Transcytotic passage of intestinal bacteria across epithelial cells under proinflammatory stress

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Competing Interest

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Abstract

Gastrointestinal epithelial layer strategically acts as a physical barrier to prevent the entry of bacteria to the body proper. Nevertheless, a leaky gut manifested by increases in transcellular and paracellular permeability of epithelial cells is observed in many clinical diseases, including inflammatory bowel disease, celiac disease, intestinal obstruction, and pathogen infection. Disorganization of intercellular tight junction (TJ) proteins and disarray of brush border (BB) contribute to the epithelial barrier dysfunctions in inflammatory disorders. Proinflammatory cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha, and interleukin-1 induced TJ disruption and increased paracellular permeability. On the other hand, accumulating evidence showed that lower doses of IFNγ (below the threshold to induce TJ impairment) caused BB fanning and bacterial transcytosis, suggesting an alternative portal for bacterial influx across epithelial cells. Both paracellular and transcellular permeability defects induced by IFNγ are dependent on the phosphorylation of epithelial myosin light chain (MLC) by myosin light chain kinase (MLCK), of which the differential regulatory mechanisms remain unclear. This article highlights the evidence of bacterial transcytosis preceding TJ damage in disease models, and discusses possible mechanisms such as splicing

Transcytotic passage of intestinal bacteria across epithelial cells under proinflammatory stress

variants of MLCK or isoforms of MLC for regulation of transepithelial bacterial influx. The epithelial recognition of intracellular bacteria following the internalization of commensals under proinflammatory stress, and the invasion of pathobionts (opportunistic pathogens converted by commensals) are also discussed. In sum, epithelial barrier defect in transcellular or paracellular pathways potentially leads to bacterial translocation to mucosa and extraintestinal organs, and may predispose the host to inflammatory disorders. The understanding of the molecular mechanisms of epithelial barrier regulation may shed light to therapeutic development for management of chronic inflammation.

Key words: intestinal barrier, bacterial internalization, tight junctions, brush borders, proinflammatory cytokines, myosin light chain kinase

Abbreviations

TJ, tight junction; BB, brush border; IFNγ, interferon gamma; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-1β; MLC, myosin light chain; MLCK, myosin light chain kinase; IBD, inflammatory bowel disease; IO, intestinal obstruction; JAM, junction-associated molecules; ZO, intracellular zonula occludens; PAMR, perijunctional actomyosin ring; TER, transepithelial electrical resistance; ROCK, Rho-associated kinase; TW, terminal web; NOD, Nucleotide-binding oligomerization domain; TLR, Toll-like receptor; iNOS, inducible nitric oxide synthase; , COX-2, cyclooxygenase-2; hBD2, human beta-defensin 2.

Transcytotic passage of intestinal bacteria across epithelial cells under proinflammatory stress
August 2017

Introduction

By acting as a portal to the outer environment for nutrient uptake, the intestinal epithelium is also constantly bombarded by undigested dietary particles and orally acquired pathogens. Commensal bacteria which reside in a massive load in the intestinal tract are luminally confined as a strategy to maintain a peaceful symbiotic relationship with the host. The epithelial layer serves as a critical physical barrier to prevent the influx of bacteria (both pathogenic and commensals) and the passage of antigenic substances into blood circulation.

Epithelial barrier damage followed by bacterial translocation to the gut mucosa, circulatory bloodstream and extraintestinal viscera, are involved in the pathogenesis of enterocolitis, systemic inflammatory syndrome, and septic complications [1, 2]. Increases of transcellular and paracellular permeability in epithelial cells are widely reported in intestinal inflammatory diseases. Increase in lactulose/mannitol or lactulose/L-rhamnose urine excretion (an indicator of paracellular permeability in small intestine) by sugar drinking test was reported in patients as a predictor of relapse in Crohn's disease [3-7] and in those with celiac disease[8-12]. Tight junction (TJ) damages in epithelial cells are documented in experimental models of inflammatory bowel disease (IBD) [13-16], celiac disease [17, 18], intestinal obstruction (IO) [19, 20], psychological stress [21-23], and intestinal

infection with pathogenic microbes and parasites [24-28]. On the other hand, commensal bacterial adherence and internalization by epithelial cells was also found in these disorders, including IBD [29, 30], celiac disease [31, 32], IO [33, 34], psychological and surgical stress [35-37], and pathogen infections [27, 38-40].

Extensive research was conducted to investigate the molecular pathways of TJ defects (see review articles [41-44]), while the mechanisms of transcellular epithelial permeability are relatively unclear. A role of bacterial transcytosis in the pathogenesis of intestinal inflammatory disorders had recently gained attention due to the findings of mucosa-associated adherent-invasive bacteria [45-49], and the accumulative evidence of bacterial translocation to aseptic viscera caused by transepithelial bacterial influx despite intact TJs [34, 50]. This review article will discuss the paracellular and transcellular permeability changes in epithelial cells caused by proinflammatory cytokines, and focus on the latest findings in the molecular mechanisms of epithelial ultrastructural and functional defects that are involved in bacterial transcytosis.

Tight junctional defects under proinflammatory stress

Subcellular ultrastructures including intercellular TJs and brush border (BB) contribute to the barrier functions of polarized epithelial cells (Figure 1). The

single-layered epithelial cells are linked by multi-protein complexes of TJs, including transmembranous proteins (e.g. claudins, occludin, junction-associated molecules (JAM)), and intracellular zonula occludens (ZO). The TJ proteins form the most narrow paracellular space with a distance of 0.009 μm between the lateral membranes of adjacent columnar epithelial cells. Microbes of an average of 0.5-1 μm diameter can hardly pass through the TJs in physiological conditions. Other intercellular junctions, such as adherent and desmosomal junctions which are located further down the lateral cell membrane are responsible for cell-cell contact, but are not the rate-limiting step for controlling the paracellular permeability. The width of paracellular space is determined by the assembly of TJ proteins and regulated by contraction of the perijunctional actomyosin ring (PAMR). Increased paracellular permeability was observed after the phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK) [51, 52], or as a result of the endocytosis of TJ proteins after activation of Rho-associated kinase (ROCK) in epithelial cells [53-55].

A number of studies have shown that mucosal immune cells and proinflammatory cytokines caused an increase in paracellular permeability, either in a cell death-dependent or –independent manner. Activation of immune cells (such as cytotoxic T cells and phagocytes) and production of free radicals triggered apoptosis-dependent TJ defects in intestinal epithelial cells, and resulted in luminal-to-serosal influx of macromolecules [19, 56-60]. Exposure to cytokines, such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin (IL)-1β, induced the reorganization of TJ proteins, without affecting the cell viability (Table 1) [53, 54, 61]. Experimental models of colitis and endotoxemia have demonstrated that IFNγ- and TNFα-induced disruption of intestinal barrier and bacterial translocation were inhibited by blockade of MLCK, suggesting the involvement of MLCK in gut permeability increase [62-67]. In addition, intraperitoneal injection of IL-1β in mice caused an increase of the luminal-to-serosal macromolecular flux in the intestine in a MLCK-dependent manner [68].

The intestinal epithelial model system *in vitro* had provided direct evidence of cytokines modulating the paracellular permeability. Previous studies have demonstrated that treatment of IFNγ at 1000-3000 IU/ml for 48 hours caused an increase in transepithelial electrical resistance (TER) and elevated paracellular fluxes of dextran probes, which associated with decreased ZO-1 expression in human intestinal epithelial Caco-2 cells [50, 69-72]. Other reports in T84 cells showed that IFNγ at 100-1000 IU/ml for 48-72 hours

caused an increase of the paracellular permeability associated with the disorganization and intracellular trafficking of TJ proteins [53, 54, 73-75]. These changes included the loss of ZO-1 expression, a diffused distribution of occludin along the lateral membrane [74, 75], and the internalization of occludin and JAM-1 into the vacuolar compartments in T84 cells [53, 54]. Moreover, TNFα at 20-100 ng/ml also induced a significant TER drop in HT29 cells after 8 hours of exposure [76]. Recent studies indicated that IL-1β at 10 ng/ml for 48 to 72 hours caused a drop of TER associated with an increase in dextran permeability, and triggered the rearrangement of occludin and ZO-1 in Caco-2 and HT29 cells [61, 77, 78].

Furthermore, a synergistic effect was observed by cytokine mixtures on induction of TJ damage. Designed to overcome the criticism regarding high doses of cytokines, the co-treatment of cytokines at lower concentrations including IFNγ (100-1000 IU/ml) and TNFα (1-10 ng/ml) caused a reduction of TER in T84 cells as early as 24 hours following treatment [79, 80]. The protein levels of TNFα receptor was found to be upregulated by IFNγ in epithelial cells, and thus, contributing to the synergistic effects on TJ disruption [81]. In addition, both IFNγ and TNFα were shown to augment the expression levels of MLCK, via promoter binding by nuclear factor kappa B (NFκB) [34, 82, 83]. Moreover, the internalization of transmembrane TJ proteins, including occludin, JAM-1, and claudin-1/4 into vacuolar-associated compartments via a ROCK-dependent process was observed after treatment with cytokine mixture of IFNγ and TNFα in T84 cells [80]. Use of cytomix (a mixture of IFNγ (1000 IU/ml), TNFα (10 ng/ml) and IL-1β (1 ng/ml)) for 24 hours decreased the expression and altered the localization of occludin and ZO-1 in Caco-2 cells [84-86]. Taken together, studies with mixtures of proinflammatory cytokines that mimic the mucosal environment in intestinal lesions provided clear evidence of a causative role of cytokines in TJ impairment for bacterial passage through paracellular spaces.

Brush border fanning and lipid raft-dependent bacterial internalization

Commensal bacteria normally reside in the colonic lumen in a safe distance from the epithelial cells separated by the inner firm mucus layer. Even with a breach of the host-microbe compartmentalization, the specialized configuration of a fence-like brush border on the epithelial cells further prevent the direct contact of gut bacteria to the cellular soma [87, 88]. The brush borders on the apical side of epithelial cells are formed by densely packed microvilli with limited intermicrovillous space (Figure 1). The microvilli core (with a diameter of ~0.1 μm) is composed of cross-linking filaments such as actin, villin, and fimbrin,

and are rooted on a cytoskeletal meshwork at the apical compartment of epithelial cells, termed the terminal web (TW), which contains myosin, fodrin and spectrin [89]. With these brush border ultrastructure as a physical barrier, epithelial cells perform absorptive and secretive acts through transporters and channels expressed on the apical membrane without uptake of bacteria in a homeostatic state.

A number of *in vitro* culture studies demonstrated that proinflammatory cytokines may induce bacterial internalization or transcytosis (Table 2). In comparison to the high dose of cytokines to impair TJs, IFNγ at a lower dose (10-100 IU/ml) is capable of induction of bacterial transcytosis in the absence of TER drop or paracellular permeability change in Caco-2 cells after treatment for 48 hours [34, 50]. Another study showed transcycotic bacterial passage as early as 8 hours post-treatment of IFNγ in T84 cells, which was dependent on extracellular signal-regulated kinase (ERK) 1/2 and ADP-ribosylation factor-6 (ARF6) signaling [90]. Metabolic and oxidative stress, such as uncoupling of mitochondrial oxidative phosphorylation[91, 92] or exposure to hypoxia [93], also trigger transcytotic bacterial passage in epithelial cells without affecting TJ integrity. A number of intestinal infection models, including the non-invasive parasite *Giardia lamblia,* or the invasive pathogen *Campylobacter jejuni,* also induced transcytosis of commensal bacteria across intestinal epithelial cells [27, 38, 94]. The proposed mechanisms of commensal transcytosis following *C. jejuni* infection included the avoidance of sorting into the lysosomal degradative pathways and the depletion of ATP [38, 95]. The *C. jejuni*-containing vacuoles deviate from the canonical endosomal-lysosomal delivery process, suggesting a perturbation of the subcellular trafficking system which may facilitate the transcytosis of commensal bacteria as a bystander [38, 94, 95].

The bacterial strains used for *in vitro* studies of transcytosis were nonpathogenic, noninvasive laboratory *Escherichia coli* such as C25, HB101, or BL21 [34, 38, 50, 90, 94]. Unlike pathogens with known virulent invasive machinery of type III secretion systems for injection of effector proteins to manipulate the host cytoskeletal actins [96], the laboratory strains of *E.coli* are commensal bacteria derived from the intestine of healthy individuals and do not possess pathogenic determinants. Therefore, ultrastructural changes of densely packed microvilli in intestinal epithelial cells must exist to allow the apical uptake of commensal bacteria. Previous studies have demonstrated that low dose IFNγ stimulation caused MLCK-dependent MLC phosphorylation in the epithelial TW region and BB fanning, thereby

increasing the intermicrovillous space for bacterial entry in Caco-2 cells [34]. The IFNγ-stimulated MLCK-dependent bacterial transcytosis associated with BB disarray was also observed in *in vivo* models of bowel obstruction[34]. Furthermore, cholesterol-rich lipid raft and caveolin-1-dependent endocytotic pathways are involved in bacterial endocytosis [34, 50]. Caveolin-1 is the main protein identified in the caveolae, which is flask-shaped membrane invaginations situated at the base of intermicrovillous clefts on epithelial cells [97, 98]. These findings provided evidence that low dose of IFNγ modulate the subcellular structures of BB and TW without affecting the integrity of TJs to widen the intermicrovillous portals for bacterial internalization through lipid rafts.

It is noteworthy that the dose of IFNγ (10-100 IU/ml) for bacterial transcytosis is much lower than the concentration (1000–3000 IU/ml) reported for rearrangement of TJ proteins in Caco-2 cells [50, 69, 70]. Similarly, the observation of low versus high concentration of IFNγ on the differential effects of transcellular and paracellular permeability was documented in T84 cells [99, 100]. Whether other cytokines also display distinct regulation on the two aspects of epithelial barriers as in IFNγ treatment remain elusive. Exposure to TNFα (20 ng/ml) for 6 hours induced translocation of bacterial across Caco-2 cells only in glutamine-free culture media, suggesting that energy depletion faciliated TNFα-induced bacterial endocytosis and low dose TNFα alone did not cause transcellular permeability increase [101]. *In vivo* animal models of bowel obstruction with upregulated mucosal IFNγ levels demonstrated that bacterial transcytosis across epithelial cells occurs before the onset of TJ damage[19, 34, 102], suggesting that low concentrations of cytokines below the threshold level for full-blown inflammation is sufficient to cause epithelial barrier dysfunction in the form of transcellular defects.

Unanswered questions about epithelial permeability change under proinflammatory stress

Abundant reports indicated co-existing transcellular and paracellular barrier defects in gut epithelium in IBD, IO, and celiac disease [103-106]. Other studies demonstrated bacterial translocation independent of paracellular permeability change in gluten-sensitive mice and bowel obstruction models at early stages [34, 107]. As mentioned previously, bacterial transcytosis preceded TJ damage in epithelial cells of IO mice [19, 34]. Whether a temporal order of the two forms of epithelial barrier defects are also present in models of IBD and celiac disease remains unclear. Electromicroscopic images have revealed disruption of microvilli and terminal web as early

mucosal lesions in patients and animal models of IBD[103, 108, 109] and in celiac disease[105, 110], suggesting ultrastructure epithelial changes in the subclinial stages of the disorders. A cause-and-effect relationship between transcytosis and paracellular passage of bacteria has yet to be determined. Moreover, the involvement of an MLCK-dependent BB fanning for bacterial internalization should be verified in experimental models of IBD and celiac disease. However, it would be hard to tease out the two transepithelial routes of bacterial influx if epithelial cell death is present in cases of overt inflammation and high oxidative stress [111-115]. Therefore, mild colitis models and early phases of gliadin-induced epithelial dysfunction are required for the understanding of transcellular permeability changes in epithelial cells.

Previous findings pointed to a common pathway for the regulation of transcellular and paracellular permeability by a single kinase, i.e. MLCK. Although a temporal affiliation is found between transcellular and paracellular bacterial passage, it remains unclear whether disorganization of TJ proteins is a downstream effect of epithelial scaffold modification due to bacterial transcytosis. The differential regulatory mechanisms underlying MLCK-dependent transcellular and paracellular epithelial permeability caused by proinflammatory cytokines is yet to be explored. Potential mechanism

includes the involvement of distinct isoforms of epithelial MLCKs or MLCs, Ca^{2+} dependency, or unknown regulatory factors for switching the pathways. So far, five splicing variants of the non-muscle form of MLCK (210kD) which is also called the long MLCK or epithelial-specific MLCK were reported in relation to variable upstream and downstream kinase activities [116-118]. Three isoforms of MLC have been documented in epithelial and muscle cells[119], suggesting another possible means for differential regulation. Further identification of regulatory signaling pathways for MLCKs would provide an explanation for the differential mechanisms between transcellular and paracellular barrier damage which may contribute to disease pathogenesis.

Bacterial transcytosis by epithelial cells in absence of TJ defects may be an early phenomenon contributing to the pathological shift towards inflammatory disorders. Although increased gut permeability and TJ disruption are seen preceding inflammation in experimental ileocolitis models [13, 120-122], clinical observation of relapse in IBD patients may or may not be accompanied with the increase of lactulose/mannitol index (a parameter of small intestinal permeability) or sucralose excretion (a parameter of colonic permeability) by the sugar drinking test [123, 124]. These findings suggest that bacterial transcytosis

uncoupled with TJ damage as an alternative portal of leakiness may have been overlooked in the pathogenesis of chronic inflammation. Moreover, it is unclear whether cytokine-induced BB fanning and transcellular permeability increase is selective for the strains of endocytosed bacteria. The finding of novel cytokines which cause transcellular permeability change without TJ damage, or vice versa, would help to exclude a cause-and-effect relationship between the two forms of proinflammatory barrier defects.

Lastly, the presence of mucosa-associated adherent-invasive bacteria in Crohn's disease patients [48, 49] implicates yet another form of epithelial barrier defects in the etiology of IBD, which may be partly due to an active role of bacteria in penetrating the cells or hijacking the endocytosis pathway. A number of commensal bacteria were found to invade epithelial monolayers in untreated conditions *in vitro*, implicating their role as "pathobionts" (opportunistic pathogens converted by commensals). These include *Escherichia coli* [125-129], *Klebsiella pneumoniae* [130, 131], *Enterococcus faecalis* [132-136], *Pseudomonas aeruginosa* [130, 137], *Staphylococcus aureus* [138-141], *Streptococcus pneumonia* [142, 143], and *Enterbacter sakazakii* [144, 145], and *Fusobacterium nucleatum* [146-149]. It remains poorly understood whether internalized commensals under proinflammatory stress and invasive pathobionts are recognized differently by intracellular molecular-associated pattern receptors and induce variable epithelial responses, such as tolerance or immunity. Nucleotide-binding oligomerization domain (NOD)-like receptors are intracellular sensors for internalized pathogens and bacterial products, whereas Toll-like receptors (TLRs) binding to bacterial products are identified mostly on the cell surface [150-153]. These innate immune receptors originally found in monocytic cell lineages were later reported to be constitutively expressed in gut epithelium at resting state or were highly expressed in intestinal epithelial cells under inflammatory conditions [150, 151, 154-157]. The opportunistic bacteria mentioned above elicited the production of human beta-defensin 2 (hBD2), inducible nitric oxide synthase (iNOS),and cytokines, via NOD1/2 or TLR2 signals in epithelial cells [129, 132, 138, 144, 158-162]. It is noteworthy that the pathobionts may induce epithelium-derived proflammatory cytokine synthesis, which could further lead to a vicious cycle for bacterial entry and bystander endocytosis. Furthermore, the NOD-dependent induction of iNOS, hBD2, IL-8 and cyclooxygenase-2 (COX-2)/prostaglandin E2 were also documented in human intestinal xenografts and intestinal epithelial cell

lines after infection with invasive pathogens such as *Salmonella enterica*, enteroinvasive *E. coli*, and *C. jejuni* [130, 163-168]. *Helicobacter pylori* as a gastric pathogen had also been reported to cause junctional destabilization[169, 170] as well as to invade gastric epithelial cells [171-173], and to activate NOD1/2 signaling [174-178]. How the invasive routes of commensal-derived pathobionts via endosomes and that of virulent pathogens through vacuoles may differentially determine the outcome of bacterial recognition and clearance warrant further studies.

Concluding remarks

The finding of proinflammatory cytokines triggering both types of epithelial permeability defects, including bacterial transcytosis and passage through impaired tight junctions, brings up the question as to whether a cause-and-effect relationship exists between the two forms of epithelial barrier breach. The understanding of the differential regulation of epithelial barrier defects may provide insights into distinct bacterial sensing responses for better management of chronic intestinal inflammation.

Transcytotic passage of intestinal bacteria across epithelial cells under proinflammatory stress

August 2017

Table 1 Paracellular junctional changes caused by proinflammatory cytokines

Cytokine	Cell lines	Dose	Readout on Tight Junctions	Ref.
IFNγ	Caco-2	100-3000 IU/ml	Reduction of TER and dextran flux	[70,71]
	T84	100-1000 IU/ml	Loss of ZO-1 expression, altered distribution of occludin, internalization of occludin and JAM-1	[53, 54, 73-75]
	HT-29	500-3000 IU/ml	Reduction of TER and mannitol flux	[69]
TNFα	Caco-2	10 ng/ml	Decreased ZO-1 expression or F-actin rearrangement	[70, 74]
	HT-29	20-100 ng/ml 10000IU/ml	Reduction of TER or cell death or up-regulation of claudin-2	[58,76]
IL-1β	Caco-2	10 ng/ml	Activation of MLCK, rearrangement of occludin and ZO-1	[61, 77, 78]
IFNγ/TNFα	T84	IFNγ (100-1000 IU/ml) and TNFα (1-10 ng/ml)	Internalization of occludin, JAM-1, and claudin-1/4, and activation of MLCK via NFκB signaling; upregulation of TNF receptor by IFNγ	[80-83]
IFNγ/TNFα/IL-1β	Caco-2	IFNγ (1000 IU/ml), TNFα (10 ng/ml), and IL-1β (1 ng/ml)	Altered localization of occludin and ZO-1, partly via induction of iNOS	[84-86]

Abbreviations: TER, transepithelial electrical resistance; ZO-1, Zonula occludens-1; JAM, Junction-associated molecule; MLCK, Myosin light-chain kinase; NFκB, nuclear factor kappa B; iNOS, inducible nitric oxide synthase.

Transcytotic passage of intestinal bacteria across epithelial cells under proinflammatory stress
August 2017

Table 2 Transcellular permeability defects caused by proinflammatory cytokines

| Cytokine	Cell lines	Dose	Mechanisms of bacterial endocytosis	Ref.
IFNγ	Caco-2	100 IU/ml	MLCK-dependent brush border fanning increased intermicrovillous space to facilitate lipid raft-dependent bacterial entry	[34]
	T84	1-10 IU/ml	Lipid raft–mediated bacterial transcytosis; Dependent on ERK1/2 and ARF6 signals	[50, 90]
TNFα	Caco-2	20 ng/ml in glutamine-free media	Cellular energy depletion	[101]

Abbreviations: MLCK, Myosin light chain kinase; ERK, Extracellular regulated kinase; ARF6, ADP-ribosylation factor-6.

Figure legend

Figure 1 Ultrastructural components of epithelial cells forming gut barrier functions.
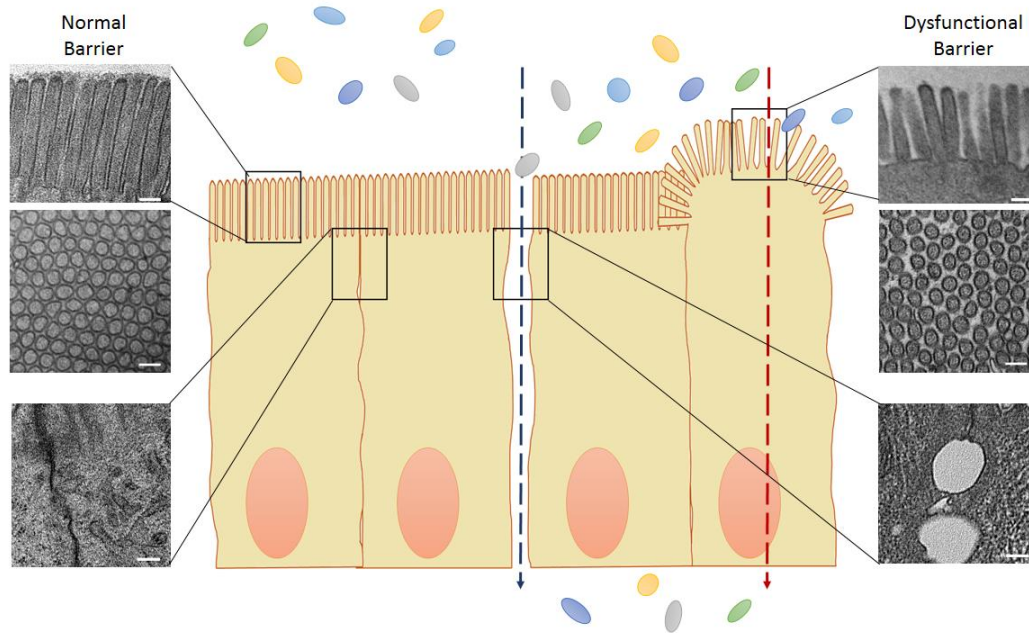
The single-layered epithelial cells are linked by multi-protein complexes of tight junction (TJ) proteins. The TJ proteins (arrows in the lower left electromicrograph images) form the most narrow paracellular space with a distance of 0.009 μm between the lateral membranes of adjacent columnar epithelial cells, and thus excluding microbes of an average of 0.5-1 μm diameter. In addition, densely packed microvilli (longitudinal and orthogonal views shown in the upper left electromicrograph images) on the apical side of epithelial cells are rooted on a cytoskeletal meshwork of terminal web, which are collectively termed brush border (BB), and form a fence-like structure to prevent direct contact between luminal bacteria and the cellular soma. Nevertheless, transepithelial bacterial influx may occur upon situations of barrier defects, such as increases of transcellular and paracellular permeability in epithelial cells (the upper and lower right images), leading to bacterial translocation to gut mucosa and extraintestinal organs, and predisposes the host to chronic inflammation Bar=200 nm.

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14

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