Epithelial barrier and dendritic cell function in the intestinal mucosa

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The enteric nervous system as regulator of intestinal epithelial barrier function in health and disease

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Abstract

The intestinal epithelia proliferate and differentiate along the crypt villus axis to constitute a barrier cell layer separating some $10^{13}$ potentially harmful bacteria from a sterile mucosal compartment. Strict regulatory mechanisms are required to maintain a balance between appropriate uptake of luminal food components and proteins, while constraining exposure of the mucosal compartment to luminal antigens and microbes. The enteric nervous system is increasingly recognised as such as regulatory housekeeper of epithelial barrier integrity, in addition to its ascribed immunomodulatory potential. Inflammation affects both epithelial integrity and barrier function and in turn, loss of barrier function perpetuates inflammatory conditions. The observation that inflammatory conditions affect enteric neurons may add to the dysregulated barrier function in chronic disease.

Here, we review recent advances in the understanding of the regulatory role of the nervous system in the maintenance of barrier function in healthy state, or during pathological conditions of for instance stress induced colitis, surgical trauma, or inflammation. We will discuss clinical potential of the advances in understanding of the role of the enteric nervous systems in this important phenomenon.
Introduction and Scope

Homeostasis of the intestinal barrier is essential to give access of nutrients and electrolytes into the interior milieu; while at the same time should control passage of pathogens. Regulation of the intestinal barrier function involves the interplay of several mechanisms, such as integrity of the epithelial layer, and a specialised immune system for surveillance, regulated phagocytosis, killing and processing of (commensal) bacteria. Disturbances in the homeostatic conditions of the intestinal barrier are an important factor in the initiation and perpetuation of pathological conditions including inflammatory bowel diseases (IBD); stress-induced disorders such as irritable bowel syndrome (IBS) and food allergies; pathogen-induced intestinal inflammation and septic morbidity after major trauma. Regulation of the intestinal barrier is complex and involves the commensal microflora, a network of immune cells and importantly, an integrated network of neuronal cells of the enteric nervous system (ENS).

It is now well established that enteric neurons participate in nearly all aspects of gastrointestinal tract (GI) function, including epithelial transport, motility, mucosal immune defense, release of gut peptides from enteroendocrine cells, and mucosal blood flow. Under normal conditions, enterocyte tight junction (TJ) proteins allow the gut epithelium to function as a barrier thus regulating access for luminal bacteria to mucosal phagocytes. Chronic stress or surgical trauma can alter expression of TJ and adherence junction (or zonula adherens) proteins and antimicrobial peptides allowing gut pathogens to adhere and cross the gut epithelium. Such proteins are composed of transmembrane proteins occludin, claudins and other junctional adherent molecules that are connected to the cytoskeleton via a complex of multiple proteins. Changes in epithelial barrier function and TJ expression also form the basis of defects in wound healing and/or increased electrolyte secretion that are often seen in IBD, and that perpetuate disease progression. Therefore, approaches aimed at protection or re-establishing epithelial barrier functions could be of interest. In this context, the role of the ENS in the
regulation of barrier function and the gut immune response is emerging. Recent advances have highlighted the ENS as playing a key role in the control of barrier functions and have indicated that alterations of the ENS could be directly associated with the development of IBD and its associated symptoms. One example thereof is that cholinergic activity has been shown to be involved in regulation of the mucosal barrier function, enterocyte endocytosis, and intestinal epithelial permeability. In addition, cholinergic signalling is implicated in intestinal permeability changes observed after chronic stress. The ENS may also exert regulation of intestinal barrier function via a network of enteric glia that interact with epithelial TJs, possibly via release of transforming growth factor (TGF)-β or other mediators.

Here, we review regulation of intestinal barrier function by the ENS, and focus mainly on the first component of intestinal barrier function: maintenance of intestinal epithelial integrity.
The epithelial barrier function in health and disease

Epithelial cell function and tight junction protein assembly

Barrier interfaces between the external environment and sterile stromal compartments are found throughout the entire body such as in skin, kidneys, and intestine. However, it has become increasingly clear that despite the presence of TJs in all epithelia, the electrical resistance of the paracellular pathway varies more than a 1000-fold between different epithelia. Hence, TJs in various tissues can have very different barrier properties. The critical nature of a cellular barrier, and consequences of barrier loss for immune homeostasis, can be demonstrated in burn patients, in whom prognosis is directly related to the surface area damaged. Burns represent a gross form of barrier loss, where there is direct damage to the epithelial cells and exposure of underlying tissues. This damage results in a markedly increased risk of infection.

The intestinal epithelial barrier (IEB) stays in close contact with the environment and is composed of a monolayer of specialised intestinal epithelial cells (IECs). The IEB has developed specific mechanisms to prevent access of luminal contents into the lamina propria, which includes the restriction of paracellular transport and the maintenance of the architecture of the epithelial barrier. This function is brought about by the apical junctional complex, which is composed of the TJ, or zonula occludens (ZO) and the subjacent adherens junction, or zonula adherens. The desmosomes, or macula adherens, are located along the lateral membranes beneath the adherens junction. Intracellular junctions including adherens junctions, desmosomes, gap junctions and TJs tightly regulate paracellular transport in conjunction. Whereas TJs seal the paracellular pathway, the adherens junctions and desmosomes provide the strong bonds necessary to maintain cellular proximity and allow TJ assembly. Adherens junctions are also critical for epithelial polarisation and differentiation, mucosal morphogenesis, and tumour suppression,
processes that occur through a variety of interactions with other proteins, including actin and β-catenin.

TJs are the most apical components of these intercellular junctions. The main functions of TJs are to prevent diffusion of membrane proteins and lipids between basolateral and apical membranes so that cell polarity is preserved (fence function) and importantly, function as a selective barrier to paracellular transport (barrier function). TJs complexes are composed of a network of proteins that are coupled to actin filaments of the cytoskeleton. Occludin (62-82kDa), several members of the claudin family (20-27kDa) and junctional adhesion molecule (JAM)-1 (36-41kDa) are proteins that make up the membrane part of TJs (see figure 1). Occludin has to be phosphorylated to become integrated into the membrane, whereas dephosphorylation directs occludin to intracellular pools. However, evidence from occludin-deficient mice indicates that occludin itself may be dispensable as these mice are still able to form well-developed TJ strands and retain normal IEB integrity. Occludin seems to be more important in cell signalling than in maintaining the IEB. Probably, claudins are the main barrier-forming proteins since different types of claudins are expressed in a restricted number of cell types or during development. It has been assumed that claudin-2 forms mainly aqueous channels, whereas the permeability of macromolecules is unaffected. Overexpression of claudin-1 and -4, however, results in an enhanced barrier function, indicating that these proteins play an important role in the TJ complex formation. Members of the ZO family are proteins that form a bridge between these membrane proteins and actin filaments, which are connected to the peri-junctional ring, a component of the cellular cytoskeleton.

Inflammatory mediators affecting epithelial TJ integrity

Increased concentrations of pro-inflammatory cytokines are present in the intestine during the active phase of IBD but also in conditions such as haemorrhagic shock or sepsis. In vitro studies in intestinal cell lines have demonstrated that pro-
inflammatory cytokines decrease the barrier function of intestinal epithelial monolayers and induce reorganisation of several TJ-associated proteins, including ZO-1, JAM-1, occludin and claudin-1, and -4. Examples of such cytokines that cause TJ rearrangements are TNF-α, IFN-γ, IL-8 and IL-1β. These cytokines influence the IEB primarily by acting on the epithelial expression and activation of myosin light chain kinase (MLCK) through PKC-activation. Upon activation, MLCK phosphorylates myosin light chain (MLC) which in turn causes contraction of the perijunctional ring, a component of the cellular cytoskeleton, so that permeability of TJs increased. IL-1β increases the intestinal permeability by the induction of MLCK gene transcription resulting in an increased MLCK protein activity, probably mediated by a rapid activation of transcription factor NF-κB. IL-1β mediates increased intestinal permeability via increased paracellular transport of luminal antigens. Also TNF-α-mediated increased intestinal permeability leads to an NF-κB dependent down-regulation of ZO-1 proteins and alteration in junctional localisation. In turn, the anti-inflammatory cytokines IL-10, TGF-β and IL-17 protect from loss of TJ proteins. The role of IL-6 in modulation of the epithelial barrier is controversial, and may depend on the specific cell type or model system used. IL-6, as well as IL-13 and TNF affect epithelial permeability and cell turnover through activation of pro-apoptotic pathways and possibly the activation of PI-3kinase dependent signalling pathways. Altogether, cytokine-mediated barrier dysfunction remains poorly defined, and cytokines modulate TJs through distinct mechanisms and intracellular signalling pathways. Data indicate that ZO-1 might be an effector molecule in this process.
Intestinal pathogens

During intestinal infections, enteric pathogens have developed several mechanisms to disrupt the IEB, which clinically often results in diarrhoea. This occurs mainly by modulating the perijunctional actomyosin ring or by interfering with TJ proteins directly. TLR triggering on enterocytes intuitively provides a protective role in epithelial integrity, which is especially evident for TLR2 and 4 activation. This link between TLR2 and TJ proteins was shown to be mediated via Connexin-43, during acute and chronic inflammatory injury of the intestinal epithelia. This protective barrier regulatory function was even put forward to account for the association of ulcerative colitis-associated TLR2-R753Q mutant genes.

Bacterial products degenerate or phosphorylate specific TJ proteins or use them as a receptor so that these proteins become dysfunctional or are reorganised and the efficiency of TJs decreases. Enteropathogenic *Escherichia coli* disrupts the IEB by changing the distribution of ZO-1 and occludin from the membrane into the cytosol in vivo. Moreover, adherent-invasive *E. coli* increases intestinal permeability by an increased macromolecular flux, a decreased TEER and redistribution of ZO-1. Besides *E. coli*, other intestinal pathogens disrupt the integrity of the IEB, such as *Salmonella*, *Campylobacter* and *Shigella*. *Salmonella thyphimurium* influences TJs by dephosphorylation of occludin and degradation of ZO-1. *Campylobacter jejuni* distributes occludin from an intercellular to an intracellular location, decreases the TEER and increases the IL-8 production by epithelial cells by activation of the NF-κB pathway. Probiotics seem to prevent disruption of the epithelial barrier. *Lactobacillus plantarum* has been found to protect the integrity of the epithelial barrier in case of an enteroinvasive infection of *E. coli* in vitro via the prevention of ZO-1, occludin, claudin-1 and JAM-1 rearrangement. Besides, Lactobacilli facilitate the maintenance of the epithelial barrier during *Shigella dysenteriae* infection in rats. The same results are found for *Lactobacillus rhamnosus* which maintain the barrier function in case of an enterohaemorrhagic *E. coli* infection. Also, proteases released from luminal
bacteria mediate intestinal barrier through cleavage of protease-activated receptor (PAR)-2. In IBD, the epithelial barrier is inflamed without obvious exogenous factors like a bacterial or viral infection. Nevertheless, colonic biopsies from Crohn’s disease (CD) patients contain decreased numbers of Lactobacillus and Bifidobacteria, whereas the mucosa and probably even the intraepithelial layer contain an increased population of adherent bacteria. Probably, the immune system itself influences the composition of TJs by the production of pro-inflammatory cytokines. Patients with CD have increased levels of the pore-forming claudin-2, whereas the sealing claudins 5 and 8 are downregulated. In colonic tissues of ulcerative colitis patients, the expression of ZO-1, JAM-1 and claudin-1 is downregulated. Moreover, in colonic epithelium, the expression of ZO-1 is lost in DSS-induced colitis in mice and also mice that are JAM deficient are more susceptible for the development of colitis.

Mast cell mediators effecting barrier function

Initially studied in the context of allergic diseases, we now know that mast cells are highly versatile cells that not only have a sentinel role in host defence but also play a central role in intestinal disorders like IBD and IBS. Mast cells can be activated by a variety of stimuli and the type of stimulus determines their mediator release profile and subsequent consequences for neighbouring cells. In reference to the long list of cytokines that may be involved in decreased barrier function (i.e. IL-10, IL-6, IL-13, TGF-β and TNF-α) it is important to notice that all of these can be expressed by mast cells. Most of them are de novo synthesised upon mast cell activation but an important exception to this is TNF-α. Results obtained by Bischoff et al. not only showed that this pro-inflammatory cytokine is constitutively expressed by these cells but also indicated that approximately 60% of TNF-α positive cells in the gut are in fact mast cells. As mentioned before, TNF-α induced barrier dysfunction depends on MLCK-mediated modulation of TJs. In addition, however, it was also shown that TNF-α potentiates histamine-induced ion secretion in enterocyte cell lines and isolated distal colon. Thus, although histamine is one of the classic mast cell
mediators involved in itch, here it was shown that its synergistic action with TNF-α induces enhanced chloride secretion across the intestinal epithelium. This is highly relevant because it may lead to excessive water secretion and subsequent diarrhoea as observed in e.g. IBD and IBS. Next to histamine, another preformed mediator relevant to barrier dysfunction is tryptase which controls paracellular permeability through PAR-2. Tryptase-mediated cleavage of the N-terminal extracellular domain of this G-protein coupled receptor not only induces the redistribution of TJ proteins via extracellular signal-related kinases (ERK1/2)\textsuperscript{45} but also via Ca\textsuperscript{2+}/calmodulin mediated activation of MLCK\textsuperscript{46}. Being far from complete, this small overview clearly shows that mast cells and their mediators are major players in direct modulation of intestinal barrier function.
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Figure 1. Model scheme of neural networks and epithelial integrity in the intestine. An integrated network of intrinsic and extrinsic neurons mediate epithelial barrier function. A) Extrinsic and intrinsic neuronal pathways may modulate neurotransmitter release in epithelia, possibly via activation of enteric glial cells (green), or inflammatory cells known to be responsive neurotransmitters, such as mast cells. B) Enterocytes may be in close contact to neurones, enteric glial cells, mast cells and phagocytes. These cells interact with each other via the release of neurotransmitters such as acetylcholine, but also (pro-inflammatory) cytokines including IL-1β, also produced by enteric glial cells. C) Epithelial tight junction integrity is known to be modulated via the action of neurotransmitters directly, or inflammatory mediators. Tight junction rearrangement may be affected via these mechanisms. Tight junctions are composed of transmembrane proteins including JAM-1, occludin and claudins, which are connected to the actin skeleton via members of the zonula occludens (ZO) family, MUPP1, MAG1 and cingulin.
The enteric nervous system in intestinal barrier function

The central nervous system (CNS) interacts with the GI tract through the brain-gut axis communicating in a bidirectional fashion largely through the enteric nervous system (ENS) 47. The autonomic ENS comprises parasympathetic and sympathetic systems that and can operate without the participation of the CNS, although the ENS receives input directly from the brain through parasympathetic nerves (i.e. the vagus nerve). The ENS is organised in several plexuses throughout the intestinal wall: the myenteric (or Auerbach’s plexus, between circular and longitudinal muscle layer) and submucosal (or Meissner’s plexus, in the submucosa) plexuses. The mucosal layer also contains nerve networks known as the mucosal plexus, which contains nerve endings that are in close contact with mucosal immune cells and enterocytes (see figure 1). The ENS contains sensory neurons, interneurons, motor neurons, which primarily control peristalsis, local changes in blood flow, and secretion of water and electrolytes 48. An important component of the ENS are the enteric glial cells (EGC), which form a large and widespread network at all levels of the GI tract 49, and serve as intermediaries in the enteric neurotransmission and information processing. They show morphologic and functional similarity to brain astrocytes and control several aspects of gut function, including motility, microvascular circulation, epithelial secretion of fluid, ions and bioactive peptides and intestinal barrier function 50.

More than 30 different neurotransmitters exist in the ENS, with most neurons expressing multiple transmitters 48. Like neurons of the CNS, ENS neurons secrete acetylcholine (Ach) and a large number of other neurotransmitters and neuropeptides including (adenosine triphosphate) ATP, NO, vasoactive intestinal peptide (VIP), tachykinins (TK), Calcitonin gene related peptide (CGRP) and neuropeptide Y (NPY) and Substance P (SP) reviewed elsewhere,47,48.

There are many indications that the GI tract’s specialised immune system intimately interacts with the neuronal mediators released from the ENS. Many cells
of the Gut-associated Lymphoid tissue (GALT) produce for instance Ach and a wide range of neuropeptides as well as the respective neuropeptides receptors. Besides the well-known role of enteric neurons in controlling electrolyte secretion and absorption, they also play a role in the control of intestinal paracellular permeability and cell proliferation. Another cell population that may contribute to this neuro-immune axis in the gut are endocrine cells (EEC), which main function is to detect changes in luminal nutrient content and signal to sensory afferents of the ENS. EEC regulate satiety, motility and pH, or fluids, electrolytes and digestive enzymes via reflex mechanisms. Mediators secreted by EEC include serotonin (5-HT), motilin, glucagon-like-peptide (GLP), neuropeptide Y (NPY) and SP which renders these cells potent activators of afferents of the sympathetic and parasympathetic nervous systems.

**Enteric glial cells as intermediates of neuronal barrier regulations**

Similar to the role of astrocytes in maintaining the blood brain barrier, enteric glia play an important role in regulating intestinal permeability. The importance of enteric glia was noted in studies using targeted ablation of EGCs in transgenic mice. Loss of EGCs was fatal in that animal model due to haemorrhagic necrosis of the small intestine. Furthermore, *in vitro* data of co-culture models of enteric glia with IECs lines have shown that glia decrease IEB permeability in part via the liberation of a recently identified glial factor, GSNO (S-nitrosoglutathione), and the regulation of ZO-1 and occludin expression. In 1998, Bush *et al.* showed that EGCs act as regulators of intestinal immunity. Ablation of ileal and jejunal glial cells in transgenic mice resulted in a fatal intestinal inflammatory phenotype and development of jejuno-ileitis similar to IBD. In subsequent studies it was demonstrated that EGCs are required to maintain mucosal barrier integrity. In cultured epithelial cells, enteric glia conditioned medium promoted intestinal barrier properties and GNSO was identified as the crucial factor in maintaining barrier homeostasis. GNSO-mediated regulation of mucosal barrier function was in part influenced by altering expression of perijunctional F-actin and association of ZO-1
and occludin with the actin-cytoskeleton. In addition, i.p. GNSO injection prevented barrier defects, cytokine induction and almost prevented enteritis caused by ablation of enteric glia. In Ussing chambers, GNSO promoted intestinal barrier function in biopsies from CD patients but not in healthy controls. However, \textit{in vitro}, at low doses GNSO promotes epithelial barrier function, whereas at higher doses GNSO that could be produced in pathological conditions disrupts epithelial barrier integrity.

Although GNSO seems to be the main factor in barrier regulation by EGCs, they also actively respond to inflammation and could be a significant source of cytokines such as IL-1β, TNF and IL-6 and also play a role in peptidergic and nitrergic neurotransmission. Moreover, EGCs were able to strongly inhibit IEC proliferation in part by their release of TGF-β1. The latter may explain the observation that ablation of EGCs \textit{in vivo} leads to an increased uptake of thymidine in IECs and crypt hyperplasia. It is unclear if the role of glia in this network is functionally pro- or anti-inflammatory. Therefore, enteric glia might alter barrier function depending on the conditions of the local microenvironment. It is has become clear however that the impact of EGCs on epithelia may be much more elaborate, also indicated by a recent study in which it was demonstrated that EGCs have a profound impact upon IEC transcriptome and induce a shift in IEC phenotype towards increased cell adhesion and cell differentiation. In addition enteric glia are neuroprotective in \textit{in vitro} or \textit{ex vivo} studies, through the factor GSH. Irrespectively, these data highlight the potential key role of EGC in the control of IEB functions.

\textbf{The vagus nerve and intestinal barrier function}

Limiting intestinal barrier injury may play an important role in decreasing the systemic inflammatory response associated with severe injury. It is generally accepted that vagal activity can have a beneficial effect on the course of intestinal inflammation, as shown for post-operative ileus, colitis, intestinal infection, septic
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shock, and pancreatitis [57]. For instance, in a model of haemorrhagic shock, CCK-dependent stimulation of vagal activity by high fat nutrition has been shown to maintain intestinal barrier integrity. Translocation of bacteria, permeability to HRP, disturbed expression of ZO-1 [58] and enterocyte damage [59] in high-lipid-treated animals was significantly reduced when compared with those in low-lipid-treated and fasted controls. The protective role of vagal nerve stimulation (VNS) has been described in several other studies. Vagal efferent activity has previously been shown to decrease histological gut injury and intestinal inflammation in a model of colitis [60,61]. VNS has also been shown to ameliorate the severity of post-operative ileus in a murine model [62], further supporting the potential of VNS to preserve the epithelial barrier function and ameliorate herewith associated intestinal inflammatory disease. Activation of vagal activity to preserve barrier function has been achieved via pharmacological, nutritional [58,59], and electrical techniques [62,63]. Alternatively, in addition to fat feeding-mediated vagal improvement in barrier function in a shock model, peripheral as well as intracerebroventricular injection with ghrelin ameliorated the disrupted barrier function in rat models of intestinal ischemia-reperfusion [64] and sepsis [64]. In both studies, vagotomy prevented the effect of ghrelin, demonstrating that these effects are depended on an intact vagus nerve.
Figure 2. Vagal nerve stimulation and enhanced bacterial Translocation. A) and B) Translocation of FITC-labelled *E. Faecium* (white dots) to the mucosal compartment of the ileum is increased in vagal nerve stimulation (VNS)-treated mice (B) compared to control mice (sham) (A). *E. faecium* is not taken up via lysosomes, but rather translocates via paracellular routes since LAMP-2 and *E. faecium* do not co-localise. For colour pictures see page 215. C) Tissue mounted after VNS did not show an increased HRP flux in Ussing chamber analyses; however HRP flux was increased by the activation of acetylcholine receptors by 1μM nicotine or 1μM carbachol. D) Translocation of aerobic and anaerobic bacteria to the mesenteric lymph nodes (MLN) is significantly increased (p<0.01 and p<0.001 respectively) in VNS-treated mice compared to the control mice (sham). Hence the vagus nerve activity may also target dendritic cell function. Tytgat Institute, Amsterdam, Unpublished data 2010.
Mechanisms of vagus nerve activity and barrier function in the gut

It is interesting to speculate on the working mechanism behind the vagal potential to affect barrier function. Vagal activity may lead to peripheral release of its principal neurotransmitter Ach, but the vagus nerve particularly projects to other post-ganglionic enteric neurons, in particular VIP 65 (studied in the duodenum) and serotonin 66. In fact, some of the functional effects of vagus nerve stimulation are counteracted by VIP antisera 67. The release of VIP by enteric neurones is shown to inhibit IEC proliferation and to maintain epithelial barrier integrity, and the effect of VIP on epithelial permeability is concomitant with a neural-induced increase in ZO-1 mRNA and protein expression in intestinal epithelial cells 68. Besides VIP, Ach increases paracellular permeability in the healthy gut 63, setting the basis for a fine 'tuning' of the barrier permeability by the ENS, either with or without the intermediate action of enteric glia (see figure 2 and 3). Irrespectively, innate immune cells express a broad range of nAchRs and mAchRs 69, and peptidergic receptors 70, and are therefore target cells for regulation through the ENS. In vitro and in vivo studies have raised interest in targeting neurotransmitter receptors on immune cells to modulate intestinal disease and counteract barrier disruption. However, very little data is available on the interactions that really occur between ENS neurotransmitters and immune cells in the intestinal tract and is subject to further investigation.

Furthermore, a likely cellular target of vagal modulation of the intestine is through the activation of enteric glia. In the respect Gulbransen et al., have recently shown that enteric neurons cause activation of EGCs through release of the neurotransmitter ATP 71. It will be interesting to investigate whether vagal nerve activity can likewise relay functional changes via activation of EGCs. This would demonstrate a link between the vagus nerve and enteric glia, and gives insight into possible mechanisms by which the CNS communicates with the ENS following gut inflammation.
Figure 3. Scheme of neurogenic influences on epithelial barrier function. The vagus nerve influences the permeability of the epithelial barrier via mast cells and other immune cells. Besides, the vagus nerve communicates with enteric glial cells (EGCs) through enteric neurons. Enteric glia produce S-nitrosoglutathione, which plays an important role in the homeostasis of the epithelial barrier. Nevertheless, enteric glia can respond to inflammation by the production of IL-1β leading to an increased epithelial permeability and activation of immune cells. Translocation of luminal antigens to the lamina propria and tissue damage result in TLR and danger-associated molecular pattern activation (DAMPs- including the pattern recognition receptor Receptor for Advanced Glycosylated Endproducts; RAGE) of immune cells followed by cytokine and chemokine release. Other immune cells, but also neurons and EGCs can show response to the cytokines and chemokines and besides the induction of inflammation, also anti-inflammatory pathways will be activated.
Epithelial barrier dysfunction as a contributor of intestinal disease pathology, focus on stress

Causes of intestinal barrier dysfunction can be quite diverse and range from intestinal infection to allergic food components, malnutrition, toxic chemicals, NSAIDs and mechanical trauma. In addition, during recent years we have gained a lot of knowledge on the possible role of psychological-stress related impairment in barrier function. Stress was shown to induce loss of barrier integrity not only in small bowel and colon but, more recently, also in oesophagus. Although the latter may explain why stress is not only associated with IBD and IBS but also with gastro-oesophageal reflux disease (GERD), focus of most research has been on the intestines. In this part of the review we will cover its role in especially IBD and IBS.

Stress-induced mast cell degranulation alters epithelial permeability

Mast cells seem to play an important role in IBD and IBS and especially in IBS-related animal models it was convincingly shown that stress-induced mast cell degranulation can induce disease phenotype. Stress leads to peripheral (next to central) release of corticotrophin releasing hormone (CRH) and, subsequently, CRH-mediated degranulation of peripheral mast cells. We discussed earlier how mast cell-derived cytokines and preformed mediators can directly modulate barrier integrity by TJ rearrangements. It was also shown that stress will lead to increased (mast cell dependent) bacterial adherence and penetration into enterocytes associated with mucosal inflammation. Importantly, although initial stress-related mast cell degranulation will depend on peripheral CRH, the subsequent flaw in barrier function evidently leads to the uptake of bacterial antigens and may thus be self-sustaining. Antigen-mediated degranulation of mast cells can lead to further breakdown of barrier function and induce a vicious circle that will have to be counteracted by interfering mechanisms. In this respect, degranulating mast cells can also activate protective mechanisms by themselves. Goblet cell secreted mucins
form a physical barrier that prevents the attachment of enteric pathogens to the epithelium\(^78\) and it is known that stress-induced mast cell degranulation can lead to increased colonic mucin release\(^79\). Further, as mentioned before, mast cell-mediated histamine-induced epithelial chloride secretion can lead to excessive intestinal water secretion. Histamine also signals to the ENS and activates a neural defence program leading to orthograde power propulsions in the small and large intestine\(^80\). These combined actions can lead to powerful expulsion of a threatening pathogen and may thus be highly beneficial to the organism as a whole. On the other hand, such mast cell-mediated mechanisms also seem to play a role in e.g. diarrhoea-predominant IBS and IBD and one can speculate whether the observed diarrhoea is either protective or detrimental (or both). In IBD diarrhoea may be initiated to expel luminal content relevant to disease pathogenesis but it is also known that stress can lead to disease relapse. IBS, being a functional disorder, is diagnosed by symptoms alone and stress is an important trigger for symptoms. Although some reports suggest subtle qualitative changes in colonic flora of IBS patients\(^81\), we do not know whether stress induced diarrhoea leads to improved intra-luminal bacterial make-up. Thus, at present, stress-induced barrier dysfunction in these disorders is being regarded as detrimental instead of beneficial.

**Stress related barrier dysfunction in IBD**

While inflammation within the gut wall can clearly influence the perception of visceral sensation by the CNS, it is also apparent that signals from the brain to the gut are crucial in modifying the course of IBD progression. This phenomenon is best exemplified by the large body of evidence that suggests psychological stress and anxiety can negatively impact on the initiation, development and recurrence of inflammation. Numerous studies in patients with IBD implicate a relationship between stress-related personality traits (e.g. obsessive-compulsive disorders) or increased life stressors and disease exacerbation. Both the sympathetic and parasympathetic nervous system has been implicated in relaying stress signals from brain to gut\(^82\). As mentioned before, stress can lead to a (mast cell dependent)
decrease in IEB function through alterations in TJ permeability. This may lead to increased influx of antigens and bacteria followed by recruitment of inflammatory cells. Indeed, in some studies the adverse effects of stress on gut physiology were shown to depend on the recruitment and activation of immune cells, most notably mast cells and lymphocytes. Mast cell degranulation and related barrier dysfunction may thus play an important role in IBD, but relevant investigations were not necessarily done in colitis models. In relation to this, it was shown that prior exposure to stress significantly enhances experimental colitis. Surprisingly, this stress-effect was associated with decreased release of protective mucins. Although these data seemingly contradict earlier reports on stress-induced mast cell degranulation and associated increase in mucin levels, this discrepancy may be explained by the observed loss of goblet cells in the colitis model. Lastly, neuronal stress signals can also negatively influence immune barrier function at another level. IgA is continuously being secreted into the lumen to contain commensal bacteria outside the epithelial barrier. Stress was shown to decrease IgA production in colonic mucosa and may thus promote bacterial adherence and result in local immune activation, inflammation and perpetuation of ongoing disease state.

**Stress related barrier dysfunction in IBS**

Abdominal pain and motility changes are key features of IBS and acute stress is a known trigger for these symptoms in patients and in animal models. Furthermore, increased intestinal permeability is present in at least part of IBS patients and, although conflicting data exist, patients may have enhanced numbers of mucosal mast cells. Even more importantly, the number of degranulated mast cells was shown to be higher compared to controls. The latter is relevant because in jejunum of IBS patients, acute stress was shown to induce release of mast cell mediators, and this was associated with enhanced permeability to albumin. These human data seemingly confirm earlier observations in animal models: stress induces peripheral mast cell degranulation and subsequent barrier dysfunction. In rats, Ait-Belgaoui et al. were able to show that stress-induced barrier dysfunction can be a direct cause
for visceral hypersensitivity (enhanced sensitivity to a normal stimulus)\textsuperscript{89}. Pre-stress treatment with a TJ blocker or MLCK inhibitor preserved barrier integrity and prevented the development of enhanced sensitivity to colonic distension. Because visceral hypersensitivity is considered a pathophysiological mechanism in IBS, these results suggest that stress-induced impairments in barrier function can be a main trigger to initiate and/or perpetuate symptoms in this disorder. Despite these evidences, a cautionary note is needed because most data on stress-related mechanisms in relation to IBS were indeed gathered in animal models. Further, although most data seem to point towards a central role for mast cells other mechanisms may also be involved. Results obtained by Demaude \textit{et al.} suggested that early post-stress epithelial barrier defects do not depend on mast cell mediators but on pancreatic trypsin instead\textsuperscript{90}. 
The enteric nervous system as regulator of intestinal epithelial barrier function

Epithelial permeability

De novo synthesis:
Pro-inflammatory cytokines
(i.e. IL-1β, TNF)
Proteases
Tryptase → PAR-2 cleavage → tight junction rearrangement
Histamine

1. Santos, J. et al., Gut 2001
2. Wallon, C. et al., Gut 2008
3. Jacob, C. et al., JBC 2005

Mast cells produce several factors that increase the epithelial permeability. Proteases and tryptase rearrange tight junction via the cleavage of PAR-2. Also histamine and pro-inflammatory cytokines including IL-1β and TNF-α increase the intestinal epithelial permeability. Stress-induced mast cell degranulation results in the production of several factors that increase the epithelial permeability. Proteases and tryptase rearrange tight junctions via the cleavage of Protease-activated receptors (PARs) resulting in leakage of the epithelial barrier. Also histamine and pro-inflammatory cytokines including IL-1β and TNF-α increase the intestinal epithelial permeability so that luminal antigens have access to the lamina propria.

Figure 4. Potential mast cell regulation of the epithelial barrier. Stress-induced mast cell degranulation results in the production of several factors that increase the epithelial permeability. Proteases and tryptase rearrange tight junction via the cleavage of PAR-2. Also histamine and pro-inflammatory cytokines including IL-1β and TNF-α increase the intestinal epithelial permeability so that luminal antigens have access to the lamina propria.
Epithelial integrity in intestinal disease

Altered intestinal epithelial permeability is not only associated with major trauma and septic states, but also perpetuates intestinal disease including IBD, stress induced conditions such as IBS and food allergy, and intestinal infections. During these pathological disorders, mechanisms such as TJ rearrangements, decreased mitochondrial function and changes in apoptosis and proliferation of the epithelium all might contribute to barrier disruption.

Inflammatory Bowel Disease

Clearly, in chronic inflammatory settings alterations in the ENS that contribute to the enhanced permeability were observed. These alterations include a change in number and size of glial cells and enteric neuronal cell bodies and their nerve fibres, and remodelling of neural synapses. Also, changes in chemical coding of the neurons occur, with alterations in VIP and NO-synthase NPY, PACAP, CHAT and also altered receptor expression. These changes occur in inflamed areas but also at remote sites and are probably responsible for the widespread nature of changed physiology in IBD. Furthermore, altered motility was observed at a remote non-inflamed site and at inflamed sites in ulcerative colitis patients or animal models of IBD. These findings suggest that the ENS may play a role not only during active inflammation, but also in the development of disease. This is illustrated by the fact that ENS disturbances will predispose to postoperative recurrence and stress induced relapses. Healthy, first-degree relatives of patients with IBD were reported to have increased intestinal permeability and altered intestinal permeability is predictive of clinical relapse in patients with clinically inactive IBD. However, ENS alterations are mostly secondary and whether changes in ENS precede gut diseases needs to be confirmed.
Barrier dysfunction in functional bowel disease

Intestinal ischemia occurs in several conditions including (non) abdominal surgery, major trauma, cardiac arrest and is responsible for the barrier disturbances and bacterial translocation to distant organs. Translocation of bacteria or endotoxins, is an important mediator in the pathogenesis of sepsis during these conditions. Ischemic conditions in the intestine result in selective intestinal actin cytoskeleton and TJ disruption, as has been shown in a model of haemorrhagic shock\textsuperscript{94}. In animal models of intestinal ischemia-reperfusion, the enzyme xanthine oxidase (XO) is activated, inducing oxidative stress\textsuperscript{95} that is the cause of morphological changes of the epithelial monolayer and alterations of enterocyte mitochondrial function\textsuperscript{96,97}. Mitochondrial dysfunction might also induce transcellular uptake of bacteria\textsuperscript{98}, but this process is not well understood thus far. Also, exaggerated local and systemic immune responses that occur during ischemic conditions might further contribute to barrier dysfunction\textsuperscript{59,99}.

Septic ileus

The occurrence of septic morbidity and even multiple organ failure in serious conditions such as surgery, trauma, ischemia reperfusion injury might be the result of a breakdown of the intestinal barrier and subsequent bacterial translocation\textsuperscript{100}. In a recent study including 927 patients over 13 years showed that bacterial translocation was associated with increased postoperative septic morbidity in surgical patients\textsuperscript{101}. However, the evidence for the so-called ‘gut origin of sepsis’ is at least in humans, controversial\textsuperscript{102}. The development of septic morbidity is multifactorial and in certain patients measures taken to prevent septic morbidity such as selective gut decontamination, the use of pre- or probiotics have not been successful.
**Post-operative Ileus**

The occurrence of barrier dysfunction following abdominal surgery may also contribute to the pathogenesis of gastrointestinal stasis and post-operative or septic ileus. Although gastric and colonic dysmotility are regarded as important contributors to ileus, small bowel motility is an important factor in the regulation of the enteric bacterial population. A delayed transit time results in bacterial overgrowth, especially in the small bowel, and predisposes to bacterial translocation. Bacteria from the intestinal lumen might activate intestinal resident macrophages, given the observation that pre-treatment with antibiotics prior to the intestinal manipulation reduced inflammatory responses. The ileus-associated bacterial translocation reflects disturbances in intestinal barrier function and does not only refer to the transepithelial passage of viable bacteria, but also (endo) toxins or antigens from the intestinal lumen. This is illustrated by the observation that in abdominal surgery, barrier dysfunction has been associated with increased postoperative septic morbidity in surgical patients undergoing laparotomy. Moreover, in an experimental model for post-operative ileus the occurrence of ileus and the reduction of small intestinal smooth muscle contractility were not observed after surgery in TLR4-deficient mice. See the proposed sequence of events leading to post-operative ileus in figure 5.
The enteric nervous system as regulator of intestinal epithelial barrier function

Figure 5. Neurogenic influences on epithelial barrier function, contributing to pathogenesis of POI. Intestinal manipulation (IM) results in impaired gastro-intestinal motility leading to post-operative ileus (POI). IM leads to degradation of mast cells, which increases the permeability of the intestinal epithelial barrier so that luminal antigens have access to the lamina propria. Residential macrophages between the circular and longitudinal muscle layers become activated by mast cells and luminal antigens, resulting in the recruitment of other immune cells. These inflammatory processes activate inhibitory neuronal pathways, which will lead to a general impairment of the gastrointestinal motility.
In human subjects, it has been shown that gut barrier loss in children undergoing major non-abdominal surgery is preceded by hypotension, mesenterial hypoperfusion and enterocyte damage. As indicated above, similar results were obtained from animal studies where surgery induces production of free oxygen radicals resulting in disturbances in enterocyte proliferation and subsequent reduction in epithelial barrier function. In conjunction, we have now unpublished data in which we observed that arterial blood pressure measured in the carotid artery is greatly reduced during manipulation of the intestine during surgery, and that this drop in blood pressure remains until the animals recover from anaesthesia. Most likely, blood pressure in the carotid artery reflects the blood pressure of the internal organs, but we cannot exclude that perfusion in the small intestine is different from the carotid artery.
Prospect of modulation of barrier function as a treatment in intestinal inflammatory disease; 5 year view

Clearly, the expression of several TJ proteins is affected in IBD patients. Patients with CD have increased levels of the pore-forming claudin-2, whereas the sealing claudins 5 and 8 are downregulated. In colonic tissues of ulcerative colitis patients, the expression of ZO-1, JAM-1 and claudin-1 is downregulated. Moreover, in colonic epithelium, the expression of ZO-1 is lost in DSS-induced colitis in mice and also mice that are JAM deficient are more susceptible for the development of colitis.\textsuperscript{40,41} The decrease in epithelial barrier contributes further to inflammatory reactions during IBD but until now, little evidence suggests that it might be a primary causative factor predisposing to disease development.\textsuperscript{91} A considerable body of evidence from animal models and patients, dated as long as 50 years ago, has emerged that demonstrate that dramatic alterations occur in the ENS under conditions such as chronic inflammation during IBD (though more pronounced in ulcerative colitis). Such changes can be apparent as gross changes in the morphology and architecture of neural ganglia and nerve cell bodies or can be seen as subtle changes in the expression of neurotransmitters or their receptors at synapses within the gut wall. Changes that occur in the ENS are clearly coupled to alterations in gut physiology and permeability and are likely to play an important role in the pathogenesis of the disease.

Treatment with chemical messengers could restore ENS function and thereby the ongoing inflammation and could also prevent relapses at remote sites. Indeed, administration of neuropeptides including VIP, NPY, cortistatin, opioids, and NK-1R and CRHR antagonists ameliorated disease in IBD animal models. Also, cholinergic agonists might have beneficial effects. In some studies with experimental animal models of intestinal inflammation, animals were treated with cholinergic agonists such as nicotine but with conflicting results. In ulcerative colitis patients, nicotine patches have shown to be effective to reduce inflammation, but due to side-effects this treatment is not applicable in patients. Therefore, a more
selective treatment might be desirable to make use of the cholinergic anti-inflammatory pathway. In one study, enhancement of sympathetic activity in ulcerative colitis was shown \(^{106}\) and treatment with an \(\alpha\)-adrenergic clonidine reduced sympathetic overactivity and was associated with clinical and endoscopical improvement as compared to placebo. The use of neuronal-derived messengers to relieve bowel disease might come from the field of psychopathologies such as anxiety and depression. The first clinical report that a CRHR1 antagonist (R121919) significantly reduces depression and anxiety scores in patients with major depression, also assigns therapeutic value to CRHR1 antagonists in the treatment of IBS patients with stress-related symptom generation \(^{107}\).

Taken together, novel therapeutic approaches designed to specifically target and disrupt neuro-immune communication in IBD would be likely to benefit in treatment of disease. The advantages of the use of neuron-derived chemical messengers are that they are short lived and act local. Importantly, in addition to the potential to ameliorate IEB, neuronal messengers also affect inflammatory processes, motility, mucus production and water and electrolyte secretion, thereby further contributing to disease amelioration.
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