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The role of intestinal epithelial barrier function in the development of NEC

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Abbreviations: AJ, adherens junctions; AJC, apical junction complex; Bb, *Bifidobacterium bifidum*; Bi, *Bifidobacterium infantis*; BAs, bile acids; EGF, epidermal growth factor; EPO, erythropoietin; IFNγ, interferon gamma; IEL, intestinal epithelial lymphocytes; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; TCRγδ, T-cell receptor gamma-delta; TJ, tight junctions; TPN, total parenteral nutrition; TGF-β, transforming growth factor-beta; TNFα, tumor necrosis factor alpha

The intestinal epithelial barrier plays an important role in maintaining host health. Breakdown of intestinal barrier function is known to play a role in many diseases such as infectious enteritis, idiopathic inflammatory bowel disease, and neonatal inflammatory bowel diseases. Recently, increasing research has demonstrated the importance of understanding how intestinal epithelial barrier function develops in the premature neonate in order to develop strategies to promote its maturation. Optimizing intestinal barrier function is thought to be key to preventing neonatal inflammatory bowel diseases such as necrotizing enterocolitis. In this review, we will first summarize the key components of the intestinal epithelial barrier, what is known about its development, and how this may explain NEC pathogenesis. Finally, we will review what therapeutic strategies may be used to promote optimal development of neonatal intestinal barrier function in order to reduce the incidence and severity of NEC.

Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in the neonatal intensive care unit (NICU), primarily affecting premature infants.[1,2] Severe NEC is characterized by hemorrhagic inflammatory necrosis of the distal ileum[3] with clinical presentation ranging from abdominal distension, pneumatosis intestinalis, occult or frank blood in stools, intestinal gangrene, bowel perforation, sepsis and shock.[4] Approximately 9,000 infants develop NEC in the United States per year and mortality rates range from 20–40%.[5-8] Disease-associated costs are significant; premature infants diagnosed with NEC remain, on average, hospitalized for an additional 43.1 days[9] with total estimated yearly costs of up to 5 billion dollars.[10] NEC patients that require surgery to remove necrotic bowel can also develop short bowel syndrome with prolonged medical expenses and chronic gastrointestinal difficulties. Further, surgical NEC is a significant predictor of neurodevelopmental morbidity in preterm infants independent of other factors.[11] Current strategies to reduce the risk of NEC include breastmilk feeds[12,13] and probiotic therapy.[14] While the major risk factors for NEC (prematurity and enteral formula feeding) are known, the pathophysiology of this disease remains poorly understood and treatment strategies are mainly supportive.

While the etiology of NEC remains unclear, immature gut host defenses and abnormal bacterial colonization are thought to play a critical role.[15,16] The neonatal intestinal barrier is immature in the preterm neonate and has been shown to mature postnatally.[17-21] Multiple factors can induce postnatal intestinal maturation of this barrier including diet,[22-24] epidermal growth factor,[25] endogenous glucocorticoids,[26] and commensal bacteria.[17,27] Commensal bacteria, in particular, are known to induce expression of tight junction proteins that can tighten the barrier.[17,28] Thus, neonates with abnormal or delayed bacterial colonization of the gut may be at increased risk for intestinal inflammation and injury due to an immature or defective intestinal barrier that allows systemic entry of microbes, their products, or toxins from the gut lumen.[29] This may explain why preterm infants with prolonged antibiotic exposure are at increased risk for NEC[30] whereas infants treated with probiotics are protected against the disease.[31] In this manuscript, we will review the role of the immature gut barrier as a predisposing factor in the pathogenesis of NEC and how active research is currently targeting the immature gut barrier in order to develop preventive therapeutics against this devastating disease.

After birth, intestinal colonization with microbes from both the birth canal and the environment occurs within 24 hours. Premature infants are often delivered via caesarean section and do not always receive breast milk immediately after birth. In the absence of these major sources of microbial colonization, detrimental microorganisms can proliferate. In addition, the intestinal barrier in neonates is more permeable, in part to allow movement of colostrum-derived antibodies into the infant's circulation. The intracellular structures that regulate intestinal permeability, tight

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junctions (TJ) and adherens junctions (AJ), as well as other barrier components that play a role in intestinal integrity have been shown to be altered in NEC. Further, inflammation, a hallmark of NEC, has been shown to adversely affect the intestinal barrier.

A comprehensive review of NEC epidemiology, clinical presentation, diagnosis, management, and pathogenesis is beyond the scope of this review. Interested readers are directed toward recent reviews on this topic.[1,2] In this manuscript, we will review the role of the immature gut barrier as a predisposing factor in the pathogenesis of NEC and how active research is currently targeting the immature gut barrier in order to develop preventive therapeutics against this devastating disease.

Structure of the Intestinal Epithelial Barrier

The intestinal epithelium is formed by a single layer of cells which separates the host (submucosal side) from the intestinal lumen (luminal side) (**Fig. 1**). This epithelial layer regulates transport of nutrients, ions and bidirectional fluid flow.[32] The luminal surface of the intestinal mucosa exists in a symbiotic eukaryotic-prokaryotic relationship with the commensal flora, which consists of a diverse ecosystem of up to 10^{11} organisms per gram of intestinal tissue. These bacteria benefit the host by metabolizing vitamins and degrading bile acids while thriving in the nutrient rich, temperature-controlled, anaerobic luminal environment.[33] Host-flora interactions are also important for appropriate development of intestinal epithelial structure and barrier function.

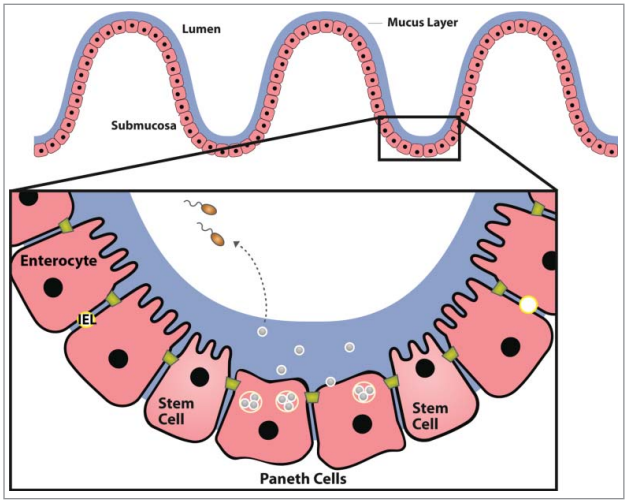

Figure 1. The intestinal epithelial barrier. A single layer of epithelia (including enterocytes, paneth cells and stem cells) separate the intestinal lumen from the submucosa (as labeled). Crypt Paneth cells secrete antimicrobial peptides which regulate microbial populations and protect neighboring stem cells. IEL are innate immune T-cells located between epithelial cells. Specialized IEL bearing the T-cell receptor (TCR) γδ (γδ IEL) have been shown to promote epithelial barrier function by preventing bacterial translocation, producing antimicrobial factors, and through interactions with the tight junction protein, occludin. Adapted with permission from Journal NeoReview, Vol. 10, Page(s) e180-e190, Copyright © 2009 by the AAP.

Epithelia are organized into crypts (invaginations) and villi (evaginations). At the base of these crypts are stem cells, which proliferate, differentiate into enterocytes, migrate to the villus tip, and eventually slough into the lumen via anoikis (a physiologic form of apoptosis). This entire process results in total reconstitution of the epithelium every 5 d.[34] Thus, one form of intestinal defense against injury is this remarkable proliferative and self-regenerating capacity.

The intestinal epithelial monolayer also protects and separates itself physically from exogenous stress by forming a thick protective layer of mucus over the intestinal mucosa. This mucus layer is composed of mucins, which are diverse, complex glycoproteins secreted by goblet cells (specialized secretory enterocytes). The mucus layer hampers direct microbial-epithelial binding, aggregates adherent bacteria, and enhances bacterial removal by reducing shear-forces of the luminal stream. Mucins also contain specific protein binding domains, which bind and stabilize critical trophic and reparative factors (e.g. epidermal growth factor (EGF) and intestinal trefoil factor) at the epithelial surface, potentially contributing to epithelial restitution.[35,36]

Chemical defenses secreted by both absorptive enterocytes and Paneth cells provide additional protection. Paneth cells are specialized secretory enterocytes located at the base of small intestinal crypts (**Fig. 1**). Adjacent to stem cells, Paneth cells protect by secreting lysozyme, phospholipase A2, and antimicrobial peptides (defensins (α and β) and cathelicidins[37,38]) that control microbial populations. Initially discovered in human neutrophils, defensins are small cationic peptides that play a key role in oxygen-independent killing of microbes.[37] Defensins 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. Once inside the microbial cell membrane, they form pores allowing the passage of anions through the membrane, thus depolarizing and killing the organism.[39] 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. Intestinal epithelial cells 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.[39] *In vitro* studies suggest that these antimicrobial peptides may contribute to host defense indirectly (by inducing host responses) as well as directly (by killing microbes).[40] Cathelicidins and defensins may have proinflammatory properties by activating chemokine release resulting in immune cell chemotaxis and differentiation. 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.[41] Future studies will be required to characterize the roles of these additional defensin and cathelicidin-induced immune modulatory activities, *in vivo*.

Epithelial integrity is further regulated by the apical junction complex (AJC), subapical intercellular contacts consisting of membrane proteins and cytoskeletal anchor proteins, which interact to form tight junctions and AJs (**Fig. 2**). Tight junctions (TJ), which seal the intercellular space between enterocytes while

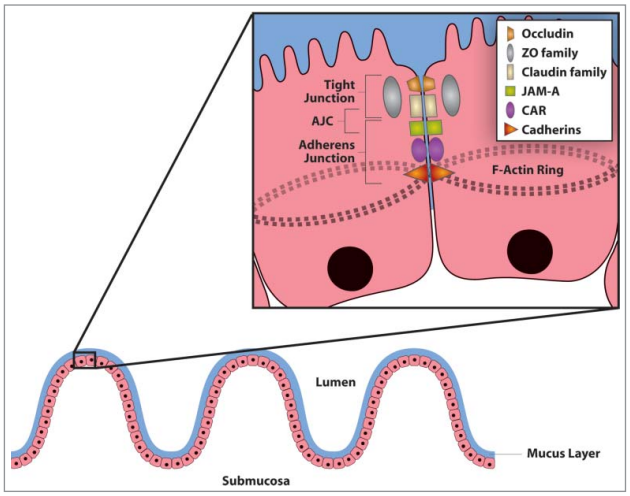

Figure 2. Structure of the apical junctional complex (AJC). Subapically located apical junctional complexes (AJC) protect the host by regulating paracellular flow. AJC is made up of tight junctions and adherens junctions. Major proteins included in the AJC are as labeled. Reproduced with permission from Journal NeoReview, Vol. 10, Page(s) e180-e190, © 2009 The AAP.

regulating paracellular permeability,[42-44] consist of over 40 transmembrane proteins including occludin, claudins, and junctional adhesion molecules such as JAM-A.[45] Major proteins comprising the AJ include E-cadherin, and α- and β-catenin and help to anchor cells to one another. The AJCs are linked to the cytoplasmic cytoskeleton to create an F-actin ring. AJCs "zipper" together intestinal epithelial cells and regulate the flow of ions and small molecules between cells.[46] The AJCs and their cytoskeletal connections are dynamic structures regulated by physiologic or pathogenic signals. In addition to regulating barrier function, the AJC proteins play important roles in epithelial cell proliferation and differentiation. The epithelial layer also regulates transcellular permeability to ions and other small molecules through alterations in expression of selective membrane ion channels and pores. Enterocytes control Cl^- and water secretion through these channels resulting in secretory diarrhea, another defense mechanism which can be used to flush unwanted pathogens or toxins from the intestinal lumen.

Innate immune cells have also recently been shown to contribute to intestinal barrier function. Intestinal epithelial lymphocytes (IEL) are innate immune T-cells located between epithelial cells that form the intestinal barrier. Specialized IEL bearing the T-cell receptor (TCR) γδ (γδ IEL) have been shown to promote epithelial barrier function by preventing bacterial translocation,[47] producing antimicrobial factors[48] and through interactions with the tight junction protein, occludin.[49] TCR γδ IEL can also regulate inflammation and foster epithelial repair.[50-53] Thus, the mature epithelium is exquisitely adapted to prevent infection and to maintain and restore barrier function in response to luminal insults using a variety of structures, secreted products and specialized cells.[54]

Development of the Intestinal Epithelial Barrier

Development of intestinal barrier function occurs both *in utero* and postnatally (**Table 1**).[17-21] The initial structural barrier of the human intestinal epithelial monolayer forms *in utero* during the first trimester. As early as 8 weeks of gestation, enterocytes appear, eventually forming the crypt-villus architecture by 12 weeks. The AJC starts to form as early as 10 weeks gestation when intercellular tight junctions can be detected. Thus, by the end of the first trimester of human gestation, the early structural barrier of the intestine is formed. However, full secretory and absorptive capabilities of the intestinal mucosa require the presence of growth and trophic factors present in amniotic fluid to induce their maturation from 26 weeks to term.[55]

Soon after the initial formation of the AJC, the epithelial barrier begins to develop additional physical and chemical barriers with the production of defensins, lysozyme and mucin gel layers. Paneth cells can be detected by 12 weeks gestation and begin to

Table 1. Immaturity of the intestinal barrier in the preterm infant

Intestinal Barrier Component	Time of Maturation	Effect of Immaturity in the Preterm Infant
Epithelial Apical Junctional Complex (AJC)	Mature structure at 12 wks gestation (in utero) Mature function at term	Increased intestinal permeability Immature absorptive capability Immature secretory capability
Paneth Cells	Detectable at 12 wks gestation Secretory capability at 13–20 wks gestation	Decreased number Decreased secretory capability (Lack of antimicrobial peptides required to regulate intestinal colonization)
Mucin (Goblet Cells)	Term	Immature mucus layer allows bacteria to contact intestinal epithelia (Lack of physical protective barrier)
Intestinal epithelial lymphyocyte (IEL)	TCRγδ subset recruited early (24 wks gestation)	Early recruitment may be important to promote immature barrier function (Important to prevent bacterial translocation, promote TJ function, regulate inflammation, and promote epithelial repair)

produce antimicrobial defensins at 13 weeks and lysozyme at 20 weeks.[56,57] Ontogeny studies have demonstrated that Paneth cells are developmentally deficient in number and function in the premature, 24-week gestation neonate.[58] TCR γδ IEL have been shown to be recruited early to the premature gut (by 24 week gestation in humans and 1 week postnatally in mice), possibly to promote barrier function while the epithelial barrier is immature.[59] Mucin expression can be detected as early as 6.5 weeks gestation, but expressional patterns continue to undergo maturation throughout gestation.[60]

While development of barrier function occurs *in utero*, there is ongoing postnatal maturation and multiple factors can induce postnatal intestinal barrier maturation including growth factors, hormones, nutrients and microbes.[17,22-25,27] Given the benefit of a mature epithelial barrier in preventing injury and inflammation, encouraging growth of the appropriate complement of commensal bacteria may be of particular benefit in premature neonates who are deprived of the benefits of an *in utero* environment.

An immature epithelial barrier causes increased intestinal permeability. This, in turn, predisposes the gut to invasion of toxins and bacteria located within the gut lumen resulting in both inflammation and injury.[61] An important characteristic of the premature gut in humans and other mammals is a leaky epithelial barrier. Over time, the intestinal barrier tightens and becomes more selective in paracellular permeability to both large and small molecules[18,19,62] Commensal colonization may be an important driving influence by which the developing intestine reduces intestinal permeability and probiotic bacteria may replicate these effects in premature infants with inappropriate intestinal microbial colonization. Both *in vitro* studies modeling the adult human intestinal epithelial barrier and *in vivo* animal studies modeling the premature intestinal epithelial barrier indicate that commensal and probiotic bacteria can improve intestinal epithelial barrier function.[17,63] A small clinical trial also confirmed that probiotics can also improve intestinal barrier function by reducing intestinal permeability in premature infants[64] and this may be a mechanism by which probiotics can reduce NEC in premature infants.[65,66] Animal models of intestinal epithelial barrier disruption during both inflammation and injury have also noted beneficial effects of probiotic bacteria on intestinal epithelial barrier function and TJ expression and localization.[67–69]

Breakdown of the Intestinal Epithelial Barrier in NEC

We propose that disruption of the intestinal epithelial barrier can predispose premature infants to NEC (**Table 2**). Premature infants are known to have diminished intestinal barrier function compared to term infants.[18,19] If an immature barrier allows invasion of microbes or toxins, this could lead to injury and inflammation in the immature gut which could further damage the already defective epithelial barrier. In fact, many studies have demonstrated how cytokines induced during intestinal inflammation, including NEC, can further weaken intestinal barrier function. For example, Prasad et. al, found the proinflammatory cytokines interferon gamma (IFNγ) and tumor necrosis factor α (TNFα) decreased claudin-2 and claudin-3, and caused redistribution of claudin 4 in T84 cells.[70] Using the same human colonic cell line, another group found redistribution of occludin, claudins 1 and 4 and ZO-1 after exposure to IFNγ.[71] In Caco 2 cells, TNFα increased permeability with down regulation and redistribution of ZO-1 protein, and interleukin-18 has been shown to disrupt occludin.[72] In animal models, injection of mice with TNFα resulted in redistribution of intestinal occludin and ZO-1 [73] and TNFα was found to play a role in the disappearance of occludin and ZO-1 during induction of experimental colitis in TNFα knockout mice.[74] Further, when neonatal rats with NEC

Table 2. Breakdown of the intestinal epithelial barrier in NEC

Intestinal Barrier Component	NEC	Effect of NEC in the Preterm Infant
Epithelial Apical Junctional Complex (AJC)	Dysfunction may predispose to NEC NEC onset causes further TJ protein perturbations (especially reduced expression and abnormal localization of occludin and claudin-3) in part caused by proinflammatory cytokines (TNFα, IFNγ, IL-18)	Further increased intestinal permeability
Paneth Cells	Deficiency may predispose to NEC NEC onset causes upregulated numbers but these cells are dysfunctional (caused in part by TNFα)	Dysfunctional Paneth cells unable to secrete antimicrobial peptides to control intestinal microbial populations
Mucin (Goblet Cells)	Deficiency may predispose to NEC NEC onset causes reduced number (caused in part by TNFα) & reduced production of mucins and trefoil factor	Compromised mucus layer allows bacteria to breach epithelial barrier
Intestinal epithelial lymphyocyte (IEL)	Deficiency may predispose to NEC NEC onset causes reduced overall number (TCRγδ subset preferentially reduced)	TCRγδ IEL deficiency further compromises barrier function (Increased risk of bacterial translocation, TJ dysfunction, and inflammation)

Table 3. Changes in TJs in NEC

		Gene Expression									Protein									
Study	**Model**	**Occludin**	**Claudin 1**	**Claudin 2**	**Claudin 3**	**Claudin 4**	**Claudin 7**	**Claudin 8**	**Claudin 14**	**ZO-1**	**Occludin**	**Claudin 1**	**Claudin 2**	**Claudin 3**	**Claudin 4**	**Claudin 7**	**Claudin 8**	**Claudin 14**	**ZO-1**	**Localization**
Clark	Rat	↑	NR	NR	↑	NR	NR	NR	NR	NR	↑	NR	NR	↑	NR	NR	NR	NR	NR	Occ, Cl^-3 to cytoplasm
Rentea	Rat	↑	↓	NR	NR	NR	NR	↑	↓	NR	↔	↑	NR	↑	NR	NR	NR	NR	NR	
Shiou	Rat	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	↔	NR	↑	NR	NR	NR	NR	↓	ZO-1 loss
Bergmann	Mouse	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	↑	NR	↓	↓	NR	NR	NR	Internalization of Occ, Cl^-2,4,7
	Human	NR	NR	NR	NR	NR	NR	NR	NR	NR	↔	NR	↑	NR	NR	NR	NR	NR	↔	Changes in CL^-2, 4
Hogberg	Rat	↑	↓	NR	NR	NR	NR	↑	↓	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	

In all studies, gene expression accessed using RT-PCR except for Hogberg, in which it was assessed via microarray analysis.
NR = not reported.
↑ = statistically significant increase in NEC versus control.
↓ = statistically significant decrease in NEC vs. control.
↔ = no statistically significant change in NEC versus control.

were injected with anti-TNFα, disease was reduced and ileal paracellular permeability was significantly decreased.[75]

In both experimental and human NEC, changes in TJs have been reported, although with considerable differences between studies (**Table 3**). One of the first studies of TJs in NEC,[76] found occludin and claudin-3 mRNA and protein elevated in ileum from neonatal rats with NEC. Histologic evaluation revealed these increased proteins were not localized at the TJ, but rather throughout the cytoplasm of the ileal enterocytes. Later studies using the same model showed increased protein levels of claudins-1 and -3, but not occludin,[77] increased expression of occludin and claudin-8, but decreased expression of claudins-1, -14, and -15,[78] and increased claudin-3 without increased claudin-1 protein with loss of histological ZO-1 in pups with NEC compared to dam-fed controls.[79] Bergmann, et al., reported increased claudin-2 in both neonatal mice with NEC as well as in human NEC surgical samples. Changes in localization of occludin, claudins -2, -4 and -7 were also observed in animals with NEC, with these TJ components found primarily in the cytoplasm, rather than at the TJ. Importantly, the concomitant increased permeability preceded over signs of NEC.[80] Further, Weitkamp et. al, reported that human NEC samples demonstrate markedly reduced occludin gene expression (compared to age-matched controls).[59]

These studies emphasize the disparities among studies with regard to which TJ components are altered. It is also difficult to fully assess these data as in most studies, it is unclear if the authors chose to report only those components where changes occurred. Taken together, however, it is apparent that during the development of NEC, changes in TJs occur and these alterations are associated with increased paracellular permeability, with occludin and claudin-3 the most likely candidates for pathophysiologic effects. While findings of increased permeability in conjunction with elevated TJ proteins may seem paradoxical, it is important to remember that the structure of the AJC is complex. Some proteins in the AJC are known to tighten the TJ (occludin, claudin 3) causing reduced permeability but others are known to cause increased permeability (claudin 2).[81] Further, correct localization of each protein to the AJC is critical to maintaining its appropriate function. In many of these studies, the increased components were not associated with the TJ structure, and thus, increased expression did not contribute to the maintenance of a healthy intestinal barrier.

The majority of studies studying the role of TJs in NEC focus on the ileum, the site of injury in this disorder. However, liver-derived proinflammatory mediators play an important role in NEC pathogenesis by increasing the levels of these potentially damaging mediators in the small intestine.[82] Changes in localization and abundance of hepatic occludin, claudins-2 and -3, and ZO-1 have been reported in neonatal rats with NEC.[83] These alterations allow leakage of TNF-α into the intestinal lumen, which exacerbates intestinal injury, and treatment of rat pups with EGF, a growth factor that can influence epithelial cells to mature, normalizes these changes.[83] In addition, bile acids (BAs) have been shown to modulate the structure of TJs and barrier functions in Caco-2 cells.[84] BAs, produced in the liver and transported into the ileal lumen, are elevated in the ileum of neonatal rats and mice with NEC,[85] and may influence intestinal TJ formation during disease development. Adherens junctions in NEC have been scantily reported, but there appears to be redistribution of ileal e-cadherin, and α and β catenin in NEC, which can be normalized with probiotic treatment[86] and changes in hepatic adherens junction proteins observed in NEC are normalized with EGF.[83]

In addition to changes in the AJC, the goblet cell products mucin and trefoil factors have been investigated in NEC. In both humans[87,88] and rats[76] with NEC, the number of mucin 2 and trefoil factor 3 goblet cells is significantly reduced and mice with genetically aberrant mucin 2 develop more severe disease than those with normal mucin.[89] In addition, ileal BAs, increased in NEC and associated with disease severity, decrease ileal mucin levels in neonatal but not adult ileum,[89] data which indicate a possible mechanism for development of NEC in premature infants. Zhang et. al, showed administration of recombinant trefoil factor 3 decreased the incidence and severity of experimental NEC.[90] TNFα injected into immature mice resulted in decreased mucin-producing goblet cells. Interestingly, much like BA's effects on mucin, adult animals showed no such effect.[87]

As early as 1998, decreased Paneth cell products were reported in human NEC. Coutinho, et. al, found decreased numbers of

Table 4. Interventions that may promote barrier function in the preterm infant

Intestinal Barrier Component	Interventions that Negatively Influence Barrier Function	Interventions to Improve Barrier Function
Epithelial Apical Junctional Complex (AJC)	Bacterial **LPS** can increase intestinal permeability **TPN** can increase proinflammatory cytokines known to negatively affect TJ protein expression and epithelial permeability **TPN** can also directly reduce TJ protein expression	**Breastmilk** reduces intestinal permeability. Components (*lactoferrin, whey, TGFβ, EGF)* have positive effects on TJ protein expression. **Probiotics** promote TJ and AJ protein expression and reduce intestinal permeability **Anti-TNFα** therapy may reverse effects of cytokine-induced increase in epithelial permeability
Paneth Cells	More research needed	More research needed
Mucin (Goblet Cells)	Cytokines negatively affect goblet cell number and mucin production	**Probiotics** can reverse these effects
Intestinal epithelial lymphyocyte (IEL)	**TPN** and lack of enteral feeds negatively influences IEL number and function	**Trophic feeds** and **glutamine** may promote IEL recruitment and function

lysozyme positive Paneth cells in babies with NEC.[91] More recently, McElroy, et. al, published Paneth cell numbers were decreased in human NEC[87] and Puiman, et. al, found Paneth cell hyperplasia with elevated defensin levels after recovery from NEC, but not at diagnosis.[92] McElroy, et. al, have also developed a novel model of NEC-like injury that specifically tests the role that Paneth cells may play in NEC pathogenesis and have discovered that TNFα may play a key role in Paneth cell defects seen in NEC.[93-95] While Salzman, et. al, showed increased Paneth cell numbers, defensin production from these cells was deficient.[58] TCRγδ IEL have also been shown to be reduced in human NEC tissue (compared to age-matched controls) and murine models of NEC-like injury confirm that deficiency of γδ IEL increases disease severity. This study also showed that reduced intestinal occludin could cause decreased intestinal barrier function through indirect effects on reducing γδ IEL recruitment to the epithelial barrier.[59]

How to Promote Barrier Function to Prevent or Treat NEC (Table 4)

In addition to examination of expression and localization of TJs in NEC, means to counteract or prevent these changes have been investigated. Human breast milk, which has long been known to decrease the risk of NEC,[13,96] improves intestinal barrier function (when compared to formula feeding) in both humans and animal models.[24] Multiple components in human milk may be responsible for these protective effects.[97] Lactoferrin has been shown to reduce increased epithelial permeability caused by lipopolysaccharide *in vitro*,[98] and whey protein and transforming growth factor-β (TGF-β) decrease intestinal permeability by upregulating claudin-4 expression.[99] In addition, casein improves intestinal barrier function by upregulating claudin-1 expression while decreasing claudin-2 expression.[100] Human milk also contains other growth factors such as EGF, which will be discussed in greater detail below. It is also important to note that lack of enteral feeds can adversely influence intestinal barrier function, in part through negative effects on IEL.[101,102] Interestingly, glutamine supplementation in parenteral nutrition may reverse these effects.[103]

Premature infants are often given total parenteral nutrition (TPN) at birth. While TPN supplies basic nutritional needs for an infant that cannot tolerate enteral feeding, it also can have adverse effects on intestinal barrier function, in part through changes in IEL. In addition, increased expression of proinflammatory cytokines known to alter the epithelial barrier are increased with TPN administration. TPN has also been shown to be associated with decreases in expression of a number of TJ components, including occludin and claudins.[101,102]

A recent meta-analysis indicate that probiotics are a promising preventive therapy against NEC.[31] The potential mechanisms by which probiotics may prevent NEC have been extensively studied in experimental animal models.[86,104-116] Specifically, their effect on TJs has been studied by a number of laboratories. In 2009, Khailova, at al., published that oral administration of *Bifidobacterium bifidum* (Bb) decreased elevated protein levels of ileal occludin and claudin-3 observed in neonatal rats with NEC. In addition, based on localization of these proteins, Bb treatment seemed to enhance formation of more functional TJs compared to untreated pups with NEC.[69] This group also demonstrated that oral therapy with Bb normalizes adherens junctions abnormalities and the number of mucin 2 and trefoil factor 3 positive cells in the distal ileum of rats with NEC.[69] A separate group showed *Bifidobacterium infantis* (Bi) preserved the intestinal barrier during experimental NEC in mice by allowing occludin and claudin-4 to localize appropriately at the TJ.[80] In a particularly elegant study, Shiou, et al., [106], found neonatal rats with NEC given conditioned media from combinations of *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium infantis* cultures were protected from intestinal barrier dysfunction and maintained ZO-1 at the TJ. A small clinical trial also confirmed that probiotics can also improve intestinal barrier function by reducing intestinal permeability in premature infants[64] and this may be a mechanism by which probiotics can reduce NEC in premature infants.[65,66]

Eukaryotic organisms rely on an effective intestinal barrier to protect against pathogenic prokaryotes while appropriately housing beneficial commensal symbiotes. In the developing immature host, an ineffective barrier can predispose to aberrant inflammatory and/or apoptotic responses to bacteria. When considering administration of probiotic therapy to premature infants who are developmentally immunodeficient, immature intestinal barrier function is particularly relevant because it potentially allows these beneficial bacteria access to the submucosa where it may exert pathologic effects. An immature intestinal epithelial barrier may contribute to the development of probiotic-associated sepsis, which has been reported in premature neonates and remains a significant concern mitigating its widespread clinical use.[117] However, probiotic and commensal bacteria can also contribute to promoting maturation of this epithelial barrier.[17] Thus timing and dosing of these probiotics may be critical to obtaining beneficial effects without unwanted side effects.

Other factors previously shown to decrease incidence and severity of experimental NEC have been investigated for their ability to normalize components of TJs altered in this disease. These include EGF,[76] and erythropoietin (EPO).[79] Much like the results observed in the use of probiotics in NEC, treatment with EGF and EPO normalized TJ components. Specifically, EGF treatment normalized expression of occludin and claudin-3, and EPO treatment normalized expression of ZO-1, when compared to untreated, NEC controls. As mentioned previously, EGF has also been shown to normalize changes in adherens junctions caused by NEC.[83]

Summary

Intestinal epithelial barrier dysfunction plays an important role not only in predisposing premature infants to NEC, but also in propagating further intestinal injury and inflammation that can increase the severity of NEC. Many studies have already confirmed the importance of optimizing expression

and localization of TJ proteins in the immature intestine in order to reduce the incidence and severity of experimental NEC. Promising therapies include promoting human milk feeds, encouraging growth of commensal bacteria, probiotics, and growth factor supplementation (EGF, TGF-β). Small clinical studies indicate that improvement in premature intestinal barrier function can be achieved with these therapies. Animal studies also indicate that anti-TNFα therapy may also be promising, but clinical trials are needed. Future research to identify changes in key components of the immature intestinal barrier that predispose to or worsen NEC may lead to the development of targeted preventive and treatment strategies as well as to the development of biomarkers to guide optimal timing of clinical interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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