supplemented with immunoglobulins, serum immunoglobulins and not SIgA was used. IgA arrives in the intestinal lumen and breast milk by transcytosis through the epithelial cells. During this process, it acquires a secretory component that protects the IgA antibodies from degradation by the gastric acids and enzymes of the digestive system. Therefore, SIgA has properties that are profoundly different from those of the serum IgA used so far in studies. Further investigations are necessary to elucidate the role of SIgA in protection from NEC, as none of the current animal models have evaluated this aspect of innate immunity in the disease.

Studies involving IL-10 have revealed slightly more information and do suggest an important role of IL-10 in protection from NEC. It has been reported that the percentage of mothers with undetectable IL-10 in their milk was significantly greater in mothers of infants who developed NEC. In addition, levels of SIgA and IL-10 in human milk are lower after very preterm delivery (<30 weeks of gestation) compared with preterm (30 to 37 weeks of gestation) and term (38 to 42 weeks of gestation) delivery. Conversely, a recent study measured increased levels of IL-10 in the serum of NEC patients relative to healthy controls. This increase could be explained by a lack of IL-10 in the maternal milk, requiring increased IL-10 production by the newborn in an attempt of the young immune system to control the disease; however, IL-10 production by the infant may be too delayed to effectively control the immune response. The G/H model has been used for investigating the role of IL-10 in the development of NEC. IL-10−/− mice develop more severe histological aspects, characterized by increased epithelial cell apoptosis and decreased organization of tight junction proteins. Additionally, the administration of IL-10 ameliorates disease, and ileal IL-10 levels are increased in rats fed with rat milk versus a cow-milk substitute. The PAF/LPS mouse model shows that the probiotic *Lactobacillus rhamnosus GG* protects mice from NEC by increasing IL-10 receptor transcription, thereby increasing the signaling abilities of both maternal milk–derived and endogenous IL-10. Increased IL-10 signaling in these studies resulted in lower levels of colonic production of tumor necrosis factor α and the neutrophil chemoattractant macrophage inflammatory protein 2, indicating that IL-10 signaling in the context of NEC leads to decreased inflammatory cytokine production, and the modulation of IL-10 may be possible through the administration of probiotics.

A low IGF level has been reported to be associated with NEC. IGF-1 can protect intestinal epithelial cells from oxidative stress–induced apoptosis and promote the development and cytotoxic activity of natural killer cells, whereas IGF-2 induces TGF-β release. NEC is associated with decreased tissue levels of TGF-β, and in mice, the disruption of TGF-β signaling results in more severe NEC-like mucosal injury in the PAF/LPS model. As TGF-β normally suppresses macrophage inflammatory responses in the developing intestine, its absence (from either a lack of breastfeeding or decreased IGF levels) can clearly impact the function of the innate immune system in premature
infants. Of note, recent data indicate that low blood TGF-β levels are associated with a higher risk for NEC.26

Innate Immune Cells

Both of the newly described chemical and infection models of NEC have suggested that there may be a defective response by innate immune cells, leading to disease (Table 2).49,50 The data suggest a destructive role for DCs in coordinating a response to an NEC-like disease and a protective role for neutrophils and macrophages. Further investigation of the phenotypes of macrophages will reveal whether they are predominantly of the IL-10-secreting M2 phenotype, and study of the subsets of DCs will determine whether there may be defects in DC subtypes that are known to control T-cell homeostasis.93,94 However, in contrast to these murine models, reports show that numerous macrophages are seen in NEC lesions and that macrophages from the human preterm intestine produce inflammatory cytokines, whereas macrophages from full-term neonates and adults do not.40,52 These macrophages may play a role in disease, as they can be suppressed by the regulatory cytokine TGF-β2, which is decreased in both patients with NEC and in preterm milk.40,82 In addition, the trinitrobenzene sulfonic acid—induced NEC model is driven by pro-inflammatory macrophages. These differences between the infection and chemical models, as well as human disease, show the difficulty in modeling a disease with an unknown etiology and that each model must be fully investigated to determine its strengths and weaknesses.

More studies of other innate immune cells are still needed in both human disease and animal models. One recent

Figure 2 The innate immune response in necrotizing enterocolitis (NEC. Top: hematoxylin and eosin stain of normal neonatal human small bowel. Middle: Mechanistic diagram of the factors that have been associated with the increased incidence of human NEC and the potential innate immune cells and molecules involved in the disease. In this diagram, prematurity, antibiotic use, method of delivery (vaginal versus Cesarean), and formal feeding can result in dysbiosis, altered barrier function, and altered growth factors and cytokines. The dashed arrows represent a reciprocal vicious cycle between dysbiosis and barrier function, and dysbiosis and altered growth factors and cytokines. All of these factors can lead to increased bacterial translocation, decreased enterocyte migration and to increased inflammation and necrosis, as evidenced in the hematoxylin and eosin panel (bottom), showing severe muscular and epithelial damage with complete loss of mucosa. EGF, epidermal growth factor; HB-EGF, heparin bound-epidermal growth factor; IGF, insulin-like growth factor; SIGA, secretory immunoglobulin A; TLR4, Toll-like receptor 4; and TGF, transforming growth factor.
publication looking at plasmacytoid DCs reported a premature morphology and decreased functional capacity, whereas another study reported diminished levels of natural killer cell activity in the peripheral blood of preterm infants. To our knowledge, there are no reports regarding the presence or function of B1 B cells (which produce natural serum antibody and T-independent antibodies) or the newly described innate lymphoid cells in the term versus the premature neonate. Because these innate cells are present in neonatal murine models and are involved in intestinal homeostasis, they may also play an important role in NEC and should be studied further.66

**Future NEC Models**

**Humanized Mouse Models**

One potential avenue that has not been explored in NEC research to date is the utilization of humanized mouse models. Due to species-specific differences in protein structure and expression and differences in various ligand specificities between species,97 humanized mouse models may provide a bridge to further enhance NEC research. Humanized TLR4 mice have recently been developed98 and may prove to be a useful model in future NEC studies. In addition, the recently developed ability to colonize germ-free mice with human fecal microbiota is a new opportunity for developing models of the disease that are based on the fecal communities of NEC and control patients, and these models may result in a dramatic new understanding of the disease while avoiding the current confounding factors of the differing microbial communities of each institution’s animal facility.99

**Novel Transgenic Technologies**

One of the main reasons scientists that gravitate toward the mouse as a model organism is the proficiency of generating genetically modified animals. The availability of this useful tool often overrides legitimate concerns regarding developmental, anatomical, and physiological differences between mice and humans. The availability of mice with cell- or tissue-specific knockouts of receptors for IgA, TGF-β, IGF, and IL-10 make the urgency for an appropriate mouse model of NEC crucial for testing the importance of these molecules in the disease. However, the increasing availability of genetically modified pigs, as well as newly described enterocolitis models in zebrafish, may enhance interest in using these novel models for further investigation of NEC.100,101

**Mathematical Modeling of NEC**

Mathematical modeling is developing as a method useful for uncovering the intricacies of human diseases. A mathematical model of NEC is appropriate because the severity of NEC depends on a complex combination of intestinal immaturity, inflammation, injury repair, and microbial communities.102 Several mathematical models of NEC have been developed, including an effort to combine independent areas of NEC research, including the inflammatory response in NEC, the dynamics of epithelial healing, the protective factors supplied by breastfeeding, and the role of pathogenic bacteria.103 However, these models have yet to translate effectively into clinical practice and must be further optimized.

**Future Perspectives**

Our understanding of NEC has increased slowly during recent years. Despite this increase in knowledge, NEC diagnosis and treatment remain challenging, indicating that the scientific and medical communities are far from optimally understanding NEC pathogenesis. The animal models described herein have proven to be invaluable resources in achieving our current level of understanding; however, the difficulty in replicating all aspects of the human disease, the lack of spontaneous disease development, and the lack of data on the involvement of the innate immune system are drawbacks common to all models (Table 1). While a shift in microbiota, breakdown in the intestinal barrier, altered sensing of and response to bacterial components, and disrupted immune factors and cells may all be involved in NEC, it is difficult to say which, if any, is the primary insult leading to disease (Figure 2). It is clear that an NEC model that more accurately replicates human disease will be required for effectively studying the disease and for improving patient outcomes.

**References**


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