The intestinal epithelial barrier: a therapeutic target?

Matthew A. Odenwald[1] *and Jerrold R. Turner*[1,2]

Abstract | A fundamental function of the intestinal epithelium is to act as a barrier that limits interactions between luminal contents such as the intestinal microbiota, the underlying immune system and the remainder of the body, while supporting vectorial transport of nutrients, water and waste products. Epithelial barrier function requires a contiguous layer of cells as well as the junctions that seal the paracellular space between epithelial cells. Compromised intestinal barrier function has been associated with a number of disease states, both intestinal and systemic. Unfortunately, most current clinical data are correlative, making it difficult to separate cause from effect in interpreting the importance of barrier loss. Some data from experimental animal models suggest that compromised epithelial integrity might have a pathogenic role in specific gastrointestinal diseases, but no FDA-approved agents that target the epithelial barrier are presently available. To develop such therapies, a deeper understanding of both disease pathogenesis and mechanisms of barrier regulation must be reached. Here, we review and discuss mechanisms of intestinal barrier loss and the role of intestinal epithelial barrier function in pathogenesis of both intestinal and systemic diseases. We conclude with a discussion of potential strategies to restore the epithelial barrier.

[1]*Department of Pathology, The University of Chicago, 5841 South Maryland, Chicago, Illinois 60637, USA.*
[2]*Departments of Pathology and Medicine (Gastroenterology), Brigham and Women's Hospital and Harvard Medical School, 20 Shattuck Street, Thorn 1428, Boston, Massachusetts 02115, USA.*

Correspondence to J.R.T.
jrturner@bwh.harvard.edu

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An essential function of the intestinal mucosa is to act as a barrier between luminal contents and the underlying immune system. The term 'intestinal barrier' is increasingly used to refer to the mucus layer or the underlying mucosal immune system, and although each of these mucosal components provides a type of barrier, the physical epithelial barrier confers the property of selective permeability to the intestinal mucosa. The term 'intestinal barrier function' will therefore be used here to refer to the ability of the intestinal epithelium to restrict free exchange of water, ions and macromolecules between the intestinal lumen and the underlying tissues. Intestinal permeability is the inverse of intestinal barrier function, and because the intestinal mucosa must simultaneously promote nutrient and water transport while serving as a protective barrier, neither property is absolute. Instead, intestinal barrier function depends on a variety of mucosal structural components that are tightly regulated in homeostasis and during disease[1–3].

The luminal surface of the intestinal mucosa is lined by a hydrated gel, composed of mucins secreted by goblet cells[4–6]. This layer prevents large particles and intact bacteria from coming into direct contact with the underlying epithelium. The importance of the mucus layer is emphasized by the observations that mucin structure is markedly altered in active enterocolitis and that knockout mice lacking the *Muc2* gene, which encodes the major component of intestinal mucin, develop spontaneous colitis[7]. However, the mucus layer does not establish a substantial barrier to transmucosal water or solute flux; that job falls to the epithelial monolayer, which is the primary determinant of mucosal barrier function[8]. The apical surface of the epithelium forms a single, continuous border as a result of the precise alignment of abutting cells. In an intact epithelium, this surface restricts passage of most hydrophilic solutes. However, to limit transmucosal flux, the paracellular space must also be sealed. The task of regulating paracellular transport is achieved by a series of intercellular junctions.

The apical junctional complex

From an apical to basal direction, the intercellular junctions are the tight junction (ZO; zonula occludens), adherens junction (zonula adherens) and desmosome (FIG. 1). Together these three types of intercellular junctions comprise the apical junctional complex[9]. The apical junctional complex is associated with a dense network of actin and myosin that encircles the apical aspect of each cell and supports the cortical actin web[10,11]. The latter supports the dense microvillus brush border, whereas the perijunctional actomyosin ring regulates epithelial barrier function (see next section).

Key points

- The intestinal epithelium is a dynamic cellular layer that serves as a barrier between luminal contents and the underlying immune system while simultaneously supporting water, nutrient and ion transport
- Tight junctions are the primary determinants of barrier function in intact epithelia and are composed of a complex network of transmembrane and cytosolic proteins accompanied by cytoskeletal and regulatory proteins
- Two distinct pathways — termed pore and leak — regulate paracellular flux in intact epithelia whereas the unrestricted flux pathway is the dominant route across ulcerated or denuded epithelia
- Reduced intestinal epithelial barrier function is associated with a variety of gastrointestinal and systemic diseases, including IBD and graft versus host disease, respectively, but is insufficient to cause disease in the absence of other insults
- Experimental evidence suggests that barrier defects contribute to IBD, as mouse models demonstrate that increased paracellular permeability accelerates experimental colitis and that preservation of tight junction barrier function delays disease progression
- Although no currently available therapeutics specifically modulate epithelial barrier function, promising approaches to target the pore, leak, and unrestricted pathways are being investigated

Adherens junctions and desmosomes provide the adhesive forces necessary for maintenance of cell–cell interactions. The most well-known component of the adherens junctions are the cadherins — single spanning transmembrane proteins that interact homotypically with the extracellular portion of cadherins on adjacent cells[12]. On the cytoplasmic face, cadherins interact directly with p120 catenin and β-catenin, which in turn interact with α-catenin[13]. Among other functions, α-catenin regulates perijunctional actin assembly, which provides further strength to these structures[14,15]. In addition, the adherens junction is necessary for efficient tight junction assembly, a function that *in vitro* studies have attributed to both epithelial cadherin (E-cadherin) and α-catenin[16,17].

The tight junction is the primary determinant of paracellular permeability. When viewed using transmission electron microscopy, the tight junction seems to eliminate the intercellular space at so-called 'kissing points,' and freeze-fracture electron microscopy clearly shows that tight junctions consist of a series of anastomosing strands[9,18]. Results from a study using direct rapid freezing methods suggested that tight junction strands might exist as pairs of inverted micelles formed by the fusion of the outer leaflets from plasma membranes of abutting cells[19,20]; however, this model has largely been abandoned with the discovery of tight-junction-associated structural and regulatory proteins[19,20]. Immunoelectron microscopy has demonstrated transmembrane proteins within tight junction strands[21]. Multiple subsequent studies have shown that tight junction proteins reside in cholesterol-rich, detergent-insoluble lipid domains[22–24]. These findings have led to speculation that dynamic fusion and fission of lipid-based tight junction strands might account for selective permeability (a detailed review considering the lipidic nature of tight junctions can be found elsewhere[20]). Specialized lipids and proteins are probably necessary components of the tight junction barrier; however, to date, far more work has been done to identify the structure and regulation of tight junction proteins.

Tight junction proteins can be broadly separated into transmembrane proteins, cytosolic plaque (scaffolding) proteins and regulatory proteins. Of the transmembrane tight junction proteins, the tetraspanning claudins are the most important, as the extracellular domains of claudins on adjacent cells form pores to regulate tight junction ion selectivity[25]. A seminal study determined that expression of a single claudin family member, claudin-2, is largely responsible for differences in transepithelial resistance between two clones of Madin Darby canine kidney cells[26]. Subsequent analyses have shown that claudin-2-driven decreases in epithelial barrier function are due to increases in paracellular ion conductance without accompanying alterations in flux of larger molecules[27–29]. Data showing that individual claudin-2-based channels are dynamically gated suggests that altering the opening and closing of claudin-2 pores is a targetable process for barrier modulation[28]. An alternative potential method of inhibiting claudin-2 function comes from the observation that prevention of casein-kinase-2-mediated occludin phosphorylation promotes assembly of a tight junction complex that blocks claudin-2 pore function, thereby reversing IL-13-induced barrier loss *in vitro*[30]. However, such therapies must be approached with caution, as *trans*-tight junction Na^+ recycling, from the lamina propria into the lumen, is necessary to support critical transcellular vectorial transport processes such as nutrient absorption[31–33].

The ZO family of proteins (ZO1, ZO2 and ZO3, encoded by the genes *TPJ1*, *TPJ2* and *TPJ3*, respectively) are multidomain scaffolding proteins that interact directly with transmembrane tight junction proteins such as claudins and the tight junction-associated MARVEL protein (TAMP) family, which includes occludin[21,34–36]. ZO proteins also interact with the actin cytoskeleton and a variety of actin regulatory elements[37]. The ZO proteins have many similar structural domains, which has led to the hypothesis that they serve similar functions[37,38]. However, these proteins must also have unique functions as knockout of either *Tjp1* or *Tjp2* genes results in embryonic lethality in mice[39,40]. Studies in humans have discovered two distinct pathogenic *TJP2* mutations[40,41]. The first mutation impairs ZO2 binding to claudins and results in an incompletely penetrant familial hypercholanaemia, which presents with elevated serum bile acid levels, pruritus and fat malabsorption[41]. The second discovered mutation in *TJP2* encodes a truncated protein and is associated with severe cholestatic liver disease that presents early in life and frequently requires liver transplantation[42]. In this case, claudin-1, but not claudin-2, fails to localize to tight junctions within canalicular and cholangiocyte membranes. Interestingly, a study of mice lacking claudin-2, which forms a paracellular Na^+ and water channel, found that these mice generated more concentrated bile and were susceptible to gallstone disease, suggesting that claudin-2-mediated paracellular water and/or Na^+ flux contributes to bile hydration[43]. The ability of this truncated ZO2 to support human life, while *Tjp2* gene knockout is lethal in mice, suggests that the shortened protein is partially functional, possibly via oligomerization with ZO1. Alternatively, the data might

indicate species differences in the redundancy between ZO1 and ZO2. In either case, these data highlight the importance of tight junction proteins in homeostasis and prevention of gastrointestinal diseases. Although barrier function has not been measured in patients with either *TJP2* mutation, the differences in localization of claudin proteins to tight junctions implies that, as in the claudin-2-knockout mouse, altered epithelial barrier function might result in hepatobiliary disease[41,42]. This finding is consistent with many other studies that have linked intestinal barrier dysfunction to hepatitis[44,45].

Paracellular permeability pathways

The tight junction barrier exhibits both size and charge selectivity with two distinct routes across an intact epithelial monolayer, termed the 'pore' and 'leak' pathways[3,8] (FIG. 2). The pore pathway refers to a high-capacity, size-selective and charge-selective route, whereas the leak pathway is a low-capacity pathway that has more limited selectivity[3,8]. Pore pathway permeability seems to be determined primarily by the subset of claudins expressed, whereas leak pathway permeability can be regulated by ZO1, occludin and myosin light chain kinase (MLCK)[8,30,46]. At sites of epithelial damage, such as erosions and ulcers, tight junctions are lost and therefore cannot contribute to local barrier function. Instead, luminal contents cross the intestinal barrier by a third pathway, termed the 'unrestricted' pathway. As its name suggests, the unrestricted pathway is high-capacity and nonselective with respect to solute size and charge. Large proteins and even whole bacteria can cross the unrestricted pathway, which partially explains the severe disease initiated by epithelial damage. In the setting of extensive epithelial injury, such as that occurring in humans with necrotizing enterocolitis or rodents treated with dextran sulfate sodium (DSS), the unrestricted pathway is often unsealed and is the predominant route of transmucosal flux[47–49]. However, during homeostasis and less active inflammatory disease, the epithelium is generally intact and barrier function primarily reflects flux across the paracellular pore and leak pathways[29,48,50].

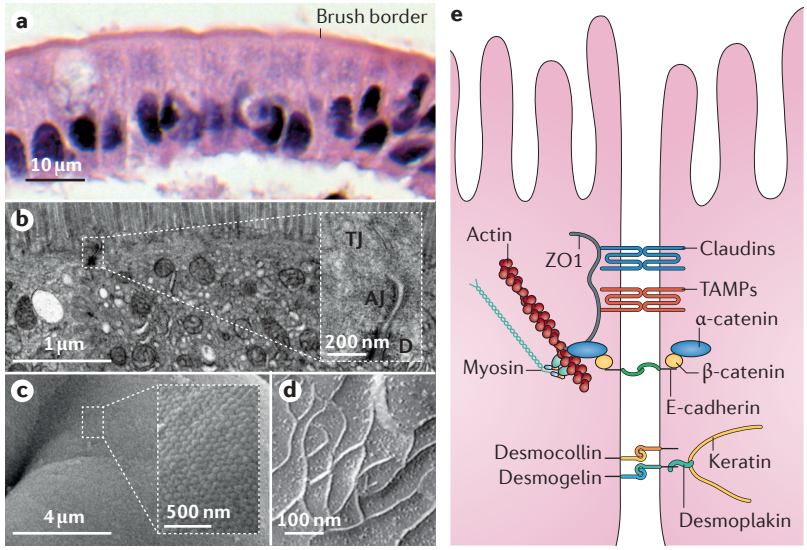

Figure 1 | **The apical junctional complex is necessary for epithelial barrier formation.** The intestinal epithelial monolayer separates luminal contents from the underlying lamina propria. **a** | Human jejunal epithelium stained with haematoxylin and eosin, showing cells forming a community brush border. Scale bar = 10 µm. **b** | Transmission electron microscopy of small intestinal epithelium shows intercellular junctions, a microvillus brush border and exclusion of organelles from the dense band of cortical actin just beneath the brush border. Scale bar = 500 nm. Inset: apical junctional complex, composed of the tight junction (TJ), adherens junction (AJ) and desmosome (D). Scale bar = 200 nm. **c** | Scanning electron microscopy shows the continuous brush border surface of the small intestine. Scale bar = 2 µm. Inset: Densely packed microvillus array. Scale bar = 500 nm. **d** | Freeze-fracture electron microscopy shows tight junction strands. Scale bar = 100 nm. **e** | Epithelial cells are held together and communicate through junctions. Schematic depicting junctional transmembrane proteins of the tight junction (claudins and tight junction-associated MARVEL proteins (TAMPs)), adherens junction (E-cadherin) and desmosome (desmogelin and desmocollin) connected to the actin cytoskeleton via cytosolic proteins (ZO1, catenins and desmoplakin). Tight and adherens junctions interact with the actin cytoskeleton, and desmosomes connect to intermediate filaments.

Regulation of the epithelial barrier

Homeostatic regulation. During homeostasis, the intestinal epithelium is a highly dynamic structure and is estimated to completely self-renew every 4–7 days[2,51–53]. Stem cells reside in the intestinal crypts where they proliferate, and daughter cells differentiate as they migrate up the crypt–villus axis to be ultimately shed into the intestinal lumen. This constant turnover presents an opportunity for potential breaches in the epithelial barrier with concomitant increases in flux across the unrestricted pathway. However, both shedding events and oligocellular wounds are accompanied by the formation and subsequent contraction of a multicellular actomyosin purse string, which drives tight junction expansion to the basal surface of the extruded cell to rapidly re-establish the contiguous epithelium and tight junction barrier[54–56].

The most studied example of physiological regulation of the tight junction barrier is that which occurs upon activation of sodium–glucose cotransport[57–61]. This cotransport leads to activation of epithelial MLCK as well as development of a transepithelial osmotic gradient. MLCK activity increases paracellular permeability via the size-selective pore pathway, and in the setting of an osmotic gradient, this increased permeability enables paracellular absorption of small nutrients (such as glucose) via solvent drag[57,59,61–64].

Pathophysiological regulation of leak and pore pathways. The pore and leak pathways are also regulated in response to pathophysiological stimuli. Perhaps the most well-studied example is flux across the pore pathway due to IL-13-induced increases in claudin-2 expression[29,65]. Notably, IL-13 is not the only immunological regulator of claudin-2 expression and pore pathway permeability, as IL-6, IL-4, IL-9 and TNF have also been reported to induce claudin-2 expression[65–71]. Although one study suggested that IL-13 causes barrier loss by inducing claudin-2 expression as well as increasing apoptosis and inhibiting wound healing[65], both *in vitro* and *in vivo* studies using lower doses of IL-13 have shown claudin-2 upregulation and claudin-2-dependent pore-pathway activation in response to IL-13 exposure without associated increases in leak or unrestricted pathway flux[29].

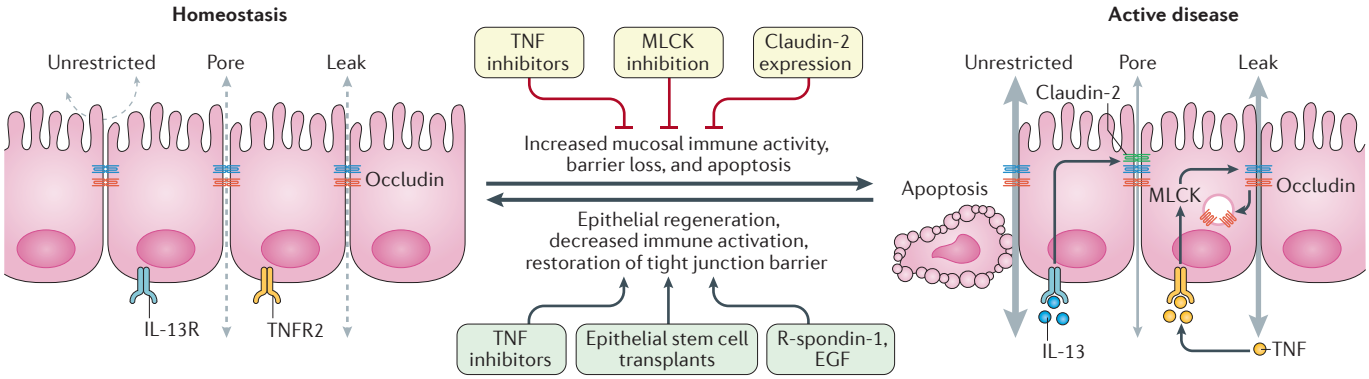

Figure 2 | **Three distinct paracellular epithelial permeability pathways are disrupted during disease pathogenesis.** During homeostasis (left) there is little underlying mucosal immune activity and the 'leak' and 'pore' pathways, regulated by tight junctions, define intestinal permeability. In the presence of an intact epithelium, cellular membranes seal the 'unrestricted' pathway, which is independent of tight junctions. During disease pathogenesis (right), increased mucosal immune activation leads to TNF and IL-13 production, which can cause increased permeability across the leak and pore pathways, respectively. TNF enhances leak pathway permeability by both increasing myosin light chain kinase (MLCK) transcription and activity at the tight junction and causing occludin endocytosis. Pore pathway permeability is increased by IL-13-dependent claudin-2 upregulation. As inflammatory disease progresses, epithelial apoptosis occurs and permeability across the high-capacity, charge and size-nonselective unrestricted pathway dominates. Upon removal of inflammatory stimuli, the epithelium regenerates to seal the unrestricted pathway and restore permeability dependent on tight junctions. Multiple therapeutic approaches targeting the intestinal epithelial barrier have been proposed (centre). These approaches aim to either inhibit initiation and progression of disease through immunosuppression or inhibition of barrier loss dependent on tight junctions, or through restoration of epithelial barriers after disease onset by inhibiting inflammation or promoting epithelial regeneration. EGF, epidermal growth factor; IL-13R, IL-13 receptor; TNFR2, TNF receptor 2.

This finding is consistent with biophysical studies demonstrating that claudin proteins such as claudin-2 form paracellular channels with exquisite size and charge selectivity, and both closed and open states, similar to transmembrane ion channels[28,72–74]. Interestingly, crypt but not surface epithelial cells express claudin-2 under normal conditions, consistent with the increased paracellular permeability of the former[75–77].

The cytokine TNF has also been shown to regulate tight junction function, and the clinically relevant role of TNF in IBD pathogenesis is clearly demonstrated by the efficacy of anti-TNF antibodies, which reduce disease severity and restore intestinal barrier function[78,79]. Repair of epithelial barrier function following anti-TNF therapy might reflect mucosal healing in the setting of a dampened immune system. However, preclinical studies have shown that TNF signalling also modulates tight junction barrier function directly[48,80–83]. This relationship was first recognized *in vitro* by the association between barrier loss and increased myosin light chain (MLC) phosphorylation in response to TNF[80]. Pharmacological inhibition of MLCK activity *in vitro* rapidly reduced MLC phosphorylation and restored tight junction barrier function[80]. Using both pharmacological and genetic methods of MLCK inhibition, TNF-induced MLC phosphorylation and tight junction barrier dysfunction was shown to be required for diarrhoea *in vivo*[84]. Subsequently, TNF was found to also upregulate claudin-2 expression, thereby enhancing pore pathway flux[68]. However, this process only occurred after many hours of TNF treatment in contrast to the rapid regulation of MLCK transcription[85], and is therefore best considered a secondary phase of TNF-induced barrier regulation that might be indirect. Notably, the expression of constitutively active MLCK (CA-MLCK) within the intestinal epithelium also upregulated claudin-2 expression *in vivo*, despite the absence of overt disease[29,48,86].

Further studies demonstrated the contribution of tight junction barrier loss to the pathogenesis of experimental colitis (discussed in the next section)[48,86]. TNF diminishes epithelial barrier function largely by inducing occludin internalization via a caveolin-1-dependent process[87], which was demonstrated *in vivo* as either pharmacological or genetic inhibition of caveolin-1 function limited both occludin internalization and TNF-mediated diarrhoea[87]. Furthermore, occludin overexpression in intestinal epithelial cells limited TNF-induced barrier loss and prevented TNF-induced diarrhoea[87]. This finding reflects the relative preservation of tight junction occludin pools, despite MLCK activation, in mice that overexpress occludin[87]. These data indicate that the removal of occludin from tight junctions, rather than some other component, leads to barrier loss. *In vitro* studies have corroborated this finding by showing that occludin depletion results in decreased barrier function and that occludin-deficient intestinal epithelial cell monolayers are resistant to further TNF-induced barrier loss[88,89]. Given the greater paracellular permeability of crypt epithelium relative to surface epithelium, it is notable that crypt epithelia have substantial intracellular occludin pools in the absence of inflammatory stimuli, whereas surface epithelia do not[75–77]. Subsequent domain analyses suggest that barrier regulation by occludin requires direct interactions between occludin

Table 1 | **Associations of representative diseases and disease models with intestinal barrier loss**

	IBD	Coeliac disease	Graft versus host disease	Type I diabetes mellitus
Structural alterations				
Pore pathway	Increased claudin-2 expression[48,65,70,114]	Increased claudin-2 expression[153,154,208]	Increased claudin-2 expression[177]	NA
Leak pathway	Reduced occludin expression[65]; increased MLCK expression and activity[48,85,115]; MLCK inactivation reduces severity[48]	Reduced occludin expression[138,153]	Reduced occludin expression[209]	NA
Unrestricted pathway	Ulceration, epithelial apoptosis[48,127,210]	NA	Epithelial apoptosis[211]	NA
Functional alterations				
Pore and/or leak pathways	Increased lactulose:mannitol ratio and PEG-400 permeability in disease[111,112,212–214], impending relapse[116–118,215] and in some healthy relatives[121,124,216]	Increased lactulose:mannitol ratio in disease[111,130,217]	Increased sucralose permeability[175]	Increased lactulose:mannitol ratio[178,179,218]
Leak and/or unrestricted pathways	Increased 4 kD dextran and Evan's blue flux in DSS-induced colitis[127,219,220]	Increased lactulose permeability; corrected by gluten-free diet[113]	Development of experimental minor mismatch disease requires intestinal damage[177]; the extent of barrier loss induced by pre-transplant conditioning correlates with disease severity[221]	Pathogenic bacteria that increase intestinal permeability accelerate disease[222]

DSS, dextran sulfate sodium; MLCK, myosin light chain kinase; PEG, polyethylene glycol.

and ZO1 (REF. 88). Unlike claudin channels that represent the structural pathway route of pore pathway conductance, the precise sites of paracellular leak pathway flux are not yet defined. The observation that overexpression of the occludin-related protein tricellulin reduces leak pathway conductance without affecting the pore pathway suggests that tricellular junctions might be the sites of leak pathway flux[90,91]. Interestingly, tricellulin is found at both tricellular and bicellular tight junctions, rather than only at the former, after occludin knockdown[88,92]. This finding raises the possibility that redistribution of tricellulin following occludin endocytosis contributes to TNF-induced barrier loss[88,91–93]. However, neither intestinal barrier defects nor intestinal disease have been described in humans or mice with tricellulin mutations[94–96]. Humans with occludin mutations have not yet been identified, but occludin-knockout mice, which are deaf, display tricellulin redistribution to bicellular tight junctions within the inner ear[97] and have been reported to lack intestinal barrier defects[98,99].

Although the leak and pore pathways represent distinct routes across the paracellular barrier, the two pathways are often affected in parallel. For example, in patients with IBD and in the SAMP1/YitFc mouse model of colitis, claudin-2 mRNA expression is increased and occludin expression is decreased, indicating that both leak and pore pathways are activated[65,100]. Mechanistic interplay between the pathways was demonstrated using mice expressing a CA-MLCK within the intestinal epithelium[29]. Colonic mucosae of these mice displayed increased cation selectivity that could not be explained by MLCK-dependent increases in leak pathway flux. Instead, *in vivo* responses to MLCK activation were shown to result in mucosal immune activation, enhanced IL-13 expression, and subsequent increases in claudin-2 expression that led to increased cation flux across the pore pathway[29].

Intestinal barrier function and disease

Impaired intestinal barrier function has been associated with an increasing variety of diseases — both intestinal and systemic (TABLE 1) — leading to the popularization of the catch-all diagnosis of so-called leaky gut syndrome[101–103]. The vast majority of these associations are merely correlative, but experimental evidence relating barrier dysfunction to disease pathogenesis exists in some cases, including IBD and coeliac disease[103]. Some bacterial pathogens are also capable of reducing tight junction barrier function including MLCK activation during enteropathogenic *Escherichia coli* infection[80,104,105], direct interactions with specific claudins by *Clostridium perfringens* enterotoxin[106,107] and Rho GTPase inhibition by *C. difficile* toxins[108,109].

IBD. The association between intestinal barrier dysfunction and intestinal disease was first recognized by studies using an *ex vivo* approach that documented increased permeability in active IBD, in both ulcerated and non-ulcerated epithelia[110–112]. Subsequent studies revealed that tight junction function, ultrastructure and protein composition are altered in patients with active IBD[71,113]. Expression and activity of MLCK as well as expression of claudin-2 are also increased in active IBD, suggesting that tight junction dysregulation might have a pathogenic role in IBD before epithelial ulceration[114,115]. Consistent with this idea, intestinal permeability has been reported as a fairly sensitive prognostic indicator of relapse to active disease in patients with Crohn's disease during clinical remission[116,117]. These results have been corroborated by a study of 43 patients with Crohn's disease, which also reported increased levels of the intestinal inflammation marker, faecal calprotectin, before relapse[118]. This finding blurs the exact role of intestinal barrier dysfunction in relapse because, as indicated by *in vitro* and *in vivo* studies,

subclinical levels of inflammation might be responsible for increased permeability. Consistent with this hypothesis, inflammatory cytokine exposure is associated with increased epithelial cell turnover *in vivo*[55], and one clinical study using confocal laser endomicroscopy reported that increased epithelial shedding and leakage of fluorescein dye across the intestinal epithelium in patients with IBD correlated directly with risk of relapse within 1 year[119]. Despite this finding, it is worth noting that the fluorescein dye flux observed was into the lumen, suggesting that barrier defect might result in local fluid efflux and, therefore, might enable only limited passive transport of luminal materials into the mucosa. Furthermore, many studies have shown relative maintenance of barrier function at sites of epithelial shedding[55,56,120].

The contribution of increased intestinal permeability to disease pathogenesis was first proposed with the realization that a subset of first-degree relatives of patients with Crohn's disease also display increased intestinal permeability[121]. Some of these individuals might also have an altered microbial metabolic state[122,123]. Although first-degree relatives do have an increased risk of developing Crohn's disease relative to the overall population, it remains to be determined if the subset with increased intestinal permeability are at greater risk than those without increased intestinal permeability. However, interestingly, relatives with increased intestinal permeability tend to carry a specific disease-associated polymorphism of *NOD2* (previously known as *CARD15*), which encodes a protein involved in bacterial recognition[124]. Although interesting in the context of disease, these studies also demonstrate that increased intestinal permeability alone is insufficient to cause overt clinical disease, as many healthy first-degree relatives also harbour this deficit[121,125]. Nevertheless, one case report identified a first-degree relative who had increased intestinal permeability before clinical presentation of Crohn's disease, suggesting a potential pathogenic role for intestinal barrier function in IBD[126]. This single case report must, however, be interpreted with caution given the individual's already increased risk of developing IBD. Furthermore, as noted, no studies have assessed long-term disease risk in first-degree relatives with increased intestinal permeability. However, a range of exciting data from experimental mouse models have provided evidence supporting the idea that intestinal barrier loss can be one component that contributes to a multifactorial mechanism of IBD pathogenesis[48,49,86,127].

Coeliac diseae. In simple terms, coeliac disease becomes apparent when genetically susceptible individuals ingest gluten-containing foods. Luminal and brush border enzymes digest gluten into gliadin, an alcohol soluble peptide. In patients with coeliac disease, gliadin drives mucosal immune activation by incompletely defined mechanisms that result in intestinal inflammation and epithelial damage[128]. To accomplish this step, gliadin must cross the epithelial barrier. Although the route by which gliadin is passed from the lumen to the lamina propria is controversial (transcellular or paracellular route), diminished intestinal barrier function is proposed to have a pathogenic role in coeliac disease. Support for the hypothesis that barrier loss contributes to coeliac disease pathogenesis first came from observations that intestinal permeability to nonmetabolizable sugars is increased during active disease and decreases to normal ranges after consumption of a gluten-free diet for several months[129]. Conversely, gluten challenge in patients with coeliac disease who have been on a gluten-free diet can increase intestinal permeability[129]. Later studies found that intestinal permeability positively correlates with disease activity and is increased in both patients with coeliac disease and their healthy relatives[130,131]. Moreover, improvements in barrier function have been shown to precede histological evidence of disease improvement after initiation of a gluten-free diet[132], and have even been reported in patients with diarrhoea-predominant IBS after introduction of a gluten-free diet[133].

Animal models of coeliac disease include a subset of Irish setter pups, which are gluten sensitive[134]. Similar to patients with coeliac disease, gluten-sensitive Irish setter pups display gluten-dependent increases in intestinal permeability that precede histological enteropathy[134]. These observations are supported by multiple studies showing increased intestinal permeability upon gluten exposure in gluten-sensitive HLA-DQ8 transgenic mice[135,136]. Each of these results can potentially be explained by immune signalling to intestinal epithelia that results in increased permeability. Consistent with this idea, removal of the immune stimulus (that is, gliadin) restores intestinal barrier function[129]. However, *in vitro* studies indicate that gliadin might have a direct effect on the intestinal epithelium, as exposure to gliadin and gliadin peptides produces a substantial reduction in barrier function of confluent intestinal epithelial cell (IEC-6) monolayers[137]. A similar result was reproduced using the human intestinal epithelial cell line Caco-2, and in this study, size-selectivity of gliadin-induced barrier defects was assessed by measuring flux of both 4 kDa and 70 kDa FITC-dextran across treated monolayers[138]. This study revealed that gliadin-exposed Caco-2 monolayers were considerably more permeable to small (4 kDa) but not large (70 kDa) dextrans, indicating an increase in leak pathway flux without increased flux across the unrestricted pathway[138].

The mechanism for gliadin-mediated reductions of epithelial barrier function has been proposed to involve upregulation of zonulin, a putative regulator of tight junction permeability. Zonulin expression is increased in patients with active coeliac disease, and a zonulin antagonist, larazotide acetate (AT-1001), inhibits gliadin-induced reductions in epithelial permeability *in vitro* and *in vivo*[139,140]. Unfortunately, although some clinical benefit has observed, trials of larazotide have not demonstrated reductions in intestinal permeability[141].

Other mechanisms of barrier loss in coeliac disease might reflect polymorphisms in myosin-IXb, which have been linked to coeliac disease[142,143]. Myosin-IXb is a Rho-GTPase-activating protein (GAP) that plays a part in

actin remodelling[144]. The myosin-IXb polymorphisms linked to coeliac disease are within the *N*-terminal portion of myosin-IXb, the region of the protein that confers Rho-GAP activity[144,145]. However, studies of myosin-IXb variants in additional populations have failed to demonstrate an association with coeliac disease[146,147]. These conflicting results might be due to population differences, unidentified environmental cofactors, or false-positive or false-negative results. Nevertheless, some support for a pathogenic role of myosin-IXb polymorphisms comes from studies linking the variants to Crohn's disease and ulcerative colitis[148–150]. Although it remains to be tested if the identified myosin-IXb variants are pathogenic, the association of polymorphisms in a single protein with multiple disease entities underscores the hypothesis that common cellular mechanisms might underlie multiple inflammatory diseases. *In vitro* studies using Caco-2 monolayers have shown an essential role of myosin-IXb in intestinal epithelial wound closure, tight junction protein localization and epithelial barrier function at steady state[151]. All of these data suggest that myosin-IXb might have an important role in maintaining the barrier by regulating both the tight junction and epithelial repair. Although intestinal permeability has not been studied in patients carrying myosin-IXb polymorphisms, it is interesting to speculate that these variants might increase disease susceptibility by enhancing flux across both tight junction leak and unrestricted pathways. Indeed, myosin-IXb-knockout mice were shown to have diminished epithelial barrier function, characterized by increased 40 kDa dextran flux[152]. These observations are probably explained by increased rates of epithelial apoptosis. However, intestinal epithelia from myosin-IXb-knockout mice also display increased subapical phosphorylated MLC and reduced ZO1 recruitment to tight junctions[152]. Other studies have identified changes in claudin protein expression that might also affect flux across the tight junction pore pathway[153,154]. Thus, as in IBD, all three flux pathways probably contribute to permeability increases in coeliac disease.

One final factor that might affect transmucosal flux in coeliac disease is the reduction in mucosal surface area as a result of villous blunting, which is often associated with reactive crypt hyperplasia[128]. Together, these events result in a skewing of the crypt:villus surface area ratio. The leak pathway of crypt tight junctions is far more permeable than in the villus[75–77], which probably increases leak pathway flux. However, pore pathway flux might also be reduced as a result of the overall loss of surface area. These changes explain the increased permeability to lactulose (as it is a leak pathway probe), decreased flux of the pore pathway probe mannitol and increased lactulose:mannitol ratio in coeliac disease[130,155,156].

Mouse models of intestinal barrier function in disease. A variety of mouse models have led to a more sophisticated understanding of the contribution of intestinal barrier function to inflammatory diseases. The critical role of epithelial barrier function in homeostasis was demonstrated in chimeric mice expressing a dominant negative N-cadherin cytoplasmic tail within intestinal epithelia[157,158]. E-Cadherin-mediated interactions were disrupted in intestinal epithelial cells expressing the N-cadherin tail, which resulted in aberrant epithelial differentiation, chronic active inflammation and dysplasia[157]. A histologically similar inflammatory process characterized by erosions and ulcerations was reported in mice lacking intestinal epithelial p120-catenin, which display marked E-cadherin downregulation owing to enhanced degradation in the absence of p120-catenin[159]. Mice with a targeted, conditional E-cadherin deletion within intestinal epithelium have also been generated[160]. These mice display altered differentiation patterns, enhanced epithelial apoptosis, bloody diarrhoea and impaired bacterial defense[160]. Disease in each of these models probably reflects marked disruption of tight junctions secondary to adherens junction disassembly, aberrant epithelial differentiation and epithelial apoptosis, and can therefore be considered a model of disease driven, at least partially, by unrestricted pathway defects. This disruption might be a component of IBD pathogenesis, but it is unlikely to reflect a primary mechanism in disease presenting after the neonatal period. Nevertheless, it is interesting that polymorphisms near the E-cadherin-encoding gene *CDH1* have been linked to ulcerative colitis[161].

Similar to human patients[162–164], colitis development in *IL10*$^{-/-}$ mice[165,166] is highly variable in penetrance, age of onset and severity. Environmental stimuli and genetic factors, including both targeted changes and strain-specific differences, contribute to the observed variation[166,167]. Moreover, enteric bacteria are necessary for colitis onset, as germ-free *IL10*$^{-/-}$ mice do not develop disease and antibiotic treatment can attenuate colitis[166,168,169], which correlates with observations of altered microbial communities in patients with IBD[170]. Although the primary defect in IL-10-deficient mice is immune, intestinal barrier defects are present before clinical evidence of disease onset and do not develop under germ-free conditions[168]. However, whether increased intestinal permeability is a key pathogenic component of colitis in *IL10*$^{-/-}$ mice or simply an early marker of mucosal injury is unclear from these data. Several studies suggest that the former might be true. First, it is now well-appreciated that the NSAID piroxicam can promote disease development in *IL10*$^{-/-}$ mice[171]. Given that NSAIDs are known to result in epithelial damage, NSAID treatment probably provokes disease by increasing flux across the unrestricted pathway. Similarly, administration of a zonulin agonist enhanced intestinal permeability and modestly increased disease severity in *IL10*$^{-/-}$ mice[172]. Conversely, a zonulin antagonist reduced intestinal permeability and disease severity in *IL10*$^{-/-}$ mice[173]. Although the mechanism of action of these agents (including their specificity) is unclear, these data do suggest that modulating intestinal permeability can affect colitis genesis in IL-10-deficient mice.

Mouse models with targeted apical junctional complex defects might shed light on the role of the tight-junction-mediated barrier in colitis development and

progression. For example, mice lacking junctional adhesion molecule-A (JAM-A), which facilitates tight junction assembly and leukocyte transmigration, display altered claudin protein expression and increases in epithelial apoptosis, proliferation and migration even in the absence of clinically apparent disease[127]. JAM-A-deficient mice are also hypersensitive to DSS injury[127]. This observation might indicate that JAM-A expression is either protective against intestinal epithelial damage or enhances regenerative capacity, but could also reflect the inability of knockout mice to mount an adequate response to DSS injury given the pre-existing chronic epithelial damage. Importantly, JAM-A is expressed in many tissues and a specific role for intestinal epithelial JAM-A has not been assessed. Notably, however, intestinal epithelial, but not endothelial, JAM-A expression is downregulated in patients with IBD[127].

A more precisely targeted model has taken advantage of the physiologically and pathophysiologically relevant tight junction regulator MLCK to increase intestinal paracellular permeability. In this model, CA-MLCK was expressed specifically within intestinal epithelia[86]. This perturbation increased intestinal paracellular permeability without affecting epithelial maturation, proliferation or turnover, much like the subset of healthy first-degree relatives of patients with Crohn's disease with increased intestinal permeability[86]. CA-MLCK transgenic mice mature normally without developing spontaneous disease, but they do exhibit subclinical immune activation with type 1 T helper (T_H1) cell polarization[86]. Furthermore, when challenged by adoptive transfer of effector T cells, disease onset is accelerated, severity is worsened and overall survival is reduced relative to nontransgenic littermates[86]. These experimental data are consistent with patient data indicating barrier dysfunction alone is insufficient to cause clinically detectable disease, and also provide direct evidence that isolated tight junction dysfunction can contribute to disease pathogenesis in susceptible hosts. As discussed earlier, the CA-MLCK-induced increase in leak pathway permeability also results in claudin-2 upregulation and enhanced pore pathway flux[29].

A subsequent study investigated the interplay between immune activation, TNF signalling, intestinal epithelial MLCK expression and intestinal barrier function using an immune-mediated adoptive transfer colitis mouse model[48]. Similar to human disease[115], intestinal epithelial MLCK expression increased as colitis progressed[48,115]. In mice, this finding was accompanied by increased intestinal epithelial transcription and expression of TNFR2 (TNF receptor 2), which had been shown to mediate TNF-induced increases in MLCK transcription *in vitro*[83]. Consistent with this finding, TNFR2-deficient mice failed to upregulate MLCK expression or MLC phosphorylation within intestinal epithelium[48]. By contrast, deletion of *TNFR1*, which often regulates signalling in immune cells, had no effect on intestinal epithelial MLCK expression or activity[48,83]. Furthermore, mice lacking either TNFR2 or epithelial MLCK were substantially protected from increases in mucosal TNF production and permeability, and deletion of either gene markedly delayed onset of colitis[48]. Interestingly, claudin-2 upregulation was also attenuated in MLCK-deficient mice[48]. Although the mice studied were generalized knockouts of the non-muscle long MLCK, the ability of intestinal epithelial CA-MLCK expression to fully restore all features of disease (including claudin-2 expression) in MLCK-deficient mice indicates that the results are a specific effect of intestinal epithelial MLCK deletion[48]. These data indicate that both TNFR2 and MLCK inhibition might be appropriate targets for future biologic therapies, and raise the possibility that TNFR2 blockade might have advantages over TNF-targeted biologic agents in terms of reduced overall immunosuppression and toxicity.

Intestinal barrier function and systemic disease. Increased intestinal permeability has been reported in patients with an array of autoimmune diseases, suggesting a link between exposure to microbial antigens and development of autoimmune disease[103]. Most notable among these associations is the link between graft versus host disease (GVHD), which develops in many patients after allogeneic stem cell (bone marrow) transplantation[174]. For many years it was known that the magnitude of intestinal barrier defects, primarily representing increased flux across the unrestricted pathway, correlated with the severity of experimental GVHD[175,176]. However, whether this finding merely represented the correlation between the extent of epithelial damage and GVHD severity or, alternatively, indicated that intestinal barrier loss played a specific causative role, was unclear. One study[177] has shown that intestinal barrier loss is not required for the development of GVHD in the context of major antigen mismatch-driven bone marrow transplantation, which is the most commonly used experimental model[47,176,177]. However, in the more clinically relevant setting of minor antigen mismatch transplantation, intestinal epithelial damage (that is, increased unrestricted pathway flux) was an essential cofactor in disease pathogenesis[177]. Remarkably, this requirement could be overcome by intraperitoneal delivery of lipopolysaccharide, suggesting that transmucosal flux of bacterial products might be the key disease-promoting event triggered by intestinal epithelial damage[177]. The specific role of barrier loss mediated by tight junctions in GVHD has not yet been defined.

Decreased intestinal barrier function has also been noted before clinical disease onset in patients with type 1 diabetes mellitus[178] and in the biobreeding diabetes-prone (BBDP) experimental rat model of type 1 diabetes mellitus[179]. One study comparing the microbiota of BBDP rats to diabetes-resistant (BBDR) rats has shown more abundant *Lactobacillus* and *Bifidobacterium* in the resistant rats[180–182]. However, whether alterations in microbiome composition are caused by diabetes or whether the alterations have a role in disease development remains unknown. In another mouse model of diabetes (the nonobese diabetic mouse model), diabetes development can be influenced by exposure to, and

ability to sense, luminal microbial stimuli[183,184]. Owing to its role as the primary regulator of interaction between the immune system and luminal antigens, the epithelial barrier is likely to be essential in preventing diabetes development. Indeed, a pathogenic link between barrier dysfunction and diabetes has been proposed to work through the negative tight junction regulator zonulin, as zonulin expression is increased in BBDP rats and administration of anti-zonulin antibodies decreases autoantibody production and the development of clinical type 1 diabetes mellitus in this model[185]. Although one study has reported that increased concentrations of serum zonulin correlate with intestinal permeability in patients with type I diabetes mellitus, a causative role of zonulin in patients has not been demonstrated[186] and this area of investigation remains controversial.

Targeting the epithelial barrier

Targeting and restoring the epithelial barrier is a tempting therapeutic goal. Unfortunately, no therapies currently exist to do so clinically, and one molecule (larazotide acetate) shown to restore epithelial barrier function in preclinical studies did not replicate barrier-protective effects in clinical trials[140,141,187]. Nevertheless, many promising approaches to target the epithelial barrier have been proposed.

Epithelial barrier restoration. Engraftment of intestinal stem cells has been proposed as a therapy for repairing damaged gastrointestinal mucosa (that is, the unrestricted pathway)[188,189]. Technological advances have made long-term culture and expansion of intestinal stem cells possible and have led many to believe that isolation, expansion and transplantation of intestinal stem cells can aid in epithelial regeneration[190–192]. This idea is supported by one study in which mice were subjected to DSS-induced epithelial damage and given either a mock enema or enema with cultured intestinal stem cells during recovery after DSS withdrawal[193]. Stem cells were able to engraft in areas of ulceration and serve as long-lived intestinal stem cells *in vivo*. However, engraftment efficiency was low and resulted in minimal immediate improvement and no long-term improvement after removal of DSS, suggesting that the most effective way to restore the barrier is to remove the disease stimulus[193]. Moreover, the $Lgr5^+$ intestinal stem cells that are expanded and engrafted have been proposed to serve as cancer stem cells[194,195], and careful characterization of enteroid gene expression over many passages has not been performed, leaving open the possibility that engrafted enteroids might harbour malignant potential. Although detailed characterization and improved culture and engraftment methods might make this method more promising, without removal of the underlying stimulus causing epithelial damage (DSS in this case), this approach is unlikely to provide meaningful benefit.

More targeted approaches have also been proposed and involve potentiating signalling pathways important for epithelial expansion[196,197]. Two factors essential for the growth and expansion of intestinal stem cells — epidermal growth factor (EGF) and R-spondin-1 — are possible therapeutic agents for restoring damaged epithelia. Activation of EGF receptor protects against TNF-induced apoptosis of epithelial cells[198], and R-spondin-1 reduces disease severity in epithelial damage models of colitis[199]. However, one might be cautious of this approach because both EGF and R-spondin-1 are mitogens, and both EGF and the R-spondin-augmented Wnt pathway are dysregulated in colon cancer[200,201]. For example, loss of a negative regulator of the EGF pathway (LRIG1) results in hyperproliferation of intestinal epithelial cells in mice[202,203]. However, one study indicated that EGF receptor signalling actually decreased colon cancer incidence and altered colonic cytokine production in IL-10-knockout colitic mice, supporting the potential of this approach in a subset of patients with colitis[204].

Tight junction regulation. An alternative approach to barrier maintenance focuses on tight junction regulation and has potential in preventing initial IBD development in susceptible individuals, and in promoting maintenance of remission[47,48]. As discussed, tight junction permeability is physiologically regulated to facilitate nutrient transport, raising concern of potential toxicity from this approach. Although further studies are necessary to characterize and mitigate these potential undesired effects, two targets are particularly enticing. One promising target is MLCK, which has a well-defined mechanism of action with respect to barrier function in physiology and pathophysiology *in vitro*, *in vivo* and in patients with IBD[115]. Additionally, studies have shown beneficial effects of MLCK inhibition in mouse models of colitis when inhibition occurs specifically in the intestinal epithelium[48,84]. However, MLCK inhibition harbours potentially detrimental off-target effects due to the fact that all MLCK isoforms share a common catalytic domain. For example, smooth muscle MLCK is essential for gastrointestinal motility, blood pressure regulation and airway contractility[205–207]. Although MLCK remains a promising target, more specific means of targeting long MLCK must be developed before considering MLCK as a drug target for treating human disease. Claudin-2 also offers a potentially druggable target by either modulating claudin-2 anchoring at the tight junction or directly targeting dynamic claudin-2 pore opening and closing events[28,30]. Unfortunately, no drug for claudin-2 modulation currently exists.

Conclusions

Currently, the best therapy for treating epithelial barrier loss is to treat the underlying disease, as increased permeability is as likely to be a consequence of the disease as it is to be a cause. For example, anti-TNF antibodies, which are successful therapies for IBD, treat the underlying immune activation while also markedly reducing intestinal permeability to near normal levels[78]. Although targeting the epithelial barrier shows promise, more research is needed to define the mechanisms of epithelial homeostasis and disease pathogenesis before therapeutically targeting the epithelial barrier.

In terms of future directions, establishing or refuting a pathogenic role for intestinal barrier dysfunction requires further investigation in both clinical studies and experimental models. Determining whether increased permeability in healthy first-degree relatives of patients with Crohn's disease is a risk factor for disease development will also be important. Delineating the contributions of pore, leak and unrestricted pathways to observations of increased intestinal permeability in both intestinal and systemic diseases will be necessary for mechanistic understanding of barrier function in disease, and subsequent rational therapeutic design. Claudin-2 and MLCK are potential therapeutic targets for modulation of tight junction pore and leak pathway permeability, respectively. However, developing the means to inhibit intestinal epithelial MLCK (to limit leak pathway flux increases) without toxicities due to systemic MLCK inhibition will be challenging. Likewise, modulating claudin-2 pores (pore pathway) without negatively affecting overall epithelial water and ion transport might also be complex. Tight junction proteins also have roles beyond barrier maintenance, including epithelial morphogenesis and differentiation. Defining the underlying structure–function relationships and their contributions to other physiological processes is a requisite precursor to targeting barrier function without detrimental effects on other systems. If these goals can be achieved, the intestinal barrier remains a promising therapeutic target in select disease states.

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