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Modulation of Intestinal Paracellular Transport by Bacterial Pathogens

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Abstract

The passive and regulated movement of ions, solutes, and water via spaces between cells of the epithelial monolayer plays a critical role in the normal intestinal functioning. This paracellular pathway displays a high level of structural and functional specialization, with the membrane-spanning complexes of the tight junctions, adherens junctions, and desmosomes ensuring its integrity. Tight junction proteins, like occludin, tricellulin, and the claudin family isoforms, play prominent roles as barriers to unrestricted paracellular transport. The past decade has witnessed major advances in our understanding of the architecture and function of epithelial tight junctions. While it has been long appreciated that microbes, notably bacterial and viral pathogens, target and disrupt junctional complexes and alter paracellular permeability, the precise mechanisms remain to be defined. Notably, renewed efforts will be required to interpret the available data on pathogen-mediated barrier disruption in the context of the most recent findings on tight junction structure and function. While much of the focus has been on pathogen-induced dysregulation of junctional complexes, commensal microbiota and their products may influence paracellular permeability and contribute to the normal physiology of the gut. Finally, microbes and their products have become important tools in exploring host systems, including the junctional properties of epithelial cells.

Introduction

The gut epithelium, with the exception of the stratified layers in the esophagus, is a single contiguous layer of cells that represents a unique barrier between the diverse luminal contents and the underlying tissue (67). The epithelium is vested with the exacting task of processing food, absorbing water and nutrition, and compacting and disposing undigested materials while, simultaneously, keeping pathogens and toxic molecules at bay. Paralleling the discrete tasks along its length, the epithelial cell composition and character, as well as the substances secreted or absorbed, differ in the various segments of the gut. Concomitantly, these niches, with distinct pH, oxygen levels, and nutrients, are home to diverse arrays of microbes. The microbial communities that thrive in these niches also intimately influence the biology of the gut and beyond. Importantly, they often bolster

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host defenses by preventing the colonization of pathogenic organisms, a feature termed as colonization resistance (52).

The epithelial cells of the gut achieve their unique tasks by maintaining apical-basal polarity (108). This is essential for the directional transport of nutrients and water, as well as the secretion of compounds such as antimicrobial peptides and enzymes into the lumen. The various junctional complexes between adjacent epithelial cells serve many functions. Most fundamentally, they tether the cells together as a monolayer, and provide mechanical strength to withstand the passage of food and water. Beyond this, they contribute to the "fence" function, that is, the maintenance of cell polarity by preventing lateral diffusion of membrane molecules between the apical and basolateral sides. Finally, they regulate the passive transport of water, ions, and molecules along the paracellular pathway, a property known as barrier function.

The apical-most tight junction (TJ) complexes are the major players in regulating the fence and barrier functions (133). Mechanical strength for the monolayer is provided by the more basal desmosomes, the "spot-welds" that hold epithelial cells together. Other junctional complexes include the adherens junctions (AJs) and gap junctions, which play various roles including the coordination of membrane events with intracellular signaling changes. The junctional complexes are variously tethered to the intracellular cytoskeletal elements, namely the actin microfilaments, microtubules, and intermediate filaments.

The organization and function of the epithelium needs to be dynamically maintained despite exposure to an array of substances and organisms in the lumen (133). In the normal intestine, there are many opportunities for breaches in the monolayer, such as luminal sampling of antigens by underlying immune cells, wounds and wound healing, programmed cell death, the extrusion of dead cells, and cell proliferation and replenishment. In relation to the last point, it is worth noting that the entire lining of the intestine renews itself once every 4 to 8 days in the adult gut. Discrete processes operate to ensure that the integrity of the paracellular pathway, and the polarity of the epithelial cells, is maintained despite these perturbations.

Polymorphisms and mutations in TJ proteins have been linked to diseases of various tissues and organs (133). For instance, claudin mutations are associated with ichthyosis (claudin-1), velo-cardial facial syndrome (claudin-5), nonsyndromic deafness (claudin-14), familial hypomagnesaemia (claudin-16 and −19), and vision loss (claudin-19), and tricellulin mutations are associated with nonsyndromic deafness. Intestinal TJ protein expression and distribution is altered in many pathologic states including inflammatory bowel disease (37). Many pathogens perturb junctional complexes and dysregulate paracellular permeability, which contributes to disease. Bacteria and viruses may bind to cell junctions, or recruit junctional proteins to regions of attachment. Often, pathogens dislocate TJ proteins from the apical junctional regions, causing their movement down along the lateral membrane, and to intracellular locations. Pathogen-mediated signaling alterations can cause contraction of the perijunctional ring of actin and increase paracellular permeability.

This overview article reviews the mechanisms by which select pathogens target intestinal epithelial TJ complexes and alter paracellular permeability. To provide context, a brief summary of the normal structure and function of TJs, and their role in regulating paracellular permeability, is included. Turner and Turner provide a comprehensive picture of the gastrointestinal tract (133), while the overview article by Madara (67) provides a review on epithelial morphology in the small intestine. For excellent overviews on TJ structure and function, the reader is referred to other recent articles (33, 155).

Overview of Cell Junctional Complexes

The apical junctional complex (AJC) is a highly organized structure located at the apex of the lateral membrane of polarized epithelial cells (97). The AJC is involved in cell-cell adhesion and paracellular permeability and, by separating the apical and basolateral domains, maintains epithelial cell polarity. It is comprised of the apical-most TJs, followed by the AJs and desmosomes. While these structures have unique compositions with relatively few common proteins, they share some overall features. Each junctional complex is composed of integral membrane proteins that form intercellular bridges, adaptor proteins that anchor the membrane components to cytoskeletal structures, and specific signaling molecules (155).

Thus, the TJ complex includes transmembrane proteins of the claudin family that mediate interactions between adjacent cells, and subjunctional adaptor proteins like cingulin and members of the zona occludens family. The protein-protein interaction domains of adaptor proteins help tether the membrane proteins to microtubules and actin filaments. Below the TJs are the AJs, containing the membrane-spanning classical E-cadherins and nectins, the cytosolic interaction proteins α- and β-catenin and p120 catenin (for E-cadherin) and afadin (for nectins). AJs are required for the assembly of the paracellular barrier and the regulation of mucosal permeability (60). Below the TJ and AJ are the desmosomes, the “spot-welds” that provide mechanical integrity to the epithelial monolayer. Desmosomes are composed of the transmembrane desmosomal cadherins desmoglein and desmocollin, and associated cytosolic plaque proteins plakophilin, plakoglobin, and desmoplakin. Desmoplakin anchors the desmosomal complex to the keratin intermediate filament network. Emerging studies suggest a role for the desmosomal junctions in potentiating epithelial barrier function (118, 137).

Structural features of tight junctions

In electron micrographs, TJs appear as focal “kissing” contacts between neighboring columnar cells, supported by electron-dense cytoplasmic plaques (67). Freeze fracture micrography, a technique that provides planar views of the hydrophobic interior of membranes, shows the TJ membrane proteins as an anastomosing meshwork of strands (155). Broadly, the number of strands correlates positively with the leakiness of the epithelium; fewer strands are present in the “leaky” epithelia of the gut compared to tight epithelia such as those of the urinary bladder (19). However, the occurrence of epithelial systems with similar number of strands, but vastly different transepithelial resistances, suggests that TJ composition also dictates barrier properties (36, 41, 120).

The TJ strands or fibrils are composed of tetraspan transmembrane proteins of the claudin family (26 isoforms in humans) and members of the TJ-associated MARVEL [myelin and lymphocyte protein (MAL) and related proteins for vesicle trafficking and membrane link] proteins, occludin, tricellulin, and MARVELD3. Occludin is primarily localized to junctions between two neighboring cells (bicellular junctions), while tricellulin and anguillins are enriched at the points of contact of three neighboring cells (tricellular junctions). Additional components localizing to the TJs include the single-span transmembrane proteins junction-adhesion molecules (JAMs) and coxsackievirus- and adenovirus-receptor (CAR), which contain immunoglobulin-like domains.

Cytosolic TJ-associated proteins, such as the zona occludens proteins ZO-1, ZO-2, and ZO-3, and cingulin, contain various protein-protein interaction motifs that facilitate their adaptor function. ZO-1, the first identified TJ protein, as well as the best studied to date, harbors N-terminal domains that facilitate interaction with the membrane-anchored claudins, occludin, and JAM, and with the transcriptional regulator ZO-1–associated nucleic acid binding proteins (ZONAB) and heat-shock protein, APG2; the C-terminal half of ZO-1 interacts with F-actin. Cytoplasmic plaque proteins may have an ordered structure, and knockout/knockdown studies suggest some level of redundancy. Also closely associated with the TJs are partitioning complexes that play a role in generating and maintaining the apical-basal polarity of epithelial cells (99). Signaling molecules localizing to the TJs, such as the small GTPases RhoA, Rac, and CDC42 and their regulators, and atypical protein kinase C (aPKC), modulate epithelial barrier function. Finally, lipids are recognized to be fundamentally important for TJ structure and function, but their precise role in the maintenance of barrier function is poorly understood (155).

Modes of paracellular permeability

The main gatekeepers of paracellular transport are TJs, which have the added role of limiting the lateral movement of membrane-associated molecules and, thereby, maintaining the polarity of the epithelial monolayer (“gate” and “fence” functions, respectively). In the simple epithelia of the gut, TJs are dynamic structures that form a semipermeable diffusion barrier to ions, solutes, and water, while restricting the passage of bacteria and toxins.

Two modes of paracellular transport, the pore and leak pathways, can be discerned (106, 138, 142). The high-capacity pore pathway is charge- and size-selective, accommodating molecules ranging from 5 to 10 Å (54, 126). The leak pathway, which has limited capacity, is not selective for charge and facilitates transport of larger molecules, with one study estimating a sieving radius of ~62.5 Å (12). Distinct mechanisms govern the pore and leak pathways, and they can be independently, even opposingly, regulated. In addition to these pathways, epithelial damage can allow mass movement of small and large molecules, as well as microbes and toxins. Movement via this unrestricted pathway, not strictly a mode of paracellular transport, may play a prominent role in the context of microbial pathogenesis, particularly during later stages of infection.

Claudins are the key determinants of TJ permeability properties (37). Claudin isoforms may offer cation-selectivity (claudin-2, −10b, and −15), anion-selectivity (claudin-10a and −17), or enhance the sealing or barrier function of TJs (claudin-1, −4, and −8). The first two

extracellular loop of claudins influence ion selectivity. One proposed mechanism for the action of barrier enhancing claudins is via the inhibition of the function of pore-forming claudins. Proteins of the MARVEL family can impact TJ strand architecture and regulate claudin channel function. For instance, occludin phosphorylation status influences claudin-2 localization at the TJs and, consequently, paracellular cation flux (98).

Regulation of TJ barrier function by the perijunctional actomyosin ring

Actin filaments within the microvilli of epithelial cells descend into the terminal web, a meshwork containing actin, actin-binding proteins, myosin, and intermediate filaments. At the cell peripheries, the terminal web condenses into a dense ring of actin at the region of the adherens and TJs (23). Filamentous actin associates with nonmuscle myosin II (NM II), which is composed of heavy chains, essential light chains, and regulatory light chains. Pharmacological inhibition of NM II by blebbistatin, or siRNA-mediated depletion of this molecule, resulted in a loss of barrier function despite apparent structural integrity of the junctional complexes (97). Mice with intestinal epithelial-specific knockout of the NM II heavy chain had altered expression/localization of junctional proteins, and increased intestinal permeability (86). Various mediators of barrier disruption, including calcium depletion and proinflammatory cytokines, activate NM II.

Phosphorylation of regulatory myosin light chain (MLC) results in unfolding of the actin-binding domain, and contraction of the peripheral actomyosin ring (97). This leads to TJ opening and, consequently, an increase in paracellular permeability. The phosphorylation status of MLC is dictated by several proteins including Rho GTPases, Rho-associated protein kinase (ROCK), MLC kinase (MLCK) and MLC phosphatase. Apart from directly phosphorylating MLC, ROCK is also an inhibitor of MLC phosphatase. Altered paracellular permeability resulting from actomyosin contraction has a normal physiological role in the intestine. For instance, activation of transcellular Na^+/glucose cotransport by the apical transporter SGLT1 results in MLCK activation and MLC phosphorylation (135). The resulting TJ opening promotes paracellular absorption of water and small nutrients (radius <3.6 Å) along the osmotic gradient. This normal physiological pathway underlies the success of oral rehydration solutions containing glucose and sodium chloride in treating diarrhea. In a pathological context, several intestinal pathogens cause diarrhea partly via the phosphorylation and activation of myosin II.

The junction-associated actin cytoskeleton undergoes constant turnover, and molecules that regulate actin dynamics, such as actin-depolymerization factor (ADF)/cofilin and actin-interacting protein 1 (AIP1), regulate the assembly and permeability of the epithelial barrier (59, 141). More recently, the actin-interacting molecule cortactin was implicated in epithelial barrier function regulation (18). Cortactin deficiency enhanced RhoA/ROCK-mediated actomyosin contraction, and ROCK1 inhibition restored barrier function. Multiple lines of evidence indicate that the maintenance of epithelial barrier integrity requires the balanced regulation of Rho GTPase activity: both the excess and depletion of active Rho GTPases can perturb paracellular permeability (60).

Assays for measuring paracellular permeability

Several complementary methods are used to test paracellular permeability (106). The most commonly used method is the measurement of transepithelial electrical resistance (TER) to assess the flux of all ions across the epithelium. A current is applied across the epithelial monolayer, the generated potential is measured, and Ohm's law is used to calculate the resistance to the flow of current. Since the small ions Na^+ and Cl^- are typically used in physiological solutions, TER measurements do not distinguish between the pore and leak pathways. Leak pathway permeability can be measured by quantitating the flux of charged or uncharged macromolecules of various sizes across the epithelium. Commonly used tracers include mannitol (molecular radius = 3.9 Å), lactulose (5.1 Å), inulin (10 Å), and varying sizes of polymers of polyethylene glycol (PEG 400 = 5.5 Å, PEG 4000 = 15.9 Å) or dextran (4 kDa = 4.9 Å, 40 kDa = 45 Å) (1, 39, 143). Charge and size selectivity of the pore pathway can be determined via dilution potential measurement and biionic substitution methods, respectively. While epithelial monolayers are known to display heterogeneity, with areas of high and low permeability (79), most measurement strategies focus on population averages. Recently, however, Weber et al. were able to use a novel patch-clamp technique to measure flux across individual claudin-2 channels (144). Most studies assessing the impact of microbes on paracellular permeability have involved TER measurements or tracer flux assays; pore selectivity measurements have seldom been performed.

Microbial Targeting of Junctional Components and Regulation of Paracellular Permeability

Many pathogenic bacteria and viruses target junctional complexes and perturb epithelial barrier function. Pathogens or their toxins may use junctional proteins as receptors. For instance, claudin family proteins serve as receptors for hepatitis C virus (HCV), as well as the *Clostridium perfringens* enterotoxin (21, 109). Even organisms like enterohemorrhagic *Escherichia coli*, which do not have an identified junctional receptor, line up along the junctions of epithelial cells ("log jam" phenotype) (76). Toxins like the *Vibrio cholerae* hemagglutinin/protease (metalloprotease) and the pore-forming aerolysin of *Aeromonas hydrophila* can directly cleave the TJ protein occludin (85).

Diverse microbes alter intracellular signaling events, leading to the posttranslational modification and displacement of junctional proteins (Fig. 1) (27). Pathogens such as enteropathogenic *E. coli* and *Helicobacter pylori* target epithelial cell polarity complexes, perturb apical-basal polarity, and alter trafficking of TJ proteins (Fig. 2) (128). In other instances, pathogen-induced changes in TJ protein expression can alter barrier function (77, 154). As an example, *Salmonella* causes the elevation of claudin-2, but not claudin3 (or claudin-7) in the colonic epithelial cells of infected mice (Fig. 3). Finally, microbe-mediated cytoskeletal changes, including MLC phosphorylation, can trigger actomyosin contraction and, thereby, alter paracellular permeability (Fig. 4).

Pathogen-induced inflammation may also be an indirect pathway to TJ alterations and barrier perturbation (60). For instance, many infectious agents trigger release of the proinflammatory molecule tumor necrosis factor α (TNFα). TNFα can stimulate leak

pathway permeability by activating MLCK (Fig. 4) and inducing occludin endocytosis (134). Typically, microbe-induced permeability alterations are mediated via multiple mechanisms, and manifest as a continuum of changes as the infection progresses. Thus, signaling changes early in infection may induce pore or leak pathway changes. The durability and persistence of these responses may be dictated by pathogen burdens or specific virulence factors, as well as cross-talk between the pore and leak pathways. One example of cross-talk was demonstrated in the context of inflammatory bowel disease, where MLCK-dependent leak pathway permeability induced IL-13 expression; IL-13-dependent claudin-2 expression, in turn, selectively activated pore pathway permeability (145). MLCK-dependent leak pathway changes respond to rapid signaling changes, such as phosphorylation; since pore pathway changes require new protein synthesis, like claudin-2 expression, the responses manifest more gradually, but persist longer. Finally, with prolonged infection or high pathogen burdens, and attendant inflammatory infiltration, epithelial erosion could precipitate a complete collapse of barrier function (Fig. 5).

The consequences of barrier disruption include diarrhea due to the paracellular loss of water and ions, and increased penetration of microbes, toxins, and other molecules to the underlying tissues (42). The loss of TJ fence function can also result in the movement of basolateral proteins to apical sites, exposing new receptors for luminal organisms or toxins (83). In some instances, diarrhea can also be a host response that serves to reduce pathogen burdens.

The following sections discuss mechanisms by which a select group of microbes exploit TJs and alter paracellular permeability. Organisms belonging to the first three groups, namely attaching/effacing (A/E) pathogens, *Salmonella enterica*, and *Shigella* spp., are Gram-negative pathogens that use a syringe-like type III secretion system (T3SS) to alter host cell signaling. While the A/E pathogens are generally considered to be extracellular pathogens, *Salmonella* spp. and *Shigella* spp. occupy intracellular niches. *Shigella* spp. grows within the host cytoplasm, while *Salmonella* spp. resides within a specialized intracellular vacuolar compartment. The Gram-negative pathogen *H. pylori* is included for discussion since it colonizes and thrives in a distinct niche—the stomach. *H. pylori* uses a distinct secretion machinery, the type IV secretion system, to deliver a virulence protein into gastric epithelial cells. In the final section, three toxins produced by the Gram-positive clostridia are considered in the context of barrier disruption. Unlike the virulence proteins of the Gram-negative pathogens introduced above, the clostridial toxins are released into the environment and can often act as autonomous agents of disease. Notably, orally ingested botulinum toxin (BoNT) has features that allow it to navigate to peripheral neurons, with an early step involving intestinal barrier disruption.

Attaching and effacing pathogens

Enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC) are diarrheagenic organisms that attach intimately to intestinal epithelial cells and efface the brush border microvilli (105). Related A/E pathogens *Citrobacter rodentium* and rabbit EPEC (REPEC) infect mice and rabbits, respectively, and are used to model human EPEC/EHEC infections (58). All A/E pathogens elaborate a T3SS, a bacterial membrane-associated syringe-like

complex that delivers specific “effector” proteins directly into the host cytosol. The secreted molecules target numerous signaling pathways and alter host cell physiology, including TJ integrity and paracellular permeability (147).

The translocated intimin receptor (Tir) is a key A/E pathogen virulence protein secreted into host cells (147). Following entry, Tir inserts into the host cell membrane, and acts as a receptor for the bacterial surface adhesin, intimin. Tir-intimin interactions stimulate actin polymerization below the regions of bacterial attachment, eventually pushing the apical membrane up to form a pedestal-like structure.

There are three noteworthy features of EPEC secreted effector molecules which, incidentally, also hold true for several other pathogens. First, effector molecules are secreted into host cells in a hierarchical fashion, with Tir being the earliest protein delivered. Subsequently, other effectors are delivered in the following order: Tir < EspZ < NleA < EspF < EspH < Map < EspG (only a subset of effectors are indicated) (80). Second, each effector molecule may interact with several host proteins, and impact multiple functions. Finally, more than one effector may target a specific host cell function, such as paracellular permeability (136). The synergistic and complementary actions of effector molecules in mediating systematic temporal alterations in host cell physiology remains to be deciphered.

A/E pathogen-induced changes in TJ integrity and paracellular permeability

—The first suggestion that A/E pathogens perturb TJs came from studies on rabbits infected with the rabbit diarrheagenic *E. coli* RDEC-1 (124). The investigators noted increased conductance across infected intestinal tissues, with no changes in the short-circuit current, and net equivalence of Na^+ and Cl^- transport. Subsequently, Canil et al. observed a drop in TER in EPEC-infected Caco-2 intestinal epithelial cells, but not in cells infected with adherence-defective mutants (13). The study, however, noted that [^{3}H] inulin penetrance across the monolayer was not altered. Based on TER and sodium/mannitol flux studies in T84 intestinal epithelial monolayers, Spitz et al. concluded that EPEC infection disrupts TJs (119). These studies were subsequently confirmed, and correlated with the redistribution of ZO-1 and occludin from the TJs to intracellular compartments in infected T84 monolayers (24, 94, 111). Coimmunoprecipitation studies demonstrated decreased association between ZO-1, claudin, and occludin, and confocal microscopy revealed the progressive displacement of TJ proteins from apex to more basolateral locations of EPEC-infected cells (84). Freeze-fracture microscopy revealed the presence of claudin-1- and occludin-containing aberrant TJ strands along the lateral membrane of infected cells (84). In addition to barrier disruption, EPEC compromises the TJ fence function, which results in the movement of basolateral proteins to apical regions; this includes β1-integrin, which serves as an alternate/additional receptor for the EPEC adhesin, intimin (83).

In vivo A/E pathogen-induced barrier disruption was first confirmed in EPEC-infected C57BL/6J mice (107). Infected ileal and colonic tissues displayed significant decreases in TER, and these changes correlated with occludin redistribution to the cytoplasm. Other investigators replicated these observations, and also showed ZO-1 redistribution and increased biotin permeability in EPEC-infected mouse intestines (152). Similarly, the murine A/E pathogen *C. rodentium* also induced T3SS-dependent claudin-1, −3, and

−5 displacement from the TJs following a 7-day infection period, and increased biotin permeability across infected monolayers, and into the lamina propria (43).

Virulence proteins involved in A/E pathogen-induced changes in TJ integrity and paracellular permeability—EPEC-induced TJ changes and barrier disruption require the T3SS-dependent delivery of effector proteins into intestinal epithelial cells. To date, five secreted molecules—EspF, Map, EspG, NleA, and EspH—have been implicated in infection-induced paracellular permeability changes (136). McNamara et al. first showed EspF to be essential for EPEC-induced occludin redistribution, TER decrease, and increased mannitol permeability in infected T84 cells (78). EspF was also required for the EPEC-induced displacement of occludin and claudin-1 from the TJs down into more lateral locations along the membrane (84). Dean et al. confirmed the requirement of EspF for EPEC-induced occludin redistribution and TER reduction in Caco-2 cells (25). Further, they showed the effector protein Map, and the adhesin intimin, to be independently required for inducing these changes.

EPEC strains lacking EspF (Δ*espF*) colonized C57BL/6J mice at levels comparable to the parent wildtype (WT) strain (107). Unlike WT EPEC, the Δ*espF* mutant failed to perturb occludin localization at the TJ, or reduce TER of the colon or ileum at 1 day postinfection. At 5 days postinfection, however, both the WT and Δ*espF* strains redistributed occludin and reduced TER, suggesting the involvement of other EPEC factors in mediating these changes. Similarly, unlike WT *C. rodentium*, an isogenic Δ*espF* mutant failed to redistribute claudin-3, or increase biotin permeabilization, in infected colonic tissue even in regions with robust colonization (43); a Δ*map* mutant, however, was similar to WT in altering claudin-3 distribution, ruling out a role for Map in this process. The investigators also found increased colonic luminal water content in *C. rodentium* WT (and Δ*map*)-infected animals relative to uninfected mice; Δ*espF*-infected animals showed an intermediate effect on luminal water content.

Role of EspF in paracellular permeability changes: EspF is a multifunctional protein that interacts with several host proteins, and has wide-ranging impacts on infected host cells (146). EspF localizes to the mitochondria and activates caspases; an L16E mutant was impaired for mitochondrial localization, but competent for barrier disruption (140). Independently, EspF interacts with sorting nexin 9 (SNX9), which regulates clathrin-mediated endocytosis (3). In a recent study, Tapia et al. demonstrated EspF-dependent redistribution of the Crumbs3 polarity complex, and the perturbation of epithelial cell polarity; site-directed EspF mutants deficient in SNX9 interaction failed to cause polarity changes (127). These mutants were not impaired for barrier disruption, suggesting independent effects of EspF on the polarity complex and on the TJ (3, 140).

EspF may regulate paracellular permeability of host cells by influencing the activation and localization of the membrane-cytoskeletal linker ezrin (110). Ezrin is phosphorylated and relocalized in LLC-PK1 (pig kidney cell line) cells infected with WT EPEC, but not a Δ*espF* mutant. Importantly, dominant-negative ezrin expression mitigated EPEC-induced ZO-1 relocalization, and TER decreases. The mechanisms by which EspF influences ezrin phosphorylation and increases its cytoskeletal association remain to be defined. Beyond

ezrin, EspF may induce broader cytoskeletal alterations. EspF harbors neuronal Wiskott-Aldrich syndrome (N-WASP) binding motifs (2), which potentiate N-WASP/Arp2/3 actin nucleation. The N-WASP binding motifs were shown to be essential for EPEC EspF-induced epithelial barrier dysfunction (26). In studies with a rabbit strain of EPEC (E22), EspF was shown to bind N-WASP, Arp2/3, actin, and profilin, relocalize multiple TJ proteins to the pedestals, and trigger a reduction in TER (93).

Role of Map in paracellular permeability changes: The mechanisms by which Map influences paracellular permeability remain undefined. Map is essential for EPEC-induced TJ changes in Caco-2 cells, and constitutive Map expression in Madin-Darby Canine Kidney (MDCK) cells decreased TER, and increased the passage of both 4 kDa and 70 kDa dextran (112). On the other hand, a *C. rodentium map* deletion strain, like the parent WT strain, was able to alter barrier function of mouse colonic tissues (43). Since *C. rodentium* Δ*map* strains are impaired for causing diarrhea, and Map expression may be temporally regulated *in vivo*, more detailed studies are required to establish its role in paracellular permeability alterations (88, 127).

Role of NleA in paracellular permeability changes: Compared to WT EPEC, an NleA-deficient strain was impaired for decreasing the TER of T84 monolayers; plasmid-encoded NleA restored the phenotype to the Δ*nleA* strains (129). Occludin was lost from the periphery in EPEC-infected Caco-2 cells, but not in cells infected with Δ*nleA*, Δ*espF*, or Δ*map* strains, suggesting that all of these molecules are required to induce TJ alterations. NleA binds to, and inhibits, the COPII protein complex, which plays a role in trafficking intracellular proteins. Inhibition of vesicle trafficking by brefeldin A, a fungal compound, functionally restored the ability of Δ*nleA* (but not Δ*espF* or Δ*map*) strains to disrupt TJs (129). NleA-dependent TJ protein redistribution and altered paracellular permeability were confirmed in a *C. rodentium*/mouse model of infection. Thus, while EspF, and possibly Map, may directly disrupt TJs, NleA may potentiate this phenotype by blocking the trafficking of newly synthesized TJ proteins to the membrane.

Role of EspG1/2 in paracellular permeability changes: The related EPEC effectors EspG1 and EspG2 (42% identical, 62% similar) contribute to EPEC-induced TER decreases across epithelial cells (72,131). EspG disrupts host microtubule networks, and this promotes cytoplasmic accumulation of occludin and delays TJ recovery following infection (40).

Other host cell pathways involved in EPEC-mediated barrier disruption: Several studies have implicated specific host cell alterations in EPEC-induced barrier disruption, but the contributing effector molecules, and the proximal signaling pathways, have not been identified. Simonovic et al. (2000) demonstrated the dephosphorylation of occludin, and its displacement into intracellular compartments in EPEC-infected T84 cells (111). While the relevant occludin phosphorylation site(s) was not defined in this study, the serine/threonine phosphatase inhibitor calyculin A prevented EPEC-induced occludin relocalization, as well as infection-induced drop in TER. The atypical protein kinase C, PKCζ, has also been implicated in EPEC-induced TJ alterations and barrier disruption (130). PKCζ colocalized with occludin in infected cells, and PKCζ inhibition prevented EPEC-induced TER

decrease. PKCζ is known to interact with the coiled-coil domain of occludin and regulate its phosphorylation and cellular localization (6, 90).

Role of actomyosin contraction in EPEC-induced barrier disruption: Early studies revealed MLC kinase to be one of the most prominent phosphoproteins in EPEC-infected cells (69). Subsequently, MLCK inhibition was shown to prevent EPEC-induced TER decrease in MDCK monolayers; a cell-permeable MLCK inhibitor also reduced infection-induced MLC phosphorylation, and attenuated EPEC-induced permeability changes, measured via TER and mannitol flux studies (150, 156). MLCK inhibition also ameliorated *C. rodentium*-induced colitis in mice (22).

It is worth highlighting that despite harboring closely related homologs and mediating similar gross alterations in host cell physiology, A/E pathogens exhibit differences at the mechanistic level. Thus, unlike EPEC, an EHEC Δ*espF* strain is not impaired for perturbing epithelial barrier function, measured as TER changes in infected T84 cells (139). EHEC *espF*, however, restored the ability of an EPEC Δ*espF* strain to decrease TER. Unlike EPEC, EHEC harbors EspF paralogs that may compensate for the absence of *espF* in the deletion strain. Also, unlike EPEC, EHEC infection had no impact on PKCζ expression or localization, and PKCζ inhibition did not impact EHEC-induced drop in TER in T84 cells (130).

Role of diarrhea in reducing pathogen burden: A recent study used a *C. rodentium*/ mouse model of infection to demonstrate the beneficial effects of diarrhea for the host (132). *C. rodentium* infection resulted in IL-22-dependent upregulation of claudin-2, and increase in paracellular transport of water and Na^+. Claudin-2-deficient mice had increased bacterial burdens, prolonged pathogen shedding, and greater tissue injury and disease; chemically-induced osmotic diarrhea reduced disease severity in these animals.

Salmonella enterica

Salmonella spp. are significant contributors to foodborne illness worldwide, with symptoms ranging from mild diarrhea to typhoid (57). These intracellular pathogens deploy two distinct T3SS, encoded on chromosomal regions called *Salmonella* pathogenicity islands (SPIs) 1 and 2, which inject virulence proteins into the host cytosol. SPI-1 T3SS effectors facilitate gut epithelial cell invasion, while SPI-2 effectors are essential for survival and growth of the bacteria within a specialized intracellular vacuolar compartment (57).

Salmonella-induced changes in TJ integrity and paracellular permeability—

Finlay et al. first noted the reduction in TER in *S.* Cholerasuis-infected MDCK monolayers (30), and they subsequently confirmed these observations, and extended them to *S. enterica* serovar Typhimurium (*S.* Typhimurium) infections, in Caco-2 monolayers (29). Other investigators confirmed the paracellular permeability changes in *Salmonella*-infected MDCK II cells, including increased inulin transport (48). While contiguous ZO-1 membrane distribution was seen in infected cells, there were gross morphological TJ distortions, including contraction of the perijunctional actomyosin ring. A follow-up study using inhibitors, however, revealed that actomyosin contraction was not required for *Salmonella*-

induced alterations in paracellular permeability (49). *S*. Typhimurium can modulate TJ integrity sufficiently to permit the migration of polymorphonuclear neutrophils across the intestinal monolayer (53).

Salmonella proteins that alter TJ integrity and paracellular permeability: SopB, SopE, SopE2, and SpiA—Initial clues to the potential mechanisms for *Salmonella*-induced barrier disruption came from Tafazoli et al., who observed that invasion-deficient mutants were unable to perturb ZO-1 or occludin distribution, increase paracellular dextran flux, or decrease TER (123). Further, the geranylger-anylltransferase 1 inhibitor GGTI-298, whose targets include Rho GTPases, blocked *S*. Typhimurium-induced TJ protein displacement, and TER decreases (123). Boyle et al. identified three SPI-1-encoded RhoGTPase-activating effectors, SopB, SopE, and SopE2, to be collectively required to mediate *S*. Typhimurium-dependent barrier alterations (11). SopB, SopE, and SopE2 induce other host cell alterations including the activation of a proinflammatory cascade that leads to neutrophil recruitment (57). While single mutations in the corresponding genes did not have a discernable impact (i.e., the mutants disrupted barrier function like the parent WT strain), a Δ*sopB/E/E2* triple mutant failed to decrease TER or increase 4 kDa- and 40 kDa-dextran permeability. A Δ*sipA/sopE/sopE2* strain was also severely impaired for disrupting barrier function. SipA is a SPI1-encoded effector that facilitates bacterial invasion via its actin bundling functions (57). The SPI-2 T3SS was dispensable for *Salmonella*-induced barrier disruption.

An earlier study presented data to suggest temporal shifts in *Salmonella*-induced TJ perturbations (7). Specifically, T84 cells infected with WT *S*. Typhimurium exhibited a rapid decrease in TER, while infection with a *sigD*-deficient mutant resulted in a delayed decrease in TER (SigD is also known as SopB). Both strains eventually lowered the TER to a similar extent. Bisindolylmaleimide, a conventional PKC inhibitor, inhibited the initial WT *Salmonella*-induced reduction in TER, but did not alter the eventual drop in resistance, suggesting a role for PKC in the acute SigD/SopB-dependent increase in paracellular permeability.

Zhang et al. observed a progressive increase in claudin-2 expression, as well as altered distribution of this protein at the TJs of *S*. Typhimurium-infected colonic epithelial cells (HT29C19A and SKCO15 cells), and in the colons of streptomycin-treated mice (154). siRNA-mediated claudin-2 knockdown attenuated infection-induced TER decrease, implicating the leak pathway in *Salmonella*-induced paracellular permeability changes. Using pharmacological inhibitors, the investigators implicated a role for the epidermal growth factor receptor pathway, and downstream c-Jun N-terminal kinases (JNK), in infection-induced claudin-2 expression. There was also a decrease in intracellular *Salmonella* in the siRNA-treated cells, implicating a direct or indirect role for claudin-2 in bacterial invasion.

A _Salmonella_ protein that limits TJ disruption and paracellular permeability: AvrA—In contrast to the TJ-disrupting activities of SopB, SopE, SopE2, and SipA, another SPI-1 effector, AvrA, stabilizes TJs, and limits paracellular permeability (63). AvrA expression stabilized occludin and ZO-1 expression in *S*. Typhimurium-infected cells

in vitro and *in vivo*, and transfected HT29C19A cells expressing AvrA had increased expression of occludin, ZO-1, and claudin-1. Compared to AvrA-deficient bacteria, strains harboring AvrA significantly decreased intestinal permeability to 3 kDa FITC-dextran in infected mice.

AvrA suppresses inflammation via its acetyltransferase activity toward specific host MAP kinases, and inhibition of the JNK/AP-1 and NF-κB signaling pathways (57). Extending the previous work, both *S*. Typhimurium and *S. enterica* Enteritidis (*S*. Enteritidis) AvrA were shown to augment TJ barrier function by stabilizing the expression and junctional localization of ZO-1 (64, 153). *S*. Enteritidis induced a dramatic decrease in TER, and this was further potentiated in Δ*avrA*-infected cells. Correspondingly, JNK phosphorylation was also greater in cells infected with the *avrA* deletion mutants. The JNK inhibitor SP600125 abolished AvrA-dependent ZO-1 stabilization at the TJs. Strains lacking *avrA* invaded epithelial cells better, and caused more pronounced pathology in a mouse gastroenteritis model of infection. AvrA also inhibited JNK pathway and stabilized ZO-1 levels in the mouse cecum. The two serovars, *S*. Typhimurium and *S*. Enteritidis did exhibit some differences; unlike *S*. Typhimurium, *S*. Enteritidis did not regulate occludin or claudin-1 and −2 levels.

It should be noted that WT *S*. Typhimurium prototype strain (14028s) expresses low levels of AvrA, and does not induce ZO-1 expression. The TJ-modulating functions of AvrA were uncovered using a 14028s derivative with an altered regulatory cascade that results in increased AvrA expression (among other alterations). The precise *in vivo* contexts in which the TJ modulatory functions of AvrA contribute to pathogenesis, therefore, remains to be defined. *S. enterica* strains associated with systemic infections have robust expression of SopB and SopE1, but do not express AvrA (63). One possible interpretation is that systemic *Salmonella* infection is facilitated by the ability to disrupt TJs.

Shigella spp.

Shigella spp. are the primary cause of bacillary dysentery, and the second leading cause (after rotavirus) of diarrheal deaths worldwide (20). *Shigella* is a primate-restricted pathogen, which distinguishes it from its close relatives. The four species of *Shigella*, *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*, vary in geographic distribution and antibiotic susceptibilities (73). These pathogens cross the epithelial monolayer via the M cells, access basolateral receptors of epithelial cells, and gain entry. They replicate within the host cytoplasm, and spread across to adjacent cells via a specialized mechanism (discussed below). A large virulence plasmid encodes most of the virulence factors including a T3SS.

Shigella-induced changes in TJ integrity and paracellular permeability—

Sakaguchi et al. first explored the possibility that *S. flexneri* applied apically to polarized T84 cells could disrupt TJs and allow for the paracellular passage of bacteria (104). Apical infection with *S. flexneri* strains decreased the expression of ZO-1, ZO-2, and E-cadherin, dephosphorylated occludin, and displaced claudin-1 from membrane fractions of infected T84 cells. Both WT and noninvasive strains initially increased TER for 10 min postinfection, and subsequently reduced it to less than 70% of nonstimulated cells. Concomitantly, the bacteria penetrated the monolayer and gained access to the basolateral

surface via the paracellular pathway. Apical infection of Caco-2 monolayers with *S. flexneri* or *S. dysenteriae* also caused the loss of ZO-1 from cell junctions and induced the hyperphosphorylation of occludin (32). These changes were accompanied by a decrease in TER, and increased paracellular permeability of 4 kDa dextran and bovine serum albumin (66 kDa).

Following entry and growth within the host cytosol, actin polymerization on one pole of the bacterium thrusts it forward, across the lateral membranes, and into adjacent cells. This allows the pathogen to spread across the epithelial monolayer without exposure to immune cells in the lumen or lamina propria. ZO-1 may be recruited to the distal portions of the *Shigella* actin tails, although the consequent implications to paracellular permeability is unknown (44). On reaching the inner surface of the host cell, the force of actin polymerization causes the bacteria to push against the membrane to form finger-like pseudopodia. Once inside the adjacent cell, the membranes are pinched off, and the bacteria are delivered into the new cell in a double-membraned compartment (4). The preferred points of cell-to-cell transit for the bacteria are junctions formed by three cells. The tricellular junction protein tricellulin is essential for cell-to-cell spread of *Shigella*, and involves a noncanonical clathrin-dependent endocytic pathway (35).

***Shigella* virulence factors that contribute to barrier disruption—**The secreted protein SepA was recently implicated in *Shigella*-induced barrier disruption (68). SepA is the major *S. flexneri* protein secreted in culture and in infected cells, and is required for bacterial invasion of epithelial monolayers. WT *S. flexneri*, but not a *sepA* deletion strain (Δ*sepA*), dramatically reduced the expression of Lim kinase 1, an inhibitor of the actin-remodeling protein, cofilin. The consequent activation of cofilin triggers actin depolymerization and severing, leading to barrier disruption. SepA-mediated TJ changes facilitate bacterial penetration across the epithelium and subsequent bacterial invasion.

Helicobacter pylori

The gastric mucosal pathogen *H. pylori* colonizes over half of the human population and causes persistent gastritis in all carriers (95). Of the infected population, 10% to 15% develop severe gastric disease including adenocarcinoma. A unique set of virulence factors facilitate *H. pylori* to colonize its particularly hostile niche and cause disease. Some of these factors also alter TJs, a possible contributing event on the path to cancer development.

***Helicobacter*-induced changes in TJ integrity and paracellular permeability—**Electron microscopy of biopsy samples from infected patients revealed the interaction of *H. pylori* (previously known as *Campylobacter coli*) with regions close to TJs, and the penetration of bacteria across the paracellular space (8, 17, 87). Freeze-fracture microscopy of antral (region close to the duodenum) biopsy samples from *H. pylori*-infected patients showed thickened, knobby, irregular, and focally fragmented TJ strands (89). *H. pylori* colonization was correlated with increased sucrose permeability, even in patients with asymptomatic gastritis (9, 34). Corpus biopsy samples from *H. pylori*-positive patients displayed greater paracellular permeability in Ussing chamber studies compared to tissues from *H. pylori*-negative individuals (74).

Helicobacter-induced alterations in permeability was recapitulated in animal models of infection. In *Helicobacter felis*-infected C57BL/6J mice, bacterial colonization and inflammation contributed to increased gastric permeability (75). Similarly, infection of C57BL/6J mice with the murine-adapted SS1 *H. pylori* strain resulted in altered distribution of cortical actin and occludin in the gastric surface epithelium. Lanthanum (4.2 A) tracer invasion assays confirmed barrier disruption of the gastric epithelium (122).

Mechanisms of H. pylori-induced alterations in paracellular permeability—In considering the impact of *H. pylori* on paracellular permeability, it is important to note that the structure and function of the gastric TJs are quite distinct from that of the intestine. Compared to the intestine, the stomach has a tighter epithelium, reflected in a greater baseline TER, and displays more TJ strands in freeze-fracture microscopy (14). Further, as in the intestine, there is heterogeneity in permeability in different parts of the gastric epithelium, with the surface epithelial cells being less permeable than those within the gastric glands (14). Some studies on the impact of *H. pylori* on paracellular permeability described below, particularly those using nongastric cells should, therefore, be interpreted with caution.

Most available gastric cell lines do not form monolayers and are, therefore, not useful for studying paracellular permeability changes. One exception is the NCI-N87 gastric cell line, which expresses claudin-18 (the most prominent isoform in gastric cells *in vivo*), forms a monolayer and has a baseline TER of about 1,000 Ω cm^2. In gastric epithelial HGE-20 cells, which are derived from NCI-N87 cells, *H. pylori* induced the phosphorylation of IL-1 receptor, which then led to ROCK activation and displacement of claudin-4 from cell junctions to the cytosol (56). The consequences to paracellular permeability were not explored in this study. In the study by Fiorentino et al., *H. pylori* infection of NCI-N87 cells resulted in the loss of ZO-1 and claudin-1 from cell junctions, and caused a progressive decrease in TER relative to uninfected cells (31). *H. pylori* infection also increased the permeability to FITC-dextran (4 kDa) and FITC-BSA (40 kDa), and these changes were dependent on the presence of live bacteria.

Role of VacA in *H. pylori*-induced barrier disruption: The key virulence factors of *H. pylori* include the vacuolating toxin VacA, the cytotoxin-associated gene A (CagA), flagellin, and the enzyme urease (95). VacA was originally recognized for its ability to induce the formation of vacuolar compartments within host cells, but has subsequently been implicated in various other host cell phenotypic alterations. All *H. pylori* strains carry VacA, but there is considerable sequence variability across the group (95). Deletion of *vacA* had no impact on *H. pylori*-induced TER decreases in NCI-N87 gastric cells, suggesting that it does not play a role in increasing paracellular permeability (31). Similarly, VacA had no impact on paracellular permeability in Caco-2 and MKN28 gastric epithelial cells (149). In MDCK and T84 cells, however, purified and acid-activated VacA decreased TER, and increased mannitol and sucrose permeability, although there was no overt evidence of alterations in TJ protein distribution (91). In a follow-up study, the investigators reported that VacA-positive *H. pylori*, but not an isogenic Δ*vacA* strain, increased paracellular permeability (92).

Role of CagA in *H. pylori*-induced barrier disruption: The *cag* pathogenicity island is present in highly virulent strains and is a strong predictor for severe disease outcome, including gastric cancer (95). Genes within this island encode a type IV secretion system (T4SS, functionally akin to the T3SS discussed above, but composed of distinct components) which delivers effector proteins directly into the host cytosol. To date, CagA is the only known substrate secreted via the *H. pylori* T4SS.

As with VacA, evidence for a role for CagA in gastric epithelial paracellular perturbations is equivocal (14). CagA was not required for *H. pylori*-dependent paracellular permeability changes in Caco-2 intestinal epithelial cells, and MKN28 or HGE20 gastric epithelial cells (56, 65, 149). On the other hand, a *cagA*$^{+}$ strain, but not an isogenic *cagA*$^{-}$ mutant, dramatically perturbed occludin and ZO-1 distribution in primary human gastric epithelial cells (55). Similarly, EM images revealed TJ alterations in AGS gastric epithelial cells infected with a *cagA*$^{+}$ strain, but not a *cagA*$^{-}$ derivative (116). Neither of these studies, however, assessed paracellular permeability changes in the infected cells. In MDCK cells, injected CagA associated with ZO-1 and JAM, and induced the ectopic assembly of TJ complexes, and perturbed barrier function as assessed by ruthenium red staining and transmission electron microscopy (5). While CagA has been implicated in claudin gene regulation in some cell lines (claudin-2 in AGS cells, claudin-3 and −4 in T84 cells, and claudin-7 in MKN28 cells), the implications of these alterations to paracellular permeability is not known (28,116,148). Claudin isoforms are differentially altered in gastric cancer, and it is speculated that these changes play a role in tumorigenesis (47). CagA also perturbs epithelial cell polarity by interacting with components of the PAR polarity complex, leading to junctional defects and initiating epithelial-to-mesenchymal transition (128).

Role of urease in *H. pylori*-induced barrier disruption: *H. pylori* urease converts urea to ammonia (and carbon dioxide), which buffers gastric acid and creates a neutral zone around the bacterial surface (81). Urease-negative *H. pylori* mutants cannot colonize the stomach. Lytton and coworkers explored a potential role for the increased ammonium (NH^{4+}) in gastric aspirates of *H. pylori*-infected patients to barrier disruption. Ammonium salt exposure, as well as WT *H. pylori* supernatants, acutely reduced the TER of Caco-2 cells, with a subsequent partial recovery. This was accompanied by TJ disruption and the expression of a low-molecular-weight form of occludin of 42 kDa. Epithelial cells exposed to supernatants of urease-deficient mutants did not exhibit a significant decrease in TER. Ammonia dampens the acid-dependent reinforcement of the epithelial barrier in gastric epithelial (HGE20) cells (70). Coculture of HE-20 cells with WT *H. pylori*, but not an isogenic urease-deficient strain (Δ*ureB*), impeded acid-induced increase in TER.

Coculture of MKN28 gastric epithelial cells with WT *H. pylori*, but not an isogenic *ureB* mutant, resulted in a progressive decrease in TER (149). *H. pylori* increased MLC phosphorylation, which was blocked by inhibition of Rho kinase or MLCK; *ureB*-deficient bacteria failed to induce MLC phosphorylation. While Rho kinase can directly phosphorylate MLC, it can also inhibit MLC phosphatase activity by phosphorylating myosin phosphatase target subunit I (MYPT1). No significant changes were seen in phosphorylated MYPT1, suggesting a direct role for Rho kinase in phosphorylating MLC in *H. pylori*-infected cells.

Clostridial toxins and barrier function

The clostridia include numerous pathogenic strains capable of causing disease in humans and animals (96). These Gram-positive spore-forming organisms are the most prolific toxin producers among bacteria, and toxins are usually the main determinants of disease. Clostridial toxins belong to three broad groups: (a) pore-forming toxins like the cholesterol-dependent cytolysins, (b) nonpore-forming molecules, including enzymes, which act on host-cell surface molecules or matrix components, and (c) toxins that enter host cells and alter signaling pathways. Many of these toxins disrupt the intestinal epithelial barrier. In the following sections, one example from each of the above functional categories is discussed in the context of barrier disruption.

C. perfringens *enterotoxin*—*C. perfringens*** enterotoxin (CPE) is responsible for the abdominal cramping and diarrhea associated with foodborne and non-foodborne enterotoxigenic *C. perfringens* strains (109). Ingested bacteria that escape killing by stomach acids multiply in the intestine and sporulate. CPE is released concomitant with spore egress from the mother cell. Katahira et al. first identified the CPE receptor, subsequently called as claudin-4, from an expression library of enterotoxin-sensitive Vero cells (51).

CPE-induced changes in TJ integrity and paracellular permeability: Sonoda et al. used CPE as a tool to probe the role of claudins in epithelial barrier function (117). The noncytotoxic C-terminal fragment of CPE (C-CPE) bound claudin-3 and −4 with high affinity, but not claudin-1 or −2, in MDCK cells. Basolateral, but not apical, C-CPE treatment of MDCK1 caused the selective displacement of claudin-4 from the TJs to the cytoplasm, and its subsequent degradation. Freeze-fracture microscopy showed aberrant TJs, with reduced number of strands and limited network complexity. Basolateral treatment of MDCK cells with C-CPE induced a ~4.5-fold drop in TER, and a twofold increase in the flux of 4 kDA and 10 kDA FITC-dextran, but not 40 kDa FITC-dextran. Removal of C-CPE resulted in the gradual recovery of TER to the levels of untreated controls. This was, in fact, the first evidence of a role for claudins in epithelial barrier function.

Members of the claudin family serve as high (claudin-3, −4, −6, −7, and 9) or low/medium (claudin-1, 2, 8, 14, and 19) affinity receptors for CPE (27). CPE is an unusual pore-forming toxin that recruits host membrane proteins, including occludin, into large membrane complexes of up to 200 kDa (114). Complex formation was shown to be essential for CPE cytotoxicity. At low concentrations (1 μg/mL), CPE forms a limited number of pores that allows passage of small cations, including the influx of Ca^{2+}, which leads to rapid activation of the intrinsic apoptosis pathway (15). In Caco-2 cells, 1 μg/mL CPE disrupted the actin cytoskeleton and induced the removal of occludin, but not ZO-1, from the TJs (113).

While the C-CPE fragment perturbed TJs and altered barrier function without a cytotoxic effect on host cells (117), this pathway may not be involved in the permeability increases mediated by the intact toxin. Based on the kinetics of killing, it has been argued that CPE perturbs barrier function primarily via its pore-forming and cytotoxic effects (27).

***Clostridium difficile* toxins A and B:** *Clostridium difficile*, the leading cause of healthcare-associated diarrhea, was responsible for nearly half a million cases in 2011 in the United

States, with 29,300 deaths (62). Ingested spores germinate into vegetative cells in the intestine in response to certain bile acids and glycine. Antibiotic-mediated alterations of the normal flora allows the vegetative cells to colonize and produce toxins. The large clostridial toxins, toxin A (TcdA) and toxin B (TcdB), are the primary mediators of disease; the binary toxin (CDT), present in some strains, is associated with increased patient mortality (38).

While TcdA and TcdB share sequence similarity, they bind to distinct receptors and have unique effects on host cells, and play different roles *in vivo* (115). Both toxins have a C-terminal combined repetitive oligopeptide repeat (CROP) domain that facilitates interactions with host cell oligosaccharides and facilitates toxin endocytosis. TcdA binds to multiple glycolipids and glycosylated proteins, while TcdB receptors include the poliovirus receptor-like protein (PVRL3, also known as nectin-3) and chondroitin sulfate proteoglycan 4. PVRL3/nectin-3 is a component of AJs which interacts with afadin, a filamentous actin-binding protein. Following entry and endosome acidification, the central cysteine protease domain and delivery domains facilitate autocatalytic processing of the toxin and egress of the N-terminal glucosyl transferase domain (GTD) into the cytosol. The GTD modifies and inactivates Rho family GTPases including Rho, Rac, and CDC42, resulting in cytoskeletal alterations and host cell death.

Rodent studies with isogenic TcdA or TcdB mutants concur on the ability of TcdB to cause severe disease in the absence of TcdA. Consistent with this, Tcd A−B+ strains have been recovered in clinical settings (2). On the other hand, data on the ability of Tcd A+B− strains to cause disease have been more variable (115). In ileal loop models, TcdA caused profound necrosis and inflammation, while even high levels of TcdB failed to induce these changes. In studies with colonic tissues, TcdB is more proinflammatory than TcdA.

***C. difficile* toxin-induced changes in TJ integrity and paracellular permeability:** Hecht and coworkers first demonstrated the disruption of barrier function by *C. difficile* TcdA and TcdB (45, 46), and Moore et al. confirmed TcdA-dependent permeability changes in *ex vivo* experiments using guinea pig ileal tissues (82). The addition of either toxin to the apical surface of T84 cells increased paracellular permeability, as measured via TER and sodium-mannitol flux studies, but TcdA-induced effects were markedly more potent and rapid (TER decreases of ~99% in 6 to 8 h for TcdA, ~74% in 72 h for TcdB, both toxins at 0.7 μg/mL) (45, 46). While the toxins caused the disassembly of perijunctional actin filaments, TcdA-dependent TJ permeability increases preceded these changes. Chen et al. explored the possibility that TcdA-mediated barrier disruption occurred prior to, and independent of Rho glucosylation (16). They demonstrated the TcdA-dependent activation of PKCα and PKCβ, increased ZO-1 translocation from TJs, and decrease in TER of T84 cells within 2 to 3 h of toxin exposure. Consistent with a role for TcdA-induced PKC activation in barrier disruption, a PKCα/β antagonist inhibited TcdA-mediated TJ protein changes and TER decrease. Other investigators have also reported the glucosyltransferase-independent impacts of the toxins on epithelial barrier function (151).

The reasons underlying the delayed TER decrease for TcdB relative to TcdA is presently not known (45, 46). TcdB, unlike TcdA, may impact barrier function only after entry into cells and subsequent Rho GTPase inhibition. It is also possible that TcdB interferes with cell-cell

interactions by binding to nectin-3 and disrupting the AJs. It should be noted, however, that nectin-3 knockout mice did not show significant alterations in paracellular permeability (125).

Based on the differential impacts of the two toxins when added to the apical surface of epithelial cells, a synergistic mode of action has been proposed (102). According to this model, TcdA-mediated barrier disruption results in the increased paracellular passage of TcdB. Interaction of TcdB with basolateral receptors, possibly PVRL3/nectin-3, then accounts for the marked increase in cytotoxic effects and further damage to the epithelium. A caveat to this model is that Tcd A−B+ *C. difficile* strains have been associated with outbreaks, and can cause disease in animal models (2, 10). Supernatants from two outbreak-associated Tcd A−B+ strains failed to decrease the TER of Caco-2 cells even after 10 h incubation, while a Tcd A+B+ strain dramatically reduced TER within 5 h (2). Thus, TcdA may facilitate TcdB translocation and intoxication, but there are likely independent pathways for TcdB to breach the epithelial barrier and cause disease.

Botulinum toxin: One of the most potent toxins known, BoNTs are produced by *Clostridium botulinum*, and cause the persistent paralysis of peripheral nerve terminals, resulting in the disease known as botulism (100). In foodborne and infant botulism, the most common forms, the ingested toxin must cross the intestinal epithelial barrier, enter the systemic circulation, and eventually reach target neurons. BoNTs proteolytically cleave peripheral nerve SNARE proteins and block neurotransmitter release. To date, more than 40 genetic variants of BoNT, differing up to 36% at the amino acid level, have been identified (101). BoNTs were traditionally classified on the basis of interactions with monovalent botulinum antitoxin into seven serotypes (A-G), but this system has come into question with the identification of newer cross-reacting BoNT types. BoNT types A, B, E, and F cause botulism in humans, while types C and D cause disease in birds and cattle, respectively.

BoNTs form large protein complexes by associating with neurotoxin-associated proteins (NAPs) (100). NAPs include nontoxic nonhemagglutinin (NTNHA) and hemagglutinin (HA), which itself is comprised of three proteins, HA1, HA2, and HA3. The large progenitor toxin complex (L-PTC) or 16S complex, which contains BoNT, NTNHA, and HA, has an oral toxicity that is several hundred folds more than that of BoNT itself (71). Nontoxic components protect BoNT from proteases and other damaging agents in the gastrointestinal tract. In addition, the HA complex facilitates toxin transport across the intestinal epithelium.

BoNT-induced changes in TJ integrity and paracellular permeability: Matsumura and coworkers showed that L-PTC added to either side of Caco-2 monolayers induced a drop in TER; the effect was more pronounced and rapid when the toxin was added basolaterally. In MDCK cells, however, L-PTC decreased the TER only when added basolaterally. In contrast, neither the M-PTC (BoNT + NTNHA) nor BoNT alone decreased the TER of Caco-2 cells, suggesting a role for the HA complex in this process. Further, L-PTC-induced paracellular permeability changes were blocked by anti-HA antiserum. Apical addition of L-PTC also disrupted the localization of occludin, ZO-1, E-cadherin, and β-catenin at cell boundaries, and increased the paracellular flux of dextran (4–150 kDa). The permeability changes were not accompanied by any toxic effects on the Caco-2 cells.

In subsequent work, this group showed the direct interaction of HA with the human, bovine and mouse isoforms of E-cadherin, but not those of rats or chickens (121). Recombinant HA caused E-cadherin internalization and disrupted cell-cell adhesion. HA also decreased the TER of MDCK cells expressing mouse, but not rat, E-cadherin. L-PTC/A and L-PTC/B, but not L-PTC/C, interacted with E-cadherin (121); correspondingly, L-PTC/A and L-PTC/B, but not L-PTC/C, decreased TER and increased 4 to 150 kDa dextran flux across Caco-2 and T84 monolayer (50).

E-cadherin, a component of the AJs, is present below the TJs, and would generally not be accessible to HA from the apical surface. This poses a conundrum: for HA to interact with E-cadherin and disrupt the barrier, it would first have to access the basolateral surface. Available data support two modes by which HA can reach the basal side: transcytosis through epithelial cells, or via M cells. HA added to the apical surface of Caco-2 cells was internalized into endosomes and transcytosed to the basolateral sides (121). HA also bound to glycoprotein 2 on microfold (M) cells in the Peyer's patches of mouse intestines, and facilitated transport of the toxin complex to the basolateral sides (71). PTC lacking HA (M-PTC) did not localize to Peyer's patches, and were two orders of magnitude less toxic when delivered orally to mice. Mice depleted for M cells, or lacking glycoprotein 2, were markedly less susceptible to orally delivered L-PTC/A. Traversing through the M cells, the toxin complex is delivered to the basolateral side.

If transcytosis can deliver the toxin to the basolateral side, is there a role for intestinal epithelial barrier disruption in the intoxication process? Lee and coworkers generated HA complexes with single-amino acid changes in two of the HA proteins which were deficient for E-cadherin binding, but not for transcytosis across epithelial monolayers (61). This complex failed to increase paracellular permeability. L-PTC/A complexes with the mutant HA proteins were severely impaired for intoxication in a mouse model, suggesting HA-dependent junctional perturbations substantially augment BoNT transport via the paracellular pathway to the target neurons. Finally, type E and type F toxins produce toxin complexes that lack HA, but still cause food-borne botulism, though they have less oral toxicity (103). Thus, the HA proteins, while not essential, can increase the oral toxicity of BoNT.

Conclusion

The maintenance of intestinal epithelial barrier function is critical for health. In the examples cited above, TJ disruption and altered barrier function not only contribute to disease symptoms but often represent a transition phase in the pathogen's virulence program. Thus, increased permeability may allow pathogens or toxins to penetrate deeper tissue, while increased luminal nutrients and water could promote pathogen growth and dispersal. Recent studies also suggest a host-protective value for increased paracellular permeability and diarrhea. Our understanding of the dynamics of the junctional barrier has been expanding rapidly over the past few years. Studies on pathogen-induced barrier disruption will have to be viewed fresh in light of the recent findings. A detailed understanding of the mechanistic and temporal parameters of TJ alterations during infections could offer novel opportunities for mitigating disease.

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Didactic Synopsis

Major teaching points

- Paracellular transport is the passive movement of ions, solutes, and water via the spaces between cells. This process is regulated by the tight junction complex.
- There are two recognized modes of paracellular transport—the pore and leak pathways—that are independently, and sometimes opposingly, regulated.
- The high-capacity pore pathway is ion- and size-selective, and is gated by the claudin family of proteins. The low-capacity, charge-insensitive, leak pathway permits passage of larger molecules.
- Many pathogenic microbes and their products target tight junctions, and alter their composition and function. The consequent perturbation of paracellular permeability contributes to disease symptoms, including diarrhea.
- Concomitant with altered permeability, the loss of epithelial cell polarity, and the potential passage of toxins and microbes into the lamina propria may exacerbate disease.
- Commensal and probiotic microbes and their products can mitigate pathogen-induced perturbations of paracellular permeability, although the molecular mechanisms are not always well defined.

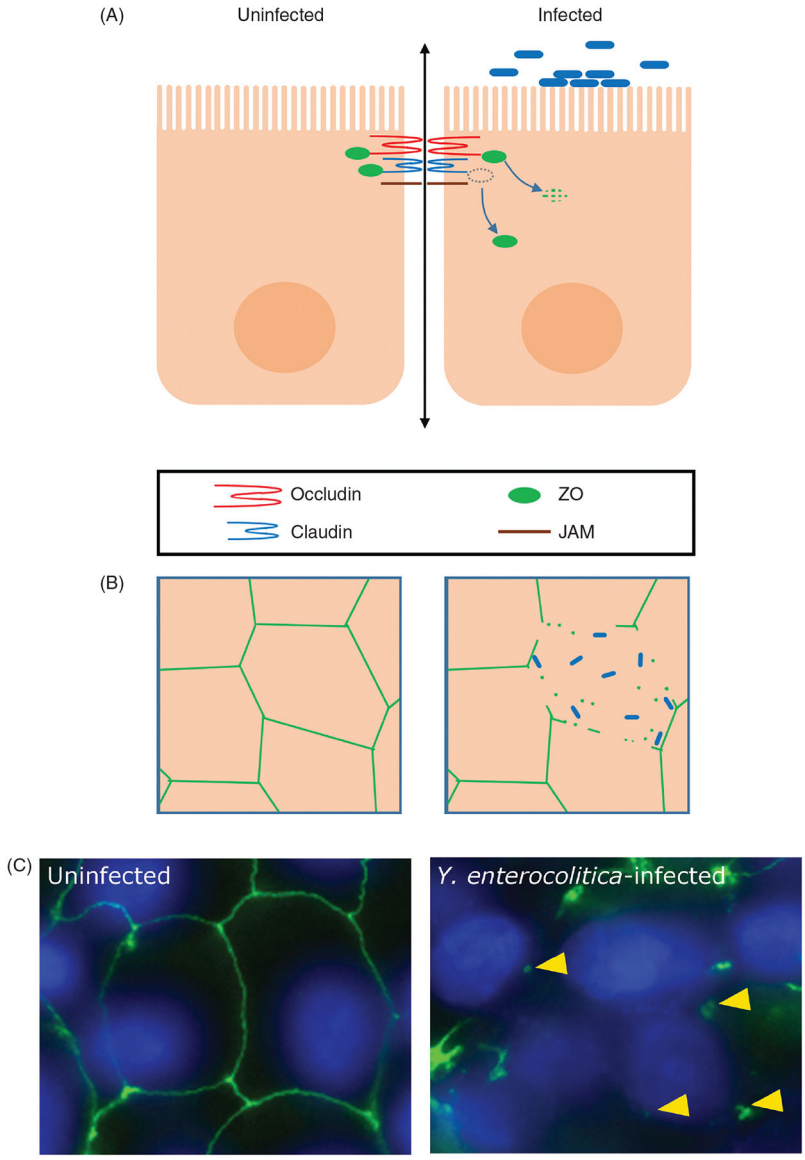

Figure 1.

Depletion and relocalization of TJ proteins and TJ-associated proteins disrupts epithelial barrier. (A) Schematic, lateral view: Pathogens may relocalize TJ proteins (occludin, claudins, JAM, and ZO proteins) to intracellular sites and/or trigger their loss. (B) Schematic, en face view: Intact peripheral staining (green) of junctional proteins in uninfected cells. Junctional staining is lost and TJ protein aggregates form in the cytoplasm of infected cells. (C) Example: ZO-1 (green) is preserved at the cell junctions of cultured human intestinal epithelial $C2_{BBe}$ cells. In cells infected with *Yersinia enterocolitica*, ZO-1 junctional staining is lost, and cytoplasmic ZO-1 aggregates (yellow arrowheads) are observed. DAPI (blue) stains DNA. Image obtained, with permission, from Jennifer Lising Roxas and V.K. Viswanathan.

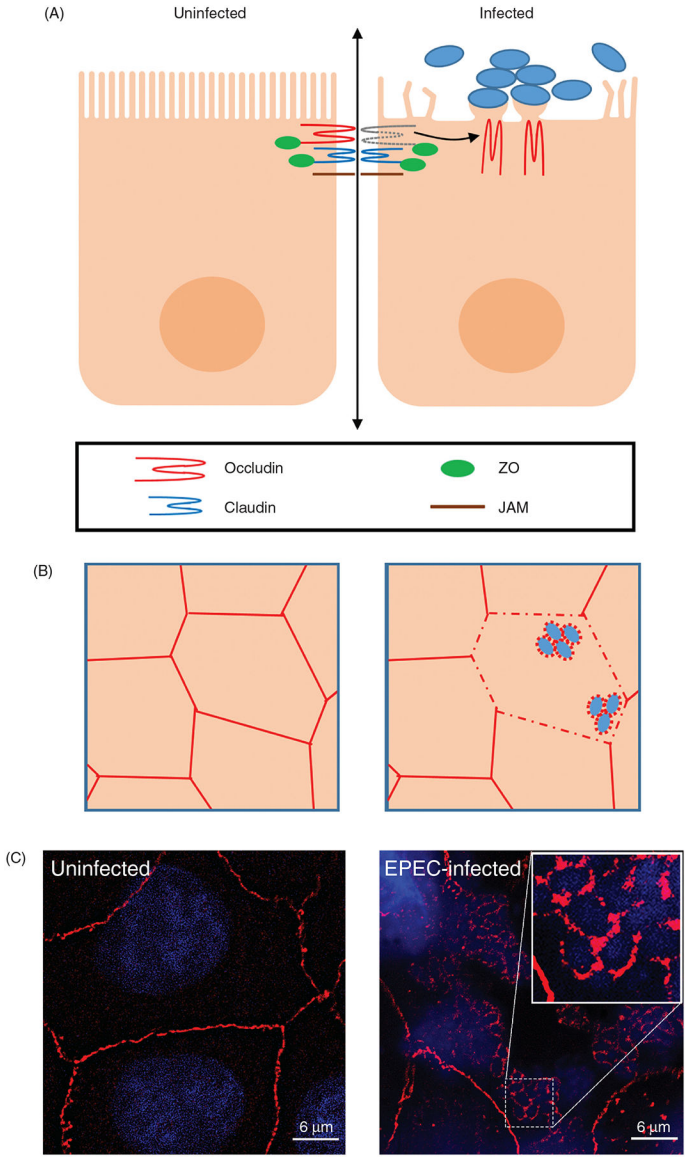

Figure 2.

Displaced TJ proteins may be deposited to alternate sites in host cells, including regions of bacterial attachment. (A) Schematic, lateral view: Beyond displacing proteins from the TJs, pathogens may also perturb epithelial cell polarity and, in some instances, recruit TJ proteins to sites of bacterial attachment. (B) Schematic, en face view: TJ proteins (red) are localized to the periphery of uninfected cells. In infected cells, TJ proteins may be recruited to sites of bacterial attachment. (C) Example: Occludin (red) is preserved at the cell junctions of $C2_{BBe}$ cells. In EPEC-infected cells, occludin is lost from the junctions, and is recruited to regions surrounding the bacteria. Inset depicts magnified view of an EPEC microcolony (blue) surrounded by occludin stain (red). DAPI (blue) stains DNA. Image obtained, with permission, from Jennifer Lising Roxas and V.K. Viswanathan.

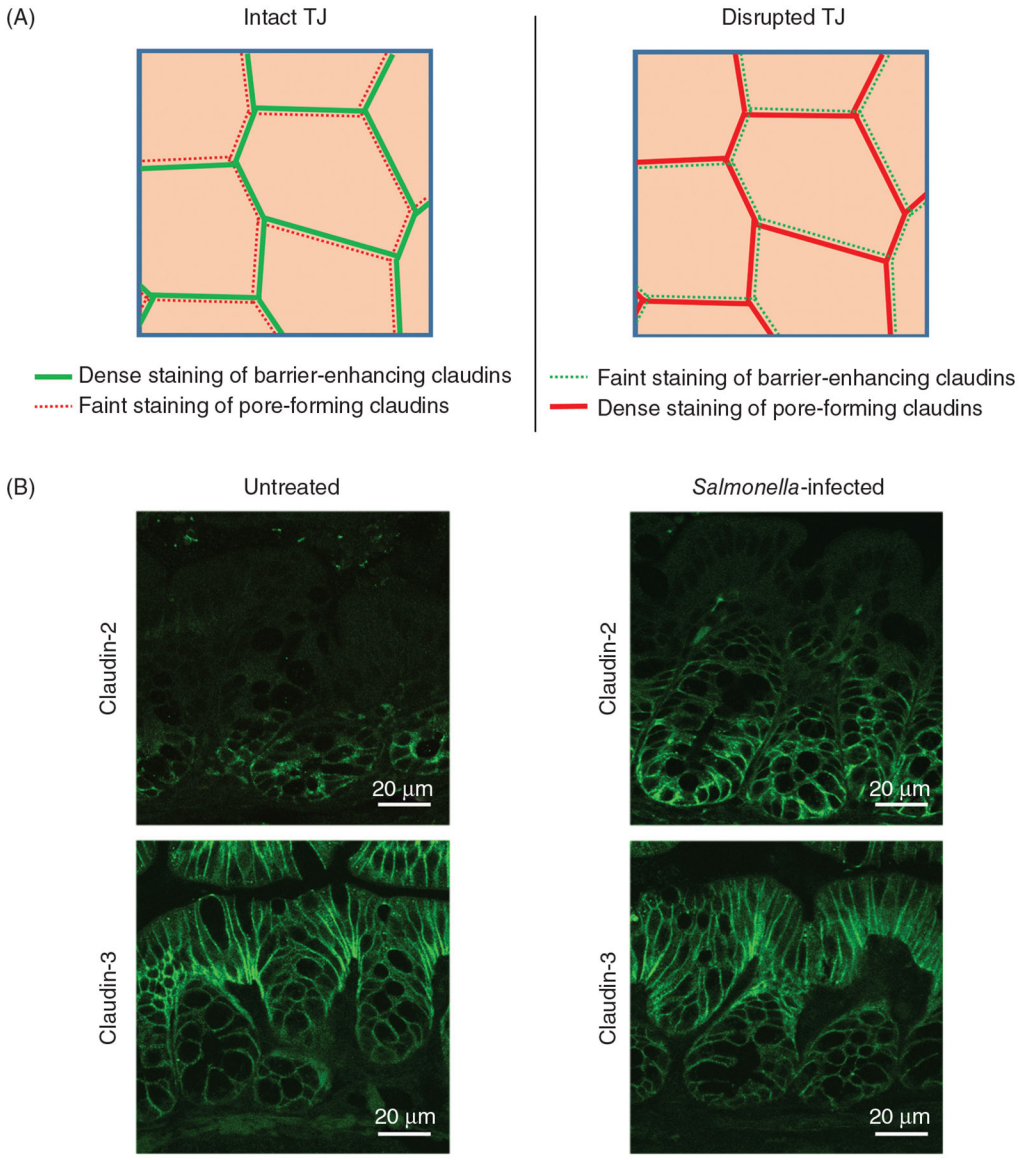

Figure 3.

Pathogens may disrupt barrier function by modulating claudin expression. (A) Schematic: Normal epithelial cells with intact tight junctions express barrier-enhancing claudins in higher abundance (green, dense solid line) compared to pore-forming claudins (faint, blue dotted line). Pathogens may downregulate expression of barrier-enhancing claudins (faint, green dotted line), and/or upregulate expression of poreforming claudins (dense, blue solid line) to increase paracellular permeability. (B) Example: *Salmonella* increases paracellular permeability by selectively upregulating claudin-2 levels. Claudin-3 is unchanged in *Salmonella*-infected cells, compared to uninfected control. Image obtained, with permission, from Yong-Guo Zhang and Jun Sun, University of Illinois at Chicago, Chicago, IL.

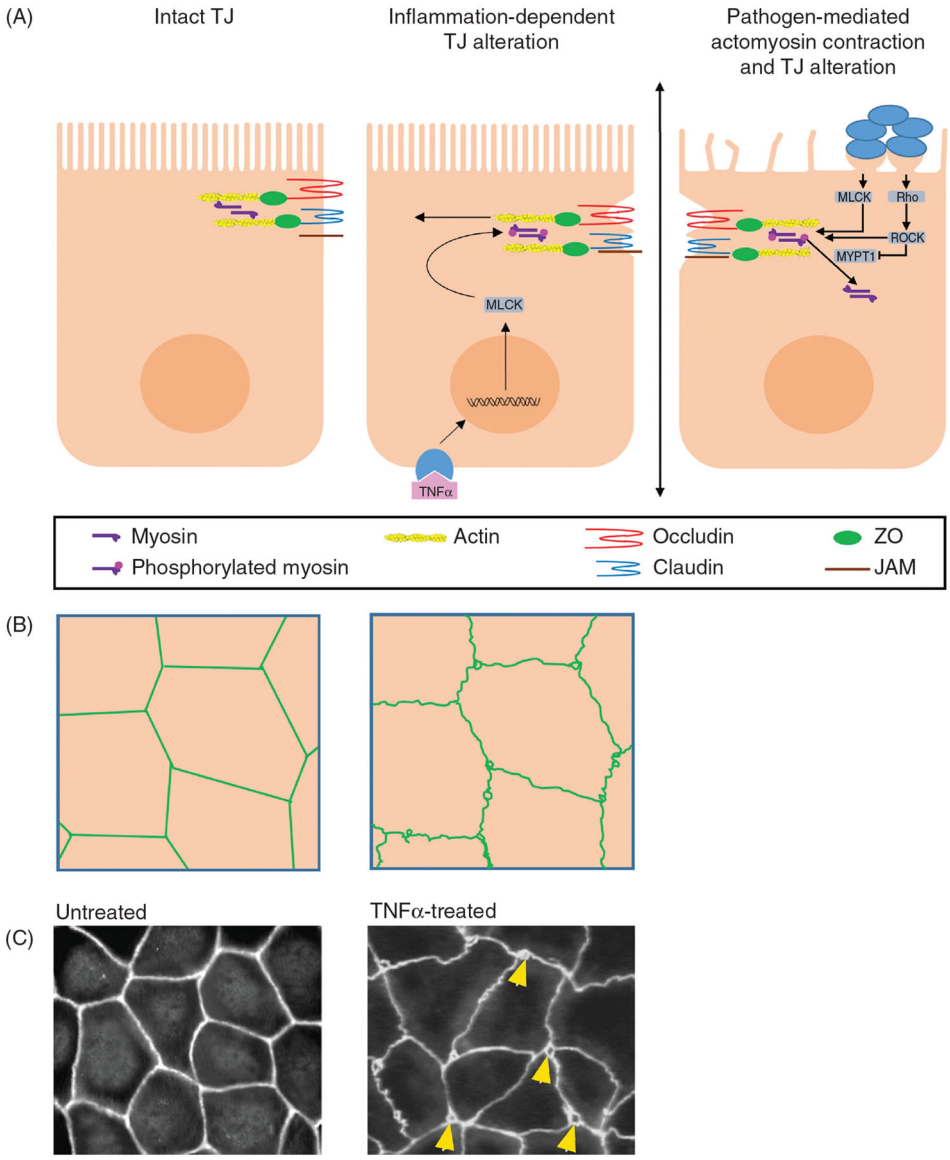

Figure 4.

Pathogens may trigger contraction of the perijunctional actomyosin ring, resulting in increased paracellular permeability by the leak pathway. (A) Schematic, lateral view: Some pathogens trigger signaling pathways that activate myosin light chain kinase (MLCK) and/or Rho-associated protein kinase (ROCK), which can both phosphorylate myosin light chain and cause actomyosin contraction. ROCK also phosphorylates myosin phosphatase target subunit 1 (MYPT1) and, thereby, inhibits MLC dephosphorylation. Alternatively, proinflammatory molecules like TNFα can also activate MLCK. (B) Schematic, en face view: Uniform distribution of TJ proteins (green) preserved at the periphery of uninfected cells. Pathogens or proinflammatory molecules like TNFα induce actomyosin contraction leading to the distortion and opening of junctional areas. Gaps may also be evident at points of cell contacts. (C) Example: Uniform ZO-1 staining at cell periphery of cultured Caco-2 monolayers. On the right, TNFα-induced disruption of ZO-1 localization, and appearance of

gaps (arrowhead) at points of cell contact. Image obtained, with permission, from Ma et al. (66).

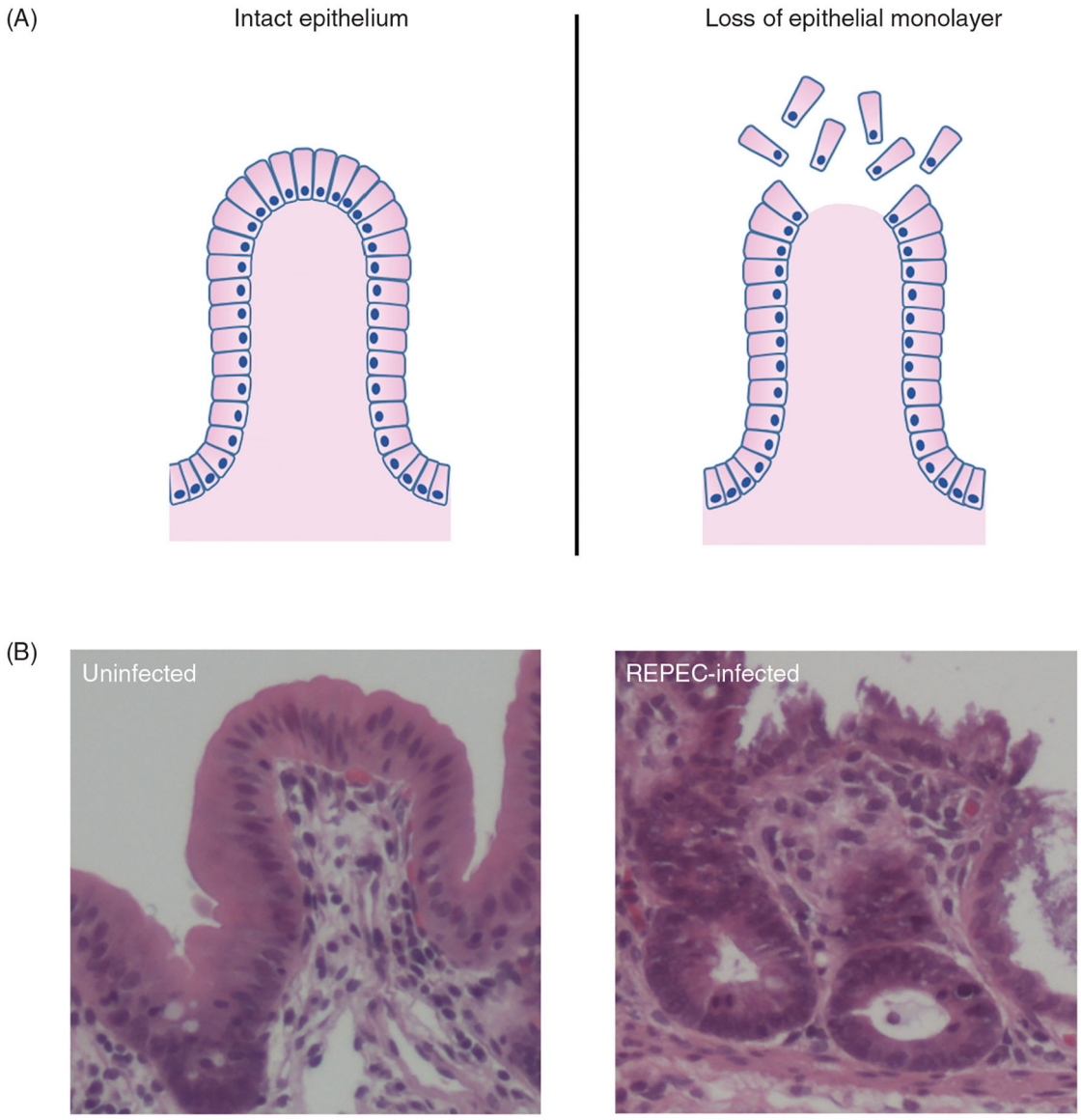

Figure 5.

Prolonged infections, or large pathogen loads, can induce large-scale loss of the epithelial monolayer, leading to barrier dysfunction. (A) Schematic: In healthy animals, the intestinal epithelium (a single villus is depicted) is a uniform monolayer lining the lamina propria. Disease states may result in gross epithelial erosion. (B) Example: An intact epithelial monolayer lines the intestine of uninfected rabbits. Infection with rabbit enteropathogenic *Escherichia coli* (REPEC) caused the gross exfoliation of intestinal epithelial cells, coincident with severe diarrhea. Image obtained, with permission, from John Scott Wilbur and V.K. Viswanathan.