



Inflammatory and Microbiota-Related Regulation of the Intestinal Epithelial Barrier

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The intestinal epithelial barrier (IEB) is one of the largest interfaces between the environment and the internal milieu of the body. It is essential to limit the passage of harmful antigens and microorganisms and, on the other side, to assure the absorption of nutrients and water. The maintenance of this delicate equilibrium is tightly regulated as it is essential for human homeostasis. Luminal solutes and ions can pass across the IEB via two main routes: the transcellular pathway or the paracellular pathway. Tight junctions (TJs) are a multi-protein complex responsible for the regulation of paracellular permeability. TJs control the passage of antigens through the IEB and have a key role in maintaining barrier integrity. Several factors, including cytokines, gut microbiota, and dietary components are known to regulate intestinal TJs. Gut microbiota participates in several human functions including the modulation of epithelial cells and immune system through the release of several metabolites, such as short-chain fatty acids (SCFAs). Mediators released by immune cells can induce epithelial cell damage and TJs dysfunction. The subsequent disruption of the IEB allows the passage of antigens into the mucosa leading to further inflammation. Growing evidence indicates that dysbiosis, immune activation, and IEB dysfunction have a role in several diseases, including irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and gluten-related conditions. Here we summarize the interplay between the IEB and gut microbiota and mucosal immune system and their involvement in IBS, IBD, and gluten-related disorders.

Keywords: intestinal epithelial barrier, mucosal immune system, gut microbiota, IBS, IBD, celiac disease, non-celiac gluten sensitivity

INTRODUCTION

The gastrointestinal (GI) tract, with a surface area of about 40 m², is one of the largest interfaces between the environment and the internal milieu of the body (1, 2). The intestinal epithelial barrier (IEB) is a structure that receives and reacts to several stimuli in a dynamic way (3). The IEB is essential as the first line of defense, limiting the passage of harmful microorganisms and antigens, and at the same time, it has to assure the correct absorption of nutrients, water, and ions. This delicate equilibrium is reached through the interaction of several elements: the mucus, the outer layer, followed by a single cell layer of epithelial cells and, finally, the lamina propria

where immune cells, such as dendritic cells, plasma cells, macrophages, lymphocytes, reside (1). The intestinal epithelium, furthermore, includes five cell lineages: absorptive enterocytes, the most abundant epithelial cells, the goblet cells producing mucus (4), the tuft cells producing IL-25 (5), the enteroendocrine cells producing hormones, and Paneth cells, which produce antimicrobial peptides or lectins (4). The dysregulation of the IEB is implicated in the pathogenesis of several intestinal [e.g., celiac disease, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colon carcinoma], and extra-intestinal diseases (e.g., chronic liver disease, type 1 diabetes, obesity). The hypothesis is that, for all these disorders, the IEB dysfunction, and consequent increase of intestinal permeability (i.e., a functional feature of the IEB, measurable by analyzing flux rates across the intestinal wall) (6), facilitate the entrance of antigens and/or microorganisms into the lamina propria with subsequent activation of the immune system and the initiation and/or maintenance of inflammatory responses (7, 8).

In the present review, we will summarize current evidence on the interplay between the IEB and immune system and microbiota and their involvement in GI pathological conditions including IBS, IBD, and gluten-related disorders.

THE INTESTINAL EPITHELIAL BARRIER

Mucus

The mucus layer covers the luminal surface of the GI tract and is the first line of defense against mechanical, chemical, and biological insults. It protects epithelial cells from bacteria, digestive enzymes, and dangerous substances coming from the outside including environmental pollutants, food antigens, toxins (9, 10). In addition, it provides lubrication for food passage and removes bacteria and debris by flowing them away in the intestinal stream. Over then acting as a physical barrier, it is also essential in the maintenance of intestinal homeostasis as small molecules, gases, ions, and water diffuse through it to reach the epithelium (11, 12). It is continuously secreted in the GI tract principally by goblet cells, but also from epithelial cells and glands (12). Interestingly, the density of goblet cells increases distally within the GI tract and reaches the peak in the colon, likely in parallel with the increase of microbiota (13, 14).

Mucus is made up of water (90–95%) (11, 15) proteins, lipids (1–2%), and electrolytes (16). Proteins, principally produced by goblet cells, include mucins, which lend the mucus its gel-like properties. Mucus comprises also antimicrobial peptides and immunoglobulin-A (IgA), giving the mucus a pivotal role in innate defense (17–19). IgA are the most abundant isotype in humans and are secreted in the lumen where they are essential to prevent infections and to assure homeostasis with gut microbiota (20). The mucins are responsible for the gel aspect of the

mucus and are characterized by mucin domains, rich in serine, threonine, and proline amino acids, that are abundantly O-glycosylated (21, 22). This post-translational modification gives these proteins the property to be soluble in water and to form a gel. In addition, the carbohydrates hide the protein core, preserving it from degradation. The glycan residues can bind the lectin-like proteins of immune cells, giving the mucus an active immunological role. In addition, mucin 2 (MUC2), the principal gel-forming mucin in the intestine, can influence dendritic cells and epithelial ones (23), contributing to oral tolerance [i.e., the unresponsiveness of the immune system induced by oral administrated innocuous antigens, including that derived from food (24) and gut homeostasis (25)]. Although mucus is present throughout the intestine, it has peculiar properties in the different tracts (26). In the small intestine, there is a single layer of mucus that is more permeable than elsewhere to contribute to nutrients' uptake (27). In addition, in this tract, mucus is discontinuous to support the release of digestive enzymes (27–30), it is not tightly attached to the epithelial cells, and it is mixed with antibacterial substances (31–33) which contribute to avoiding the contact between bacteria and epithelium (29, 34–36). In the large intestine, the mucus is divided into two layers: the inner and the outer ones. The inner layer is organized in lamellar layers made up of MUC2 multimers anchored to the epithelium and not accessible to bacteria (22, 27). In humans, at a distance of 200 μm from the epithelium, the action of proteases changes the structure of the inner mucus, producing the outer one. The outer layer (i.e., non-attached to the epithelium) is less dense than the inner mucus, has larger pores, and is the place where commensal bacteria live (37–39).

Some years ago, Kamphuis et al., proposed a new model of mucus. They characterized the colonic mucus barrier in rodents by using histological and FISH techniques. They demonstrated that in the distal colon, mucus covers the feces instead of epithelial cells confining the microbiota to the feces. On the other side, this organization is lost in the proximal colon, suggesting that mucus organization depends on the presence of colonic content (40).

The high polysaccharide content of the mucus represents a source of energy for bacteria, although only a subset of gut microbiota can hydrolyze mucus carbohydrates in physiological conditions (41, 42). Interestingly, in the case of a diet poor in fibers, there is a change in the microbiota composition, with an increase in the abundance of mucus-degrading bacteria that are capable to use glycosylated residues as a source of energy (43). Gut microbiota and its metabolites have a key role in the formation and correct folding of the mucus. As a matter of fact, germ-free mice showed a similar organization of the mucus layer to conventionally raised (ConvR) mice, but the inner mucus was more penetrable, and small intestinal mucus was tightly attached to the epithelium. Following the colonization of germ-free mice intestine with ConvR microbiota, colonic inner mucus became impermeable, and small intestinal mucus acquired the typical characteristic of being easily detachable (44). The mechanisms through which microbiota can influence mucus production and properties are still a matter of study. One possible way is that gut microbiota can influence mucin glycosylation

Abbreviations: IEB, Intestinal epithelial barrier; GI, Gastrointestinal; TJs, tight junctions; IBS, irritable bowel syndrome; IBD, inflammatory bowel diseases; MUC2, mucin 2; AJs, adherens junctions; JAM-A, junctional adhesion molecule-A; ZO, zonula occludens; CD, celiac disease; NCGS, non-celiac gluten sensitivity; PAR-2, Proteinase-activated receptor 2; SCFAs, short-chain fatty acids; TLRs, Toll-like receptors; FGIDs, Functional Gastrointestinal Disorders; HLAs, human leucocyte antigens; GFD, gluten-free diet; UC, ulcerative colitis.

(21) and the expression of glycosyltransferase. On the other hand, the gut-microbiota composition is influenced by mucus glycosylation pattern, which differs among species, establishing an equilibrium that ensures nutrients to microbiota and avoids mucus degradation (45). The role of mucus as a barrier is crucial for the maintenance of health. Reduction or abnormalities in the mucus production or in the glycosylation of the mucins are associated with gut inflammation and ulcerative colitis (46, 47) as well as in the colonization of the inner mucus by bacteria in both patients with ulcerative colitis and in the murine model of colitis (48). Qualitative and quantitative changes in the mucus layer in response to inflammation, have been demonstrated also in Crohn's disease (49–52) and colorectal cancer (53, 54). Finally, mucus is the first barrier for pathogens to enter the intestinal mucosa and start an infectious (55). Lipopolysaccharide (LPS) expressed on the outer membrane of Gram-negative bacteria, lipoic acid on the membrane of Gram-positive bacteria and flagellin, can activate MUC2 expression by the activation of toll-like receptors (TLRs) as reported in animal studies and in studies using cell lines, including goblet cell line and HT-29 (56–61). In conclusion, on the basis of the above evidence, the integrity of the mucus and the correct balance between mucus production by goblet cells and mucus degradation by intestinal microbiota, are essential for the maintenance of a health state.

Tight Junctions

Beneath the mucus layer, there is a columnar monolayer of intestinal epithelial cells that represents an additional line of interface, between the outside environment and intestinal mucosa.

Alterations of the IEB allow the excessive passage of food and microbiota antigens which elicit immune activation involved in the pathogenesis of the intestinal and systemic disease (e.g., diabetes, obesity, non-alcoholic liver diseases). The functioning of the IEB depends on the presence of a series of intercellular junctions composed by the apical junctional complex (AJC), including tight junctions (TJs) and adherens junctions (AJs), and desmosomes. Under physiological conditions, only water and solutes like electrolytes can cross the epithelium through the paracellular way. TJs, the principal regulators of paracellular permeability (62), are a network of proteins located at the apex of the lateral membrane of epithelial cells, including claudins (63), tight junction-associated marvel proteins (TAMPs) such as occludin (64), junctional adhesion molecule-A (JAM-A) (65), and intracellular scaffold proteins, such as zonula occludens (ZO), and tricellulin (66).

Claudins are transmembrane proteins that form two extracellular loops interacting with their counterparts from the neighboring cell (67, 68).

Claudins are a group of proteins with molecular weights ranging from 21 to 34 kDa (68–70). Intestinal claudins exist in two functionally different classes: sealing claudins, responsible for the tightness, and pore-forming claudins (71). Sealing tight junction proteins include claudins-1, -3, -4, -5, -7, -8, -11, -14, -18, and -19 (68) and form a selective barrier to macromolecules and ions, whereas claudins-2, -10a/-10b, -15, -16, and -17 form selective pores to ions and water (72). Each pore-forming claudin

has different specificity for cations or anions as well as selectivity for ionic size. The characteristics of each claudin are shown in **Table 1**.

Occludin is a transmembrane protein with a molecular weight of around 65 kDa. It presents a long C-terminal cytoplasmic domain, two loops, and a short N-terminal cytoplasmic portion (64). The carboxy-terminal of occludin contains the binding site for zonula occludens (ZO)-1. Occludin is a phosphorylated protein and it has been reported that its phosphorylation correlates with the localization at the TJs (105). ZO-1 (~220 kDa), ZO-2 (~160 kDa), and ZO-3 (~130 kDa) are scaffold proteins, localized to the cytoplasm (106). ZO-1, -2, and -3 have three PDZ domains and a guanylate kinase-like domain (GUK) (107). PDZ1 binds the C-terminus of claudins (106) while the GUK domain interacts with occludin (107). C-terminal regions of ZOs interact with actin and serve as scaffolds linking TJ strands with the cytoskeleton (108–110). The association of the cytoskeleton with the TJ structure is crucial for the regulation and maintenance of TJs function (111). Junctional adhesion molecules (JAM, ~40 kDa) are transmembrane molecules localized to apical cell-cell contacts and associated with TJs. They are members of the immunoglobulin superfamily and include 3 transmembrane proteins JAM-A, -B, and -C (also called JAM-1, -2, and -3) characterized by a short N-terminal portion, two extracellular immunoglobulin-like loops, a transmembrane portion, a short cytoplasm fragment containing phosphorylation sites and a C-terminal PDZ domain involved in cell-cell adhesion (67, 112). The latter domain is involved in the binding of ZO-1 and is fundamental for protein-protein interactions (112–114). Among the JAM proteins, JAM-A is the best characterized in the regulation of TJ barrier function. It's expressed in intestinal epithelial cells and has also been implicated in cell proliferation and migration (115–118). The characteristics of ZO and JAM proteins are shown in **Table 2**.

Cingulin is a cytoplasmic protein with a molecular weight of 140–160 kDa. It has a structural role, binding ZOs and cytoskeletal proteins (125), and a signaling one, being involved in the control of epithelial proliferation (114, 126). TJs are linked to microtubules and microfilaments of the cytoskeleton. An important role in the regulation of TJ opening/closing is attributed to the microfilaments of actin and myosin (127, 128). Myosin is mainly involved in the regulation of TJ assembly and tone (1, 129), while actin binds to the cytoplasmic scaffold proteins (130). In addition, actin polymerizing proteins and regulators of actin, are central in TJ changes (114).

The composition and stability of the IEB depend not only on TJs but also on their interaction with AJs and desmosomes (131, 132). AJs establish cell-cell contacts, which are essential for TJs maturation and maintenance. E-cadherin is the main transmembrane protein involved in AJ assembly (133). Its intracellular domain is associated with p120, b-catenin, and a-catenin, forming a complex that is bound to actin filaments (134). Moreover, regulating the organization of the underlying actin cytoskeleton, it establishes a hub for cell signaling and regulation of gene transcription (135).

Desmosomes provide mechanical strength to the intercellular cell-cell contact in the epithelium. Their composition

TABLE 1 | Characteristics of claudins and their changes in intestinal diseases.

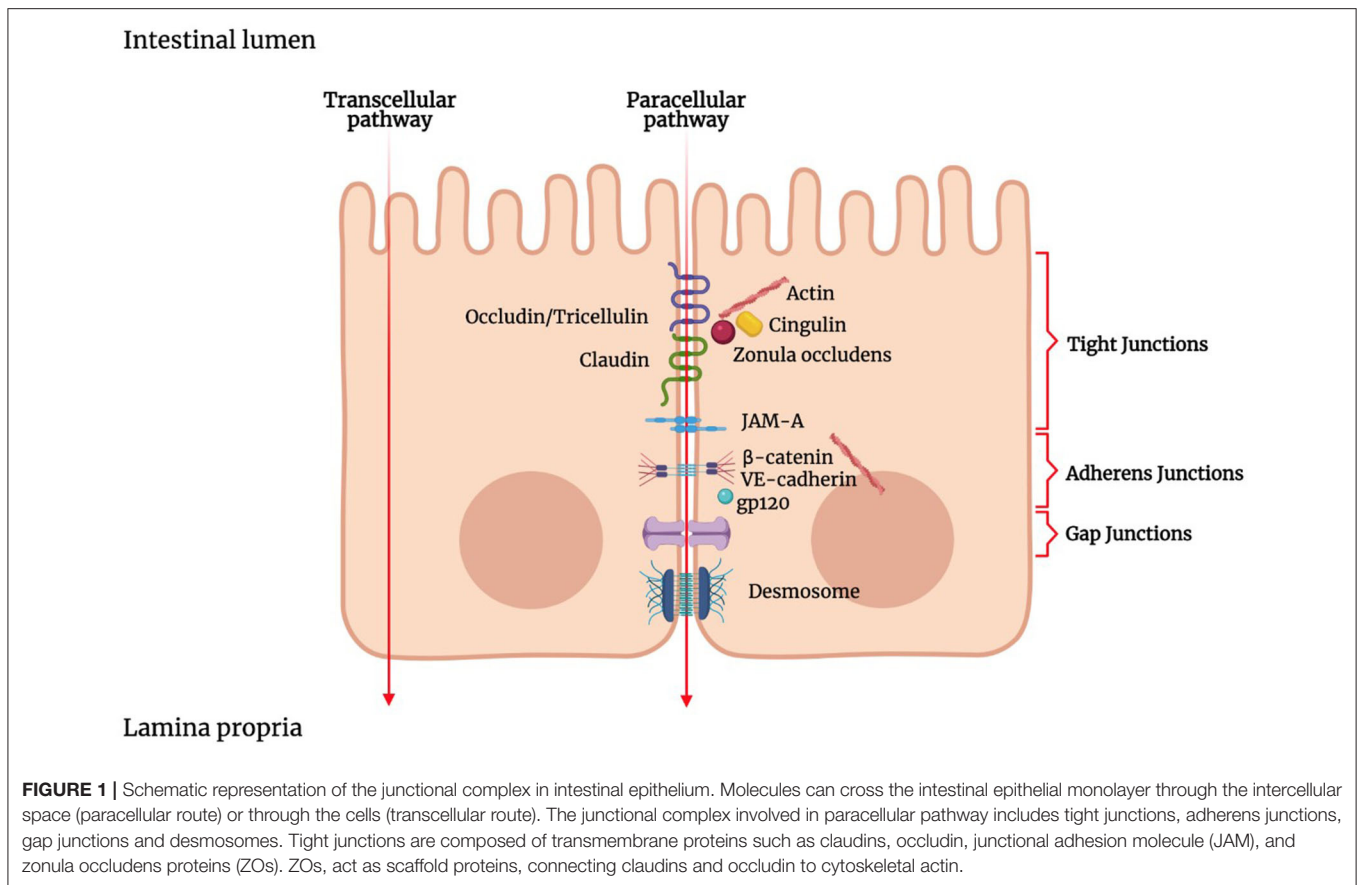
Claudin	Size (kDa) (68)	Localization	Functions	Ions permeability	Interactions (68)	Role in disease
1	22.8	Ubiquitary (63)	Barrier forming	↓ Cations	It plays a general role in preventing the loss of water and macromolecules	↓ In UC (73–75), food allergy (76), liver cirrhosis (77), and IBS-D (78)
2	24.4	Intestinal crypts (79), Proximal renal tubule (80), choroid plexus (81), Human ovarian surface epithelium (82)	Pore forming	↑ Cations	It is involved in the regulation of Na ⁺ , Cl ⁻ , Ca ²⁺ and water	↑ UC (83), celiac disease (84), IBS-D (85) ↑↓ Crohn's disease (86)
3	23.3	Human gallbladder (87) Brain capillary endothelium (88) Tighter segments of nephron (89) Liver/intestinal epithelial cells (79)	Barrier forming	↓ Cations	It is involved in the reduction of paracellular permeability of large molecules and in the formation of the blood-brain barrier	↓ Crohn's disease, acute colitis (90) ↑ Celiac disease (84)
4	22.1	Kidney and lung (68) Human gallbladder (87) Stomach, small intestine and colon (91)	Barrier forming	-	It can act as a Na ⁺ barrier or, interacting with claudin-8, as an anion (Cl ⁻) pore. In the colon strengthens tight junctions	↓ Acute colitis (73, 83) ↑ NCGS (92)
5	31.6	Endothelial tissue: endothelial cells (93) and brain capillary (94) Epithelial tissue: Human ovarian surface epithelium (82) Human colon epithelium (95)	Barrier forming	↓ Cations	It is involved in permeability of small molecules (~800 Da)	↓ Acute colitis, Crohn's disease (86), Celiac disease (84, 96)
7	22.4	Epithelial tissue: Duodenum, jejunum, ileum, colon (97) Human palatine tonsillar epithelium (98) Nephron segments primarily at the basolateral membrane (99)	Barrier forming	↓ Anions	It plays a role in the regulation of Na ⁺ , Cl ⁻ , K ⁺ and water	↓ UC (83), Celiac disease (84, 96)
8	24.8	Epithelial tissue: Duodenum, jejunum, ileum, colon (100) Distal nephron (99)	Barrier forming	↓ Cations	It can act as a Na ⁺ barrier or Cl ⁻ pore, depending on the cell type	↓ Crohn's disease (86)
12	27.1	Brain endothelial cells (94) Duodenum, jejunum, ileum, colon (97)	-	↑ Cations	It increases permeability to Ca ²⁺	↑↓ Crohn's disease (101)
15	24.4	Kidney endothelial cells (89) Intestine (duodenum, jejunum, ileum, colon) (97)	Pore forming	↑ Cations	It can act as a Na ⁺ channel or Cl ⁻ barrier, depending on the cell type; it is involved in paracellular K ⁺ absorption and Na ⁺ secretion	↑ Celiac disease (84, 96)
18-2	27.7	Stomach (102) and bone cells (103)	Barrier forming	↓ Cations	It acts as selective barrier against Na ⁺ and H ⁺ , protecting the epithelium from low pH	↓ Gastric cancer (104)

TABLE 2 | Characteristics of zonula occludens (ZO) proteins and junctional adhesion molecules (JAM) and their changes in intestinal diseases.

Tight junction protein	Size (kDa)	Interactions	Role in disease
ZO-1	~220 kDa	Claudin, JAM-A, occludin, F-actin, ZO-2,-3, cingulin	↓ Crohn's disease (119) IBS-D (120), IBS-A (121) Celiac disease (122)
ZO-2	~160 kDa	Claudin, occludin, F-actin, ZO-1,-3, cingulin	↓ IBS-D (120)
ZO-3	~130 kDa	Claudin, occludin, ZO-1, cingulin	↓ IBS-D (120)
JAM-A	~40 kDa	Occludin, JAM-A, ZO-1, cingulin	↓ In IBD (75, 83, 123) IBS-D and IBS-A (124)
JAM-B	~40 kDa	JAM-C, ZO-1	-
JAM-C	~40 kDa	JAM-B, JAM-C, ZO-1	-

includes two subtypes of transmembrane cadherins, desmogleins, and desmocollins. These proteins through the binding with plakophilin and desmoplakin (136) can

bind intermediate filaments providing junction stability to mechanical stress due to peristaltic movement of the gut (137).



Zonulin, the pre-haptoglobin 2, is an endogenous modulator of intestinal permeability.

The binding of gliadin to the chemokine receptor C-X-C Motif Chemokine Receptor 3 (CXCR3) on epithelial cells, likely induces the release of zonulin through a MyD88-dependent pathway. Zonulin disassembles tight junctions, through the transactivation of EGF receptor *via* proteinase-activated receptor 2 (PAR-2) activation (138, 139). Interestingly, zonulin serum levels are altered in conditions characterized by IEB alterations, including celiac disease (CD), type 1 diabetes, and non-celiac gluten sensitivity (NCGS) (138, 140, 141). It is to note that zonulin belongs to a family of structurally and functionally related proteins named “zonulin family peptides” that can affect intestinal permeability. In addition, it cannot be excluded that different members of this family could be detected by commercially available enzyme-linked immunosorbent assay (ELISA) kit (142).

Transcellular and Paracellular Pathways

Luminal solutes and ions can pass across the IEB *via* two main pathways: the transcellular pathway (i.e., through the cells) or the paracellular pathway (i.e., between the cells) (Figure 1). The transcellular transport implicates the passage across the cell membrane, which generically occurs by passive transport, passive diffusion by efflux pumps, active transport, or endocytosis (143). Apolar compounds like soluble lipids can pass through

the phospholipid bilayer by simple diffusion. Small hydrophilic compounds and nutrients mainly use channels and transporters located on cellular membranes to cross the cell membrane (144). Some channels belong to the group of the ligand-gated channels, which requires the binding of a ligand to the receptor region to modify their conformation and be opened or closed (145, 146). Carrier-mediated transport involves the binding of the target molecules to the receptor portion of the transporters, which in turn produces a conformational change in the carrier and allows the translocation of the compound (147).

Larger molecules, such as proteins and bacterial products, penetrate by the mechanism of receptor-mediated endocytosis, which implies the invagination of the plasma membrane and the subsequent formation of vesicles, as a consequence of proteins or peptides binding to specific cell surface receptors (148). The substances thus incorporated are transported through the cytoplasm by transcytosis. This transport is essential for antigen surveillance in the GI tract (149).

Endocytosis in epithelial cells occurs differently depending on the substance interacting with the epithelium. Immunoglobulins and viruses penetrate the epithelium *via* clathrin-mediated endocytosis. This is a highly specific receptor-mediated process in which molecules, after binding the receptor, are internalized *via* clathrin-coated vesicles (150). Transcytosis is a mechanism through which IgA can cross the IEB and arrives into the lumen. In particular, epithelial cells express the polymeric IgA-receptor

(pIgA-R) that binds IgA. The extracellular portion of pIgA-R is cleaved at the apical side and released into the lumen with IgA (151), and the so generated secretory IgA (SIgA) complexes play multiple protective roles (152). Bacterial and food antigens delivery across the intestinal epithelium takes place throughout the transcellular pathway. At the level of Peyer's patches, SIgA can mediate antigens selective translocation, first binding them and then promoting their apical-to-basal transepithelial migration *via* M cells, dendritic cells, and T cells (20, 153–156). Whereas, the food allergens translocation throughout the intestinal epithelium, during the initial phase, involves transcytosis of IgE-allergen complexes mediated by the CD23 IgE receptor in epithelial cells (157, 158). Indeed, the IgE-allergen complexes binding to the CD23, located at the apical side of polarized epithelial cells, promote IgE-allergen-CD23 migration toward the basolateral surface of the cells with AP2-dependent endocytosis *via* clathrin-coated (159, 160).

Phagocytosis is another pathway that allows the uptake of antigens. In particular, enterocytes are able to carry out TLR-mediated phagocytosis to internalizing gram-negative bacteria (161). Moreover, phagocytosis is a route through which bacteria, viruses, and particles can enter enterocytes, after binding to different receptors (162, 163). Through the non-specific process of micropinocytosis, extracellular fluids are internalized, as well as dissolved molecules, viruses, and apoptotic cell fragments. Finally, there is the mechanism mediated by lipid rafts/caveolae, that seems to be involved in the internalization of some enterotoxins and viruses into enterocytes (151). This mechanism involves the invagination of cholesterol-rich areas of the plasma membrane that contain a coating protein, caveolin (164).

TJs are responsible for the sealing of the paracellular space between cells and, therefore, strictly limiting the transport of hydrophilic molecules (165–167). TJs have the so-called “gate and fence” function as they regulate the size and charge selectivity of the paracellular pathway. Indeed, TJs allow the paracellular transport of some medium-sized hydrophilic molecules, ions, or positively charged molecules but prevent the transport of proteins, such as antigenic macromolecules, lipids, and microbial-derived peptides (168).

GUT MICROBIOTA-EPITHELIAL BARRIER INTERPLAY

The human gut harbors a community of about 10^{14} microorganisms resulting from thousands of years of co-evolution with the host, with an intricate and mutually beneficial relationship (169). Indeed, gut microbiota participates in digestive functions, shapes the host immune system (170, 171) modulates host metabolisms, and influences local and systemic processes, such as vitamin intake and nutrient transformation (172, 173). The intestinal microbial population also protects against pathogens, by competing with them for nutrient uptake and regulating host immunity (174–178). To avoid aberrant immune responses, the IEB separates microbes and immune cells, leading to the establishment of host-commensal mutualism (171). Despite this physical separation, gut microbiota can

deeply modulate epithelial cells and the immune system (38) through the production of a series of metabolites. Microbial metabolites can be classified as metabolites generated by the host and then modified by the gut microbes into the lumen, such as secondary bile acids, dietary product-derived metabolites, such as compound K, or *de novo* synthesized metabolites such as short-chain fatty acids (SCFAs). SCFAs including butyrate, acetate, and propionate, profoundly influence aspects of GI physiology, such as contractility, visceral pain, epithelial proliferation, barrier function, host immunity, but also bacterial pathogenesis (179–181).

SCFAs can modulate the expression profile of epithelial cells, enhancing the production of proteins involved in the biosynthesis of mucin (182). Specifically, butyrate enhances MUC2 expression both activating the MUC2 promoter and enhancing histone acetylation by HDAC inhibition in cell cultures (183). Moreover, the intestinal epithelial cells express receptors activated by SCFAs. These are members of the G-protein coupled receptors (GPR) and include GPR41, GPR43, and GPR109a. The SCFAs binding to GPR41 and GPR43 stimulates colonic epithelium to release chemokines and cytokines (184). The butyrate depending activation of GPR109a enhances IL-18 excretion in epithelial cells, protecting the colon against inflammation and carcinogenesis (185). Other microbial metabolites that significantly influence the maintenance of gut barrier integrity include indole derivatives, bile acid metabolites, conjugated fatty acids, polyamines, and polyphenolic derivatives. Their role in regulating gut barrier function was recently assessed in an extensive review (186).

TLRs are single-pass membrane-spanning receptors playing a key role in the innate immune system (187). They are expressed on the membranes of immune and non-immune cells, including epithelial cells, and recognize molecules that are broadly shared by microbes. In particular, TLR recognition of microbial fractions improves the IEB function, the secretion of mucus, and the production of antimicrobial peptides, promoting immune tolerance toward the gut microbiota. The role of epithelial TLRs in gut homeostasis and disease was recently reviewed (188).

Bacteria-epithelial cell interactions play an essential role in regulating epithelial permeability through the modulation of TJs expression and assembly (189). Animal studies clearly underlined the interplay between the intestinal microbiota and the IEB homeostasis. Germ-free mice had a higher colonic expression of claudin-1 and occludin as compared with conventional mice, with lower paracellular uptake of a standard probe, suggesting that commensal microbiota controls colonic TJs proteins and paracellular permeability (190). Interestingly, transplantation of fecal microbiota from healthy humans restores the IEB features of conventional mice within a week. In particular, colonization reestablishes physiological colonic paracellular permeability, maturation of colonic barrier structure, and reduces systemic microbial antigen exposure (190). Altogether these data suggest that gut microbiota is crucial in preserving the integrity of the IEB, thus preventing the systemic spreading of potentially harmful antigens.

Specific gut microbiota components may differently modulate the IEB permeability. For example, colonization of germ-free mice with *Bacteroides thetaiotaomicron* (191) or *Escherichia coli* Nissle 1917 (EcN) (192) led to up-regulation of genes encoding for proteins such as small proline-rich protein-2 (sprr2a) and ZO-1 involved in improving cellular adhesion. Interestingly, we have recently demonstrated that the increase in paracellular permeability elicited by supernatants obtained from patients with IBS could be ameliorated by EcN (193). Furthermore, EcN treatment abolished the correlations between increased permeability induced by IBS supernatants with abdominal pain and distension referred by IBS patients, paving the way to further explore clinical applicability of this probiotic in IBS human studies (193). In contrast, other bacteria had no impact on IEB permeability or could impair the IEB. For the latter, not only pathogens, such as enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (194), enterotoxigenic *Escherichia coli* (ETEC) K88 and *Salmonella typhimurium* SL1344 (195), but also non-pathogenic commensal bacteria, such as *Escherichia coli* C25 (196), can be involved.

Growing evidence indicated that microbiota dysbiosis, occurring when the diversity, composition, and/or functions of the intestinal microbiome are disrupted, could contribute to the alteration of the IEB with implications in the development, progression, and symptom flare-up of several diseases. Among these, inflammatory conditions, such as IBD, and functional bowel disorders, such as IBS, are both characterized by a well-established microbiota dysbiosis associated with impairment of intestinal permeability. The interplay between the host, and in particular the semipermeable multi-layer ecosystem where intestinal epithelial cells exert a critical role, and the gut microbiota is constantly challenged by numerous factors, including genetics, age, environment, food, and immunological factors. A recent report of the Rome Foundation on the Intestinal Microenvironment and Functional Gastrointestinal Disorders (FGIDs) provided an excellent overview of the importance of the environment, including food, diet, microbiota, and its metabolic interactions, in the pathophysiology and symptom generation of patients with FGIDs (197). Patients with IBD display a loss of biodiversity (mostly Firmicutes) and stability, while an increase of Proteobacteria such as *Enterobacteriaceae*, *Bifidobacteria*, and certain members of Bacteroidetes [for review, see (198)]. In addition, *Akkermansia muciniphila*, a mucolytic commensal, is generally reduced in the gut of these patients, with a consequent increase of the overall mucosal bacteria population (42). Furthermore, patients with IBD display a reduction in SCFA-producing bacteria such as *Faecalibacterium prausnitzii* (199) that is well-known to have anti-inflammatory properties through its ability to produce butyrate, allowing for T regulatory cell and T helper 17 regulation (200). Altogether, these changes may lead to a loss or reduction of key functions necessary for maintaining IEB integrity, potentially resulting in increased immune responses and the diffusion of pathogens into the intestinal tissues. In addition, bacterial translocation induces the production of inflammatory cytokines, which promote the disassembly of TJs, enhancing IEB permeability. However, if these changes are a cause or consequence of these diseases,

this is virtually unknown at this time. Strategies to improve the relationship between host and intestinal microbiota to enhance epithelial mucosal permeability include the use of probiotics and dietary interventions, although there is still uncertainty on the potential benefits (6). In this context, we have recently demonstrated that *Lactobacillus paracasei* CNCM I-1572 modulates gut microbiota structure and function and reduces intestinal immune activation in patients with IBS. Interestingly, the most robust result was obtained for a marked reduction of interleukin 15 (IL-15) that affects the integrity of the IEB, suggesting that this probiotic may play an important role in the restoration of mucosal integrity (201).

GUT IMMUNE SYSTEM-EPITHELIAL BARRIER INTERPLAY

The integrity of the IEB depends on the delicate balance between differentiation and renewal of intestinal epithelial cells, the response to signals coming from the lumen including microbiota and their end products, and nutritional factors introduced with daily diet as well as signals coming from the mucosal immune system (202). Several factors, including cytokines, proteases, growth factors, gut microbiota, and dietary components are known to regulate intestinal TJs opening (6). Immune dysregulation in several disorders of the gastrointestinal tract such as IBD, celiac disease, colonic cancer, and IBS is often associated with impaired IEB integrity or dysfunction. Indeed, the IEB may represent the target of mediators released by inflammatory cells in the lamina propria. This elicits epithelial cell damage and TJ dysfunction, mucus structural and functional alterations, ultimately leading to increased intestinal permeability (6). Disruption of the IEB would then allow the passage of antigens, bacterial products in the mucosa leading to further inflammation hence creating a self-maintaining pathological inflammatory process (6). The involvement of the immune system in conditions affecting the gastrointestinal tract can be greatly different for magnitude as well as the type of immune cells. Conditions characterized by mucosal inflammation often show the involvement of both innate as well as adaptive immunity.

Intestinal epithelial cells and mononuclear phagocytes sense bacteria or their products mainly through pattern recognition receptors such as TLRs (166). Downstream of activation of innate responses is the production of cytokines, including the IL-1 family cytokines such as IL-1 and IL-18 which lead to pro-inflammatory effects in the context of intestinal inflammation. IL-1 can further promote Th17 cell differentiation and IFN- γ production from T cells (203) and its two isoforms (IL-1 α and - β) seem to be involved in epithelial repair/regeneration (204). IL-1R1 is a receptor expressed on different cell types of the colon such as innate lymphoid cells (ILCs) and GREM+ mesenchymal cells (205). Cox et al. demonstrated that in a mice model of DSS-induced colitis, the binding of IL-1 α/β to IL-1R1 in ILCs induces the production of IL-22, a cytokine involved in progenitor cell proliferation. Conversely, in a model of *C. rodentium* infection, the activation of IL-1R1 occurs both in ILCs

and in GREM+ mesenchymal cells, inducing the production of R-spondin 3 (RSPO3), an intestinal stem cell self-renewal activator. The IL-1R1-dependent response thus appears to reveal a damage-specific reparative capacity, which opens the door to IL-1-based therapeutic approaches (206).

Other important cytokines in the orchestration of innate immunity-related intestinal inflammation, include IL-6, IL-33, TNF- α (202). The release of TNF- α and IFN- γ in the intestinal mucosa has been associated with IEB disruption in patients with IBD (207–210) as well as IBS (211). In line with this concept, incubation of intestinal epithelial cell monolayers with TNF- α and IFN- γ elicits profound changes of claudins, occludin, ZO-1, JAM-A, leading to increased epithelial permeability (212). During intestinal inflammation, TJs show strand breaks, and changes in TJ proteins composition and function as well as impaired structure and remodeling of apical junctions (213).

During inflammatory processes neutrophils, mast cells, and macrophages release proteases (214). Besides degrading the extracellular matrix and proteins, proteases act as signaling molecules *via* specific receptors (215). Proteases may deeply influence the integrity of the IEB either through excessive proteolysis determined by direct cleavage of intercellular junction proteins, or by a functional TJ opening elicited by the activation of protease-activated receptors (216). Mast cells are very proficient producers of proteases and mast cell activation has been proposed as underlying pathogenetic mechanisms in different gastrointestinal disorders, including IBS (217, 218).

CLINICAL IMPLICATIONS

Irritable Bowel Syndrome

Differently from IBD and celiac disease, IBS is not associated with overt mucosal inflammation, and evidence supporting the potential role of cytokines in these patients has been so far controversial (Figure 2) (219–221). However, we previously reported that IBS is associated with a marked increase in IFN- γ gene and IFN- γ protein expression in the colonic mucosa (211), a finding also supported by evidence of increased IFN- γ release in the lumen (222). As IFN- γ is known to increase paracellular permeability through disruption of TJs (223). This cytokine could well be involved in increased paracellular permeability described in patients with IBS (224). In addition to IFN- γ , other players could be of relevance in the dysregulation of TJs. IL-9 is abundantly produced by mast cells as well as innate lymphoid cells and T helper cells. Evidence suggests that IL-9 amplifies intestinal mastocytosis involved in several inflammatory diseases of the gastrointestinal tract (225). Recently, it has been shown that IL-9 modulates IEB function, modifying TJs proteins expression. In particular, IL-9 increases intestinal permeability through the upregulation of claudin-2 expression in an experimental model of ulcerative colitis (226). This concept is further supported by recent studies showing that in murine models of colitis, the TJ protein claudin-1 showed lower expression levels in IL-9 knock-out mice (227).

We have previously shown that the permeability of colonic biopsies was significantly higher in patients with IBS compared to healthy subjects (228). Trypsin, a key serine protease released

by mast cells upon degranulation, was abundantly increased in mucosal biopsy supernatants of patients with IBS compared to healthy controls (229–231). Interestingly, the application of mucosal biopsy supernatants of IBS patients elicited a marked increase in paracellular permeability in Caco-2 epithelial cell monolayers (193). The importance of proteases in the pathogenesis of IEB dysfunction described in IBS is further supported by the evidence that transfer of fecal supernatants from patients with diarrhea-predominant IBS evoked increased mucosal permeability in mice and mucosal factors obtained from IBS evoked IEB dysfunction and TJ disruption in isolated epithelial monolayers (228). The effect of fecal supernatants on the epithelial barrier was absent in mice lacking PAR-2 (232). These results suggest that mucosal or luminal mediators impact negatively IEB by increasing epithelial permeability through a protease, PAR-2-dependent pathway and open new avenues for therapeutic intervention (232).

Contrary to the long-lasting belief that IBS is not associated with organic changes, there is now growing evidence supporting that structural, although subtle, changes may be found in the gastrointestinal tract of large subgroups of these patients (197). The observation that IBS can develop after an episode of acute infectious gastroenteritis further supports the organic as opposed to the functional nature of this condition (233). Many studies showed that the intestinal mucosa of IBS patients contains larger numbers of immune cells and evidence of a higher state of activation of the immune system (218). Interestingly, growing evidence supports the concept that IEB dysfunction may be key in the initiation and progression of immune activation and that this may eventually contribute to brain-gut axis dysfunction and symptom generation (228, 234). Accordingly, this evidence has generated a new dogma in the pathophysiology of IBS. More in detail, microenvironmental factors (e.g., food, microbiota, bile acids) would permeate in excess through a leaky IEB, allowing amplification of signaling from the lumen to deeper mucosal and muscle layers, including overstimulation of the mucosal immune system (197). These factors may determine abnormal signaling to neural circuits (intrinsic primary afferent nerves and extrinsic primary afferent nerves), which in turn may affect intestinal physiology and sensory perception (197).

Nonetheless, evidence indicates that there is a poor correlation between pathogenetic mechanisms and symptom generation in IBS. More likely, only the combination of peripheral (i.e., intestinal) factors, in conjunction with central nervous system mechanisms is necessary for the full expression of symptom perception. For this reason, IBS, like many other functional gastrointestinal disorders, is now better defined with the new term of Disorders of Gut-Brain Interaction (DGBI). On the same line, a single phenomenon is insufficient to explain the complexity of the protean symptom experience of IBS. One example in line with these concepts was our previous demonstration that the number of mucosal mast cells was increased in the colonic mucosa of patients with IBS (229). However, this phenomenon alone was not correlated to any symptom experienced by the patients. Nonetheless, when this parameter was associated with the presence of activated mast cells in close proximity with nerve endings in the

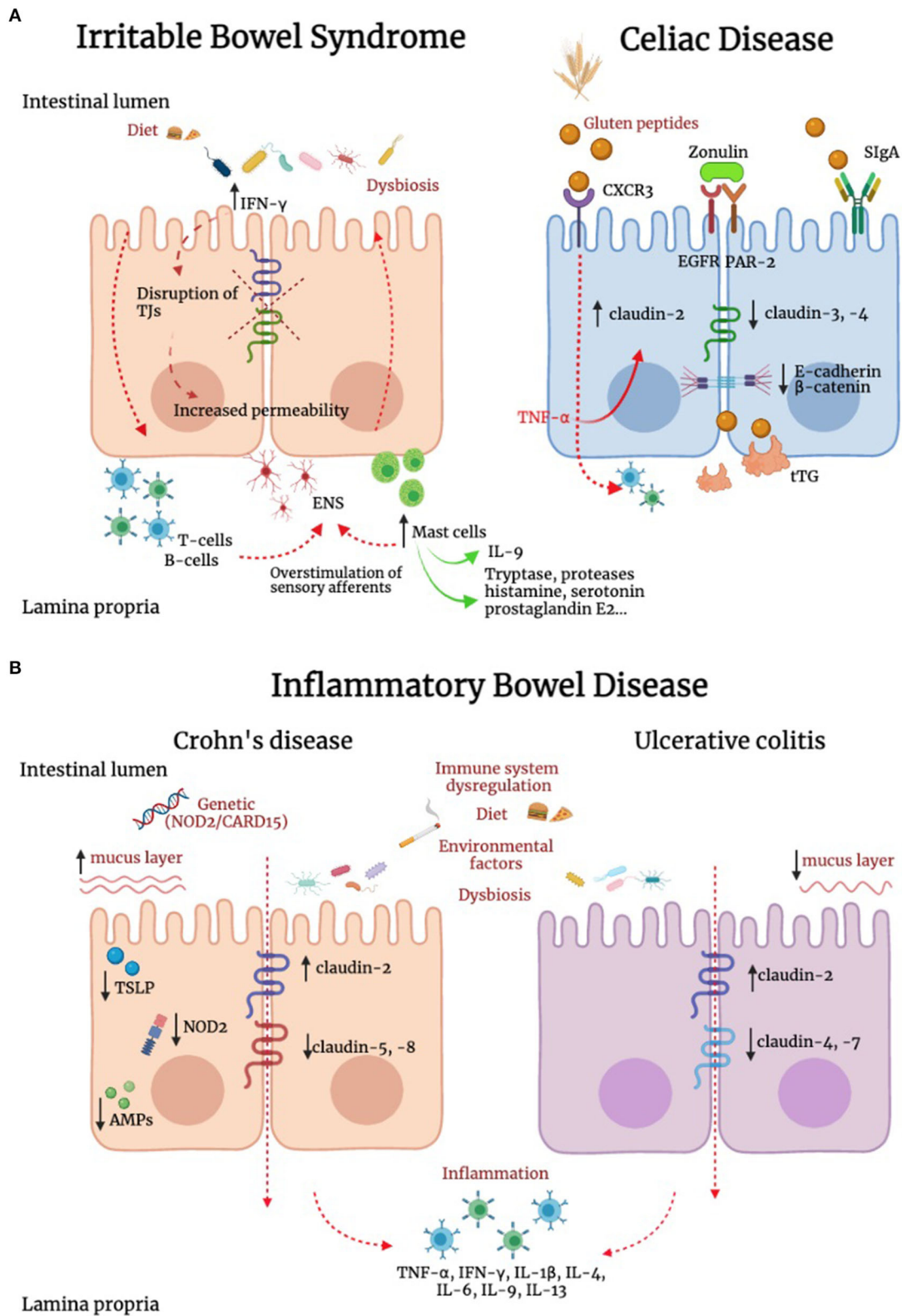


FIGURE 2 | Alteration of IEB in irritable bowel syndrome, inflammatory bowel disease and celiac disease. **(A)** In irritable bowel syndrome (IBS), tryptase, histamine, and interferon-γ (IFN-γ) are increased and can contribute to TJ disruption. In addition, IL-9 is produced by innate lymphoid cells, T helper cells and mast cells. This latter (Continued)

FIGURE 2 | cell population has a key role in IBS pathophysiology, and can modify TJ protein expression. In addition, immune mediators, including histamine, serotonin and proteases evoke sensory afferent over-stimulation and contribute to symptom generation. In celiac disease (CD), intestinal epithelial cells recognize gluten peptides through CXCR3, which increases IEB permeability through zonulin release and its transactivation of epidermal growth factor receptor (EGFR) and protease activated receptor 2 (PAR-2). Transcytosis of gluten peptides occurs after peptide recognition by secretory immunoglobulin A (SIgA). A reduction in the expression of E-cadherin and β -catenin was found in intestinal biopsies of CD patients. Active tissue transglutaminase2 (tTG2) deamidates gluten peptides, contributing to the development of a cell mediated pro-inflammatory immune response. **(B)** Genetics, environment, diet, immune system dysregulation and dysbiosis represent some of the complex mechanisms responsible for inflammatory bowel diseases (IBD). Inflammation down-regulates TJs proteins contributing to IEB alteration. In IBD patients has been reported an upregulation of the pore-forming claudin-2 and downregulation of occludin. Channel-forming claudins are up-regulated by cytokines including TNF- α and IL-13, leading to an increased permeability for ions and water. Occludin is down-regulated by inflammatory processes (e.g., TNF- α and IFN- γ), leading to increased paracellular permeability for macromolecules. Moreover, it has been shown a downregulation of claudin-5 and -8 in Crohn's disease and claudin-4 and -7 in Ulcerative Colitis (UC). In Crohn's disease, the stimulation of NOD2, a sensor of Gram-positive bacteria, induces the production of pro-inflammatory mediators, which concur to IEB dysfunction. Differently from UC, in Crohn's disease the mucus layer is thicker, suggesting an increase in MUC2 expression and goblet cells hyperplasia. In patients with Crohn's disease, intestinal epithelial cells (IECs) failed to produce thymic stromal lymphopoietin (TSLP), with consequent inability to control IL-12, produced by dendritic cells and involved in the development of Thelper 2 cells, resulting in alteration of intestinal homeostasis. Compared to UC, in which the antimicrobial peptides (AMPs) system seems to be adequately induced, Crohn's disease is characterized by lower levels of AMPs. Contrasting evidence are available on the role of smoking in UC and Crohn's disease.

mucosa of the intestine, we found a stringent correlation with abdominal pain (229). In addition, there is wide redundancy in pathogenetic mechanisms, and the different pathogenetic mechanisms influence each other and therefore should be always considered in context. For example, on one hand, changes in gut microbiota can promote IEB dysfunction which can lead to mucosal inflammation. On the other hand, inflammatory mediators can increase mucosal permeability and influence gut microbiota. This generates a vicious and integrated circle that cannot be separated into fully independent compartments.

With all the above considerations in mind, one of the most common observations in the studies evaluating the gut IEB and IBS relates to the correlation between increased intestinal permeability and abdominal pain (234). The link between epithelial permeability and pain likely reflects the abovementioned redundancy of the system involving the gut microbiota overstimulating the immune system through a leaky IEB, leading to the release of immune mediators, such as histamine, tryptase, serotonin, polyunsaturated fatty acids (12-hydroperoxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid, 5-oxoeicosatetraenoic acid, and leukotriene B4) (235, 236) known to evoke sensory afferent over-stimulation and pain (230).

Although some tantalizing findings have been reported, the final proof to demonstrate the key role of increased intestinal permeability in IBS has yet to be provided. There is a need for longitudinal studies that include assessment of the IEB function over time and its correlation with symptoms in well-characterized IBS populations. In addition, further studies assessing the role of IEB modulation in IBS are now needed. In this perspective, a recent study using a medical device containing xyloglucan, pea protein and tannins from grape seed extract, and xylo-oligosaccharides, acting together to protect and reinforce the IEB, effectively controlled diarrhea and alleviated clinical symptoms in patients with IBS-D (237).

Gluten-Related Disorders

Gluten-related disorders comprise distinct clinical entities, including celiac disease (CD), wheat-associated allergy, and NCGS, characterized by distinct pathophysiological pathways,

with altered permeability following gluten ingestion as a possible common shape.

CD is a chronic systemic disorder triggered by the abnormal response of human immunity to gluten ingestion in genetically pre-disposed individuals (238). The activation of the immune system against gluten peptides begins after their transfer to the lamina propria where they are deamidated by the tissue transglutaminase enzyme and subsequently bounded to human leucocyte antigens (HLAs) expressed by antigen-presenting cells (APCs), which activate the inflammatory cascade (**Figure 2**) (239, 240). However, the presence of gluten and genetic background does not fully explain CD pathogenesis. Indeed, in studies on twins, in one-fourth of cases, only one twin developed CD (241). Mounting evidence suggests that CD onset is favored under the influence of triggering environmental factors, such as viral infections and dysbiosis (242–247) which activate innate immunity leading to IEB disruption (248). Several studies suggest that the *primum movens* for CD onset depends on an increased intestinal permeability, which leads to gluten passage from the lumen to the mucosal layer and innate cytotoxic immune activation on epithelial cells further enhancing gluten migration (249). Although the IEB has been extensively investigated in the context of CD pathogenesis, to date is still unclear whether the IEB impairment is a cause or consequence of CD. On the other hand, in the last decades a novel clinically entity has been defined in patients without CD complaining extraintestinal and gastrointestinal symptoms referring to a clear benefit by avoiding gluten from their diet and/or symptom worsening upon gluten reintroduction, namely NCGS (141). The pathophysiology of NCGS is incompletely known, although some evidence suggests a role for permeability alterations, microbiota changes, and immune activation (141). The Salerno criteria are the gold standard to diagnose NCGS. These criteria imply a double-blind placebo-controlled gluten challenge (250), which is cumbersome and unfeasible to perform in clinical practice. In addition, there are no available biomarkers for NCGS. For all these reasons, diagnosis is currently based on self-reported symptoms by patients. There is a great overlap in symptoms between NCGS and IBS which makes a challenge the differential diagnosis between these two conditions. We have recently

reported that zonulin serum levels are significantly increased in NCGS compared to IBS. In addition, the combination of zonulin levels, gender, and abdominal symptoms, can differentiate NCGS from IBS with a diagnostic accuracy of 89% (141).

Studies carried out using epithelial cell lines and animal models showed that gliadin was able to induce apoptosis of intestinal epithelial cells through a direct toxic effect (251), including enterocyte atrophy, villi shortening, decreased epithelial disaccharidase activity, increased expression of HLA molecules, and intraepithelial inflammatory activation. Among other effects, gliadin fraction p31-43 is able to mimic epidermal growth factors in human intestinal epithelial cell lines, finally leading to barrier defects (252). This peptide is usually detoxified during the transport in healthy subjects, whereas in active CD patients most of these peptides are transported intact in the serosal compartment; this process may be explained by the binding between gluten peptides with anti-gliadin secretory IgA finally forming large complexes in the intestinal lumen which in turn bind the CD71 receptor, namely the endocytic receptor for transferrin, thus activating the transport across enterocytes escaping lysosomal degradation (156). It is unclear whether this process is involved in CD development since it has been postulated that the entrance of IgA-gliadin complexes may break local immune tolerance (253). However, it is more likely that this process may perpetuate the immune reaction against gluten once the disease started. Besides, the effect of gliadin on the enterocyte actin cytoskeleton was studied on rat intestinal epithelial (IEC-6) cell cultures, finding that it was able to reversibly stimulate tyrosine phosphorylation of actin filaments resulting in filaments polymerization, cytoskeleton reorganization, and tight junction opening (254). Other authors suggested that gliadin was able to bind the mono-sialic ganglioside 1 (GM1) or the receptors for the proinflammatory chemokine 3 (CXCR3) in mouse models, leading to the release of zonulin which in turn decreases electrical resistance of epithelial layers, finally resulting in increased epithelial permeability (139, 255). Indeed, zonulin release can induce cytoskeleton reorganization and zonula occludens-1 and occludin downregulation (139, 256). More interestingly, the receptors for the proinflammatory chemokine CXCR3 were also activated by the overexpression of its chemokine ligand 10 (CXCL10), which in turn is upregulated *in vitro* by viral infections (256).

Macroscopic alterations in intestinal permeability have been studied since the 60s (257) using tests able to measure the absorption of two inert probes of different dimensions by the IEB (257). The first probe was mannitol, which was taught to get through the IEB freely; the second was lactulose, which was able to get through the IEB only when its integrity was lost. An increased ratio between urinary lactulose/mannitol concentrations 6 and 12 h after oral administration indicated increased intestinal permeability, which has been extensively reported in CD patients (258–260). These alterations were also correlated to villus atrophy and intraepithelial lymphocyte count. Indeed, a further study showed that permeability increase was dependent on the release of IFN- γ by intraepithelial lymphocytes (207).

Structural changes consisting of dilatation, destruction, and reduced number of TJ strands were found in CD patients from ancillary (261) to more recent studies using transmission electron microscopy of duodenal biopsies (262). In CD has been widely reported an increased expression of pore-forming proteins such as occludin and claudin-2, together with a decrease of pore-sealing proteins such as claudin-3 and -4 and ZO-1 (84, 262–264). Claudin-2 expression is also induced by TNF- α , which in turn is upregulated in CD (86) and responsible for increased transcellular permeability in CD. These alterations are simultaneous to the loss of the penta-laminar ultrastructure and dilatation of TJs found in CD (262). As matter of fact, the overexpression of pore-forming and under-expression of pore-sealing TJ proteins is responsible for the dilatation of TJs (262).

CD patients have been found to have also alterations in adherence junction protein expression. A reduction in the expression of E-cadherin and β -catenin was found using transcription analysis in both intestinal biopsies of CD patients and Caco-2 cells (265). Moreover, an impaired interaction between ZO-1, mostly not phosphorylated in CD patients, and β -catenin has been reported, finally, leading to an absent connection between β -catenin and occludin in CD patients (263). On the other hand, β -catenin has been reported to be highly phosphorylated in CD, thus not binding E-cadherin which in turn is free to bind intra-epithelial lymphocytes responsible for inflammatory activation (263). Together with barrier impairment, epithelial polarization may also affect permeability since it regulates the function of proteins partition defective-3 (PARD-3) and protein phosphatase-2 (PP-1) which are involved in tight junctions formation (266).

Although these alterations are not specific for CD and may merely depend on mucosal inflammation, tight junctions' abnormalities have been found even in asymptomatic and first-degree relatives of CD patients, thus underlying a possible genetic background for permeability defects in CD (267). Supporting this hypothesis, polymorphisms in tight junctions encoding genes, such as PAR-3, membrane-associated guanylate kinase WW, and PDZ domain containing 2 (MAGI-2) and myosin IXb (MYO9B) have been found in CD patients (268–270). A subsequent study did not confirm a direct causal relationship between genetic polymorphisms and increased intestinal permeability (271). However, more recent studies evaluating CD loci showed a possible role of other genes involved in barrier functions and cell-cell adhesion (272–274).

To date, the only effective treatment for CD is a gluten-free diet (GFD). GFD is able to partially restore TJs abnormalities found in CD since TJs numbers remained low in crypts (261). Other studies found a normalization of ZO-1 (264) expression after gluten removal *in vitro* and of claudin expression (262) after 6 months of GFD. In parallel, *in vitro* models showed that β -catenin and E-cadherin alterations were reversible after gluten removal (265, 275). These microscopic findings were further confirmed by the recovery of a normal lactulose/mannitol ratio after histological mucosal healing due to long-term GFD (276). This evidence additionally confirmed that most intestinal permeability alterations were reversible, thus excluding the previous hypothesis of a causal role for genetic background.

Additional or alternative therapeutic options to GFD focused on the reduction of intestinal permeability have been proposed for CD patients. Gluten detoxification through proteolysis or gliadin sequestrants has been proposed for overcoming the gliadin-related toxic effect across the epithelium (277, 278). A randomized controlled trial carried out in 2007 aimed to improve intestinal permeability using larazotide acetate (AT-1001), which is an inhibitor of paracellular permeability derived from a protein secreted by *Vibrio cholerae* and analogs of human zonulin (279). The authors concluded that larazotide acetate reduced IEB permeability assessed by lactulose/mannitol permeability test, proinflammatory cytokine production, and gastrointestinal symptoms in CD after gluten exposure (279). Two further phase II trials (280, 281) with larazotide acetate failed in finding an improvement in intestinal permeability, whereas the last trial published (282) underlined possible usefulness in refractory CD, defined as malabsorption and continued villous atrophy on duodenal biopsies despite strict avoidance of gluten for a minimum of 12 months (283). Another promising approach for reducing intestinal permeability is the use of probiotics, which however deserve further studies (244, 284).

Inflammatory Bowel Diseases

Dysfunctional IEB has been implicated as a pathogenic factor in IBD in the last 30 years (285, 286). IBD includes a spectrum of disorders such as Crohn's disease and ulcerative colitis (UC), representing chronic remittent or progressive conditions determined by non-specific inflammation and intestinal tissue damage. It has been characterized by exaggerated and inappropriate mucosal immune responses that can involve the entire mucosal wall, as in Crohn's disease, or be confined to the submucosa, as in UC (287). The pathogenesis of IBD is complex and multifactorial and it is not fully understood, involving a complex dysregulated interaction between different factors (**Figure 2**). Particularly genetic pre-disposition, gut microbiota, and innate and adaptive immune responses represent fundamental elements (288). Some authors suggest that the alteration of the balance between these three components would be responsible for the triggering of the inflammatory environment needed to induce IBD (289). Otherwise many researchers reported that many other factors are involved in the pathogenesis of IBD such as dysfunction of intercellular transport mechanisms (290, 291), associated with factors responsible for the exacerbation in IBD (i.e., cigarette smoking, diet, stress, microbial dysbiosis, and food additives) (292–297). In fact, urbanization of societies is associated with changes in diet, antibiotic use, hygiene status, microbial exposures, and pollution, which have been implicated as potential environmental risk factors for IBD (298). Although these factors have been explored, the data available are still inconclusive (299). It is still a matter of debate the role of smoking in IBD. Evidence reported in literature shows smoking is associated with opposing risks in Crohn's disease and UC (300–302).

A meta-analysis focused on the effects of smoking behaviors and IBD has demonstrated that different races may have varying degrees of susceptibility to IBD (300). Smoking may play differing roles in the development of Crohn's disease compared with

UC. Studies that involve Israeli populations, for example, have consistently failed to demonstrate a positive association between smoking and Crohn's disease, yet these same studies have shown a protective relationship between smoking and the development of UC (300). Conversely, a recent paper showed current smokers compared to never-smokers had an ~2-fold risk of UC and Crohn's disease (303).

Another study analyzed systemic concentrations of key chemokines and cytokines in IBD patients with a different range of disease activity compared to levels found in healthy donors (304). The result shows that there was a significant increase of chemokines including macrophage migration factor (MIF), CCL25, CCL23, CXCL5, CXCL13, CXCL10, CXCL11, MCP1, and CCL21 in IBD patients as compared to normal healthy donors. Further, has been reported an increase in the inflammatory cytokines IL-16, IFN- γ , IL-1 β , and TNF- α in IBD patients when compared to healthy donors ($P < 0.05$). These data clearly indicate an increase in circulating levels of specific chemokines and cytokines that are known to modulate systemic levels through immune cells, results in affecting local intestinal inflammation and tissue damage in IBD patients (304). More recently it has been studied the role of antimicrobial peptides (AMPs) in IBD patients. Between this group of molecules, the most important ones are produced in the gut epithelium and are α -defensins HD5 and HD6, produced by the small-intestinal Paneth cells, and β -defensins (constitutive HBD1 and inducible HBD2 and HBD3), mostly in the gastric and colonic epithelium (305). The changes in the microbiome composition and the bacterial contamination of the mucosa as well as the inner layer of the mucus may well be mediated by defects in this chemical defense (306). Compared to ulcerative colitis, in which the AMP system seems to be adequately induced, colonic Crohn's disease is characterized by low HBD1, regulated by peroxisome proliferator-activated receptor gamma (PPAR γ), and a compromised induction of HBD2 and HBD3 (307, 308). Considering the important role played by the intestinal epithelium, which establishes a tightly regulated barrier, its integrity defects are frequently reported during intestinal inflammation (309). Microscopic analysis of intestinal tissue from IBD patients reveals a decrease in goblet cells (50), reduction thickness of mucus layer, and defective defensin production (310), with an altered composition of some components such as mucins and phosphatidylcholine (311). The alteration of the mucus protective barrier plays a key role in the onset of IBD (312). A recent case-control study reported that mucus abnormalities contribute to UC pathogenesis, demonstrating how core mucus structural components were reduced in active UC (313). These alterations were associated with attenuation of the goblet cell secretory response to microbial challenge and occurred independently of local inflammation. Another important mechanism involved in the alteration of the mucus protective barrier in IBD is the invasion by bacteria directly in contact with the epithelium and their penetration into the crypts and epithelial cells (314). This mechanism has been demonstrated in mice lacking MUC2 mucin (27). In these animal models, bacterial invasion induces the response of the colonic immune system resulting in inflammation, spontaneous

colitis, diarrhea, rectal and colon prolapse, rectal bleeding, and an increased risk of colon cancer development. Differently from UC, in Crohn's disease, the mucus layer is thicker, suggesting an increase in MUC2 expression and goblet cells hyperplasia. Nevertheless, the structure of MUC2 is altered due to a reduction in the oligosaccharide chain length by 50%, leading to a loss of mucus viscoelastic properties and consequently a loss of protective function (315). Furthermore, indirect data suggest that barrier defect might precede the onset of disease (316) and up to 40% of first-degree relatives of patients with Crohn's disease demonstrate an altered small intestinal permeability (317). Moreover, it was shown that patients that had increased intestinal permeability were at greater risk of successive disease relapse (318, 319).

The mechanisms involved in the proper functioning of the IEB include the transport of molecules across the intestinal mucosa through two distinct mechanisms: paracellular diffusion through TJs between adjacent intestinal epithelial cells and transcellular transport involving the transcytosis of materials, eventually mediated by membrane receptors. In IBD patients has been observed increased paracellular permeability with abnormal TJ structure and a down-regulation and redistribution of many TJ proteins or other adherents junctions (320, 321). Both paracellular hyperpermeability [demonstrated by abnormal TJ expression and upregulation of myosin light chain kinase (MLCK) activity (86, 322–324)] and transcellular hyperpermeability [represented by bacterial internalization to epithelia (325)] were documented in mucosal biopsies of patients with Crohn's disease and UC. Disruptions of TJ proteins could lead to an alteration of the IEB, allowing entry of luminal bacteria. In fact in IBD has been reported an upregulation of the pore-forming claudin-2 and down-regulation of occludin, particularly claudin-5 and -8 in Crohn's disease and claudin-4 and -7 in UC (83, 86, 326). Increased expression of claudin-2 determines an increased number of pores responsible for the paracellular movement of small molecules, characteristic of both UC and Crohn's disease (1). This rupture in the IEB can result in inflammatory infiltrate resulting in a production of cytokines and other mediators that can further contribute to the impaired functioning of the IEB. Different proinflammatory cytokines are implicated in IEB dysfunction through the increased intestinal permeability along paracellular pathways (327). In particular cytokines such as TNF- α , IL-4, IL-13, interferon- γ (IFN- γ), IL-1 β , IL-9, and IL-6 increase intestinal permeability, whereas has been shown that IL-10 has a protective role, maintain IEB function (321, 328–330). This is supported by the fact that IFN- γ and TNF- α are elevated in the mucosa of IBD patients contributing to a pro-inflammatory cascade and IEB disruption (75, 331).

Regarding the important role of genetic susceptibility in IBD patients, most genetic loci that confer susceptibility to Crohn's disease and UC, have been linked to defects in IEB function (332–335). The first Crohn's disease susceptibility gene identified was NOD2/CARD15 (336). It was demonstrated that patients with a mutation of NOD2 have altered cell-cell epithelial contacts (337). Furthermore, epithelial cells with mutated NOD2, have an inappropriate response to a sensor of a cell wall component

of Gram-positive bacteria (muramyl dipeptide, MDP) whose stimulation determines the production of pro-inflammatory mediators (338–341). Moreover, it was shown that mutations in the NOD2 gene increased susceptibility to intestinal permeability in the healthy relatives compared to control subjects (342).

The intestinal microbiota has an important role as a regulator of epithelial-immune cell communication and patients with IBD often show dysbiosis (309). In fact in IBD have been documented changes in the commensal gut microbiota, represented by reduced complexity of commensals bacteria that are beneficial for the host or a greater representation of a specific phylum (343). Currently, it is not clear if these disorders are the cause or consequence of the manifestation of IBD. The cause of this condition seems to be disturbances in the recognition of pathogens microorganisms in the human intestine determined by alterations in the expression of pathogen recognition receptors, which include the already mentioned NOD2 (336, 340) and also TLRs and Rig-I like receptors. Intestinal epithelial cells (IECs) and innate immune cells, such as dendritic cells and macrophages, are equipped with these receptors to distinguish between components of pathogens and beneficial commensals (344). IECs have also the role to produce thymic stromal lymphopoietin (TSLP), IL-2 cytokine member, in response to commensals, such as Gram-negative *Escherichia coli* or Gram-positive *Lactobacillus rhamnosus* (345). Interestingly it was shown that in patients with Crohn's disease, IECs failed to produce TSLP, with consequent inability to control IL-12, produced by dendritic cells and involved in the development of Thelper 2 cells, resulting in alteration of intestinal homeostasis (346). These dysregulated epithelial-immune cell communication support the evidence of disturbed microbiota in patients with IBD (309).

Despite the involvement of immune intrinsic and barrier intrinsic dysfunction in the pathogenesis of IBD, none of these mechanisms alone would be able to explain all the characteristics of IBD. At the same time, data available in the literature, do not fully support defect in the IEB as a primary etiologic factor leading to the manifestation of IBD and it is unclear whether increased intestinal permeability is a consequence of the inflammatory process (347, 348) or anticipates and contributes to intestinal inflammation (317). Considering that current anti-inflammatory therapy (steroids and immunosuppressant drugs) remains unsatisfactory due to substantial side effects and uncontrolled relapses (349), understand the interaction between gut microbiota and IEB, particularly at the early subclinical phase of inflammatory disease, could be used to identify novel therapeutic approaches in chronic intestinal inflammation.

THERAPEUTIC IMPLICATIONS

Although several dietary factors, such as gluten, bile acids, fructose, ethanol, and emulsifiers are well-known agents damaging the IEB, other dietary components, such as fibers, SCFAs, glutamine, and vitamin D, may improve the IEB. Similarly, prebiotics, symbiotics, and probiotics may act on IEB function fortifying it. Recent extensive reviews have analyzed

the effects of nutrients and supplements on IEB function (350, 351). Now, we will focus on nutritional approaches exerting a protective effect on the IEB, in particular on those that are better defined and studied such as glutamine, vitamin D, and SCFAs, particularly butyrate. Interestingly, these approaches have been proposed in the management of patients with IBS or IBD, conditions characterized by altered IEB function.

Glutamine is an essential amino acid in humans and one of the main energy sources for rapidly dividing epithelial cells of the gastrointestinal tract (352). Its depletion during illness or infection leads to intestinal epithelial cells atrophy resulting in increased intestinal permeability (352). Pioneer studies assessing the effect of enteral supplement with glutamine granules on intestinal IEB function in severely burned patients, showed that this compound can improve intestinal mucosal permeability, decrease plasma endotoxin levels, reduce hospital stay and costs (353, 354). A recent randomized controlled trial (RCT) assessing the efficacy and safety of oral glutamine supplementation in patients with post-infection IBS with diarrhea and increased intestinal permeability, showed that this treatment significantly improves all IBS symptoms and IEB function (355). However, due to the small sample size and the suboptimal experimental study design with significant methodological limitations, these results require caution and need to be replicated in larger well-performed clinical trials.

Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of electrolytes including calcium, magnesium, and phosphate (356). Among the many other biological effects, vitamin D may activate the innate and modulate the adaptive immune systems with antibacterial, antiviral, and anti-inflammatory effects (357, 358). Vitamin D receptors are expressed by both epithelial and a large number of immune cells in the gastrointestinal tract (357, 358). Although *in vitro* studies showed that vitamin D is involved in the regulation of TJ molecules (357, 359, 360), few randomized controlled intervention studies have investigated its clinical efficacy or mechanisms of action in gastrointestinal diseases. Low levels of vitamin D are associated with IBD. A recent systematic review and meta-analysis of vitamin D therapy in patients with IBD and vitamin D deficiency has shown that this supplementation is effective not only for the correction of vitamin D levels but also for improving scores of clinical disease activity and biochemical markers (361). Although the mechanism of action is virtually unknown at this time, preliminary clinical data suggest that vitamin D supplementation in Crohn's disease patients in remission may have a prominent role in intestinal permeability maintenance over time (362).

SCFAs are essential molecules involved not only in host metabolism and immunity but also in IEB function (180, 363, 364). In addition, they are an energy source for intestinal cells and serve as signaling molecules with a beneficial role in intestinal homeostasis (180, 363, 364). Among SCFAs, butyrate is considered the most beneficial. Butyrate can be produced by a wide variety of bacteria and the most important butyrate-producing microorganisms in the human gut belong to the genera *Faecalibacterium* (in particular the species

Faecalibacterium prausnitzii) and *Roseburia* (in particular the species *Roseburia intestinalis*) (365, 366). Sources for butyrate production include sugars, lactate, acetate, and amino acids, such as lysine (367). Although a lack of dietary fibers and SCFAs production can compromise both IEB integrity and mucus production, altering gut permeability, the role of SCFAs in DGBI pathophysiology is controversial (368). Similarly, although *in vitro* studies suggested that butyrate can improve paracellular permeability (183, 185), its role in restoring human intestinal permeability in clinical practice is very poorly defined. Preliminary data suggest that supplementation of sodium butyrate may improve IBS symptoms, particularly abdominal pain (369). In a recent proof-of-concept study performed in 40 patients with IBS, we showed that *Lactobacillus paracasei* CNCM I-1572 improves IBS symptoms through a significant reduction in genus *Ruminococcus* associated with a reduction of IL-15, linked with the modulation of IEB, and a significant increase in the fecal SCFAs acetate and butyrate (201). However, if nutritional butyrate exerts a protective effect on the IEB and may be effective in the management of patients with common gastrointestinal disorders, this should be demonstrated in *ad hoc* studies.

CONCLUSIONS

IEB dysfunction is a common element of numerous intestinal and extra-intestinal diseases. Several factors can alter IEB including gut microbiota metabolites and immune system mediators. The intricate relationship among all these elements is still a matter of study. If IEB dysfunction is a cause or a consequence of the pathogenesis of common diseases including IBS, IBD, CD and the emerging NCGS is a challenge for the researcher. Solving this dilemma and deciphering the underlying molecular mechanisms will open the way to the development of new therapies and the optimization of the diagnostic process.

AUTHOR CONTRIBUTIONS

GB and VS designed the review. GB, MRB, DF, MP, FF, CC, and GM performed literature search and drafted the manuscript. All authors critically revised and approved the final version of the manuscript.

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