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Review

# Tight junctions as targets of infectious agents

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ABSTRACT

The epithelial barrier is a critical border that segregates luminal material from entering tissues. Essential components of this epithelial fence are physical intercellular structures termed tight junctions. These junctions use a variety of transmembrane proteins coupled with cytoplasmic adaptors, and the actin cytoskeleton, to attach adjacent cells together thereby forming intercellular seals. Breaching of this barrier has profound effects on human health and disease, as barrier deficiencies have been linked with the onset of inflammation, diarrhea generation and pathogenic effects. Although tight junctions efficiently restrict most microbes from penetrating into deeper tissues and contain the microbiota, some pathogens have developed specific strategies to alter or disrupt these structures as part of their pathogenesis, resulting in either pathogen penetration, or other consequences such as diarrhea. Understanding the strategies that microorganisms use to commandeer the functions of tight junctions is an active area of research in microbial pathogenesis. In this review we highlight and overview the tactics bacteria and viruses use to alter tight junctions during disease. Additionally, these studies have identified novel tight junction protein functions by using pathogens and their virulence factors as tools to study the cell biology of junctional structures.



**Contents**



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## 1. Body of paper

Polarized epithelial cells are positioned at the interface of the lumen and the deep cell layers of organs. Of all the cells in the body, intestinal epithelial cells are the prototypic polarized cell type displaying distinct apices that contain microvilli and bases that interact with the basal lamina, where they anchor into components of the extracellular matrix. Laterally, adjacent cells attach together through intercellular junctions. There are four different types of junctions commonly found at these sites. At the most apical intercellular membranes, three individual junctions (a tight junction followed by an adherens junction then a desmosome) form an apical junction complex (Fig. 1) [1]. Components of the apical junction complex interact with cytoskeletal elements to strengthen their interactions. Tight and adherens junctions form junction belts that course throughout tissues and are involved in numerous signaling events (reviewed by: [2–4]). These junctions attach to actin filaments through cytoplasmic adaptor proteins. Consequently, there are three distinct sub-components that form the individual junctions of the apical junction complex; 1) transmembrane proteins, 2) cytoskeletal elements and 3) cytoplasmic scaffolding proteins that attach the two together.

## 2. Tight junction overview

Tight junctions are the most apically located intercellular junctions and appear as membrane fusions by electron microscopy. Despite their appearance, transmembrane proteins span the intercellular space to restrict the paracellular zone and are crucial for their function. Often extremely small openings exist extracellularly at these sites that allow the intercellular passage of nanometer-sized molecules [5], however the seal can be much tighter to even restrict water molecules [6]. Through altering the function of certain tight junction proteins, dehydration through the epidermal barrier has been observed [6]. Tight junction alterations have also been proposed to be involved in diarrhea generation through a “leak-flux” mechanism in which water enters the lumen of the intestine through the passive movement of both water and ions following a break in the intestinal barrier [7]. Although tight junctions have also been proposed to function in the segregation of apical membrane-bound proteins from those at the basolateral membranes, recent studies in polarized epithelial cells completely devoid of tight junctions have clearly shown that protein segregation is maintained, thus challenging the membrane protein gating function of tight junctions [8].

Thus far there are four primary groups of transmembrane proteins that have been described at conventional epithelial tight junctions; occludin, members of the claudin family, the junction-adhesion-molecules (JAMs) and the Coxsackievirus and Adenovirus Receptor (CAR) proteins. Excellent reviews have described the structure and function of these components and should be referred to for additional background [9–11]. Briefly, these proteins can be grouped into 2 main categories based on their structure. Occludin and the claudins span the membrane four times and interact with adjacent cells through their extracellular loops whereas the JAMs and CAR contain extracellular IgG-like domains that are important for their extracellular attachment.

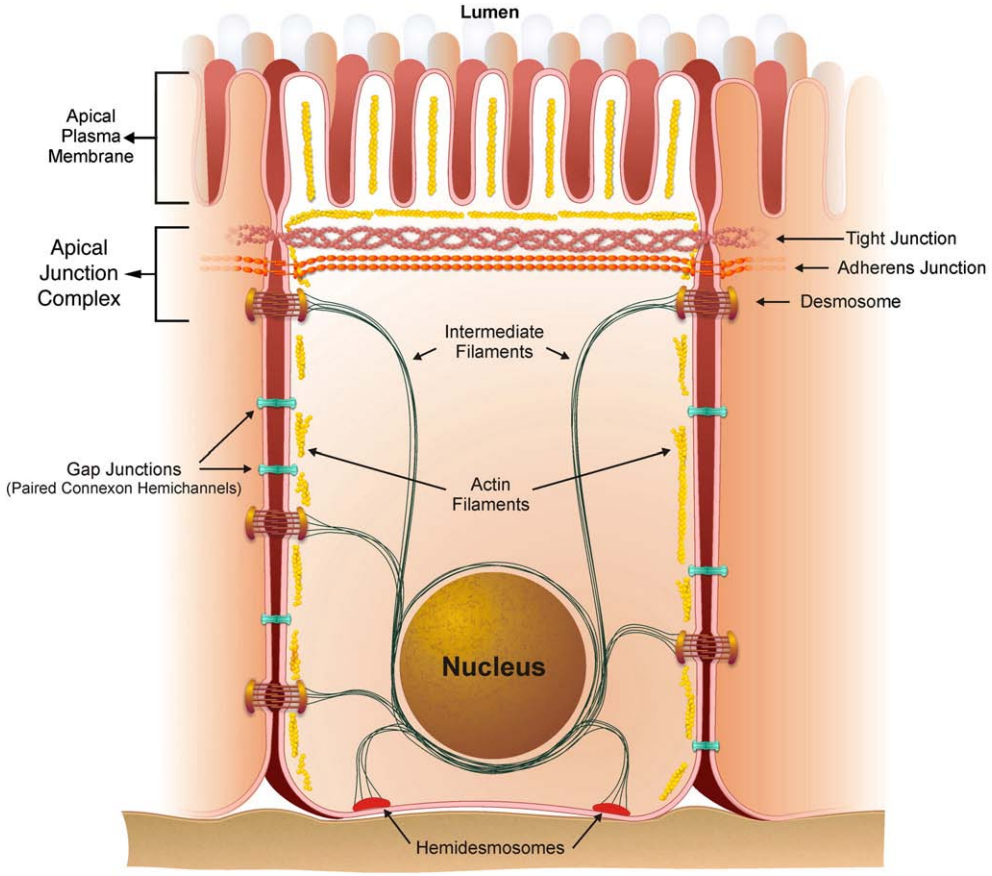


**Fig. 1.** Prototypic arrangement of junctions in polarized epithelial cells. The apical junction complex is formed from the tight junction, adherens junction and the most apically located desmosome. Gap junctions and additional desmosomes associate beneath the apical junction complex along the remainder of the lateral cell membranes. Hemidesmosomes interact with the basal lamina at the base of the cells. Intermediate filaments dock into desmosomes and hemidesmosomes whereas actin filaments attach to both tight and adherens junctions.

## 3. Zonula occludens adaptor proteins

Common to all of these transmembrane proteins are their ability to interact with members of the membrane-associated guanylate kinase (MAGUK) family of cytoplasmic adaptors. The most prominently studied MAGUK proteins at tight junctions are the zonula occludens proteins (ZO-1, ZO-2 and ZO-3). ZO-1 was the first protein discovered exclusively at mature tight junctions [12]. It can interact directly with each of the tight junction transmembrane proteins as well as the actin cytoskeleton.

## 4. Pathogenic organisms and the epithelial barrier

The interactions between pathogenic microorganisms and their hosts have provided researchers with a plethora of scientific puzzles to explore to try to understand various mechanisms of infectious diseases. Although human cells have incorporated epithelial barriers to block organisms that covet access to deeper cell layers within tissues, certain pathogens have evolved to exploit, and thus control, tight junctions to alter this barrier. These pathogens use an array of tactics to commandeer junctional structures for their advantage. Some pathogens use tight junction proteins as receptors for their attachment and subsequent internalization. Others destroy the junctions thereby providing a gateway to the underlying tissue. Infectious enteric agents that alter tight junctions often elicit inflammatory cascades and cause diarrhea. Some of the strategies bacteria and viruses use to hijack tight junction are outlined below.

## 5. Bacteria

### 5.1. Pathogenic Escherichia coli

Enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are members of a family of extracellular pathogens called attaching and effacing (A/E) bacteria that induce the production of diarrhea in their infected hosts. These pathogens use a syringe-like delivery apparatus, called a type-three secretion system (T3SS), to inject pathogenic effector proteins from the bacterial cytosol directly into the cytoplasm of host cells to collapse (efface) localized microvilli (reviewed by:[13,14]), control the functions of organelles [15], rearrange the cytoskeleton and membrane channels (reviewed by: [16]), and disrupt intercellular tight junctions [17–20].

Tight junction alterations have been documented during these infections for decades as exemplified by decreases in trans-epithelial resistance (TER) during incubations of cultured cells (MDCK, T84 and Caco-2) with EPEC. However, as TER indiscriminately calculates the electrical conductance between apical and basal compartments through the measurement of ion conductance and because a difference in [$^3$H]inulin penetration across infected monolayers did not occur during the initial study, despite a decrease in TER in polarized Caco-2 and MDCK cells infected for up to 10 h with EPEC, the authors rightfully did not conclude a breach in the tight junction barrier due to a lack of additional evidence [21]. The conclusion that tight junctions were broken during these infections came from Spitz et al. [22] who used TER as well as sodium/mannitol flux studies on 6 hour EPEC infected T84 cells. Subsequent work confirmed this conclusion and has since demonstrated that occludin [19], ZO-1 [23] and importantly the barrier forming claudin proteins [18] are dissociated from the cell periphery during these infections. Electron microscopic analysis has also shown that tight junction strands are extensively morphologically altered by EPEC during these infections [18]. Three multifunctional EPEC effectors have been implicated in disrupting tight junctions; *E. coli* secreted protein F (EspF) [17], *E. coli* secreted protein G (EspG) [24] and the mitochondrial associated protein (Map) [25] (Fig. 2). During infections using EPEC strains mutated in individual effectors, each of these has demonstrated improvement in tight junction integrity either through microscopic observation, molecular tracer permeability of infected cell monolayers or TER.

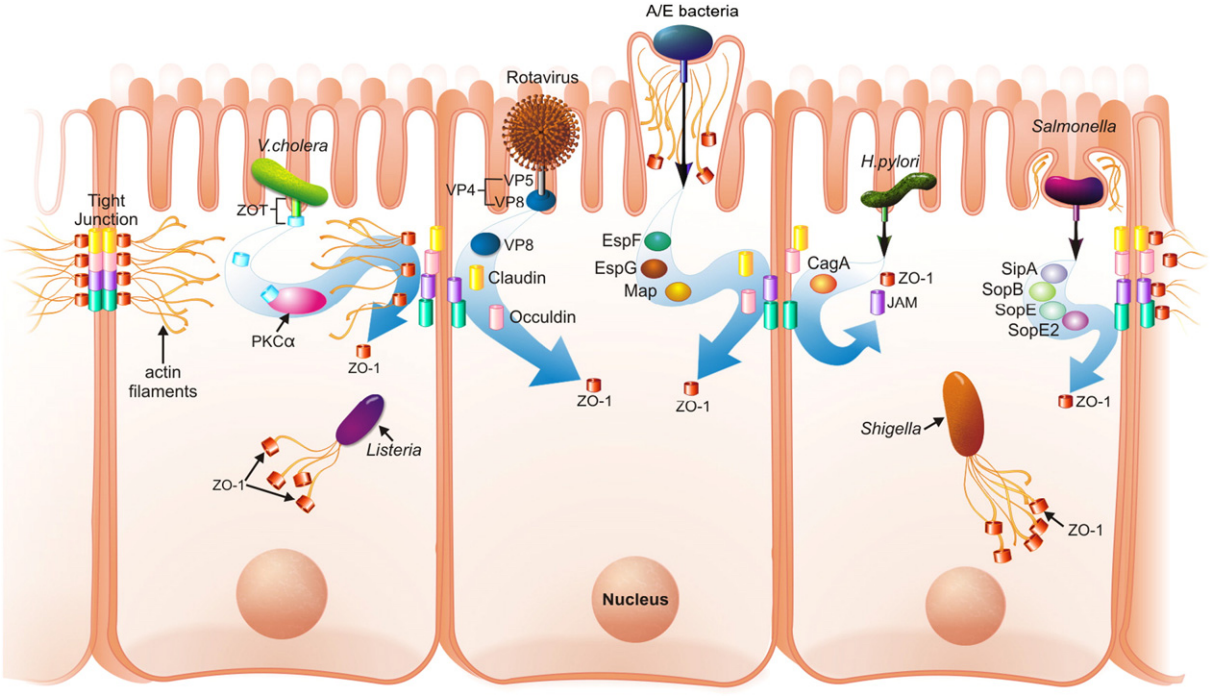


**Fig. 2.** Strategies bacterial and viral pathogens use to alter tight junctions. Transmembrane tight junction components associate with actin filaments through ZO-1. *V. cholera* uses a cleavage product from the ZOT protein to alter tight junctions. This protein activates PKCα, which acts on junction associated actin filaments. ZO-1 is also dissociated from the cell periphery. Rotaviruses use the VP8 fragment of VP4 to alter tight junction components. The A/E bacteria use the translocated effector proteins EspF, EspG and Map to alter tight junction integrity. *Salmonella* modifies tight junctions through the use of 4 effectors (SipA, SopB SopE and SopE2). *H. pylori* translocates the effector CagA that alters the localization of ZO-1 from the junctions. Both ZO-1 and JAM are found beneath *H. pylori* when this bacterium is bound to non-junctional sites. ZO-1 also associates with the distal portions of actin filaments associated with *Listeria* and *Shigella* actin tails as well as actin-rich pedestals formed by the A/E pathogen enteropathogenic *E. coli* (EPEC).

Because actin filaments interface with tight junctions, the actions of molecular motors that use this filament system as tracts can impact the downstream effects on junctions. Consequently, studies have found that during these infections tight junctions can be influenced through the actions of myosin II following the activation of myosin II light chain kinase (MLCK). In this model, MLCK (a protein responsible for phosphorylating the light chains of myosin) activates the mechanoenzyme, inducing the contraction of the actin filaments docked to the junctions, thus "pulling open" the junction. The work testing this proposal during EPEC infections comes primarily from examinations using the pharmacological MLCK inhibitor ML-9 on 6–8 hour infected T84 cells [26]. Studies have correlated a preservation of tight junction integrity, through TER measurements, during ML-9 treatment thus implicating MLCK in the observed tight junction alterations. However a significant drawback of this drug treatment is the non-specific effects inherent in pharmacological studies. To overcome these challenges and determine the precise role of MLCK on tight junctions in general, Shen et al. [27] recently performed an elegant and extensive study using an inducible construct of MLCK in a polarized Caco-2 epithelial cell line. They determined that although occludin, ZO-1 and TER were altered, these alterations did not correspond to an expected alteration in claudins or tight junction morphology, both of which remained unchanged. Thus, because it is currently understood that claudins and likely not occludin or ZO-1 are crucial for maintaining the barrier function of tight junctions, and evidence shows that tight junctions are morphologically disrupted and claudins are extensively altered during EPEC infections a re-evaluation of the specific role of MLCK during this disease is required.

EPEC and EHEC are primarily human pathogens and consequently do not colonize mice to appreciable levels or cause any discernable disease phenotypes [28]. In order to translate the phenotypes seen *in vitro* to the whole animal, researchers have used another related A/E pathogen, *Citrobacter rodentium*, as an *in vivo* model of these infections. *C. rodentium* are natural murine pathogens that colonize the colons of infected mice and like the human A/E pathogens, generate diarrhea as part of their disease manifestations [29]. These bacteria contain homologous effectors to EPEC and EHEC thus allowing for the evaluation of effector functions *in vivo*. Recently, it was shown that the barrier function of tight junctions, claudin alterations and morphological disruption seen during *in vitro* studies also occur in the course of these *in vivo* infections [30,31]. *C. rodentium*-based tight junction modifications were dependent on the bacterial protein EspF but not Map or EspG (unpublished), as bacteria mutated in *espF* retained fully functional and morphologically intact tight junctions [30].

Evidence suggests that the mechanisms of A/E bacterially-induced tight junction alterations are likely not through the direct function of effectors on tight junction proteins themselves. Studies aimed at localizing EspF in infected cells failed to localize this multifunctional protein at the cell periphery, but rather localized it at mitochondria [15]. This is not terribly surprising because if EspF were to bind to the junction to cause its effects, one could predict that the junction proteins involved would likely be removed from those sites upon EspF/tight junction protein interaction. A recent in depth examination of the role of EspF during Rabbit EPEC (REPEC) infections has further analyzed the properties of this effector. In that study, the authors found that REPEC EspF has ~30% homology with the actin associated proteins WASP, N-WASP, WAVE-Scar and WIP [32]. Through additional investigation they were able to immunoprecipitate both actin and profilin using anti-EspF antibodies during short-term (1–3 h) infections on rabbit kidney cells (RK13 cells). Actin was also immunoprecipitated by the anti-EspF antibodies using purified actin and EspF proteins [32].

During REPEC infections on RK13 cells EspF remained localized within the cytoplasm where it was likely associating with organelles. These infections generated an increase in cytoplasmically located claudin and occludin, and together with ZO-1 and ZO-2, these four proteins were all recruited to actin-rich sites generated beneath these A/E bacteria called pedestals [32]. This recruitment corroborates work by Hanajima-Ozawa et al. [33] that showed that ZO-1 localized to EPEC pedestals which was primarily concentrated at the most distal portions of these structures (Fig. 2). ZO-1 at EPEC pedestals was dependent on the proline-rich region of ZO-1 as constructs with that region deleted from ZO-1 that were transfected into HeLa cells then infected with EPEC did not get recruited to these structures. Conversely, when the proline-rich region alone was transfected into cells then infected, this portion of ZO-1 was again found at the distal portion of pedestals [33]. This surprising discovery was also evident in other bacterially-induced actin-rich structures including actin comet tails generated by *Listeria monocytogenes* and *Shigella flexneri* (Fig. 2).

Despite not knowing the detailed mechanisms of tight junction alteration during A/E infections, recent work has demonstrated that probiotic pre-treatment of cultured MDCK and T84 cells with strain GG of *Lactobacillus rhamnosus* prior to 3 h EHEC infections prevented tight junction barrier alterations [34]. Although the presence of *L. rhamnosus* did not alter the growth of EHEC, presented evidence showed few (if any) any attached bacteria. Even though this work does not advance our knowledge on the mechanism of tight junction alterations caused by EHEC, it does present an important step in potentially treating this disease.

### 5.2. Salmonella

Salmonellae are the causative agents of a variety of diseases ranging from diarrhea-generating gastroenteritis to systemic typhoid fever. These pathogens actively invade non-phagocytic cells through the use of effector proteins encoded on a *Salmonella* Pathogenicity Island (SPI1) that they inject into the host cells through a needle-like apparatus, similar to that of the A/E bacteria. Certain effectors found within SPI1 act on small GTPases to induce actin dynamics that result in the generation of host cell membrane ruffling in the area of the extracellular *Salmonella* bacteria (Fig. 2). This ruffling leads to the engulfment of these bacteria, thus delivering them into the host cell within a membrane containing vacuole that serves as a protective niche from lysosomal degradation. *Salmonella* also induces the disruption of host cell tight junctions during these infections. This is exemplified through decreases in TER, increases in tracer permeability and tight junction protein alterations when assessed in a variety of cell lines including MDCK, CaCo-2 and T84 cells [35–40]. *In vitro* experiments examining specific tight junction protein alterations have found that *Salmonella* causes a decrease in both ZO-1 expression and in the amount of phosphorylated occludin by 2 h of T84 cell infections [38]. Occludin is thought to be phosphorylated when at tight junctions, thus this reduction implies that less occludin is present at tight junctions during these infections. Although ZO-1 and occludin are likely not crucial for the barrier function, the claudins are. However, the surprising finding of an increase in the presence of claudin-1 at the cell border was recently documented during *Salmonella* infections [38]. These experiments did not co-localize the *Salmonella* bacteria in the cells, thus we are unable to be certain that the cells with the increased intensity of fluorescent claudin-1 staining were being actively infected.

Studies aimed at elucidating the specific bacterial proteins involved in the documented *Salmonella*-induced tight junction alterations have primarily used mutated strains of *Salmonella* to identify those that do not alter TER, ZO-1 and occludin localization. Through these experiments, the SPI1 effectors; SopB (SigD), SopE, SopE2 and SipA have all been implicated in tight junction alterations [37,39] (Fig. 2). This suggests that synergistic and potentially redundant mechanisms are in place to ensure tight junctions are modified as part of the disease imparted by *Salmonella*.

A recent manuscript has identified another T3SS effector, AvrA, as a tight junction stabilizer. T84 cells that were infected with *Salmonella enterica serovar typhimurium* (*Salmonella* Typhimurium) mutated in *avrA* showed decreased expression of ZO-1, claudin-1 and occludin. When claudin and ZO-1 proteins were immunolocalized on AvrA$^-$ S. Typhimurium infected T84 monolayers, ZO-1 remained localized at the cell periphery as did Claudin-1, however the authors commented that ZO-1 appeared thinner and claudin more disorganized [41]. There are numerous *in vivo* animal models of *Salmonella*-based infections. Two of the most well-characterized utilize mice. One models the gastroenteritis disease [42] and the other mimics systemic typhoid disease (reviewed by: [43]). AvrA was also analyzed using the *in vivo* gastroenteritis model [41]. Although similar alterations to those found during cultured cell infections were reported by the authors, in the presented images throughout the entire manuscript there was a severe lack of bacteria interacting with the cells or tissues [41]. Consequently, it will be important to confirm and present evidence that these findings occur to cells that are being actively infected by these pathogens.

### 5.3. Helicobacter pylori

The bacterial pathogen *H. pylori* increase the risk of gastroduodenal ulcers and gastritis. Like *Salmonella* and pathogenic *E. coli*, these organisms also deliver effector proteins into host cells, however these pathogens use an alternative delivery apparatus called a type-four secretion system (T4SS), which employs a different set of apparatus proteins (reviewed by: [44]). During their pathogenesis, *H. pylori* preferentially attach at the intercellular boundaries of epithelial cells and disrupt the function of tight junctions [45]. Consequently, these infections also result in the mis-localization of the overall homogeneous distribution of ZO-1 that is normally found at the cell borders, resulting in disjointed staining at the cell periphery [45,46]. During infections ranging from 7 min to 8 h on MDCK and AGS (gastric adenocarcinoma) cell lines, some bacteria also attach at non-junctional sites and recruit ZO-1 and JAM beneath the area of bacterial contact [45] (Fig. 2). Key to the tight junction alterations are two bacterial effectors, CagA and VacA (vacuolating toxin). Infections with CagA/VacA double mutants retain an intact epithelial barrier where as single mutant *H. pylori* infections cause barrier defects in MDCK cell monolayers particularly during long-term (1–10 days) *in vitro* infections [45].

Although the combination of CagA and VacA synergistically influence tight junctions, evidence suggests that both factors can also alter the function of the junctions independently. In studies that examined the role of CagA during tight junction formation, CagA mutant *H. pylori* infections permitted tight junctions to re-form following Ca2+ switch experiments, whereas wild-type *H. pylori* did not [45]. ZO-1 recruitment to bacteria attached to non-junctional sites also occurred in a CagA dependant manner [45]. These mutant bacteria also did not concentrate at the cell boundaries during infections [45]. Transfection experiments that focused on examining the specific functions of the CagA protein alone, demonstrated that GFP-tagged CagA, delivered into epithelial cells, severely mis-localized ZO-1 to the basal membrane of the cell [47].

The extent of the influence of VacA on tight junctions comes primarily from studies using purified VacA. VacA induced a decrease in TER in MDCK cells during short exposures (<1 h) and T84 as well as epH4 cells during 5–7 h VacA treatments. This protein did not alter TER in CaCo-2 cells even after 20 h. VacA also caused an increase in MDCK and T84 monolayer permeability to small molecular weight molecules and ions when treated for 1.5 h until 24 h time points [48,49]. Despite these physiological measurements, the localization and the abundance of ZO-1, occludin and cingulin remained unaltered even during 24 h treatments [48].

The tight junction protein alterations induced by *H. pylori* appear to be strain specific. A study by Fedwick et al. [50] found that using an alternative strain (strain SS1) a redistribution of occludin, claudin-4 and -5 was detected and a decrease in abundance of these proteins was also evident during *in vitro* cultured cell infections with this strain of *H. pylori*. This decrease also resulted in tight junction barrier deficits. Surprisingly, ZO-1 and JAM were unaffected during these infections and VacA or CagA did not influence observed alterations. They also showed that during *in vivo* murine infections a barrier defect occurs [50].

### 5.4. S. flexneri

*S. flexneri* are pathogens that actively invade non-phagocytic cells through the use of T3SS-delivered effectors and cause severe inflammatory diarrhea, including dysentery. Although few studies have examined the effects *Shigella* have on tight junctions, one detailed examination by Sakaguchi et al. [51] revealed that it, extensively disrupts these structures. In polarized cultured T84 epithelial cells, ZO-1, claudin-1 and the phosphorylation status of occludin were all affected by *S. flexneri* [51]. Importantly during the early stages of the infections (10 min) with *S. flexneri* exposed to the apical aspects of the cells, claudin-1 protein expression was initially severely depressed. The claudin-1 protein levels associated with the membrane returned to approximately 50% of the control levels by 90 min of *S. flexneri* exposure. Despite a general decrease in TER by the 90 minute time point, TER levels during 10 and 30 minute exposures of *S. flexneri* to the apices of these cells resulted in an increase in TER over baseline standards [51]. Even with the clear evidence that numerous aspects of tight junction function and molecular architecture are influenced by these diarrhea-generating pathogens much more work is needed to explain how the inconsistencies in TER,

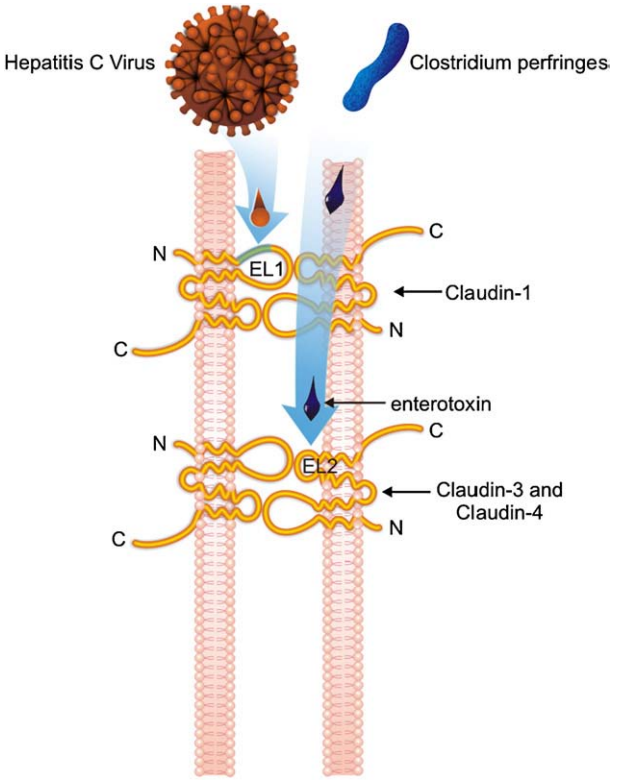


**Fig. 3.** Regions of claudin proteins that are involved in hepatitis C virus and *Clostridium perfringens* disease. The hepatitis C virus specifically interacts with the N-terminal 1/3 of the first extracellular loop (EL1) of claudin-1. The second extracellular loop (EL2) of claudins-3 and -4 are the sites of *Clostridium perfringens* enterotoxin action.

barrier function and molecular components are all influenced by this pathogen. Additionally, the role of specific effectors has not been clearly assessed. It will be important to elucidate this crucial interplay to understand the role tight junction modifications truly play during these infections.

### 5.5. Clostridium perfringens

Although numerous bacteria have been implicated in altering tight junctions, few pathogenic factors have been shown to directly interact with tight junction proteins. The most in depth analysis of direct tight junction protein interaction by a bacterial factor comes from analysis of *C. perfringens*, a pathogen that causes food poisoning. This bacterium uses a potent enterotoxin to bind to claudins-3 and -4 [52]. Claudins interact with their family members through two heterogeneous extracellular loops, EL1 and EL2 (Fig. 3). EL1 from a claudin protein in one cell associates with EL2 from the adjacent cell. Detailed analysis has identified residues 290–319 of this toxin as the region that binds to the second extracellular loop of claudins-3 and -4 [53,54] (Fig. 3). The claudin binding site of this toxin has been crystallized by using residues 194–319 as residues 290–319 resulted in an unstable fragment [55]. This data will provide important structural information for specific therapeutic intervention.

## 6. Viruses

Tight junction transmembrane proteins are the most exposed intercellular junctions components that pathogens encounter during their interactions with host cells. Consequently, some viruses have exploited these proteins as receptors for their subsequent internalization.

### 6.1. Hepatitis C virus (HCV)

HCV is a microbial agent responsible for cirrhosis of the liver. These pathogens use the claudin transmembrane proteins as receptors for their internalization. In addition to requiring other membrane factors, this virus targets the N-terminal third of extracellular loop 1 of claudin-1 as a co-receptor for entry into eukaryotic cells [56,57] (Fig. 3). This interaction is highly specific, as an alteration of 2 amino

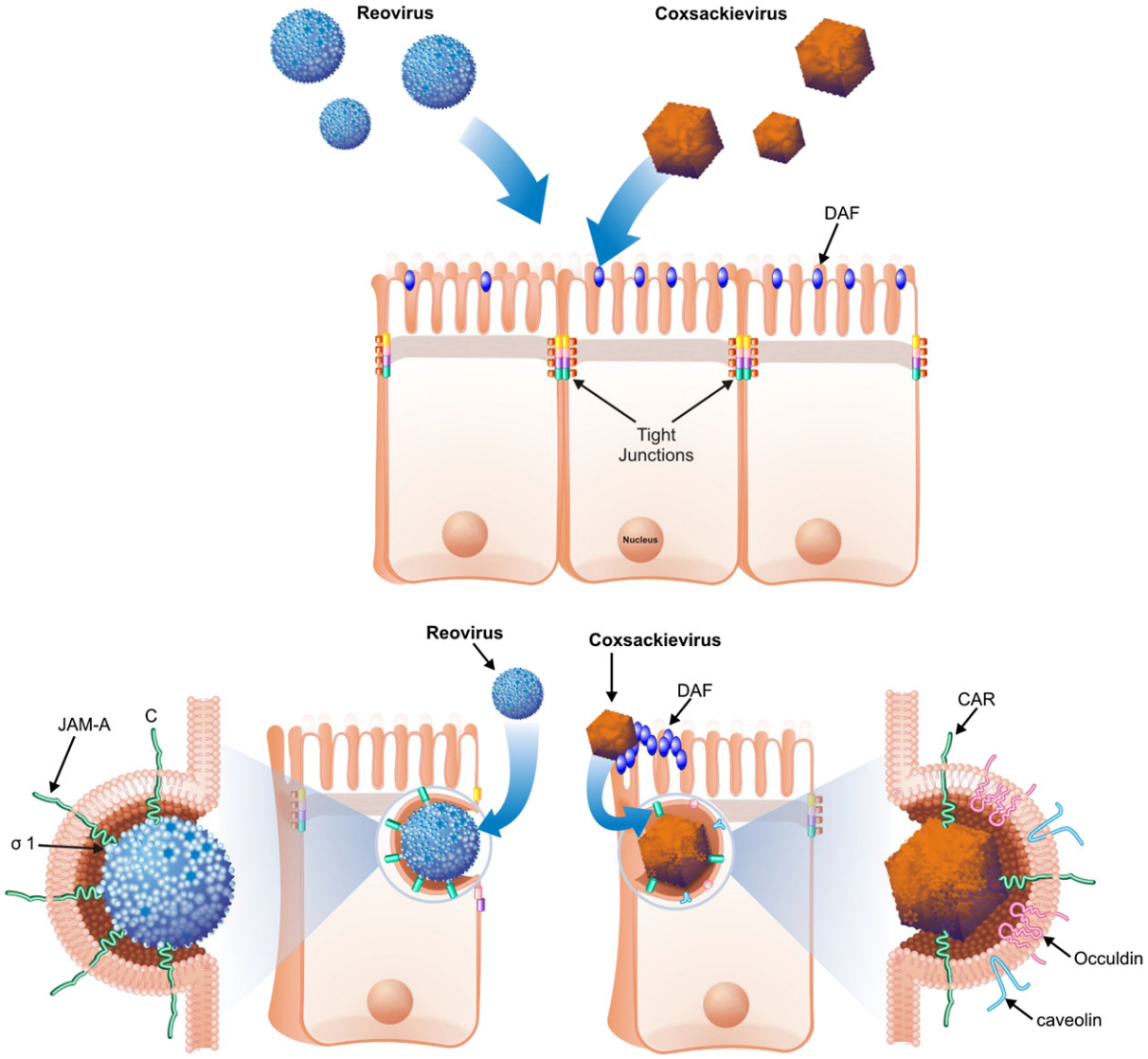


**Fig. 4.** Reovirus and coxsackievirus association at tight junctions. The reovirus surface protein σ1 associates with the N-terminal domain of the tight junction protein JAM-A. JAM-A is used as a receptor for reovirus internalization. The coxsackievirus initially associates with the membrane protein DAF. This interaction induces the clustering of DAF and the subsequent movement of the coxsackievirus towards the tight junctions. At tight junctions the coxsackievirus interacts with CAR and is internalized through caveolin-mediated internalization. This internalization requires the presence of occludin.

acids (I32 and E48) within this extracellular loop of claudin-1 abolishes the internalization of HCV into cells [56]. Very recent work has also shown that in endothelial cells, claudins-6 and -9 are also suitable co-factors for HCV entry [58]. However, these claudins do not efficiently allow HCV invasion into hepatoma cells [58].

### 6.2. Reovirus

Reoviruses are prevalent infectious agents in children causing respiratory and gastrointestinal diseases. In infected mice, these pathogens can cause encephalitis. These viruses contains 10 segments of double-stranded RNA encapsulated in a protein coat and use JAM-A as a receptor for their attachment and subsequent internalization into host cells [59–61] (Fig. 4). This virus/host cell interaction occurs through the direct association of the viral surface protein σ1 to the amino (N-) terminal domain of JAM-A [59,62] (Fig. 4).

### 6.3. Rotavirus

Rotaviruses are a major cause of viral gastroenteritis leading to diarrhea and morbidity in mammals. These organisms consist of a 3 layered protein core, housing double stranded RNA segments. 60 protein spikes surround this core. Each spike is formed by a VP8 and VP5 subunits, which together are referred to as VP4. Tryptic cleavage of VP8 from VP5 allows the VP8 fragment to alter the localization of claudin-3, ZO-1 and occludin, which consequently leads to the disruption of the barrier integrity of tight junctions during these infections [63–66]. VP8 can also block the formation of tight junctions in MDCK cells [63].

Rotaviruses also produce a toxin called NSP4. This viral toxin has also been shown to block the formation of tight junctions in polarized MDCK cells [67]. Additionally, this toxin inhibited the localization of ZO-1 to MDCK cell borders prior to monolayer confluency [67]. These

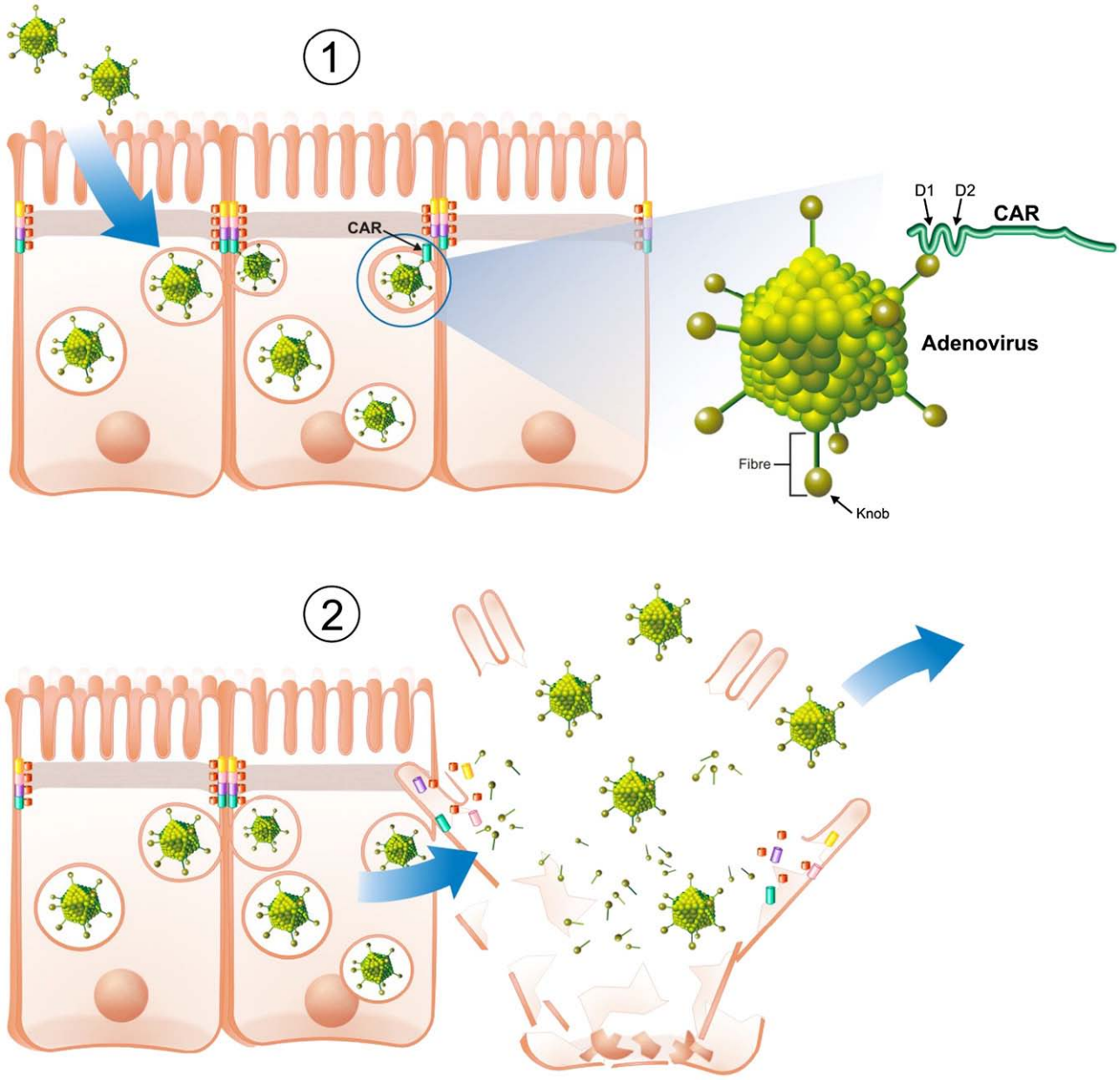


**Fig. 5.** Adenoviruses interact with the D1 domain of CAR through the knob region of the adenovirus "Fibre" protein. This association is used for both virus internalization (1) and is involved in virus release (2).

disruptions may not only influence the diarrhea phenotype of this infection, but also exposes the deeper membrane associated integrin proteins to rotavirus, which act as receptors for these pathogens (reviewed by: [68]).

### 6.4. Adenovirus

Adenoviruses-2 and -5 use the Coxsackievirus and Adenovirus Receptor protein both as a receptor [69] for their internalization and to break the epithelial barrier during viral escape [70] (Fig. 5). These viruses accomplish this through interactions of the most distal proteins projecting from the virus core, the knob region of the fibre protein, by directly binding to the N-terminal, D1 extracellular component of CAR (Fig. 5). The CAR/knob interaction is a higher affinity interaction than CAR/CAR dimer attachment thus providing a mechanism of CAR-based tight junction disruption. Concurrent with the CAR alterations is a decrease in TER, suggesting functional tight junction breakdown by these viruses.

### 6.5. Coxsackievirus

Coxsackieviruses are the causative agents of meningitis and myocarditis. During infections by coxsackievirus-B, these pathogens use CAR the as a co-receptor for their internalization [69]. In order for coxsackievirus-B to infect polarized cell layers these viruses initially attach to the decay-accelerating factor (DAF) surface proteins [71]. These proteins are abundantly dispersed along the apical membranes of epithelial cells and cluster upon contact with this virus (Fig. 4). This clustering leads to the activation of the src kinases, Abl and Fyn [71]. Abl activates the small G-protein Rac within the host cells that leads to the re-arrangement of the actin cytoskeleton that enables the viruses to move to the tight junctions. The viruses subsequently interact with CAR, and are internalized through a caveolin-based mechanism [72] (Fig. 4). Their internalization also requires occludin as cells deficient in occludin do not efficiently internalize coxsackievirus-B [72] (Fig. 4).

### 6.6. Discovery of zonulin from Vibrio cholerae's zonula occludens toxin (ZOT)

*V. cholerae*, the causative agent of severe watery diarrhea, induces tight junction alterations through the use of a bacterial surface protein called the zonula occludens toxin (ZOT). This protein caused a decrease in epithelial resistance in the ilium of rabbits and the mislocalization of occludin and ZO-1 in CaCo-2 cells [73–76]. The mechanism that this pathogenic protein uses to reversibly alter tight junction integrity is likely through the cleavage of a 12 kDa C-terminal fragment, which is excreted upon contact with intestinal epithelial cells [77]. Thus far a direct modification to the tight junction proteins responsible for the barrier function has not been identified, however ZOT does alter the actin filament regulatory protein PKCα, which is proposed to influence tight junction permeability [78].

The characterization of ZOT led Wang et al. [79] to postulate that a mammalian homologue may exist that controls tight junction paracellular permeability. Through purification of cellular proteins using anti-ZOT antibodies a homologue of ZOT, zonulin, was identified in the small intestine, heart and brain, but not the colon [79]. During pathogenic *E. coli* and *Salmonella* infections *ex vivo*, increases in zonulin production from the small intestine were evident [80].

## 7. Conclusion

Cellular microbiologists continue to elucidate the strategies pathogens use to exploit host cells. We have overviewed how bacteria and viruses modify tight junctions, however apart from few instances the detailed mechanisms that these microorganisms employ to induce these alterations remain elusive. Although advances have led to an overall greater understanding of these junctional modifications, there are still controversies that need to be rectified. It is fairly evident based on current data, that there will not be a unified strategy employed by all pathogens. Consequently, discovering the binding partners of effectors that influence tight junctions are crucial as many virulence factors likely act upstream of the junctions themselves. The knowledge we will gain through determining these modes of action will not only be applicable to tight junction alterations caused by pathogens, but will also be relevant to general tight junction physiology.

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