Intestinal mucosal barrier function in health and disease

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Abstract | Mucosal surfaces are lined by epithelial cells. These cells establish a barrier between sometimes hostile external environments and the internal milieu. However, mucosae are also responsible for nutrient absorption and waste secretion, which require a selectively permeable barrier. These functions place the mucosal epithelium at the centre of interactions between the mucosal immune system and luminal contents, including dietary antigens and microbial products. Recent advances have uncovered mechanisms by which the intestinal mucosal barrier is regulated in response to physiological and immunological stimuli. Here I discuss these discoveries along with evidence that this regulation shapes mucosal immune responses in the gut and, when dysfunctional, may contribute to disease.

Mucosa-associated lymphoid tissue
(MALT). The collections of B cells, T cells, plasma cells, macrophages and other antigen-presenting cells found in the mucosal linings of organs including the gastrointestinal tract, lungs, salivary glands and conjuctiva.

Tight junction
Also known as the zonula occludens, this is a site of close apposition of adjacent epithelial cell membranes — kiss points — that create a barrier against the free diffusion of water and solutes.

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Complex multicellular organisms interface with their external environments at multiple sites, including mucosae of the airways, oral cavity, digestive tract and genitourinary tract, and the skin. Although the skin is the most visible site of interface, the combined area of the mucosal surfaces is much greater than that of the skin. Mucosae are also the primary sites at which the mucosa-associated lymphoid tissue (MALT) is exposed to and interacts with the external environment. The magnitude of these interactions is greatest in the gastrointestinal tract, which is the largest mucosal surface and is also in continuous contact with dietary antigens and diverse microorganisms. Thus, mucosal surfaces, particularly in the intestines, are crucial sites of innate and adaptive immune regulation.

A central mediator of interactions between MALT and the external environment, including the intestinal lumen, is the epithelium that covers the mucosa. Epithelial cells establish and maintain the barrier, which in the skin is a tight, although not impermeant, seal. By contrast, most mucosal epithelial cells form leaky barriers. This is necessary to support universal functions that include fluid exchange, which occurs at nearly all mucosal surfaces, as well as essential tissue-specific functions. Thus, the precise permeability characteristics of each barrier type differ based on the functions supported. For example, the ion-selective properties of the barrier vary considerably along the length of a nephron to promote or restrict transport of specific ions in each segment[1]. Similarly, the ability of tight junctions to discriminate between and restrict passage of solutes based on size (a characteristic known as size selectivity) varies with location in the intestine, as permeability to larger solutes decreases from the crypt to the villus[2]. In addition to these fixed differences, mucosal permeability of many tissues is adaptable and may be regulated in response to extracellular stimuli, such as nutrients, cytokines and bacteria.

Recent advances have uncovered some of the mechanisms by which physiological and immunological stimuli affect cellular and extracellular components of the intestinal barrier. In this article I review our current understanding of the mechanisms that regulate intestinal barrier integrity, and discuss the hypothesis that the mucosal barrier can shape pro-inflammatory and immunoregulatory responses in the context of homeostasis and disease.

Although they may affect barrier function, dendritic cells (DCs), which extend dendritic processes across the tight junctions in the distal small intestine[3,4], and intraepithelial lymphocytes are not discussed here. In addition, the functions of M cells, which are specialized epithelial cells that deliver antigens directly to intraepithelial lymphocytes and to subepithelial lymphoid tissues by transepithelial vesicular transport from the gut lumen, are reviewed elsewhere[5]. Finally, detailed analyses of the interactions between epithelial cells and luminal microorganisms, as well as the intricacies of mucosal immune regulation, have also been recently reviewed elsewhere[6,7]. This Review therefore focuses on the roles of the epithelial cell barrier in health and disease, with particular emphasis on the junctional complexes between intestinal epithelial cells, which have a crucial role in barrier regulation.

Mucins
A family of heavily glycosylated proteins that are secreted as large aggregates by mucous epithelial cells.

Unstirred layer
A thin layer of fluid at epithelial cell surfaces that is separated from the mixing forces created by luminal flow and, in the intestine, peristalsis.

Coeliac disease
A chronic inflammatory condition of the upper small intestine in humans that is caused by immunological hypersensitivity to the α-gliadin component of wheat gluten. It can cause severe villous atrophy, which can lead to malabsorption and malnutrition if gluten-containing foods are not removed from the diet.

Anatomy of mucosal barriers

Extracellular components of the barrier. Most mucosal surfaces are covered by a hydrated gel formed by mucins (FIG. 1a). Mucins are secreted by specialized epithelial cells, such as gastric foveolar mucous cells and intestinal goblet cells, and create a barrier that prevents large particles, including most bacteria, from directly contacting the epithelial cell layer[8]. The importance of mucus gel hydration is shown by cystic fibrosis, in which the production of hyperviscous mucus contributes to pulmonary, pancreatic and intestinal disease[9]. Defective mucus production has also been reported in various immune-mediated diseases, and spontaneous colitis develops in mice that lack specific mucin genes[10].

Although small molecules pass through the heavily glycosylated mucus layer with relative ease, bulk fluid flow is limited and thereby contributes to the development of an unstirred layer of fluid at the epithelial cell surface. As the unstirred layer is protected from convective mixing forces, the diffusion of ions and small solutes is slowed. In the stomach, this property of the unstirred layer works with epithelial cell bicarbonate secretion to maintain a zone of relative alkalinity at the mucosal surface[11]. The unstirred layer of the small intestine slows nutrient absorption by reducing the rate at which nutrients reach the transporting protein-rich microvillus brush border, but may also contribute to absorption by limiting the extent to which small nutrients released by the activities of brush border digestive enzymes are lost by diffusion into the lumen. Unstirred layer defects have not been linked to specific diseases. However, it is interesting to note that increased unstirred layer thickness has been reported in coeliac disease[12], in which it may contribute to nutrient malabsorption.

Cellular components of the mucosal barrier. The primary responsibility for mucosal barrier function resides with the epithelial cell plasma membrane, which is impermeable to most hydrophilic solutes in the absence of specific transporters. Accordingly, direct epithelial cell damage, such as that induced by mucosal irritants or cytotoxic agents, including some drugs used for cancer chemotherapy, results in a marked loss of barrier function. However, in the presence of an intact epithelial cell layer, the paracellular pathway between cells must be sealed. This function is mediated by the apical junctional complex, which is composed of the tight junction and subjacent adherens junction (FIG. 1b). Both tight and

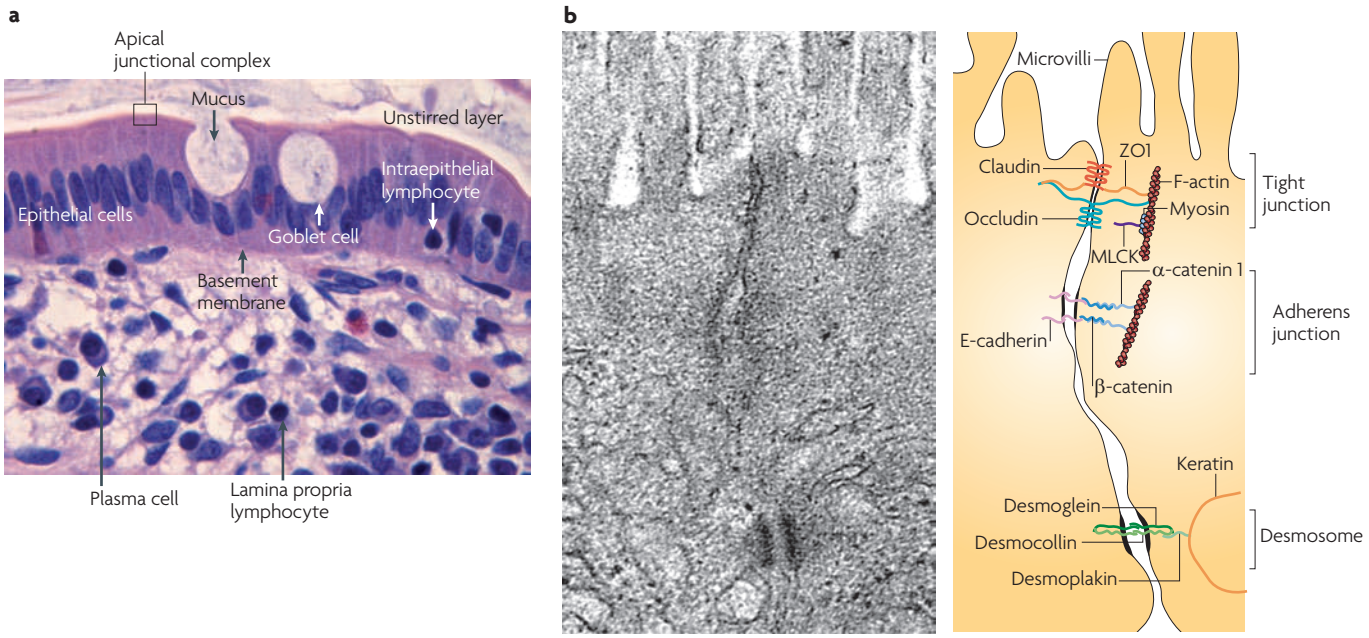


Figure 1 | **Anatomy of the mucosal barrier. a** | The human intestinal mucosa is composed of a simple layer of columnar epithelial cells, as well as the underlying lamina propria and muscular mucosa. Goblet cells, which synthesize and release mucin, as well as other differentiated epithelial cell types, are present. The unstirred layer, which cannot be seen histologically, is located immediately above the epithelial cells. The tight junction, a component of the apical junctional complex, seals the paracellular space between epithelial cells. Intraepithelial lymphocytes are located above the basement membrane, but are subjacent to the tight junction. The lamina propria is located beneath the basement membrane and contains immune cells, including macrophages, dendritic cells, plasma cells, lamina propria lymphocytes and, in some cases, neutrophils. **b** | An electron micrograph and corresponding line drawing of the junctional complex of an intestinal epithelial cell. Just below the base of the microvilli, the plasma membranes of adjacent cells seem to fuse at the tight junction, where claudins, zonula occludens 1 (ZO1), occludin and F-actin interact. E-cadherin, α-catenin 1, β-catenin, catenin δ1 (also known as p120 catenin; not shown) and F-actin interact to form the adherens junction. Myosin light chain kinase (MLCK) is associated with the perijunctional actomyosin ring. Desmosomes, which are located beneath the apical junctional complex, are formed by interactions between desmoglein, desmocollin, desmoplakin and keratin filaments.

adherens junctions are supported by a dense perijunctional ring of actin and myosin that, as discussed below, can regulate barrier function.

As implied by the name, the adherens junctions, along with desmosomes, provide the strong adhesive bonds that maintain cellular proximity and are also a site of intercellular communication. Loss of adherens junctions results in disruption of cell–cell and cell–matrix contacts, ineffective epithelial cell polarization and differentiation, and premature apoptosis[13]. Adherens junctions are composed of cadherins, a family of transmembrane proteins that form strong, homotypic interactions with molecules on adjacent cells. The cytoplasmic tail of the epithelial cadherin, E-cadherin (also known as cadherin-1), interacts directly with catenin δ1 (also known as p120 catenin) and β-catenin. In turn, β-catenin binds to α-catenin 1, which regulates local actin assembly and contributes to development of the perijunctional actomyosin ring.

Adherens junctions are required for assembly of the tight junction, which seals the paracellular space. Tight junctions are multi-protein complexes composed of transmembrane proteins, peripheral membrane (scaffolding) proteins and regulatory molecules that include kinases. The most important of the transmembrane proteins are members of the claudin family, which define several aspects of tight junction permeability, as discussed below. Claudins are expressed in a tissue-specific manner, and mutation or deletion of individual family members can have profound effects on organ function. The role of occludin, a transmembrane tight junction protein that interacts directly with claudins and actin, is less well understood. Peripheral membrane proteins, such as zonula occludens 1 (ZO1) and ZO2, are crucial to tight junction assembly and maintenance, partly owing to the fact that these proteins include multiple domains for interaction with other proteins, including claudins, occludin and actin.

The tight junction limits solute flux along the paracellular pathway, which is typically more permeable than the transcellular pathway. The tight junction is, therefore, the rate-limiting step in transepithelial transport and the principal determinant of mucosal permeability. Thus, it is important to understand the specific barrier properties of the tight junction, which can be defined in terms of size selectivity and charge selectivity.

At least two routes allow transport across the tight junction, and emerging data suggest that the relative contributions of these types of paracellular transport may be regulated independently[2,14,15]. One route, the leak pathway, allows paracellular transport of large solutes, including limited flux of proteins and bacterial lipopolysaccharides[14,15]. Although the size at which particles are excluded from the leak pathway has not been precisely defined, it is clear that materials as large as whole bacteria cannot pass. As might be expected for a route that allows large solutes to cross, the leak pathway does not show charge selectivity. Flux across the leak pathway may be increased by cytokines, including interferon-γ (IFNγ) *in vitro* and tumour necrosis factor (TNF) *in vitro* and *in vivo*[15–17].

A second pathway is characterized by small pores that are thought to be defined by tight junction-associated claudin proteins, which are also primary determinants of charge selectivity[18–20]. These pores have a radius that excludes molecules larger than 4 Å[14,15]. Expression of specific claudins varies between organs and even within different regions of a single organ and, as detailed below, can be modified by external stimuli, such as cytokines. Thus, tight junctions show both size selectivity and charge selectivity, and these properties may be regulated individually or jointly by physiological or pathophysiological stimuli.

Interdependence of transport routes

Transepithelial transport requires a selectively permeable barrier. The vectorial nature of transcellular transport, which is required for effective absorption and secretion, generates a transepithelial concentration gradient. Transepithelial transport would, therefore, be ineffective without a tight junction barrier, as diffusion would allow equalization of concentrations on both sides of the epithelium. Thus, active transcellular transport depends on the presence of an intact tight junction barrier. The selective permeability of the tight junction barrier also allows transepithelial gradients to drive passive paracellular transport of ions and water[17,20]. For example, claudin-16, which is necessary for tight junction cation selectivity, is mutated in the human disease familial hypomagnesaemia with hypercalciuria and nephrocalcinosis[20]. Paracellular absorption of Mg^{2+} and Ca^{2+} in the thick ascending limb of Henle requires a cation-selective tight junction barrier, the absence of which results in urinary loss of Mg^{2+} and Ca^{2+} (REF. 20). Similarly, apical Na^+–H^+-exchange protein 3 (NHE3) is required for transcellular Na^+ absorption in the renal tubule and intestine, and also contributes to the transepithelial Na^+ gradient that drives paracellular water absorption (BOX 1). The tight junction barrier to paracellular Na^+ efflux prevents dissipation of the gradient established by NHE3-mediated transcellular transport.

Tight junction barrier regulation modifies absorption. In addition to providing the driving force for paracellular transport, transcellular transport can activate intracellular signalling events that regulate the tight junction barrier. The best studied physiological example of this is the increased intestinal paracellular permeability that is induced by apical Na^+–nutrient co-transport[21]. This allows passive paracellular absorption of nutrients and water to amplify transcellular nutrient absorption, particularly when high luminal nutrient concentrations exceed the capacity of apical Na^+–nutrient co-transporters[22]. Ultrastructural analyses of intestinal epithelial cells during Na^+–nutrient co-transport revealed condensation of the perijunctional actomyosin ring, suggesting that cytoskeletal contraction could be involved in this physiological process[21]. Subsequent studies confirmed this hypothesis and showed that the Ca^{2+}–calmodulin-dependent serine–threonine protein kinase myosin light chain kinase (MLCK) is essential for Na^+–nutrient co-transport-induced tight junction regulation[23,24]. Moreover, MLCK activation alone is sufficient to increase tight junction permeability, both *in vitro* and *in vivo*[25,26]. The mechanisms that mediate this

Paracellular pathway
The route of transepithelial transport that involves passive movement through the space between adjacent cells.

Adherens junction
Also known as the zonula adherens, this junction is immediately subjacent to the tight junction and requires the activity of lineage-specific Ca^{2+}-dependent adhesion proteins, termed cadherins.

Desmosome
An adhesive junction that connects adjacent epithelial cells. These junctions are composed of multiple protein subunits and are the points where keratin filaments attach to the plasma membrane.

Claudin
From the Latin *claudere*, meaning 'to close', members of this family of transmembrane proteins are variably expressed by specific epithelial cell types and thereby contribute to the unique barrier properties of different tissues.

Occludin
The first transmembrane tight junction protein identified. The function of occludin remains controversial but it is likely to have roles in barrier regulation and tumour suppression. It also serves as a cofactor in hepatitis C virus entry.

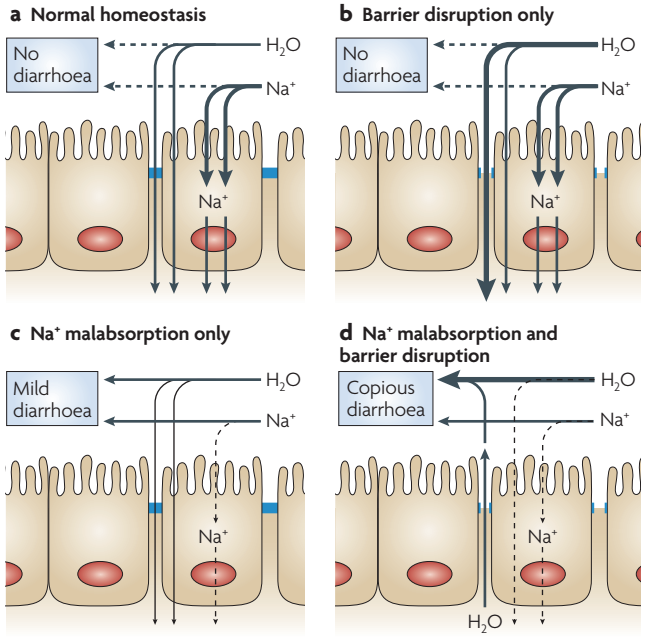
Zonula occludens 1
A peripheral membrane, or plaque, protein containing multiple protein interaction domains that, along with the related protein zonula occludens 2, is required for tight junction assembly.

Transcellular pathway
The route of transepithelial transport that involves active or passive movement across cell membranes, usually as a result of the action of specific transport channels.

Transepithelial transport
The sum of transport through the transcellular and paracellular pathways.

Box 1 | **Coordination of transcellular and paracellular transport**

The signal transduction pathway that enhances paracellular permeability following initiation of Na^+–glucose co-transport has been characterized and includes activation of mitogen-activated protein kinase cascades, trafficking of Na^+–H^+-exchange protein 3 (NHE3) to the apical membrane and myosin light chain kinase (MLCK) activation[23–24,107]. Increased NHE3 activity at the apical membrane enhances Na^+ absorption, which (along with the Na^+ absorbed as a result of Na^+–glucose co-transport) increases the transcellular Na^+ gradient and promotes paracellular water absorption (see the figure, part **a**). The MLCK-dependent increase in tight junction permeability enhances paracellular absorption of water and small solutes, such as glucose, that are concentrated in the unstirred layer. This might contribute to the observation that rehydration after infectious diarrhoea, or even exercise, is more effective when oral rehydration solutions contain Na^+ and carbohydrates[108] (see the figure, part **b**). By contrast, tumour necrosis factor activates both protein kinase Cα (PKCα) and MLCK to cause diarrhoea. NHE3 inhibition, mediated by PKCα, reduces the transcellular Na^+ gradient that normally drives water absorption (see the figure, part **c**). This synergizes with MLCK-dependent increases in tight junction permeability to allow water to flow into the lumen, thereby causing large-volume diarrhoea[17] (see the figure, part **d**).



MLCK-dependent tight junction regulation have been studied in detail owing to their contributions to nutrient and water absorption under normal physiological conditions and to the diarrhoea associated with acute barrier loss (BOX 1).

Barrier regulation by immune stimuli

The ability of cytokines, such as TNF and IFNγ, to regulate the function of the tight junction barrier was first described 20 years ago[27]. Since then, increased tight junction protein transcription, vesicular removal of proteins from the tight junction, tight junction protein degradation, kinase activation and cytoskeletal modulation have all been proposed to mediate cytokine-induced loss of tight junction barrier function. Although extensive apoptosis of epithelial cells may also cause barrier loss, the relevance of single-cell apoptosis to barrier dysfunction remains controversial owing to differing results in diverse experimental systems.

Tight junction regulation through the cytoskeleton. TNF and IFNγ modify tight junction barrier function in intestinal[27,28], renal[29], pulmonary[30] and salivary gland[31] epithelia as well as between endothelial cells[32]. The effects of TNF on barrier integrity have been best studied in the gut, where this cytokine has a central role in many diseases associated with intestinal epithelial barrier dysfunction, including inflammatory bowel disease[33], intestinal ischaemia[28,34] and graft-versus-host disease[35]. For example, although the effect of therapy with TNF-specific antibodies may be largely due to the overall reduction in inflammation, it is notable that this treatment corrects barrier dysfunction in patients with Crohn's disease[36]. Increased mucosal TNF production may also contribute to increased intestinal permeability and susceptibility to colitis in mice with defective mucin biosynthesis[10,37].

MLCK has been shown to have a central role in TNF-induced epithelial and endothelial barrier dysregulation, both *in vitro* and *in vivo*[16,38–41]. Similar to Na^+–nutrient co-transport, TNF-induced MLCK activation seems to increase paracellular flux through the leak pathway[16–17,23]. This MLCK activation occurs as a result of increased enzymatic activity and increased MLCK transcription and translation, both *in vitro* and *in vivo*[16,40,42]. Similarly, MLCK expression and activity are increased in intestinal epithelial cells of patients with inflammatory bowel disease[43]. The degree to which MLCK expression and activity are increased correlates with local disease activity, suggesting that these processes may be regulated by local cytokine signalling in these patients[43]. MLCK is also a fundamental intermediate in barrier dysfunction induced by the TNF family member LIGHT (also known as TNFSF14)[17,44], interleukin-1β (IL-1β)[45], enteropathogenic *Escherichia coli* infection[39], *Helicobacter pylori* infection[46], giardiasis[47], lipopolysaccharide[48,49] and the ethanol metabolite acetaldehyde[50]. Thus, MLCK activation can be viewed as a common final pathway of acute tight junction regulation in response to a broad range of immune and infectious stimuli.

Thick ascending limb of Henle
The portion of the nephron just proximal to the distal tubule. This is a site of active Na^+, K^+ and Cl^- reabsorption, which generates an electrochemical gradient that drives paracellular reabsorption of Mg^{2+} and Ca^{2+}.

Myosin light chain kinase
The Ca^{2+}–calmodulin-dependent kinase that phosphorylates myosin II regulatory light chain at serine 19 and threonine 18 to activate myosin ATPase.

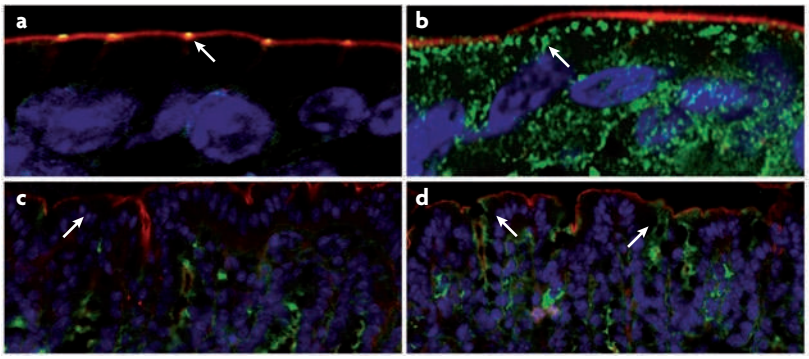


Figure 2 | **Differential effects of cytokines on tight junction structure and function. a** | Occludin is normally concentrated at the tight junction (arrow) in jejunal villus epithelium. The perijunctional actomyosin ring (red) and nuclei (blue) are shown for reference. **b** | Exogenous tumour necrosis factor increases myosin light chain kinase activity, which causes perijunctional myosin II regulatory light chain phosphorylation and triggers occludin (green) endocytosis (arrow). This increases flux across the tight junction leak pathway and enhances paracellular permeability to large solutes. **c** | Claudin-2 expression (green) is limited to crypt epithelial cells; it is not expressed by epithelial cells at the mucosal surface in the normal colon. F-actin (red) and nuclei (blue) are shown for reference. **d** | Interleukin-13 can stimulate claudin-2 expression (green) in surface epithelial cells (arrows). This increases flux across small tight junction pores, thereby enhancing paracellular cation permeability.

Although it is clear that MLCK phosphorylates myosin II regulatory light chain (MLC) within the perijunctional actomyosin ring to activate myosin ATPase activity, the subsequent molecular events that cause increased permeability are poorly defined. However, recent work suggests that ZO1, which interacts directly with actin, occludin, claudins and other proteins, may be an essential effector of perijunctional actomyosin ring-mediated tight junction regulation[25,51,52]. Endocytic removal of the transmembrane protein occludin from the tight junction is also common in actomyosin-dependent, cytokine-mediated tight junction regulation[38,53] (FIG. 2). *In vitro* studies of tight junction regulation induced by LIGHT suggest that occludin endocytosis occurs via caveolae and that inhibition of this process can prevent loss of barrier integrity despite MLCK activation[44]. Caveolar endocytosis of occludin has also been associated with loss of epithelial and endothelial tight junction barrier function in response to actin disruption[54] and chemokine signalling[55], respectively, *in vitro*. However, other mechanisms of occludin removal may also be involved as *in vitro* studies have shown that IFNγ-induced occludin internalization is mediated by myosin ATPase-dependent macropinocytosis[53,56], and occludin cleavage may even modify the barrier to enhance transepithelial migration of inflammatory cells in the lung[57]. Nevertheless, further work is necessary to define the contributions of occludin and endocytosis to *in vivo* tight junction regulation. This is particularly important as, despite numerous *in vitro* studies demonstrating a role for occludin in tight junction function, intestinal barrier function is intact in occludin-deficient mice[58]. Future analyses of the response of occludin-deficient mice to stress, with particular reference to intestinal tight junction function, as well as studies of potential compensatory changes in other, perhaps not yet discovered, proteins that allow these mice to avoid intestinal disease will be of great interest.

Caveolae
Specialized flask-shaped invaginations of the plasma membrane that contain the protein caveolin-1 and cholesterol. These proteins mediate uptake of some extracellular materials and are involved in cell signalling.

Although MLCK activation is clearly important, it is not the only means of cytoskeletal tight junction regulation. Other mediators include myosin ATPase[51], the activity of which is regulated by MLC phosphorylation[59–60]; members of the Rho kinase family[41,51,53,61], which can both phosphorylate MLC directly[59] and inhibit MLC phosphatase[62]; and AMP-activated protein kinase[52], which is activated during stress and can also directly phosphorylate MLC[63]. Moreover, Rho kinases and AMP-activated protein kinase each have diverse effects that are separate from myosin function, and it is likely that at least some of these contribute to tight junction regulation.

Tight junction permeability and claudin expression. As discussed above, regulation of perijunctional actomyosin provides a means of rapidly and reversibly regulating the paracellular leak pathway. By contrast, synthesis and trafficking of claudin proteins provides a means of regulating tight junction pores over longer periods. The perijunctional actomyosin ring does not seem to be directly involved in this mechanism of barrier regulation, which is consistent with the fact that claudins do not interact with actin directly. Expression of specific claudin proteins changes during development, differentiation and disease and in response to stressors — including cytokines — in intestinal[64], renal[65] and alveolar[66] epithelial cells. In addition to affecting barrier function, altered patterns of claudin expression may have other consequences. For example, claudin proteins have been associated with control of cell and organ growth. It may be, therefore, that some changes in claudin protein expression enhance cell proliferation and regeneration, as might be necessary to compensate for cell loss in colitis. This may explain the increased claudin-1 expression by intestinal epithelial cells of patients with inflammatory bowel disease[67]. However, claudin-1 has also been shown to enhance neoplastic transformation, tumour growth and metastasis in experimental models[68]. Thus, changes in claudin expression may have undesired consequences.

One common change in claudin expression that directly affects barrier function is the increased claudin-2 expression by intestinal epithelial cells in animal models of colitis (FIG. 2) and patients with inflammatory bowel disease[64]. Consistent with this, IL-13 and IL-17, which are increased in the mucosa of patients with colitis[64,69], reduce barrier function and increase claudin-2 expression in cultured intestinal epithelium monolayers[64,70]. *In vitro* studies have shown that claudin-2 expression increases the number of pores that allow paracellular flux of cations, predominantly Na^+, and small molecules with radii less than 4 Å[14,71], and recent analyses have provided new insight into the structure of these pores[72]. However, it is not clear whether increased claudin-2 expression contributes to disease progression or, as proposed above for claudin-1, is an adaptive

Table 1 | **Barrier defects associated with intestinal disease**

Disease or model	Cause of barrier defect	Timing of barrier defect	Effects on tight junction proteins and cytoskeleton	Role of commensal microorganisms	Refs
Crohn's disease	Unknown, associated with a frameshift insertion at nucleotide 3020 of *NOD2*	Before clinical onset and before clinical relapse	Claudin-2 upregulation, MLCK activation and occludin downregulation	Antibiotics can be helpful in maintaining remission	43,64,109
Ulcerative colitis	Unknown	Not well studied	Claudin-2 upregulation, MLCK activation and occludin downregulation	Not defined	43,64,109
IL-10-deficient mice	Immune signalling (IL-10 deficiency)	Before clinical onset	Not defined	Eradication of microorganisms prevents disease	76,97
Coeliac disease	Not well studied	Not well studied	Not defined	No demonstrated role	110,111
Systemic T cell activation	MLCK activation	Associated with acute diarrhoea	MLCK activation and occludin endocytosis	Not defined	38
$CD4^+CD45RB^{hi}$ T cell adoptive transfer model of colitis	Cytokine release and epithelial cell damage	With clinical onset	MLCK activation, claudin-2 upregulation and occludin endocytosis	Eradication of microorganisms reduces severity	112–114
SAMP1/yit mice	Unknown	Before clinical onset	Claudin-2 upregulation and occludin downregulation	Not defined	77
DSS-induced colitis	Epithelial cell damage	After DSS treatment but before clinical onset	Not defined	Eradication of microorganisms exacerbates disease	80
TNBS-induced colitis	Immune signalling	Not well studied	Claudin-18 upregulation	Probiotics can reduce disease severity	92
Mucin-2-deficient mice	Not well studied	Not well studied	Not defined	Not defined	10,37
MDR1A-deficent mice	Unknown, follows increased CCL2 production	After immune cell activation	Reduced occludin phosphorylation	Increased epithelial cell response to LPS precedes disease	115,116
JAM-A-deficient mice	JAM-A deficiency	Not well studied	JAM-A deficiency	Not defined	89,90
Clostridium difficile-induced colitis	Actomyosin disruption and glucosylation of Rho proteins	With release of toxin and disease onset	Loss of ZO1 and ZO2	Antibiotics predispose to disease	117
EPEC infection	Type III secretion (of bacterial proteins)	After infection	MLCK activation and occludin endocytosis	Not defined	118
Graft-versus-host disease	Associated with elevated TNF production	After clinical onset	Not defined	Eradication of microorganisms limits disease	35

CCL2, CC-chemokine ligand 2; DSS, dextran-sulphate sodium; EPEC, enteropathogenic *Escherichia coli*; IL-10, interleukin-10; JAM-A, junctional adhesion molecule-A; LPS, lipopolysaccharide; MDR1A, multidrug resistance protein 1a; MLCK, myosin light chain kinase; *NOD*, nucleotide-binding oligomerization domain; TNF, tumour necrosis factor; TNBS, trinitrobenzene sulphonic acid; ZO, zonula occludens.

response that promotes homeostasis. Nevertheless, the consistency of intestinal epithelial claudin-2 upregulation in disease suggests that this should be a subject of further study.

Barrier function and immunity

Barrier loss is associated with disease risk. A large body of circumstantial evidence suggests that intestinal barrier dysfunction is associated with the pathogenesis of Crohn's disease. This includes the observation that a subset of first-degree relatives of patients with Crohn's disease has increased intestinal permeability despite being completely healthy[73]. Interestingly, genetic analyses have linked increased intestinal permeability in these healthy relatives to the Crohn's disease-associated frameshift insertion at nucleotide 3020 of the gene encoding the cytoplasmic sensor nucleotide-binding oligomerization domain-containing 2 (*NOD2*)[74] (TABLE 1), and the same *NOD2* mutation has been associated with decreased IL-10 production by peripheral blood mononuclear cells[75]. Thus, one explanation for the increased intestinal permeability observed in some of these healthy relatives is that they have subclinical mucosal immune activation, perhaps with increased TNF production, that leads to barrier dysfunction without overt disease. This hypothesis would also explain why IL-10-deficient and SAMP1/yit mice, which develop spontaneous colitis and enteritis, respectively, show increased intestinal permeability before disease onset[76,77]. The suggestion that permeability is merely a sensitive indicator of mucosal

SAMP1/yit mice
An outbred mouse strain that spontaneously develops a chronic intestinal inflammation similar to human Crohn's disease.

immune activation would also explain the observation that, during clinical remission, increased intestinal permeability is a predictor of relapse in patients with Crohn's disease[78]. However, barrier dysfunction must also be regarded as a potential contributor to disease progression.

The classic experiment showing a role for epithelial cell function in maintaining mucosal immune homeostasis used chimeric mice in which dominant-negative cadherin was expressed in some intestinal epithelial stem cells, resulting in disruption of the adherens junctions[13]. This led to profound epithelial cell defects that included incomplete polarization, brush border and actin cytoskeletal disruption, accelerated crypt–villus migration and premature apoptosis[13]. Moreover, the mucus layer overlying epithelial cells expressing dominant-negative cadherin was disrupted and numerous adherent bacteria were present at these sites[13]. Patchy, transmural enteritis developed in these mice and was limited to regions where epithelial cells expressed dominant-negative cadherin[79]. In addition, epithelial cell dysplasia was occasionally present in these areas[79]. This study has often been cited as evidence that tight junction barrier loss is sufficient to cause inflammatory bowel disease and, although tight junctions were not specifically examined, they were likely to be defective. However, although this important study shows that apical junctional complex disruption causes enteritis, the broad range of epithelial cell defects induced makes it impossible to draw conclusions regarding the specific contributions of the tight junction to homeostasis or disease. Similarly, although dextran-sulphate sodium (DSS)-induced colitis is associated with increased intestinal permeability, this is the result of widespread epithelial cell damage[80] rather than targeted dysfunction of the tight junction barrier. Altered sensitivity of genetically modified mice to DSS must therefore be viewed in the context of epithelial cell injury and repair[81–83] and cannot be interpreted as a function of disrupted tight junction permeability alone.

Isolated barrier loss is insufficient to cause disease. One study has reported an *in vivo* model of barrier dysfunction induced by direct activation of endogenous tight junction regulatory mechanisms[26]. Although no overt developmental defects or disease developed in mice in which intestinal epithelial cells transgenically expressed constitutively active MLCK, increased intestinal tight junction permeability was observed[26]. This increase in paracellular permeability was quantitatively similar to that induced by activation of Na^+–glucose co-transport and was not an indiscriminate loss of barrier function[26], as occurs with exposure to DSS or dominant-negative cadherin expression. In addition, MLCK inhibition normalized MLC phosphorylation in epithelial cells and paracellular permeability[26], suggesting that compensatory changes in pathways that regulate MLC phosphorylation or barrier function were not induced. Moreover, growth of these mice was normal, and intestinal morphology, enterocyte structure, tight junction and adherens junction organization, actomyosin and brush border architecture, and epithelial proliferation, migration and apoptosis were all similar to that observed in wild-type mice[26]. This suggests that the effects of constitutively active MLCK expression in these mice were specific to its effect on the tight junction. Thus, consistent with the presence of barrier dysfunction in healthy relatives of patients with Crohn's disease, these mice provide evidence to suggest that increased tight junction permeability in the absence of more extensive epithelial cell dysfunction is insufficient to cause intestinal disease. Further analysis of mice expressing constitutively active MLCK did, however, provide evidence of mucosal immune cell activation, including increased numbers of lamina propria T cells, enhanced mucosal IFNγ, TNF and IL-10 transcription and repositioning of $CD11c^+$ DCs to the superficial lamina propria[26]. Thus, despite being insufficient to cause disease, chronic increases in intestinal permeability as a result of continuous activation of a physiological pathway of tight junction regulation does lead to mucosal immune cell activation.

Because a subset of healthy first-degree relatives of patients with Crohn's disease will ultimately develop the disease, and because a similar fraction of these healthy relatives have increased intestinal permeability, it has been suggested that tight junction barrier dysfunction is one factor that contributes to the development of inflammatory bowel disease[84]. This hypothesis is consistent with a case report documenting increased intestinal permeability 8 years prior to disease onset in a healthy relative of a patient with Crohn's disease[85]. However, given that the subject of the case report had two first-degree relatives with Crohn's disease and was, therefore, at increased risk of developing the disease regardless of intestinal permeability measures, this report does not provide evidence that barrier dysfunction itself is related to disease onset.

The relationship between barrier dysfunction and disease has been assessed using mice with a constitutively active MLCK transgene, which have increases in permeability that are quantitatively and qualitatively similar to those in healthy relatives of patients with Crohn's disease. Recombination-activating gene 1-knockout mice (which lack mature B and T cells) expressing constitutively active MLCK, as well as littermates that did not express constitutively active MLCK, received $CD4^+CD45RB^{hi}$ naive T cells from wild-type mice (the $CD4^+CD45RB^{hi}$ T cell adoptive transfer colitis model). Both groups of mice developed colitis. However, the disease developed more rapidly and was more severe, in clinical, biochemical and histological terms, in the mice that expressed constitutively active MLCK[26]. Although one could argue that this accelerated disease progression was due to effects of constitutively active MLCK expression beyond tight junction regulation, other epithelial cell defects were not apparent in these mice (see above), perhaps because constitutively active MLCK targets an endogenous signalling pathway. Thus, targeted increases in intestinal epithelial tight junction permeability through constitutive activation of the pathophysiologically relevant MLCK pathway are sufficient to accelerate the onset and enhance the severity of immune-mediated colitis.

$CD4^+CD45RB^{hi}$ T cell adoptive transfer colitis model
A well-characterized model of chronic colitis induced by transfer of $CD4^+CD45RB^{hi}$ (naive) T cells from healthy wild-type mice into immunodeficient syngeneic recipients.

Conversely, the data above also suggest that inhibition of MLCK, which is sufficient to reverse acute TNF-induced barrier loss[38], may have therapeutic benefit in immune-mediated colitis. This notion is supported by a preliminary *in vivo* report using mice with a knockout of non-muscle MLCK[86]. However, as non-muscle MLCK has many functions, including an essential role in neutrophil transendothelial migration[87], the results of this report may reflect changes beyond tight junction barrier preservation. Another recent study showed that a peptide capable of enhancing small-intestinal barrier function reduced the extent of mucosal inflammation in IL-10-deficient mice[88]. However, this result must be interpreted with caution, as the peptide used (which is thought to antagonize the putative extracellular pathway signalling molecule zonulin) activates an undefined regulatory pathway and the overall physiology of peptide-treated mice has not been examined in detail. Thus, although the finding is intriguing, further investigation is necessary to determine whether barrier preservation, by MLCK inhibition or other means, can prevent or reverse intestinal disease.

Two studies have recently reported that junctional adhesion molecule-A (JAM-A)-deficient mice have reduced intestinal barrier function and increased rates of intestinal epithelial cell proliferation and apoptosis[89,90]. These mice also have intestinal mucosal immune cell activation as defined by increased numbers of neutrophils in the intestinal mucosa[89,90]. In addition, one study reported an increase in the number of distal-colonic lymphoid aggregates in JAM-A-deficient mice[90], although neither lymphoid aggregates nor enhanced mucosal cytokine expression was detected in a subsequent analysis[89]. However, as JAM-A is widely expressed by epithelial cells, endothelial cells, platelets, antigen-presenting cells, circulating neutrophils, monocytes, lymphocytes and platelets, and as the mice studied were not epithelial cell-specific knockouts, it is not clear whether the observed effects were due to altered tight junction integrity, changes in epithelial cell shape[91], an increased rate of epithelial cell apoptosis[90] or trapping of neutrophils within mucosal vessels owing to the loss of endothelial cell-expressed JAM-A. Nevertheless, it is intriguing that JAM-A-deficient mice showed increased sensitivity to DSS-induced colitis[89,90]. By contrast, the sensitivity of mice with an endothelium-specific JAM-A deficiency to DSS was similar to that of wild-type control mice, indicating that the response of mice with universal JAM-A deficiency was not solely due to loss of endothelial JAM-A[89]. Unfortunately, the use of mice with a universal JAM-A deficiency and the presence of other epithelial cell abnormalities, particularly increased epithelial cell turnover, in the absence of exogenous stimuli limits interpretation of these studies. Even so, data from JAM-A-deficient mice do support the hypothesis that mucosal barrier loss can enhance the severity of colitis.

Overall, data from both human subjects and mouse experimental models show that defects in tight junction barrier function are insufficient to cause disease. However, several lines of evidence suggest that increased paracellular permeability can increase mucosal immune activity, enhance disease progression and severity and, possibly, be a risk factor for development of disease. Finally, although much more investigation is needed, early reports indicate that restoration of tight junction barrier function may be effective, either alone or in combination with other agents, in preventing disease in at-risk individuals or maintaining remission in patients with inflammatory bowel disease.

Barrier loss activates immunoregulatory processes. Why is tight junction barrier dysfunction alone insufficient to cause disease? Endoscopic mucosal resection, which removes the epithelium and mucosa completely and therefore causes barrier loss far greater than that caused by targeted tight junction dysfunction, is insufficient to cause chronic disease. Therefore, immunoregulatory mechanisms must be induced by the host following barrier loss to prevent inappropriate inflammatory responses, as mucosal damage is a daily occurrence in the gastrointestinal tract. Moreover, these immunoregulatory mechanisms must be robust because, even in patients with inflammatory bowel disease, endoscopic mucosal resection is insufficient to initiate a relapse to active disease.

The increased transcription of IL-10 in the intestinal mucosa of mice with a constitutively active MLCK transgene may provide a clue to which immunoregulatory processes are triggered by barrier dysfunction. In addition, responses to transient barrier loss have been used to explore mucosal immunoregulation[92]. In this model, intrarectal administration of ethanol caused transient epithelial cell damage, mucosal erosion and barrier loss. The induction of barrier loss was followed by an increase in the numbers of IFNγ- and IL-10-producing lamina propria mononuclear cells and lamina propria $CD4^+CD25^+$ T cells that express latency-associated peptide (LAP) on their surface[92]. Remarkably, such ethanol administration conferred protection from subsequent trinitrobenzene sulphonic acid (TNBS)-induced colitis, and this protection required the presence of LAP^+ T cells[92]. The induction of these LAP^+ T cells was shown to depend on $CD11c^+$ DCs, Toll-like receptor 2 signalling and a normal luminal microorganism population[92].

Although further study is needed to understand the mechanisms of LAP^+ T cell induction by transient mucosal damage, it is interesting that in different experimental systems increased intestinal epithelial cell tight junction permeability increased the number of $CD11c^+$ DCs in the superficial lamina propria[86], and that interactions with intestinal epithelial cells enhance the ability of bone marrow-derived $CD11c^+$ DCs to induce the differentiation of regulatory T cells[93]. Together, these observations support the hypothesis that the interactions between $CD11c^+$ DCs and luminal materials are regulated by tight junction permeability and are also central to mucosal immune homeostasis.

Other data also support important roles for luminal material, particularly microorganisms and their products, in mucosal immune regulation[94]. For example, antibiotics can be helpful in the management of Crohn's disease[95]. Although the mechanisms by which antibiotics

Latency-associated peptide
A small peptide derived from the N-terminal region of the TGFβ precursor protein; it can modulate TGFβ signalling.

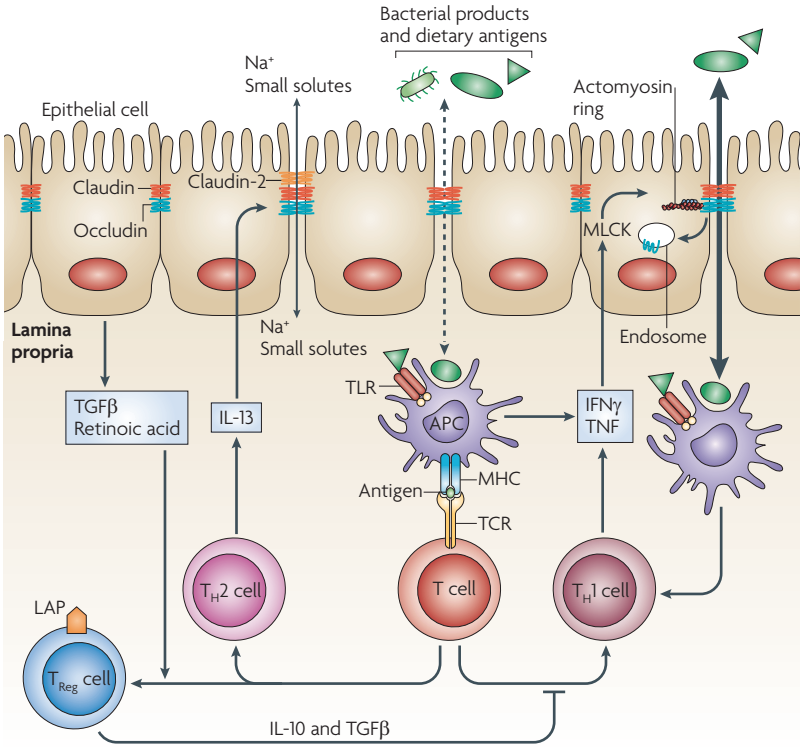


Figure 3 | **The epithelium and tight junction as integrators of mucosal homeostasis.** Minor barrier defects allow bacterial products and dietary antigens to cross the epithelium and enter the lamina propria. This can lead to disease or homeostasis. If the foreign materials are taken up by antigen-presenting cells (APCs), such as dendritic cells, that direct the differentiation of T helper 1 (T_H1) or T_H2 cells, disease can develop. In this process, APCs and T_H1 cells can release tumour necrosis factor (TNF) and interferon-γ (IFNγ), which signal to epithelial cells to increase flux across the tight junction leak pathway, thereby allowing further leakage of bacterial products and dietary antigens from the lumen into the lamina propria and amplifying the cycle of inflammation. This may, ultimately, culminate in established disease. Alternatively, interleukin-13 (IL-13) released by T_H2 cells increases flux across small cation-selective pores, potentially contributing to ongoing disease. Conversely, homeostasis may dominate if APCs promote regulatory T (T_{Reg}) cell differentiation, which can be enhanced by epithelial cell-derived transforming growth factor-β (TGFβ) and retinoic acid. The T_{Reg} cells display latency-associated peptide (LAP) on their surfaces and may secrete IL-10 and TGFβ to prevent disease. MLCK, myosin light chain kinase; TLR, Toll-like receptor; TCR, T cell receptor.

promote maintenance of remission in patients with Crohn's disease are not defined, they may be related to the observation that luminal microorganisms are required in experimental models of inflammatory bowel disease that include IL-10-deficient mice[96,97] and adoptive transfer colitis. Microorganisms are also necessary for the development of increased intestinal permeability before the onset of overt disease in IL-10-deficient mice[76]. Although this observation is not entirely understood, one interpretation is that the limited flux of microbial products that normally occurs across intact intestinal epithelial tight junctions is sufficient to trigger mucosal immune activation in IL-10-deficient mice. This mucosal immune activation could, in turn, result in the release of cytokines that cause increased intestinal permeability and accelerate disease progression (FIG. 3). However, in addition to immune cells, epithelial cells can respond to foreign materials. Lipopolysaccharide, for example, enhances paracellular permeability in pulmonary and intestinal epithelium[48,49]. Conversely, epithelial Toll-like receptor 2 activation may restore barrier function[98]. It will therefore be of interest to define the microbial products, dietary components and other factors that influence mucosal immune status following alterations in intestinal epithelial tight junction permeability.

Tight junctions integrate mucosal homeostasis

One interpretation of the available data is that the tight junction barrier integrates the relationship between luminal material and mucosal immune function (FIG. 3). In most individuals, this is a healthy relationship in which regulated increases in tight junction permeability or transient epithelial cell damage trigger the release of pro-inflammatory cytokines, such as TNF and IFNγ, as well as immunoregulatory responses. Immunoregulatory responses may include DC conditioning by epithelial cell-derived transforming growth factor-β (TGFβ) and retinoic acid, both of which can enhance regulatory T cell differentiation[99,100]. This precarious balance between pro-inflammatory and immunoregulatory responses can fail if there are exaggerated responses to pro-inflammatory cytokines, as may be associated with mutations in the endoplasmic reticulum stress response transcription factor X-box-binding protein 1 (XBP1) (REF. 101), insufficient IL-10 production (as in IL-10-deficient mice and, possibly, in patients with *IL10* promoter polymorphisms[102]) or inadequate immune tolerance to luminal antigens and microbial products[94] (potentially because of *NOD2* mutations in patients with inflammatory bowel disease[103–106]). As a result, mucosal immune cell activation may proceed unchecked and the release of cytokines, including TNF and IL-13, may enhance barrier loss that, in turn, allows further leakage of luminal material and perpetuates the pro-inflammatory cycle. This model highlights the roles of a susceptible host and defective epithelial cell barrier function as key components of the pathogenesis of intestinal inflammatory disease and explains the crucial role of the epithelial barrier in moulding mucosal immune responses.

Conclusions

Significant progress has been made in understanding the processes by which physiological and pathophysiological stimuli, including cytokines, regulate the tight junction. Early reports suggest that restoration of tight junction barrier function may have benefit[38,86]. However, the mechanisms of tight junction regulation will have to be defined in greater detail if they are to be viable pharmacological targets. Conversely, recent data have emphasized the presence of immune mechanisms that maintain mucosal homeostasis despite barrier dysfunction, and some data suggest that the epithelium orchestrates these immunoregulatory events through direct interactions with innate immune cells. Future elucidation of the processes that integrate mucosal barrier function, or dysfunction, and immune regulation to prevent or perpetuate disease may lead to novel therapeutic approaches for diseases associated with increased mucosal permeability.

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DATABASES
UniProtKB: http://www.uniprot.org
α-catenin 1 | β-catenin | catenin δ1 | claudin-1 | claudin-2 | claudin-16 | E-cadherin | IFNγ | IL-1β | IL-10 | JAM-A | LAP | LIGHT | MLCK | NHE3 | TNF | XBP1 | ZO1 | ZO2

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