Intestinal Epithelial Barrier Function and Necrotizing Enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is a major cause of morbidity and mortality in premature infants. NEC is characterized by intestinal tissue inflammation and necrosis. The intestinal barrier is altered in NEC, which potentially contributes to its pathogenesis by promoting intestinal bacterial translocation and stimulating the inflammatory response. In premature infants, many components of the intestinal barrier are immature. This article reviews the different components of the intestinal barrier and how their immaturity contributes to intestinal barrier dysfunction and NEC.

Keywords
Intestinal barrier; Necrotizing enterocolitis; Preterm neonate

INTRODUCTION

Necrotizing enterocolitis (NEC) is a disease affecting the gastrointestinal (GI) tract of premature infants thought to result from an immature immune system, impaired microvasculature development, and an impaired mucosal barrier. In this review, the different components of the intestinal barrier and their functions are discussed (Fig. 1), with emphasis on how these are affected by the NEC disease process and by factors and interventions known to protect against NEC. The GI tract is a highly vascularized organ where the exchange of water and nutrients occurs via a single layer of epithelial cells. Precise regulation of the gut barrier function is essential to maintain the critical balance between...
its absorptive function and its role at preventing potentially harmful digestive enzymes, bile acids, and bacteria present in the lumen to get into the tissues. Breakdown of the intestinal barrier in neonates is thought to be a critical step in the development and the progression of NEC.

In human neonates, intestinal permeability to sugar decreases during the first week of life.\(^1\) This is more pronounced in breastfed newborns, compared to those that are fed formula,\(^1\) suggesting a beneficial role of breast milk on the intestinal barrier. The intestinal barrier has been shown to be impaired during NEC in humans\(^2\) and animals.\(^3,4\) We showed that the intestinal permeability is increased in response to NEC-inducing stresses prior to the development of intestinal injury in a mouse model of NEC,\(^5\) which suggests that alterations of the intestinal barrier may play a role in NEC pathogenesis. In adults, an increase in intestinal permeability has been shown to precede Crohn’s disease\(^6\) and its relapse,\(^7\) and is thought to contribute to the disease. In premature infants, several components of the intestinal barrier are immature and therefore may predispose them to NEC.

**THE MUCUS LAYER**

### The Mucus

The intestinal epithelium is protected against harmful luminal bacteria and toxins by a thick gelatinous mucus layer secreted by specialized epithelial cells called goblet cells. In the colon, while bacteria reside and thrive in the outer mucus layer,\(^8\) the inner mucus layer is physically impenetrable to bacteria.\(^9\) However, in the small intestine, the mucus layer is loose, unattached, and can easily be penetrated by microorganisms.\(^10\) To decrease the risk of infection, the small intestinal mucus layer is regularly flushed by a process consisting of liquid secretion and motor activity. The migrating motor complex generated by the enteric nervous system likely allows the loose mucus to migrate to the colon.\(^10\) The mucus is composed of mucins, lipids, and water. Mucins are large glycoproteins made of a central protein backbone rich in proline, threonine, and serine which are \(O\)-glycosylated with large glycans.\(^11\) These glycans cannot be digested by digestive enzymes thus protecting the central mucins from degradation by endogenous proteases. Mucins are produced, stored, and released by goblet cells. Once secreted, gel-forming mucins lubricate and protect the gastrointestinal tract. While Muc2 is the main secreted mucin in the small intestine,\(^12\) others are MUC5AC, MUC5B, MUC6, and MUC7.\(^10\) As opposed to secreted mucins, membrane-bound mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, and MUC17) have a transmembrane domain that enables them to be anchored into the cell membrane. These play a role in protection, apical cell surface sensing, and signaling.\(^10\)

In the fetus, Muc2 mRNA is expressed at 12 weeks of gestation in the jejunum, ileum, and colon.\(^12\) Muc2 was found to be rapidly synthesized in the small intestine of preterm infants who have undergone an enterostomy for necrotizing enterocolitis.\(^13\) While Muc2 developmental regulation is not fully known, a few studies suggest that its deficiency may play a role in NEC. In the intestinal tissue samples of patients with NEC, the number of mucus-containing small intestinal goblet cells is decreased compared to age-matched control samples.\(^14\) In a neonatal rat NEC model, Muc2 mRNA and protein have been found to be decreased as well as the number of Muc2 positive cells when compared to
In immature but not mature mice, TNF injection resulted in the loss of mucus-containing goblet cells but induced Muc2 and Muc3 mRNA upregulation in the mature ileum. Several recent studies correlate increased Muc2 production with decreased NEC severity. In addition, in premature neonates, the immaturity of the enteric nervous system and of the migrating motor complexes may delay the normal migration of the bacteria-containing mucous layer from the small intestine to the colon. Both of these mechanisms may increase the risk of bacterial product translocation through the intestinal mucosa and contribute to NEC pathogenesis.

**Antibacterial Products, Enzymes, and Soluble Factors**

The mucus layer not only limits the diffusion of toxins but allows the generation of a gradient of antibacterial products secreted by Paneth cells and enterocytes. Paneth cells secrete different antibacterial products such as alpha-defensins, cathelicidins, lysozyme, and secreted phospholipase A2 (sPLA2). The production of Paneth cell antimicrobial peptides is affected by the composition of the microbiota and increases with age. Paneth cells reside at the base of the crypt in close contact with the epithelial stem cell compartment. The potent cocktail of antimicrobial products produced by paneth cells is therefore thought to play a role in the protection of these vital cells from bacterial invasion. Mice lacking Paneth cells have increased bacterial translocation possibly predisposing them to the development of NEC. Conflicting reports exist on the number of Paneth cells in NEC patients. As antibacterial peptide detection is used to identify Paneth cells and immature Paneth cells do not yet produce antibacterial peptides, discrepancies in the number of these cells reported in the literature may ultimately be due to a variable degree of maturation of this cell population or to degranulation of the Paneth cells during the NEC process. NEC typically occurs in the neonatal period when these peptides are not being produced at high levels thus supporting the premise that decreased Paneth cell differentiation and maturation may be a predisposing factor for NEC. Interestingly, Paneth cell metaplasia and increased expression of the paneth cell product alpha-defensin upon recovery from NEC has been reported. A recent study suggests that Paneth cells may play an important role in NEC as immature mice (P14–16) treated with the zinc chelator dithizone to ablate Paneth cells develop NEC-like disease when infected with *Klebsiella pneumoniae*.

**The alpha-defensins** (called cryptdins in mice) are the most abundant antimicrobial peptides made by Paneth cells. These are produced in an inactive form and converted into an active peptide after cleavage by proteases such as matrilysin (also called MMP-7). Active defensins are able to permeabilize gram-positive and gram-negative bacterial cell membranes. Altered alpha-defensin expression has been shown in NEC.

**Cathelicidins and beta-defensin** are antimicrobial peptides produced by different cell types including epithelial cells and several populations of immune cells such as neutrophils, NK cells, B cells, and monocytes. They differ in structure from the alpha-defensins but have similar cationic amphipathic properties and are also effective against gram-positive and gram-negative organisms. In addition, they present chemotactic activity for neutrophils, monocytes, and T cells. In mice, their expression is extremely high during the neonatal period and decreases with maturity concomitant to an increase in Paneth
cell maturity and antimicrobial peptide production. In a rat model of NEC, treatment with human beta-defensin-3 improved NEC and promoted mucosal integrity by reducing inflammatory mediators and reduced autophagy-activated proteins. Furthermore, rats treated with *Bifidobacterium* increased beta-defensin-2 which provided protection from NEC. However, whether deficient production of these antimicrobial peptides contributes to NEC remains unknown.

**Lysozyme** is a highly cationic protein and enzyme which cleaves $\beta$-1–4 glycosidic bonds of gram-positive bacteria. This causes the destabilization of the bacterial peptidoglycans leading to cell lysis. Gram-negative bacteria are resistant to this mechanism due to their outer shell that encases their peptidoglycan layer. Lysozyme has been shown to be absent in the Paneth cells of newborn infants with NEC and may play an important role in preventing bacterial invasion in the neonatal intestine.

**Secreted phospholipase A2 (sPLA2)** is another antimicrobial protein produced by Paneth cells. It acts on gram-positive bacteria through a mechanism that hydrolyzes phospholipids. Group II phospholipase A2 is developmentally regulated after birth and was found to increase from D15 to D21 in the neonatal rat ileum. The expression of sPLA2 has been shown to be increased in a neonatal mouse NEC model. Phospholipase A2 is an enzyme critical for the production of platelet-activating factor (PAF), which has been shown to mediate intestinal injury and experimental NEC.

**Intestinal alkaline phosphatase (IAP)** is a brush border enzyme produced by intestinal epithelial cells. It is expressed in decreasing concentrations from the duodenum to the ileum. IAP has multiple functions including the regulation of lipid metabolism, the regulation of bicarbonate secretion, as well as the detoxification of bacterial lipopolysaccharide (LPS). By dephosphorylating the Lipid-A moiety of LPS, IAP prevents its interaction with toll-like receptor 4 (TLR-4). The level of IAP expression has been found to be decreased in NEC. Furthermore, IAP supplementation in experimental NEC models decreased the severity of the disease, attenuated the systemic inflammatory response, and increased barrier function with upregulation of claudins-1, and −3, as well as occludin and ZO-1 following treatment. In the stool of premature infants with NEC, relative IAP content was increased but had biochemical dysfunction.

**Trefoil Factor 3 (TFF3)**, like Muc2, is an important protein secreted by goblet cells. TFF3 is thought to play a role in the maintenance of the mucus layer and surface integrity by facilitating mucin polymerization. Furthermore, TFFs play a significant role in epithelial cell restitution following injury promoting enterocyte migration, proliferation, and survival. Mice lacking TFF and subjected to experimental DSS colitis die of complications due to impaired restitution. Large amounts of TFF3 are present in breastmilk and TFF3 expression has been found to be decreased in NEC tissues. Treatment of mice with recombinant human TFF3 during experimental NEC reduced inflammation which suggests a protective role of TFF3 in NEC.

**IgA** molecules are transported from the basolateral to apical epithelial surface and secreted. These antibodies act in the mucus layer to inhibit attachment of microorganisms to the
epithelial cells. In term neonates, the synthesis of secretory IgA is very low and takes 2 weeks or more after birth for normal production.\textsuperscript{50} Colostrum is rich in IgA.\textsuperscript{51} Breast milk from mothers of preterm infants was found to have higher IgA compared to those of term infants.\textsuperscript{52} Breastmilk-derived IgA has been shown to shape the host-microbiota relationship of preterm neonates\textsuperscript{53} and pups reared by IgA-deficient mothers are more susceptible to experimental NEC, suggesting that IgA is critical for preventing NEC in a mouse model.\textsuperscript{53}

**Lactoferrin** is an iron-binding protein present in breast milk and in most exocrine fluids such as tears, saliva, bile, and pancreatic secretions.\textsuperscript{54} Lactoferrin provides protection against bacterial translocation via several mechanisms: (1) by binding iron which is necessary for bacterial growth; (2) via the toxic effect of its metabolite, lactoferricin, which disrupts gram-negative bacteria cell membranes; (3) by binding microbe-associated molecular pattern (MAMP) such as endotoxin, CpG, flagellin, and secondary inflammation; (4) by promoting the growth of probiotics;\textsuperscript{55} (5) by promoting intestinal epithelial cell proliferation.\textsuperscript{56} Lactoferrin concentration is very high in human colostrum.\textsuperscript{57} Oral lactoferrin prophylaxis has been found to reduce the incidence of late-onset sepsis in infants weighing less than 1500 g.\textsuperscript{58} Recent work describes its effect on neonatal myeloid cells in their conversion to myeloid-derived-suppressor-cells, thus blocking intestinal inflammation and experimental NEC.\textsuperscript{59} While large-scale randomized clinical trials are needed, current evidence does not support a protective effect against NEC of exogenous lactoferrin when given alone.\textsuperscript{58–60}

**THE EPITHELIAL LAYER**

**Paracellular Permeability**

Paracellular permeability is the passage of molecules across intercellular structures. Indeed, adjacent cells of the intestinal epithelial barrier are secured by several complex structures that are named, from luminal to basolateral side, the tight junction complex, the adherens junction complex, and the desmosome (Fig. 2). Besides a recent study showing that desmoglein-2, a component of desmosome, is increased with increased NEC severity in a pig model,\textsuperscript{61} no current data exist on desmosomes in NEC. Detailed studies looking at tight junction and adherens junction complexes have been performed and are discussed below.

**The Tight Junction Complexes**—Intercellular tight junctions are protein structures formed on the apical surface between adjacent cells of the epithelial barrier which regulates the passage of water, ions, and large solutes across the epithelium via the paracellular pathway (Fig. 3).\textsuperscript{62,63} Tight junctions are made of several structural and functional proteins which regulate their function, such as occludin and claudins.\textsuperscript{64} Their extracellular domains interact with the proteins on the adjacent cell membrane while their cytoplasmic tails associate with scaffold proteins called zonula occludens (ZO-1, ZO-2, and ZO-3).\textsuperscript{65} These protein complexes associate with a variety of kinases and cytoskeletal proteins such as actin and myosin to regulate barrier function. The apical surface of the epithelial cell is circled by a belt of actin and myosin. Upon phosphorylation of the light chain of myosin (MLC) by activated myosin light chain kinase (MLCK), the actin-myosin ring contracts leading to tight junction reorganization and increased paracellular permeability.\textsuperscript{66} Several
inflammatory mediators such as TNF and IL-6 have been found to increase paracellular permeability.\textsuperscript{67,68} TNF for example has been shown to activate MLCK.\textsuperscript{69} During human development, TJ complexes have been detected in the fetal intestine as early as 10 weeks of gestation.\textsuperscript{70} We have shown that pups exposed to an experimental NEC protocol have TJ restructuring with increased apical-to-basal tight junction length prior to the development of intestinal injury.\textsuperscript{5}

The claudin family of proteins has been shown to control charge selectivity, and ion and small molecule permeability\textsuperscript{64,71} and mutations in claudins have been shown to disrupt paracellular transport.\textsuperscript{72} Several members of the claudin family are expressed in the intestine. While the role of claudins 7, 12, and 15 is unclear, claudins 1, 3, 4, 5, and 8 have been shown to tighten the barrier or decrease permeability. In contrast, claudin-2 is pore-forming and its expression leads to increased permeability. In mice, the gene expression of the intestinal claudins has been shown to be developmentally regulated.\textsuperscript{73} In neonatal rat pups, hypoxia/reoxygenation induced the downregulation of claudin 1, 14, and 15 and the upregulation of claudin 8 and of the gap junction protein, beta 3.\textsuperscript{74} In a neonatal rat model of NEC, the expression of claudin 3 and occludin has been found to be increased at 96 hours in the intestine of stressed pups compared to controls.\textsuperscript{3} This may be a compensatory mechanism of the intestine to re-establish its barrier function. Indeed, in neonatal mice, intestinal claudin 3 expression has been found to increase in the first 2–3 weeks after birth and this increase to be associated with a decreased in intestinal barrier permeability.\textsuperscript{75} Claudin 3 increase was dependent upon bacterial colonization.\textsuperscript{75} In humans, when measured in the urine, claudin 3 has been shown to be a useful diagnostic marker for the detection of NEC in infants.\textsuperscript{76} In a neonatal mouse NEC model, we found that claudin 2 expression is increased at 6 hours while claudin 4 and 7 are decreased at 24 hours.\textsuperscript{5} Claudin 2 protein is increased in the intestine of neonatal mice submitted to a NEC protocol for 48 hours and in human NEC tissues compared to controls.\textsuperscript{5} Interestingly, claudin 2 is also increased in the intestinal tissues of patients with inflammatory bowel disease\textsuperscript{77,78} and to be positively correlated with inflammatory activity.\textsuperscript{78} IL-6 has been shown to increase the expression of claudin 2, thus increasing TJ permeability.\textsuperscript{79} Whether the increase in claudin 2 is injurious or serves as a compensatory protective mechanism remains to be determined. Indeed, claudin 2 increases paracellular channel-like permeability to monovalent cations such as sodium.\textsuperscript{80} The resulting absorption of nutrients such as glucose and amino acids may prevent malnutrition during a time of stress, and therefore may be protective. Another potential protective mechanism may be, by increasing intestinal water loss, help flushing pathogens from the intestinal lumen. The distribution of TJ proteins is altered during several disease processes.\textsuperscript{77,81} In patients with ulcerative colitis, claudin 4 association with TJs has been noted to be lost in the colonic epithelium.\textsuperscript{77} In a neonatal rat model of NEC, the association of ZO-1 with TJ has been found to be lost at day 5.\textsuperscript{82} In neonatal mice, we found that claudins 2, 4 and occludin are densely localized to TJ structures and claudin 7 is mainly associated with enterocyte lateral membranes.\textsuperscript{5} When neonatal mice were exposed to a NEC protocol for 12 hours, which is a time when injury has typically not yet occurred, the association of claudin 4 with TJ structures was markedly decreased, and occludin and claudins 4 were mainly found in the cytoplasm.\textsuperscript{5} Furthermore, administration
of *Bifidobacterium* (*B.*) *infantis* preserved claudin 4 and occludin distribution at TJ, significantly attenuated stress-induced intestinal permeability and NEC in a mouse model.\(^5\)

**The Adherens Junction Complexes**—The adherens junction complexes are made of transmembrane epithelial cadherin (E-cadherin) proteins and cytosolic proteins named alpha and beta-catenin. E-cadherin forms homophilic interactions with the neighboring cell thus conferring bonds that are necessary for epithelial strength and support. Additionally, adherens junctions are critical in directing cell polarity along the apical-basolateral axis.\(^83\) While their role in NEC is not known, these proteins have been shown to be differently localized in the intestine of pups submitted to a NEC protocol compared to dam fed controls,\(^15\) and this change was prevented by the inoculation of the probiotic *Bifidobacterium bifidum*.\(^15\) E-cadherin expression has been shown to be decreased in human NEC and experimental NEC, where it was shown to be internalized.\(^84\) Also, the cytoskeletal protein vinculin, known to be associated with adherens junctions, has been found to be decreased in formula-fed neonatal mice compared to dam-fed controls.\(^85\)

**Transcellular Permeability**

The intestinal epithelium has multiple transport mechanisms for molecules to gain entry into the cellular compartment (Fig. 3). Depending on the specific molecules involved, it can occur by simple diffusion through the cell membrane, by diffusion through channels and pores, by facilitated diffusion utilizing transport proteins, or by osmosis. Passive transport is driven by concentration gradients. In contrast, active transport is able to move molecules against a concentration gradient and requires the use of energy. Large particles or macromolecules can gain entry into a cell by an active transport process consisting of endocytosis which may or may not be receptor mediated. Transcytosis is the transcellular transport process that involves endocytosis followed by vesicular transport across the cell to the opposite membrane where exocytosis occurs. Transcytosis thus provides a route of entry from the intestinal lumen to the underlying lamina propria. This process is particularly important in the transport of maternal IgG to the infant, conferring immunologic protection prior to the maturity of their own adaptive immune system.\(^86\) In addition, many pathogens are known to exploit this mechanism leading to dysfunction of the epithelial barrier,\(^87\) and *E. coli* transcytosis has been hypothesized to be an initiating event in necrotizing enterocolitis.\(^88\) Alpha-haemolysin from *E. coli* has been shown to induce focal leaks in colonic epithelial cells which causes increase bacterial translocation.\(^89\) Several strains of commensal bacteria and probiotics have been shown to increase TJ proteins at the cell boundaries and in some cases prevents or reverses the adverse effects of pathogens.\(^90\) In addition, many nutrients have been shown to impact barrier permeability.\(^90\) Luminal antigens that reach the lamina propria have the potential to initiate an immune response. This response includes the production of inflammatory cytokines that may further facilitate transcellular permeability. Specifically, TNF can increase endosomal uptake and enhance transcellular transport.\(^91\) Also, interferon-\(\gamma\) (IFN\(\gamma\)) which has been shown to play an important role in NEC\(^92\) enhances transcytosis of macromolecules.\(^93\) Increased transcytosis of luminal molecules occurs in conjunction with tight junction reorganization and increased paracellular permeability\(^91\) and barrier function may be affected through alterations of both transcellular and paracellular transport.\(^94\)
Although epithelial cells are able to capture antigens and microbes, transcellular transport is mainly thought to occur at the level of M-cells (M for microfold) which cover isolated lymphoid follicles or Peyer’s patches. M-cells are specialized epithelial cells of the follicle-associated epithelium. They take up antigens and microorganisms from the intestinal lumen via transcytosis and present them to dendritic cells and other immune cells such as macrophages and lymphocytes. As their glycocalyx (protective outer cell layer composed of glycoproteins and glycolipids) is thinner than enterocytes, M-cells constitute a functional opening in the intestinal mucosal barrier. During inflammation, there is increased apoptosis of M cells that may contribute to the breakdown of the intestinal barrier. However, not much is known about the developmental maturation of M cells and their potential role in NEC.

**Epithelial Cell Layer Integrity**

The single layer of epithelial cells is a highly regulated barrier. The cells are replaced approximately every 4–5 days through a process of proliferation, differentiation, migration, and apoptosis. This process is initiated by rapidly cycling epithelial stem cells situated at the base of the small intestinal crypt. Signaling events instruct these newly created immature transit amplifying cells to differentiate into one of four main cell types of the small intestine. Enterocytes are the most numerous and perform the absorptive functions of the barrier. Paneth, goblet, and enteroendocrine cells are of secretory lineage. Paneth cells migrate downward surrounding the stem cells. They are relatively long lived, with a turn-over time of 57 days in mice. The other cell types migrate upward toward the villus tip where they eventually undergo apoptosis and are replaced. During villous tip shedding, MLC gets phosphorylated and tight junction reorganization occurs. This process is vital to the normal turnover of enterocytes and does not compromise the barrier. However, when this process is impaired, tight junction function may be affected, impacting barrier permeability. To maintain the integrity of the intestinal epithelial layer, the proliferation and differentiation of intestinal stem cells, and the migration and apoptosis of intestinal epithelial cells are tightly regulated and synchronized. Upregulated apoptotic rate increases permeability and bacterial translocation. Unbalanced epithelial proliferation and apoptosis may be a contributing factor in the loss of the intestinal barrier function and in NEC development. Indeed, NEC has been associated with a decrease in intestinal epithelial cell proliferation and migration and with an increase in intestinal epithelial cell apoptosis. LPS, the ligand of TLR-4, has been shown to play an important role in NEC and to inhibit enterocyte migration via increased expression and function of the adhesion molecule alpha 3- and beta-1 integrin and via autophagy. Also, TLR4 expressed on intestinal stem cells regulates enterocyte proliferation and apoptosis and may contribute to the pathogenesis of NEC. Indeed, TLR4 activation has been shown to inhibit beta-catenin signaling via GSK3β activation thus reducing enterocyte proliferation. An inhibitory interaction between TLR4 and NOD2 signaling in enterocytes leads to the regulation of enterocyte apoptosis and NOD2 may have a protective effect on NEC.
Non epithelial influences

Immune cells and inflammatory mediators

Other cell types may affect intestinal barrier permeability indirectly. Indeed, during inflammation, activated macrophages inhibited enterocyte migration and mucosal healing via the release of nitric oxide and the activation of RhoA. IFNγ inhibits enterocyte migration by impairing enterocyte gap junctions, which are intercellular channels composed of connexin43 (Cx43) monomers. Mesenchymal stem cells have been shown to enhance the viability and proliferation of human fetal intestinal epithelial cells following hypoxic injury via paracrine mechanisms.

In the intestine, dendritic cells and CX3CR1+ macrophages maintain direct contact with epithelial cells through dendrites that extend from the mucosa to the lumen to sample antigens in the external environment. These cells express tight junction proteins allowing them to span the intercellular space while maintaining an intact barrier. The close proximity of epithelial and immune cells facilitates the cytokine and chemokine signaling necessary to initiate an immune response upon a barrier breach.

While the exact mechanism is unknown, the interaction of inflammatory cells with tight junctions may contribute to the TJ restructuring and barrier dysfunction seen in diseases such as NEC. Activated dendritic cells and macrophages secrete a variety of cytokines, which are increased in human and experimental NEC. Many of these factors are essential for an appropriate immune response but may also have a negative impact on barrier function. Specifically, TNF at high doses has been shown to induce apoptosis and shedding due to a major redistribution of the tight and adherens junction structures. Low-dose TNF in contrast does not induce cell shedding yet impacts barrier permeability through MLCK activation and endocytosis of occludin. IL-1β is also known to increase tight junction permeability in an MLCK and NF-κB-dependent pathway without causing apoptosis and IL-6 has been shown to increase intestinal permeability via the upregulation of claudin 2 mRNA and protein expression both in vitro and in vivo.

During inflammation, effector cells are recruited to the site of injury via the secretion of chemokines such as CCL20 and CCL2 by epithelial and innate immune cells. Recruitment of dendritic cells through the CCL20/CCR6 axis was shown to be responsible for intestinal epithelial damage in a model of NEC. Recruited T cells secrete Th1 cytokines such as IFNγ and the Th2 cytokine IL-13. Epithelial cells respond to IL-13 by upregulating claudin 2 and thereby increasing permeability. IL-13 also induces epithelial cell apoptosis and decreases proliferation, causing epithelial microerosions and bacterial translocation. IFNγ has been shown to increase the intestinal epithelial permeability to macromolecules via the Src kinase pathway. In addition, IFNγ was found to synergize with TNF to induce barrier dysfunction.

Many pro-inflammatory cytokines are under the control of the transcription factor NF-κB, which has been shown to be a major effector molecule in NEC. NF-κB is essential for signaling in both epithelial and immune cells. TNF, IL-1β, and TLR ligands activate the NF-κB signaling pathway and can trigger amplification of the immune response. Also, NF-κB is
known to protect the cell against apoptosis and inhibition of NF-κB specifically in epithelial cells leads to a loss of barrier function by inducing apoptosis and bacterial translocation. While increased intestinal permeability may be necessary to induce intestinal injury in experimental NEC, it is not sufficient as we found that blocking NF-κB activation in monocytes prevented against NEC without impacting intestinal permeability.

Cytokines produced by immune cells can alter barrier function not only by causing tight junction structure alteration, but also by altering transcytotic mechanisms and by causing apoptotic leaks and mucosal gross lesions.

**Commensal Bacteria and Probiotics**

Commensal bacteria and probiotics upregulate TJ proteins and may prevent the adverse effects of pathogens on intestinal barrier. The beneficial effect of probiotics such as *Lactobacillus acidophilus* and *B. infantis* in human NEC may be mediated by its effect on intestinal permeability. In human preterm neonates, supplementation of formula with *Bifidobacterium lactis* decreased intestinal permeability at day 30 of life. In a neonatal mouse model of NEC, *B. infantis* has been found to attenuate the increase in intestinal permeability observed at 24 hours and to decrease the incidence of NEC. In this same model, *B. infantis* preserved claudin 2, 4 and occludin integrity at TJ structures and claudin 7 at lateral membranes. In vitro, when T84 cells (human colon carcinoma cell line) were treated with *B. infantis* conditioned medium, the expression of claudin 4, ZO-1 and occludin was increased, claudin 2 was decreased and the IFNγ-induced rearrangement of occludin and claudin 1 was prevented. Also, *B. bifidum* reduces intestinal epithelial cell apoptosis in a neonatal rat NEC model. Conditioned medium from *B. infantis* has been found to decrease apoptosis and maintain epithelial cell proliferation in a model of neonatal intestinal inflammation induced by *Cronobacter sakazakii*.

**Amniotic Fluid**

During development, the intestinal epithelium is exposed to a diversity of bioactive molecules present in the amniotic fluid such as growth factors, which promote mucosal morphogenesis, and cytokines, which have immunomodulatory and anti-inflammatory properties. These molecules have protective effects on barrier function, and therefore, postnatal enteral administration of amniotic fluid has been hypothesized to protect against NEC. Amniotic fluid has been shown to increase cell migration, proliferation, and cell survival in vitro and these effects were dependent on PI3Kinase and were reproduced by HGF treatment. Stem cells isolated from the amniotic fluid have been shown to improve enterocyte cell survival and enhance repair of damaged intestine in NEC via a COX-2 dependent mechanism. Furthermore, isolated amniotic stem cells restored tight junction protein expression in mice.

**Breast Milk**

Preterm infants who received the majority of feeding as human milk had significantly lower intestinal permeability when compared to infants receiving minimal or no human milk. Breast milk contains many molecules such as trefoil factor and lysozyme that may improve intestinal permeability and protect the neonatal intestine against injury. Several
components of breast milk such as hyaluronan 35kD,\textsuperscript{141} lactadherin,\textsuperscript{61} TIMP-1,\textsuperscript{142} and exosomes\textsuperscript{143} have been shown to protect TJ proteins. Also, breast milk oligosaccarides protect against NEC by inhibition of TLR4 signaling\textsuperscript{144} and were also found to interact with bacterial receptors, inhibiting the binding of pathogenic bacteria with intestinal epithelial cells and preventing bacterial invasion.\textsuperscript{145} Breast milk oligosaccharides can also serve as a food source for commensal bacteria promoting their growth and therefore limiting the growth of pathogenic species or preventing intestinal inflammation.\textsuperscript{146,147} Heat Shock Protein 70 is induced in enterocytes by exposure to breast milk and has has been shown to preserve barrier function.\textsuperscript{148} In addition, breast milk contains many immune cells (such as monocytes and lymphocytes) which downregulate the inflammatory response of the immature intestine.\textsuperscript{133}

**Growth Factors**

Both amniotic fluid and breast milk contain growth hormones which promote the integrity of the intestinal barrier:

**Epithelial Growth Factor (EGF)—**EGF, which present in breast milk,\textsuperscript{149} has been shown to protect against NEC in neonatal rats.\textsuperscript{150} It might be mediated via a protective mechanism on the intestinal barrier. Indeed, in a neonatal rat model of NEC, EGF has been found to abrogate the increase in intestinal occludin and claudin 3 found in the intestine of pups exposed to the NEC model.\textsuperscript{3} In caco-2 monolayers, EGF has been found to reverse the increase in epithelial permeability and occludin dephosphorylation and rearrangement induced by bile acids.\textsuperscript{151}

**Heparin-binding EGF (Hb-EGF)—**Hb-EGF is a member of the EGF family produced by macrophages which is present in breast milk.\textsuperscript{152} It has been found to increase enterocyte proliferation and migration in a rat model of NEC\textsuperscript{153} and has been found to protect against NEC.\textsuperscript{154}

**Transforming-growth Factor-beta (TGF-beta)—**TGF-\(\beta\) is an extracellular peptide which has anti-inflammatory properties and promotes cell differentiation, migration, and cell death in the intestine. TGF-\(\beta\) is present in breast milk\textsuperscript{155} and is secreted by many cell types including immune cells. Monocytes from infants with NEC have reduced TGF-\(\beta\) expression.\textsuperscript{156} In rodents, TGF-\(\beta\) administration has been shown to decrease the severity of experimental NEC.\textsuperscript{157} TGF-\(\beta\) has been shown to be associated with the restoration of intestinal morphology and barrier function in pigs following weaning stress.\textsuperscript{158}

**Erythropoietin—**Erythropoietin is a glycoprotein present in breast milk that controls erythropoiesis.\textsuperscript{159} It has also been shown to protect epithelial cells against autophagy and apoptosis thus preserving intestinal barrier function to protect against NEC.\textsuperscript{82,160}

**Conclusion**

In humans, many mechanisms contribute to tighten the intestinal barrier. In premature infants, several of these mechanisms are immature, which affect the intestinal epithelial barrier function. These include decreased mucus production, decreased amounts of
antibacterial peptides, and decreased intestinal motility due to an immature enteric nervous system. This may lead to bacterial translocation, with subsequent activation of NF-κB in lamina propria immune cells, causing them to secrete pro-inflammatory mediators such as chemokines (CXCL2), cytokines (TNF, IL1β), prostanoids, platelet-activating factor, and nitric oxide (Fig. 4). These inflammatory agents further recruit inflammatory cells inducing reactive oxygen species production, and causing further damage to the intestinal barrier resulting in the translocation of bacteria and their products, intestinal epithelial injury, impairment of epithelial cell restitution, apoptosis and mucosal necrosis. In severe NEC, a vicious cycle is thus created where gut barrier failure causes bacterial invasion, immune activation and uncontrolled inflammation, production of reactive oxygen and nitrogen species, vasoconstriction, secondary ischemia-reperfusion injury, intestinal necrosis, sepsis and shock. While the pathogenesis of NEC is multifactorial with involvement of the immune system and the microvasculature, measures aimed at improving barrier function in premature infants may prevent NEC and/or slow down the progression of the disease.

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Intestinal permeability is increased prior to the development of experimental NEC and breakdown of the intestinal barrier is thought to be a critical step in the development and the progression of necrotizing enterocolitis (NEC).

Immature mucins and decreased antibacterial products such as alpha-defensins, cathelicidins, lysosyme, and secreted PLA2 due to decreased Paneth cell differentiation and maturation may be a predisposing factor for NEC.

Peri-junctional cytoskeletal condensation occurs at the TJ complex in NEC prior to the development of intestinal injury and tight junction dysfunction may contribute to the increase in permeability preceding NEC.

Increased apoptosis and decreased epithelial cell restitution may play a role in the altered barrier function seen during NEC.

Breast milk and amniotic fluid improve intestinal barrier and thus protect the intestinal mucosa against NEC.
Fig. 1:
Components of the intestinal barrier
Fig. 2:
Intercellular structures playing a role in paracellular permeability
Fig. 3:
Transcellular mechanisms responsible for transcellular permeability
Fig. 4:
Illustration of our current understanding of NEC pathogenicity