No Static at All

A New Perspective on Molecular Architecture of the Tight Junction

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Permeability of the intestinal epithelial barrier is regulated in response to physiological and pathophysiological stimuli. Recent work has characterized a critical role of acute tight junction regulation in diarrhea secondary to T cell activation and cytokine release. The intracellular mediators of the ensuing barrier dysfunction include myosin light chain kinase, which phosphorylates myosin II regulatory light chain and triggers structural tight junction reorganization. While the molecular intermediates in this reorganization are not defined, the new discovery that individual tight junction–associated proteins are highly dynamic at steady state may provide insight into the mechanisms of regulation.

Key words: **occludin; myosin; TNF; FRAP**

Epithelial surfaces function to define the boundary between various tissue compartments. While many roles are similar, there are some diverse, tissue-specific functions. For example, the skin maintains homeostasis and prevents systemic infections.[1] In contrast, the epithelium in the renal tubule forms a barrier to maintain gradients between the renal interstitium and the sterile tubular lumen in order to support transport and regulate urine composition.[2] The intestinal epithelium is unique in this regard because, like the skin, it must provide a barrier to prevent contamination of the interstitial tissue from the harsh environment of the intestinal lumen; but, like the renal tubule, it must also support nutrient and water transport.[3–5]

The intestinal epithelial barrier is formed by the intestinal epithelial cell membranes. These membranes are impermeable to solutes in the absence of specific transporters. The potential shunt between cells is sealed by intercellular junctions, thus limiting paracellular flux. These junctions comprise two major protein complexes, the tight junctions and the adherens junctions. Adherens junctions are basal to the tight junctions and primarily formed by E-cadheren, catenins, and are actin filaments. These do not create a barrier but do provide the strength necessary to hold the cells together and form a continuous epithelium.[6] Conversely, the tight junctions, which are composed of claudin family proteins, other transmembrane proteins, such as occludin, and a broad spectrum of cytoplasmic peripheral membrane proteins,[7–11] form the barrier but provide little strength to this intercellular bond.[12,13] Thus, the intestinal tight junction must balance the need for a barrier with the necessity of water, salt, and nutrient transport. As a result, intestinal tight junction function is a remarkable example of precisely-regulated function.[14]

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Barrier Regulation in Health and Disease

The modifications of tight junction barrier function that occur in response to physiological and pathophysiological stimuli make this an ideal model in which to study regulatory mechanisms.[14] Although the existence of this regulation, or even the possibility of transport across the tight junction, was not appreciated intially[15–19] there is a growing understanding of the critical role paracellular transport plays in health and disease.[20–27] For example, activation of Na^+-nutrient cotransport, most significantly by the Na^+-glucose cotransporter SGLT1, increases paracellular permeability and allows paracellular water and nutrient absorption to amplify the saturable transcellular pathways.[5,12,21,28,29] Increased paracellular permeability is also well recognized in patients with inflammatory bowel disease.[25,26] This has been most carefully studied in patients with Crohn's disease (CD) involving the small intestine because, until recently, probes were available to measure small intestinal, but not colonic, barrier function in human subjects *in vivo*. Notably, increased permeability is present in both CD patients and a subset of their healthy first-degree relatives, leading to the hypothesis that reduced intestinal barrier function may contribute to CD pathogenesis.[24] This is supported by the observation that, during clinical remission, increased permeability is associated with disease relapse in CD patients.[30] However, the observation that barrier dysfunction is genetically linked to mutations in NOD2/CARD15 supports the alternative hypothesis that increased permeability in CD is the result, rather than cause, of disease.[31] This clinical observation is also consistent with reports that barrier dysfunction precedes clinical disease in IL-10 knockout mice.[32]

MLCK Mediates TNF-Induced Barrier Loss

The mechanisms of barrier dysfunction in humans and mice are not well understood, but it is clear from studies of human patients and murine models of intestinal disease that tumor necrosis factor-α (TNF) is a critical component of these processes.[33–35] This is consistent with the clinical utility of anti-TNF therapy in CD, although immune cells are likely to be the major target of this treatment.

The molecular events surrounding TNF-mediated tight junction regulation are becoming clear. Exposure of cultured epithelial monolayers to TNF *in vitro* can cause barrier loss.[36] This tight junction regulation occurs within hours and, in the acute interval, is mediated by myosin light chain kinase (MLCK), which phosphorylates myosin II regulatory light chain (MLC).[37] Remarkably, specific inhibition of MLCK can prevent *in vitro* tight junction barrier dysfunction.[37–39] The mechanisms by which TNF activates MLCK may include initiation of an increase in intracellular Ca^{2+}, but this has not been observed in intestinal epithelium. TNF also induces transcriptional activation of MLCK, both in human intestinal epithelial cell lines *in vitro* and in mouse enterocytes *in vivo*.[38,40] Moreover, this process appears to be active in human inflammatory bowel disease patients *in vivo*, as both MLCK expression and MLC phosphorylation are increased in association with active disease (Fig. 1).[41] Interestingly, in human patients, the extent of increased MLCK expression and MLC phosphorylation correlate with the magnitude and severity of disease, suggesting a relationship between MLCK expression and disease activity.[41]

In addition to the above data, *in vivo* studies of immune-mediated diarrheal disease demonstrate the crucial role of barrier function in this process. Acute, systemic T cell activation using an anti-CD3 antibody causes a self-limited, T cell mediated diarrhea in humans and mice.[42–44] When analyzed in detail, using an *in vivo* perfusion approach, we found that systemic T cell activation induces net reversal of water flow, from absorption to secretion (diarrhea) that is associated with defective epithelial barrier function.[43] This barrier dysfunction was not associated with ulceration of the

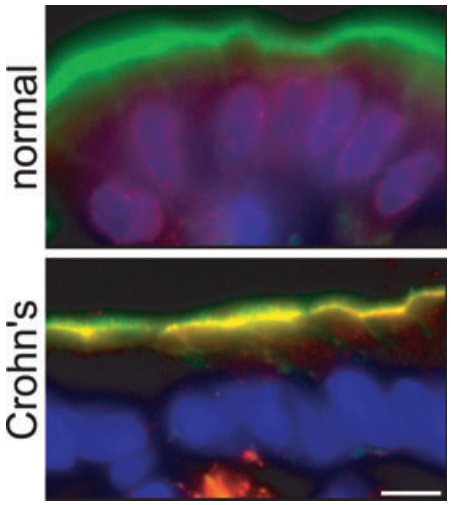

Figure 1. *B+W Version:* Epithelial MLCK expression is increased in CD. MLCK expression is limited, though detectable, in the cytoplasm and at the apical actomyosin ring, of ileal enterocytes in a normal subject. Enterocyte MLCK expression is increased, primarily within the perijunctional actomyosin ring, in active CD. Bar = 5 μm. (With permission from Blair et al.[41]) *Color Version:* Epithelial MLCK expression is increased in CD. MLCK expression (red) is limited, though detectable, in the cytoplasm and at the apical actomyosin ring (green), of ileal enterocytes in a normal subject. Enterocyte MLCK expression is increased, primarily within the perijunctional actomyosin ring, in active CD. Nuclei are shown in blue. Bar = 5 μm. (With permission from Blair et al.[41])

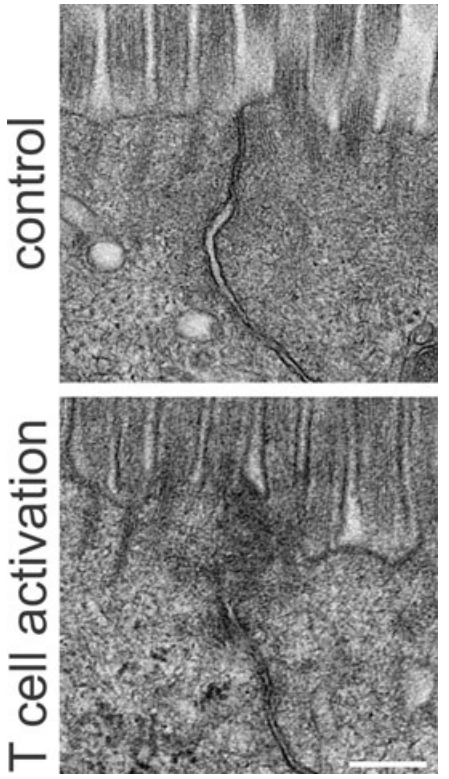

Figure 2. Systemic T cell activation induces cytoskeletal condensation at the tight junction. Electron micrographs of villous enterocytes of control and anti-CD3–treated mice demonstrate marked perijunctional cytoskeletal condensation after T cell activation. Bar = 250 nm). (With permission from Clayburgh et al.[43])

mucosa, nor epithelial apoptosis, and, therefore, could be localized to the tight junction.

Ultrastructural examination of enterocytes before and after *in vivo* T cell activation demonstrated condensation of the perijunctional cytoskeleton (Fig. 2) that was remarkably similar to the condensation associated with Na^+-nutrient cotransport-dependent tight junction regulation.[20,22] Since Na^+-nutrient cotransport-dependent tight junction regulation is mediated by MLCK-mediated phosphorylation of perijunctional MLC,[45–47] we asked if MLC phosphorylation was also triggered by anti-CD3.[43] This was in fact the case, and either pharmacologic or genetic MLCK inhibition was able to completely prevent TNF-induced barrier loss. Perhaps more critically, MLCK inhibition also restored net water absorption, providing data supporting the sometimes controversial concept of paracellular water transport.

Coordination of Paracellular and Transcellular Transport

Despite this central role of cytoskeletally mediated tight junction regulation in acute diarrhea, the observation that MLCK inhibition completely restored barrier function after T cell activation but only partially restored water absorption suggested that other factors were involved. We therefore examined the effects of individual T cell-derived cytokines.

Direct injection of recombinant TNF caused diarrhea that was qualitatively and quantitatively similar to that induced by anti-CD3.[27] In contrast, the TNF core family member LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells), which is also released following anti-CD3-induced T cell activation,[27] induced MLCK-dependent barrier dysfunction but enhanced water absorption.[27] This marked difference was due to the inhibition of NHE3-mediated Na^+ absorption by TNF, but not LIGHT,[27] and emphasizes the precisely orchestrated interplay between transcellular and paracellular transport that occurs in the intestine.

Mechanisms of Cytokine-induced Tight Junction Reorganization

To better understand the molecular mechanisms of *in vivo* tight junction regulation, we examined the distribution of individual tight junction proteins before and after exposure to T cell-derived cytokines. The distribution of most proteins was unchanged.[43] However, minor changes were observed in the distributions of ZO-1 and JAM-A. More strikingly, occludin was almost completely removed from the tight junction and internalized into cytoplasmic vesicles.[43] Remarkably, this *in vivo* tight junction reorganization almost perfectly matched that induced by acute treatment of cultured monolayers with the actin-depolymerizing drug latrunculin A.[48] *In vitro* latrunculin A-induced occludin internalization required dynamin II-dependent caveolar endocytosis.[48] However, *in vitro* occludin endocytosis has also been reported to occur via macropinocytosis and clathrin-mediated endocytosis. Thus, the relevant pathophysiological pathway of occludin endocytosis is of great interest. Detailed examination of occludin internalization induced by LIGHT in cultured intestinal epithelial monolayers demonstrated that this occurs via caveolae; internalized occludin colocalized with caveolin-1 and both LIGHT-induced occludin endocytosis and barrier loss were prevented by inhibition of caveolar endocytosis.[49] This inhibition of endocytosis does not prevent enzymatic activation of MLCK and, importantly, inhibitors of macropinocytosis and clathrin-mediated endocytosis did not prevent either endocytosis or barrier loss induced by LIGHT.[49] This finding is important, as it demonstrates the functional requirement for caveolar endocytosis in barrier dysfunction induced by proinflammatory cytokines.

While this evidence for a functional role of endocytosis in the barrier dysfunction induced by TNF is important in furthering our understanding of the effect of TNF on the tight junction and its role in the pathogenesis of diseases characterized by intestinal barrier defects, the mechanism of TNF-induced *in vivo* occludin internalization and means by which this affects barrier function remain unknown. In the past, occludin internalization following TNF treatment has been observed in fixed tissue from murine jejunal epithelium collected several hours after TNF treatment, after barrier loss has occurred. Although live imaging of tight junctions in intact intestinal mucosa has not yet been observed, this has been reported in cultured epithelial monolayers.[48,50–52] For example, one study noted claudin endocytosis during cell movement within an epithelial sheet.[50] Another study noted that the intensity of fluorescent-tagged occludin seemed to waiver in stable tight junctions.[48] Together, these studies emphasize the dynamic nature of tight junctions and the potential of live imaging to advance our understanding of tight junction function.

Tight Junction Structure Is Dynamic at Steady State

Recently, we have used monolayers of cultured epithelial cells to explore dynamics of tight junction structure at steady

state. This had not been studied previously because the large number of direct and indirect interactions between tight junction proteins led most to conclude that the tight junction is stable at steady state.[11] This view was supported by limited fluorescence recovery after photobleaching (FRAP) studies performed in fibroblasts transfected with claudin-1-EGFP (enhanced green fluorescent protein).[52] While the strands formed by claudin-1-EGFP were dynamic, FRAP analysis suggested that claudin molecules were not mobile within these strands.[52] However, even though claudin-1-EGFP formed strands, it is important to recognize that fibroblasts do not form tight junctions, even after transfection of claudin proteins.[52] Moreover, the presence of EGFP at the carboxy terminus of these fusion proteins prevented association with ZO-1 and ZO-2, which is necessary for efficient delivery of claudins to tight junctions.[9]

We developed fusion proteins of claudin-1, occludin, and ZO-1.[48] In each case, the fluorescent tag was placed at the amino terminus of the tight junction protein, away from defined protein interaction domains. The fusion proteins co-localized with their endogenous counterparts, both morphologically and biochemically, and did not disrupt normal tight junction function.[48] Moreover, expression of the ZO-1 and occludin constructs in enterocytes of transgenic mice had no effect on normal intestinal function. Thus, particularly in monolayers in which the fusion proteins were expressed at levels similar to or less than the endogenous proteins, these fluorescent-tagged proteins represent a powerful tool for the analysis of tight junction structure and function.[48,53]

Monolayers expressing these validated claudin-1, occludin, ZO-1, and β-actin EGFP fusion proteins were studied by FRAP.[53] The results showed that approximately 70% of occludin and ZO-1 are mobile within the tight junction (Fig. 3). In contrast, only 30% of claudin-1 is mobile. Detailed analysis showed that the FRAP behavior of these proteins was differentially affected by exogenous stimuli. Occludin, but not ZO-1, mobility was inhibited by disruption of membrane fluidity by reduced temperature[54] and cholesterol depletion. In contrast, ZO-1, but not occludin, exchange was blocked by metabolic depletion. These observations were explained by subsequent FRAP and fluorescence loss in photobleaching (FLIP) studies showing that occludin and claudin-1 FRAP behavior is due to diffusion within the plasma membrane, while ZO-1 exchanges with a large cytoplasmic pool.[53]

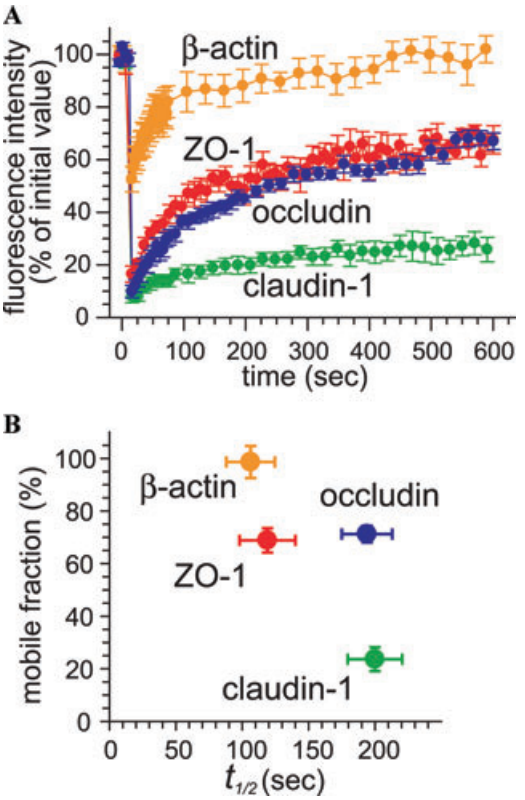

Figure 3. Individual tight junction proteins display distinct patterns of fluorescent recovery after photobleaching (FRAP) in polarized epithelia. (**A**) Quantitative analysis of FRAP in cells expressing EGFP-tagged fluorescent occludin, claudin-1, ZO-1, and β-actin. (**B**) The mobile fraction and $t_{1/2}$ of recovery for each protein are unique. (With permission from Shen et al.[53])

Mathematical modeling of FRAP and FLIP data suggests that occludin and claudin-1 may diffuse within the membrane at similar rates, but that all of occludin, as opposed to only 40% of claudin-1, is available for exchange.[53]

Thus, the remaining 60% of tight junction-associated claudin-1 is immobile. Modeling of ZO-1 showed that only 40% of total cellular ZO-1 is present at the tight junction. The remaining 60% is present in an intracellular, likely cytoplasmic, pool. This was a surprising result, as images of EGFP-ZO-1 as well as immunofluorescent ZO-1 labeling rarely detect cytoplasmic ZO-1. Of the 40% of ZO-1 present at the tight junction, ~60% is available for exchange and ~40% is nonexchangeable.[53]

The factors that anchor the nonexchangeable pools of ZO-1 and claudin-1 at the tight junction are unknown. However, since ZO-1 and claudin-1 can bind to one another directly, we considered the possibility that these might anchor one another. To address this experimentally, the motif at the carboxy terminus of claudin-1 that mediates binding to ZO-1 was deleted. As expected, this claudin-1 mutant was inefficiently delivered to tight junctions.[9,53] However, the portion of mutant claudin delivered to the tight junction was at least as stable as full-length claudin-1.[53] Thus, direct interactions with ZO-1 are not required for claudin-1 stabilization at the tight junction. However, as these cells also expressed endogenous full-length claudin-1, it remains possible that the mutant claudin-1 molecules were stabilized as part of a complex with endogenous claudin-1 and ZO-1. Consistent with this hypothesis, some claudins have been shown to form homophilic interactions with one another.[55]

When considered as a whole, these recent discoveries suggest a new dynamic view of the tight junction at steady state.[53,56] This ongoing molecular remodeling may set the stage for the rapid functional and structural responses of the tight junction to extracellular and intracellular stimuli such as TNF (Fig. 4).[18,19,38,45,57–60] Thus, it will be important to define the structural factors and signaling events that regulate tight junction protein stability. Such discoveries hold the promise of providing molecular understanding of tight junction pores at a resolution currently beyond our reach and should be a major goal of future tight junction research.

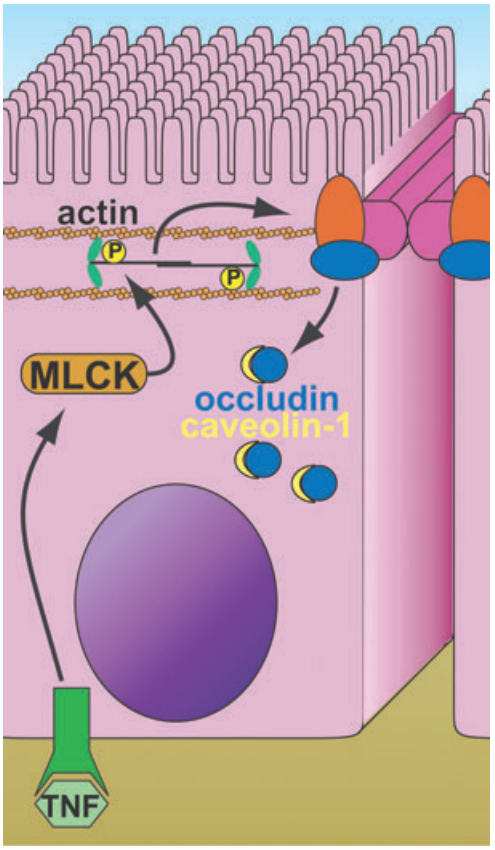

Figure 4. Model of TNF-mediated tight junction regulation. TNF interacts with its receptor to activate MLCK. MLCK phosphorylates myosin regulatory light chain resulting in actin remodeling and tight junction protein reorganization. These events are associated with caevolar endocytosis of occludin and increased tight junction permeability.

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Conflicts of Interest

The authors declare no conflicts of interest.

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