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REVIEW

Intestinal epithelium, intraepithelial lymphocytes and the gut microbiota - Key players in the pathogenesis of celiac disease

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Abstract

Celiac disease (CD) is a chronic immune-mediated disorder triggered by the ingestion of gluten in genetically predisposed individuals. Before activating the immune system, gluten peptides are transferred by the epithelial barrier to the mucosal lamina propria, where they are deamidated by intestinal tissue transglutaminase 2. As a result, they strongly bind to human leucocyte antigens (HLAs), especially HLA-DQ2 and HLA-DQ8, expressed on antigen-presenting cells. This induces an inflammatory response, which results in small bowel enteropathy. Although gluten is the main external trigger activating both innate and adaptive (specific) immunity, its presence in the intestinal lumen does not fully explain CD pathogenesis. It has been hypothesized that an early disruption of the gut barrier in genetically susceptible individuals, which would result in an increased intestinal permeability, could precede the onset of gluten-induced immune events. The intestinal barrier is a complex functional structure, whose functioning is dependent on intestinal microbiota

homeostasis, epithelial layer integrity, and the gut-associated lymphoid tissue with its intraepithelial lymphocytes (IELs). The aim of this paper was to review the current literature and summarize the role of the gut microbiota, epithelial cells and their intercellular junctions, and IELs in CD development.

Key words: Celiac disease; Intestinal microbiota; Epithelium; Intraepithelial lymphocytes; Intestinal barrier

Core tip: There is evidence that the host-microbiota homeostasis is disrupted in celiac disease (CD) patients. Dysbiosis, meaning an imbalance in the gut microbiota and its metabolome, may activate innate immunity leading to pro-inflammatory changes, which induces intraepithelial lymphocyte infiltration and epithelial barrier damage, ultimately resulting in increased transfer of gluten peptides and inflammatory activation leading to CD development. The intestinal microbiota also has a direct effect on the breakdown of gluten and formation of immunogenic peptides. As colonization of the gut with microorganisms may be dependent on genetic factors, future prophylactic strategies may focus on gut microbiota modulation in genetically predisposed infants.

Cukrowska B, Sowińska A, Bierła JB, Czarnowska E, Rybak A, Grzybowska-Chlebowczyk U. Intestinal epithelium, intraepithelial lymphocytes and the gut microbiota - Key players in the pathogenesis of celiac disease. *World J Gastroenterol* 2017; 23(42): 7505-7518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i42/7505.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i42.7505

INTRODUCTION

Celiac disease (CD) is a chronic immune-mediated disorder triggered by the ingestion of gluten in genetically predisposed individuals[1]. Gluten is a storage protein that consists of alcohol-insoluble glutenins and soluble prolamines, such as gliadin in wheat, secalin in rye, and hordein in barley. CD development requires the presence of gluten, the intestinal enzyme tissue transglutaminase 2 (TTG2), which modifies gluten peptides, and the genes encoding human leucocyte antigen (HLA)-DQ2 or HLA-DQ8[2]. Gluten from food products is degraded by gastrointestinal tract enzymes into peptides, which then are transferred through the epithelial barrier into the mucosal lamina propria.

In CD individuals, some of these peptides can bind to HLA-DQ2 or HLA-DQ8 heterodimers expressed on the surface of antigen-presenting cells (*e.g.,* macrophages, lymphocytes or dendritic cells) and, after triggering T-cell responses, lead to local tissue damage[3]. TTG2 converts glutamine residues present in gluten peptides into glutamic acid, and this conversion generates deamidated gluten peptides (DGP), which strongly bind to HLA-DQ2/-DQ8 molecules. Consequently, increased gluten antigenicity amplifies a gluten-specific T-cell response.

Gluten-activated T cells release pro-inflammatory cytokines [(mainly interferon-gamma (IFN-γ), interleukin (IL)-21 and IL-17)], which induce mucosal inflammation and have a direct cytotoxic effect on the epithelium, all of which finally leads to villous atrophy in the small intestine. Moreover, specific T cells induce B cells to produce antibodies directed against DGP and TTG2[4]. Thus, this adaptive (specific) T-cell response is a requirement for CD development. Nonetheless, innate immunity also plays an important role in CD development. The increased transfer of gluten peptides through the epithelial barrier could be a consequence of earlier activation of innate (non-specific) immunity, dependent on the function of both the epithelium and the lymphocytes located between epithelial cells, *i.e.* intraepithelial lymphocytes (IELs)[5].

Some of the gluten peptides can directly react with epithelial cells and activate production of pro-inflammatory cytokines, especially IL-15. IL-15 plays a key role in enhanced cytolytic activity of IELs *via* increasing the expression of both intestinal epithelial cell surface ligands (such as MICA and MICB, *i.e.* major histocompatibility complex class I chain-related molecules), which are targeted by cytotoxic, natural killer (NK)-like IELs, and NK receptors, such as NKG2D and CD94/NKG2C, on the surface of IELs. Finally, IL-15 activation leads to innate cytotoxic disruption of epithelial cells, resulting in increased intestinal permeability to different luminal macromolecules, including immunogenic gluten peptides[6].

Although gluten is the main external trigger of CD, gluten ingestion does not fully explain CD pathogenesis. Introduction of gluten into the diet starts in early childhood, but CD can develop at any point during a person's lifetime. The role of both breastfeeding and the time when gluten is first introduced into the diet in the risk of CD has long been debated. Retrospective data from Sweden indicated that introducing gluten in small amounts to breastfed infants at the age between 4 mo and 6 mo reduced the risk of CD compared with introducing gluten in larger amounts at older ages[7,8]. However, a recently published systematic review with meta-analysis of studies that assessed the effect of gluten consumption on CD development showed that for infants at high genetic risk of CD, gluten introduction at the age of 4 mo, 6 mo or 12 mo, resulted in similar rates of CD diagnosis in childhood, and neither breastfeeding as such (at any time during an infant's life) nor breastfeeding during gluten introduction were shown to reduce the risk of CD[9]. Also, the recently

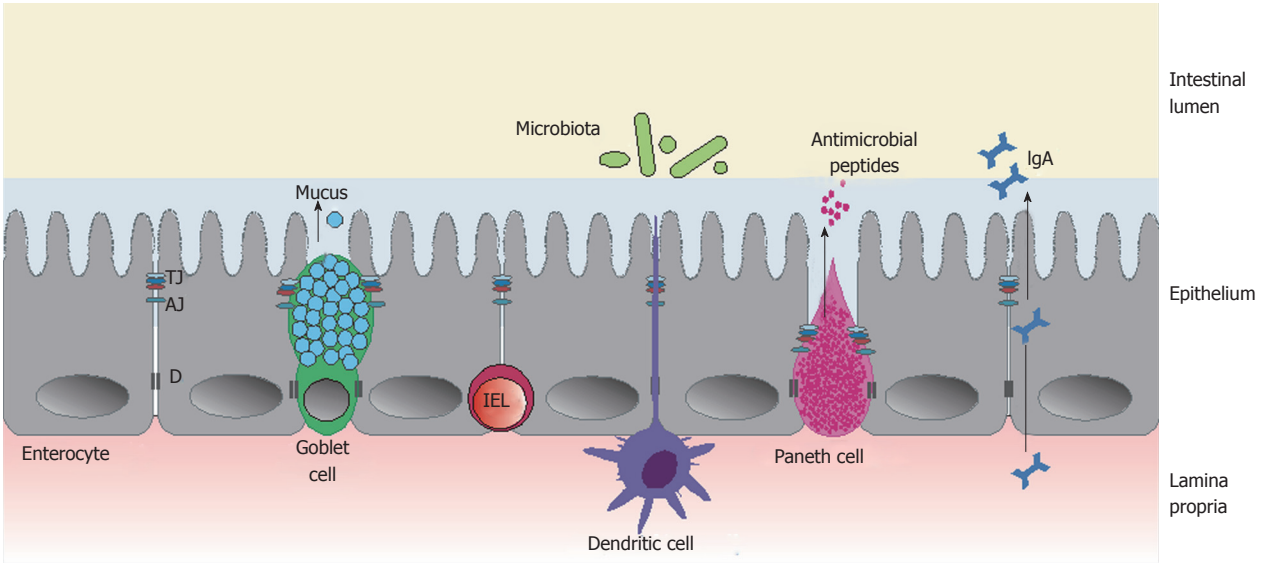

Figure 1 Schematic illustration of the intestinal barrier. The three main components of the intestinal barrier: the microbiota; epithelium, with its specialized cells (goblet cells, Paneth cells and enterocytes), together with a layer of mucus; and gut-associated lymphoid tissue cells, including IELs and dendritic cells. AJ: Adherens junction; D: Desmosome; IEL: Intraepithelial lymphocyte; TJ: Tight junction.

published prospective PreventCD cohort study showed that neither the gluten consumption pattern nor the amount of gluten consumed at the age of 11 mo to 36 mo influenced CD development in children with a genetic risk[10].

Thus, the time of gluten introduction into the diet seems not to play a key role in CD development. In addition, gluten-free diet (GFD) has been reported to improve mucosal lesions and decrease specific antibody levels, but not to correct the increased activation of pro-inflammatory mediators, which is characteristic for CD[11]. That is why it has been hypothesized that an early disruption of the gut barrier in genetically susceptible individuals, which is not associated with gluten peptides and results in an increased intestinal permeability, could precede the onset of gluten-induced immune events.

The intestinal barrier is a complex structure that separates the internal milieu from the luminal environment[12]. It consists of three main functional components: the microbiota that colonize the intestines; the epithelium, with its specialized mucus-producing cells and cells producing antimicrobial peptides; and gut-associated lymphoid tissue, composed of various immune cells (including IELs, which come in direct contact with gut luminal antigens, and lamina propria cells, producing secretory IgA) (Figure 1).

This review summarizes the role of epithelial cells and their intercellular junctions as well as IELs and the gut microbiota in the activation of early processes leading to the pathomechanisms associated with CD.

EPITHELIAL JUNCTIONS - STRUCTURES RESPONSIBLE FOR GUT PERMEABILITY

The small intestinal epithelium is organized into a monolayer of specialized cells: enterocytes (constituting approximately 80%), goblet cells (secreting mucus), Paneth cells (synthesizing defensins and other antimicrobial agents), endocrine cells (secreting hormones), and intestinal stem cells (responsible for epithelial cell homeostasis and regeneration)[13,14]. Epithelial cells form a continuous layer thanks to being sealed together by intercellular junctions, including tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions[15].

The ultrastructure of epithelial junctions is presented in Figure 2. TJs and AJs are supported by a dense perijunctional ring of actin and myosin, and they form the apical junctional complex and regulate epithelial paracellular permeability[16,17]. TJs are located near the apical surface of enterocytes and they act as a gate in the paracellular transport of ions, solutes, water, and cells. TJs are highly dynamic structures, whose degree of sealing varies in response to external stimuli as well as physiological and pathological conditions. TJs are composed of transmembrane proteins: occludin, claudins, junction adhesion molecules, tricellulin, and scaffold proteins - zonula occludens (ZO-1, ZO-2 and ZO-3)[17].

Occludin is an integral membrane protein with two extracellular loops, a short cytoplasmic N-terminal region, and a long cytoplasmic C-terminal region,

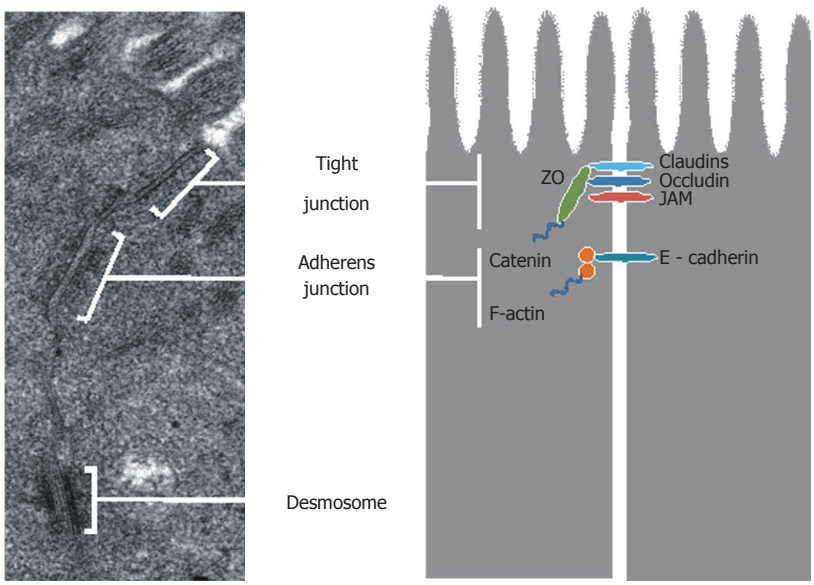

Figure 2 Ultrastructure and corresponding schematic representation of intercellular junctions. Transmission electron microscopy (JEOL JEM-1011, Japan; × 60000) was used to show the ultrastructure of intercellular junctions in the human small intestine. The transmission electron micrograph comes from our own research. JAM: Junction adhesion molecule.

which interacts with a ZO-1 protein that links occludin to the actin cytoskeleton[18,19]. Occludin plays a role in TJ maintenance and assembly, which are regulated by phosphorylation of serine (Ser), threonine (Thr), and tyrosine (Tyr) residues[20]. In an intact epithelium, occludin is highly phosphorylated on Ser and Thr residues[21,22] and poorly phosphorylated on Tyr residues[23]. Dephosphorylation of Ser/Thr residues and increased phosphorylation of Tyr residues reduces occludin's interaction with ZO-1, leading to its separation from the junctional complex and TJ disruption[24,25].

The claudin family can be divided into sealing proteins (claudins 1, 3, 4, 5 and 8), which reduce permeability, and pore-forming proteins (claudins 2, 7, 10 and 12), which increase permeability[26]. Thus, claudins 1, 3, 4, 5 and 8 strengthen the intestinal barrier, whereas claudins 2, 7, 10 and 12 weaken it. The extracellular loops of claudins are involved in the formation of ion-selective channels[27], while the intracellular C-terminal domain is connected to the cytoskeleton *via* a domain containing ZO-1, ZO-2 and ZO-3[28,29]. ZO-1, ZO-2 and ZO-3 are multidomain bridging proteins that function as cross-linkers, anchoring the TJ strand proteins to the actin cytoskeleton[30].

Recently, tricellulin has been identified as a co-mponent maintaining TJ structure and regulating the passage of macromolecules through the junctions[31]. TJ development may be dependent on AJ formation, since the ability of ZO-1 proteins to migrate apically to join occludin was observed only after AJ assembly[32]. The main component of AJ is E-cadherin, a transmembrane protein that forms homodimers with other cadherin molecules on adjacent cells. This protein is connected to the actin cytoskeleton by a complex of cytoplasmic proteins: α-, β- and γ-catenins[33].

Despite the major progress in knowledge on TJ structure and function, the mechanisms regulating TJs are still incompletely understood. The discovery of the *Vibrio cholerae*-derived Zonula occludens toxin, which reversibly regulates TJ permeability, helped identify its intestinal mammalian analogue - a human protein named zonulin[34,35]. Zonulin was identified as pre-haptoglobin 2. Structural analysis of this protein revealed similarities with several growth factors, such as hepatocyte growth factor or epidermal growth factor, which affect intercellular TJ integrity[36,37].

Zonulin was shown to induce TJ disassembly and a subsequent increase in intestinal permeability. Zonulin transactivates the epidermal growth factor receptor through proteinase-activated receptor 2, and then activates phospholipase C, which hydrolyzes phosphatidylinositol to release inositol 1, 4, 5-tris phosphate and diacylglycerol[38, 39]. Protein kinase Cα is then activated, either directly (*via* diacylglycerol) or through the release of intracellular calcium ions (*via* inositol 1, 4, 5-tris phosphate). Membrane-associated, activated protein kinase Cα catalyzes the phosphorylation of target proteins, including ZO-1 and myosin 1C, as well as polymerization of soluble G-actin in F-actin. This polymerization results in actin filament rearrangement and subsequent displacement of proteins (including ZO-1) from the junctional complex. As result, intestinal TJs become looser, which increases the paracellular transport of luminal molecules[35].

Zonulin is over-expressed in tissues and sera of subjects affected by autoimmune diseases, including CD[35]. *In vitro* studies showed that increased zonulin release in the small intestine can be triggered by both gluten peptides[38,39] and enteric bacteria[40].

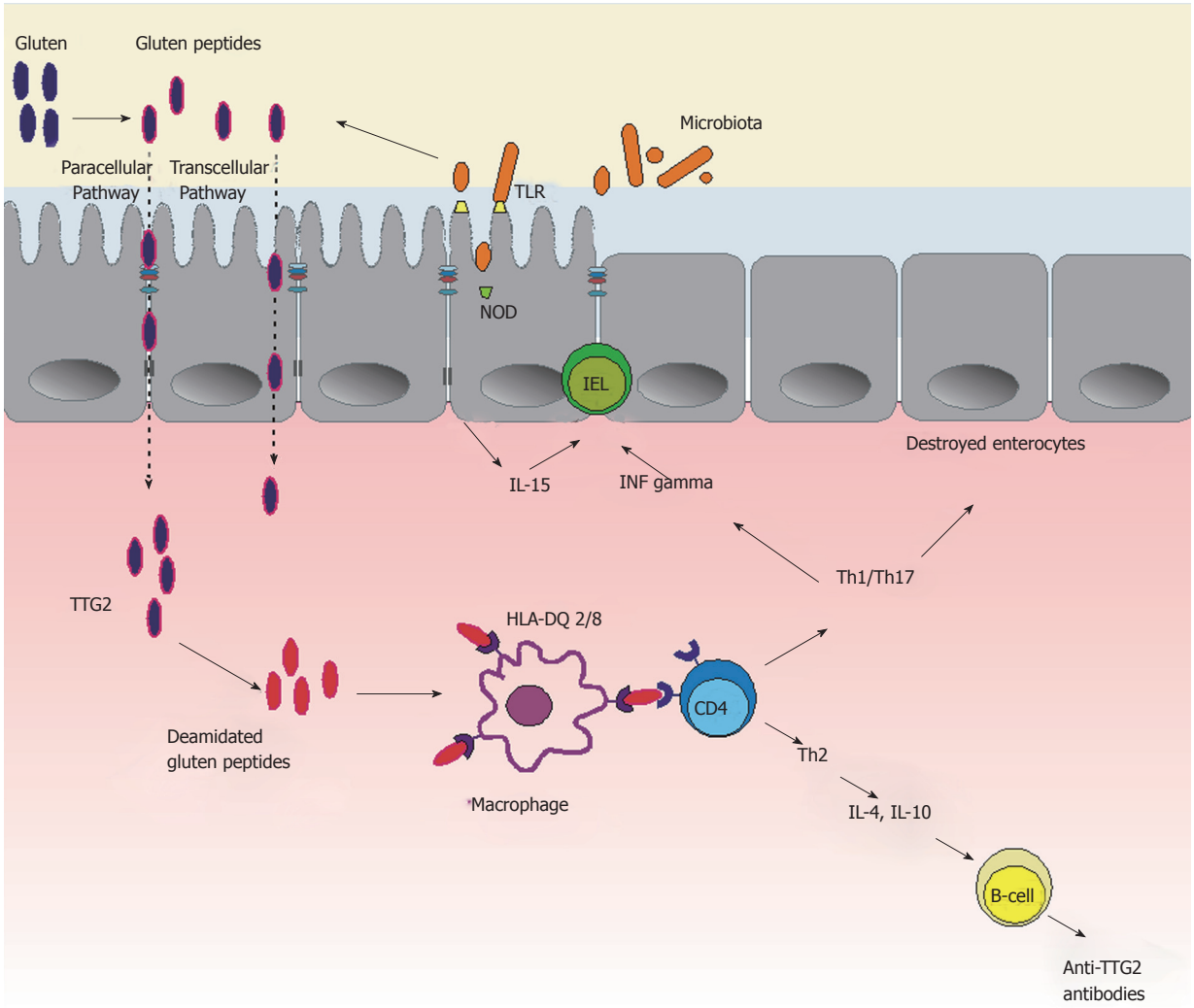

Figure 3 Schematic illustration of celiac disease pathogenesis. Microbiota dysbiosis activates innate immunity resulting in pro-inflammatory changes, which leads to IEL infiltration and epithelial barrier disruption. This ultimately results in an increased paracellular and transcellular transfer of immunogenic gluten peptides and activation of adaptive pro-inflammatory Th1/Th17 pathways, leading to villous atrophy and production of autoantibodies against intestinal TTG2. HLA: Human leucocyte antigen; IEL: Intraepithelial lymphocyte; IL: Interleukin; INF: Interferon; NOD: Nucleotide-binding oligomerization domain; Th: T helper; TLR: Toll-like receptor; TTG2: Tissue transglutaminase 2.

Zonulin secretion has been demonstrated to be independent of either the species or the virulence of the microorganisms tested[40]. However, recently an association of low serum zonulin levels with lower quantities of *Bacteroidaceae* and *Veillonellaceae* and higher quantities of *Faecalibacterium* has been found in overweight pregnant women[41]. Thus, this *in vivo* study suggests that zonulin release could be affected by changes in gut microbiota composition.

Recently, epithelial polarity regulators, especially the Par-3 protein, have been reported to be likely involved in regulating TJ permeability[42]. Par-3 and other proteins regulating cell polarity, such as Par-6 and atypical protein kinase C, form the apical polarity complex that orchestrates the formation of apical junctional complex. In addition, Par-3 located in the junctional complex together with ZO-1 and catenins is able to affect TJs by rearranging the actin cytoskeleton. Schumann *et al*[43] in 2012 found a reduced level of Par-3 and a defect in performing lateral exclusion of Par-3 in the epithelial cells of CD patients. In this context, genetic studies on non-HLA gene candidates associated with CD seem to be very interesting. Wapenaar *et al*[44] in 2008 found two candidate genes: Par-3 and Magi2, encoding the proteins regulating of epithelial polarity. However, this study involved a homogenous Dutch population, and further genome-wide association studies did not confirm this association[45].

DYSFUNCTION OF EPITHELIAL JUNCTIONS IN CD PATIENTS

One of the first studies on the structure of epithelial junctions using freeze-fracture electron micrographs presented severely altered TJs with strand discontinuities and a reduced number of strands in

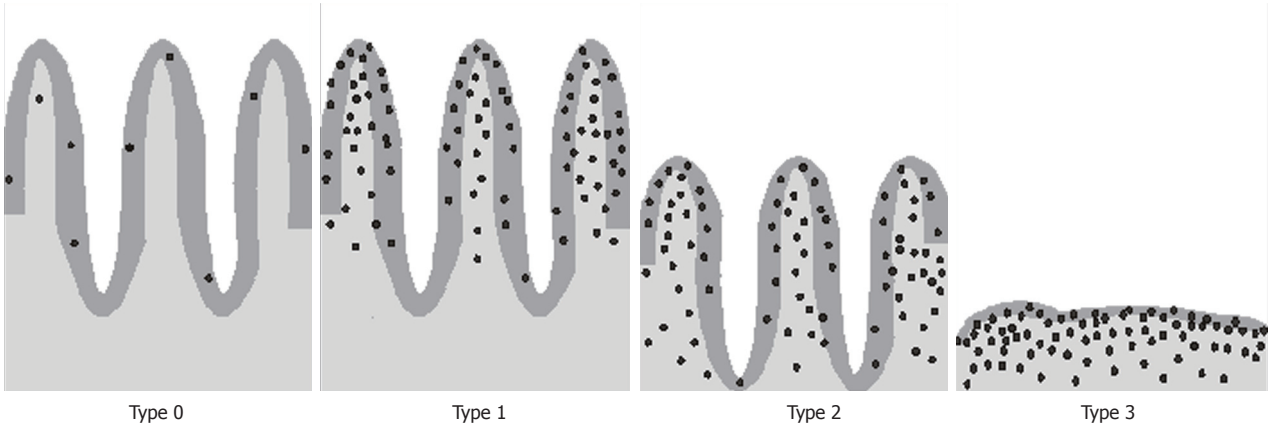

Figure 4 A schematic illustration of progressive histopathological changes in the small intestine according to the modified Marsh-Oberhuber grading scale. Type 0: Normal mucosa with IEL count < 25 per 100 enterocytes; Type 1: Normal mucosa with an increased IEL count; Types 2 and 3 show increased IEL counts and lymphocytes in the lamina propria. IELs are presented as black dots. IEL: Intraepithelial lymphocyte.

children with active CD[46]. GFD improved these abnormalities, but only partially - strand numbers were restored to normal at the surface, but remained low in the crypts. The recent transmission electron microscopy analyses on duodenal biopsies of CD patients also showed changes in TJ ultrastructure: dilatation (saccular or fusiform) and destruction of pentalaminar structures[47]. Interestingly, ultrastructural abnormalities of TJs were also found in asymptomatic and serologically negative first-degree relatives of CD patients[48].

Furthermore, over-expression of occludin and the pore-forming protein claudin-2 was demonstrated in CD patients, as well as an under-expression of pore-sealing proteins claudin-3 and 4, and scaffold protein ZO-1[47,49,50]. After introduction of a GFD, normalization of claudin expression was observed. No improvement after GFD introduction was reported in about 3% of the patients with refractory CD, whose mucosa undergoes a constant inflammatory process[51]. Other studies indicated a subcellular localization and downregulation of claudin 4 and claudin 5 in refractory CD patients[52].

Alterations in AJ structure were also reported. The expression of E-cadherin and β catenin - proteins required for TJ formation - was shown to be reduced in the duodenal epithelium of children with CD. Ciccocioppo *et al*[50] in 2006 showed that a lack of ZO-1 phosphorylation in active CD led to TJ disruption. The authors suggested that non-phosphorylated ZO-1 was unable to detach from β-catenin and to connect with occludin. It was also found that a higher phosphorylation of β-catenin was responsible for the absence of membranous E-cadherin. On the other hand, highly phosphorylated β-catenin was unable to connect with E-cadherin, which, in turn, could bind to the αEβ7-integrin of IELs. However, the levels of both E-cadherin and β-catenin returned to normal following GFD introduction[53,54]. Interestingly, a recent study by Mishra *et al*[48] in 2015 indicated the presence of altered ZO-1 and occludin expression not only in active CD patients but also in asymptomatic and serologically negative first-degree relatives of CD patients.

Fasano *et al*[35] in 2000 tried to explain the increased expression of zonulin found in CD patients. Some studies suggested that gliadin, by binding to the proinflammatory chemokine CXCR3 receptor on the intestinal epithelium, initiates the release of zonulin, which induces cytoskeleton rearrangement, ZO-1 and occludin down-regulation, leading to disruption of TJ integrity and finally to an increase in epithelial permeability[38,55]. Thus, the receptor CXCR3 could be involved in early TJ dysfunction, preceding the immune cascade of events observed in CD patients. Recently, Bondar *et al*[55] in 2014 showed that CXCL10 - a ligand for CXCR - is over-expressed in the small intestine of CD patients and strongly activated by poly I:C (an experimental model of viral infections) and IL-15 in non-CD controls. Thus, it cannot be excluded that the CXCR3/CXCL10 axis activated by infectious agents may play a role in initiating gluten-induced inflammatory processes in the small intestinal mucosa.

Overall, the presented results show that epithelial barrier impairment occurring in CD patients can play an important role in CD development. Because epithelial function is regulated by microorganisms colonizing the intestines[56], there is a hypothesis that dysbiosis, *i.e*. disturbances in both the quantity and composition of the gut microbiota, is a critical factor for the activation of innate immunity, leading to epithelial barrier dysfunctions.

GUT MICROBIOTA: THE MAINSTAY OF EPITHELIAL AND IMMUNE HOMEOSTASIS

The microbiota colonizing the gut after birth reaches the pattern found in adults within 2-3 years of life. Eventually, the human intestine is colonized with more than 1000 species categorized into subgroups

of phyla, classes, orders, families and genera, with *Firmicutes* and *Bacteroidetes* constituting the most abundant phyla[57]. The number of bacteria in the gut microbiota is similar to the number of cells making up the human body[58], and microbiota genes (microbiome) outnumber those in the human genome by approximately 100-fold. This complex microbial community adjusts the immune system, protects the body against pathogens, harvests nutrients and energy from the diet, and ferments non-digestible carbohydrates.

Extensive studies in germ-free (GF) animals, *i.e.* animals deprived of the gut microbiota, have demonstrated an indispensable role of microbiota in shaping the local mucosal gut-associated lymphoid tissue as well as systemic immunity[59,60]. In contrast to conventionally raised (CV) mice, GF mice have hypoplastic Peyer's patches and decreased number of both IgA-secreting plasma cells and lymphocytes located in the lamina propria. Colonization of GF animals with components of the gut microbiota induces production of secretory immunoglobulins A (sIgA). sIgAs are natural antibodies that constitute the first line of defense by reacting with a wide spectrum of microorganisms and toxic molecules, which directly affects the composition of the gut microbiota[61]. Experimental data have shown that sIgAs cooperate with innate defense factors to reinforce the epithelial barrier[62].

Epithelial barrier integrity also depends on homeostatic regulatory mechanisms, including mucosal induction of regulatory T (Treg) cells, and the gut microbiota plays a decisive role in this process[63]. According to some reports, gut-colonizing commensals are responsible for differentiation of effector T helper (Th) 1, Th17, and Treg cells responsible for Th1/Th2/Th17 homeostasis[64]. Colonization of GF mice with components of conventional microbiota also induced the recruitment and activation of IELs, some of which (especially γδ IELs) were reported to be involved in epithelial cell generation and differentiation[65,66]. Thus, the gut microbiota seems capable of protecting the epithelium and strengthening its barrier function[59].

Recently, using transmission electron microscopy, we found ultrastructural differences of enterocytes and epithelial junctions in GF mice, CV or specific pathogen-free (SPF) mice, and mice inoculated with a mixture of *Lactobacillus* strains obtained from stools of healthy children[61]. Brush borders of GF-mouse enterocytes were irregularly arranged and exhibited decreased numbers of cytoskeletal microfilaments and a lack of elongation into the terminal web. The AJ region was significantly broader and shorter in GF animals compared both with that in CV mice and in mice colonized with *Lactobacillus* strains. Consistent with other reports[67,68], we observed that the gut microbiota and *Lactobacillus* strains significantly increased the expression of TJ proteins: occludin and ZO-1[61]. On the other hand, there is experimental evidence that certain components of the gut microbiota, such as *Escherichia coli, Klebsiella pneumoniae* and *Streptococcus viridans,* are able to increase gut permeability[69].

The gut microbiota interacts with the host *via* pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) expressed on the surface of epithelial and dendritic cells. Recognition of specific microbial structures, called microorganism-associated molecular patterns, by PRRs induces signaling cascades that eventually result in immune response activation and the production of cytokines responsible for intestinal barrier strengthening (*e.g.,* TGF-β and IL-10) or weakening (*e.g.,* IL-15, TNF-α and IFN-γ)[70]. Alterations in TLR4 and TLR2 expression, as well as functional single-nucleotide polymorphisms in the genes expressed upon TLR4 activation, have also been associated with CD[45,71,72].

Interestingly, epithelial barrier function may be controlled indirectly by the intestinal metabolome, *e.g.,* gut microbiota metabolites in the form of low-molecular weight chemical intermediates[73]. Soluble dietary fibers (such as fructans, pectin, inulin and xylans) and resistant starches can be actively fermented by commensal microbiota in the human colon, producing biologically active short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate. These SCFAs are the main metabolites produced by gut-colonizing bacteria and a major source of energy for intestinal epithelial cells[74]. Acetate and propionate are predominantly produced by bacterial species of the phylum *Bacteroidetes*, whereas butyrate is primarily produced by those of the phylum *Firmicutes*. SCFAs serve as specific activators of orphan G-protein-coupled receptors, such as GPR43 and GPR41, predominantly expressed in intestinal epithelial cells[75,76]. GPR43 deficiency leads to expansion of *Firmicutes* in the gut microbiota and consequently raises fecal SCFAs and plasma acetate levels. Indoles, produced from tryptophan by various Gram-positive and Gram-negative intestinal bacteria, and acetate, produced by *Bifidobacterium* strains, enhance epithelial defense functions and suppress intestinal inflammation[77-79]. Microbe-derived SCFAs also have an impact on terminal differentiation of CD4+ Th cells[80].

The gut microbiota is responsible not only for immune homeostasis and epithelial barrier function, but also can have direct impact on gluten digestion in the intestinal tract. There is evidence that certain bacterial strains isolated from feces, *e.g.*, *Bifidobacterium* and *Bacteroides fragilis*, are capable of digesting immunogenic gliadin peptides, which are rich in proline residues but resistant to human enzymes[81,82].

GUT MICROBIOTA AND METABOLOME IN CD

Several studies addressed the phenomenon of gut dysbiosis in CD patients with active untreated disease and those on a GFD. Fecal analyses in untreated CD patients showed an imbalance in the composition of intestinal microbiota characterized by an increase in the number of *Bacteroides* species and reduced numbers of *Bifidobacterium* species[83-86]. In addition, CD patients, both untreated and treated with a GFD, demonstrated a lesser diversity of *Bacteroides* species in biopsy samples of the duodenal microbiota in comparison with controls[87]. The numbers of *Escherichia coli* and *Staphylococcus* bacteria were also higher in fecal and biopsy specimens of untreated CD children than in controls[88]. *Escherichia coli* strains from CD children carried a higher number of virulence genes than those from healthy children. Nadal *et al*[89] in 2007 reported a significantly lower ratio of harmless Gram-positive bacteria (*Lactobacillus* and *Bifidobacterium*) to potentially harmful Gram-negative bacteria (*Bacteroides/Prevotella* and *Escherichia coli*) in CD patients compared to controls, with no distinction between active and inactive CD. The numbers of bacteria of *Streptococcus* and *Prevotella* genera were found to be lower both in adults and children with untreated CD in comparison with healthy controls.

The disturbances in intestinal microbiota composition found in CD patients have been associated with changes in the metabolome[90]. Metabolic profiles of serum, urine and feces in celiac patients revealed a significantly altered profile of volatile organic compounds (*e.g.,* phenols and ketones), SCFAs and amino acids (*e.g.,* proline, methionine, histidine and tryptophan)[91,92]. CD patients were also characterized by higher urine levels of certain gut microbiota-derived metabolites, such as indoxyl sulfate, meta-[hydroxyphenyl] propionic acid and phenylacetylglycine, which were associated with untreated CD[92]. Interestingly, metabolic abnormalities found in celiac patients and "potential" celiac patients (*i.e.* individuals with a positive antibody test but no evidence of intestinal damage) were similar, indicating that CD-related dysmetabolism/dysbiosis precedes the intestinal damage[93].

Only a few serum metabolites can help differentiate between potential and overt CD; none of these metabolites are related to energy metabolism. Glycolysis appears to be somehow impaired in potential CD patients, just as is the case in overt CD patients. This is consistent with the hypothesis that the gut microbiota of CD patients is altered or contains specific species with their distinctive microbial metabolome. Schirmer *et al*[94] in 2016 reported that TNF-α and IFN-γ production was associated with specific microbial metabolic pathways: palmitoleic acid metabolism and tryptophan degradation to tryptophol.

Low doses of pro-inflammatory cytokines, such as IFN-γ, were shown not to affect TJ protein expression but to activate bacterial endocytosis by epithelial cells[95]. This process is dependent on extracellular signal-regulated kinase Cζ and ADP-ribosylation factor-6 signaling[96]. Thus, some commensal bacteria might interact with certain intracellular PRRs, namely, nucleotide-binding oligomerization domain (NOD)-like receptors, and activate epithelium-derived pro-inflammatory cytokines and free radicals that may cause secondary TJ damage[96,97]. An increased activity and expression of inducible nitric oxide synthase in human duodenal enterocytes has been reported in CD patients[97].

Thus, dysbiosis, which can follow viral or bacterial infections or antibiotic therapy, may activate innate immunity leading to pro-inflammatory changes, with the resulting IEL infiltration, epithelial barrier disruption, and increased transfer of immunogenic gluten peptides, which in turn activate inflammation leading to CD development (Figure 3).

ROLE OF IELs IN EPITHELIAL BARRIER HOMEOSTASIS

The typical histopathological presentation of CD is small intestinal enteropathy characterized by an increase in IELs, crypt hyperplasia, and villous atrophy. The changes develop gradually over time. The increased number of IELs is one of the earliest signs of CD[98] and may herald the impending disease[99]. Histological changes in the small intestine can be graded using the Marsh classification[100] modified by Oberhuber[101] (Figure 4). The Marsh-Oberhuber classification includes four categories of CD-associated lesions: infiltrative (type 1), infiltrative-hyperplastic (type 2), flat-destructive (type 3) and atrophic-hypoplastic (type 4)[101].

Irrespective of the type of changes found in CD patients, an increase in the number of IELs is considered to be the most sensitive histopathological marker of CD. The upper limit of normal for IELs in duodenal or jejunal mucosa is 25 IELs per 100 enterocytes. An IEL count between 25 and 29 is considered to be "borderline intraepithelial lymphocytosis", and 30 or more means "pathological lymphocytosis" in the duodenum[103,104]. IELs are classified into two major subgroups based on their phenotypical and functional characteristics: one bears the αβ T-cell receptor (αβ-IEL), while the other bears the γδ T-cell receptor (γδ-IEL). When it comes to the typical composition of the small-intestinal IEL population, approximately 75% of it consists of CD8-positive αβ T cells, 10% constitute CD4-positive αβ T cells, and 15% constitute γδ T cells which are CD4- and either CD8- or CD8+[5].

In CD sensitive patients, gluten exposure causes rapid activation of both αβ-IELs and γδ-IELs[105],

while a GFD lowers both αβ-IEL and γδ-IEL counts; however, lowering of the latter IEL subtype takes months or even years[106]. It is believed that CD8+ αβ-IELs represent the effector T cell subset that mediates epithelial cell destruction (after IL-15 up-regulation) and, ultimately, induces villous atrophy in CD. The role of γδ-IELs in CD pathogenesis remains unclear.

A recent study showed that IEL expansion can be modulated by the host microbiota. Mice deficient in NOD2 (receptors recognizing bacterial molecules)[107] exhibited a significant reduction in IEL counts and IL-15 expression in the epithelium, with the residual IELs displaying reduced proliferation and increased apoptosis. Moreover, *Lactobacillus* strains were able to decrease the number of IELs activated by TLR3 after an experimentally induced viral infection (poly I:C). They also significantly reduced the levels of pro-inflammatory cytokines, such as TNF-α and IL-15, and increased serum and intestinal regulatory IL-10 levels[108]. Finally, the immunomodulatory capacity of *Lactobacilli* helped significantly reduce intestinal tissue damage.

The data above indicate that IEL homeostasis is controlled by commensal microbiota, which affects cytokine production by epithelial cells *via* PRR activation. Moreover, increased IEL counts in CD patients, which lead to epithelial barrier disturbances, may be primarily induced by microbiota dysbiosis.

ROLE OF THE GUT MICROBIOTA PROGRAMMING IN CD DEVELOPMENT

Recent research has shown that early bacterial colonization may affect the risk of developing CD later in life. This phenomenon is called microbial programming[109]. There is evidence indicating that the pioneer microbiota of the neonatal gut is essential for gut maturation as well as for metabolic and immunologic programming[109,110]. Establishment of the human gut microbiota is a complex, stepwise process. The composition of microbiota within the 1st year of life is characterized by low diversity, high instability, and high inter-individual variation[111]. By the age of 2-3 years, the microbiota becomes stable, more diverse, and resembles that found in adults, with *Firmicutes* and *Bacteroidetes* as the predominant phyla. Gut microbiota formation after the birth is dependent on different environmental factors, such as the mode of delivery, breast or formula feeding, or antibiotic therapy[111].

Although the evidence that the perinatal environment influences CD development is still only circumstantial[112], there have been studies showing that cesarean sections and antibiotic treatment in infancy increased the risk of CD[113-115]. There is also evidence that colonization of the gut with microorganisms may be dependent on genetic factors[116,117]. The hypothesis that gut microbiota composition is affected by host genes has been confirmed by studies in twins, showing that fecal microbiota of monozygotic twins was much more similar than that of dizygotic twins[118]. Recent microbiome analyses performed on 22 infants demonstrated that certain HLA genes predisposing to CD could affect microbiota composition[119]. The infants at high genetic risk of CD, *i.e.* those with an HLA-DQ2 genotype, showed a higher proportion of *Firmicutes* and *Proteobacteria* and lower proportion of *Actinobacteria* than those at low genetic risk. At the genus level, the gut microbiota of high-risk infants had a significantly lower proportion of *Bifidobacterium* and unclassified *Bifidobacteriaceae* and a higher proportion of *Corynebacterium*, *Gemella*, *Clostridium*, unclassified *Clostridiaceae*, unclassified *Enterobacteriaceae* and *Raoultella*. Sellitto *et al*[120] in 2012 reported an overall lack of bacteria of the phylum *Bacteriodetes*, with a high abundance of *Firmicutes*, in infants genetically predisposed to CD compared with microbiota composition of low-risk infants. Those differences were stable until 2 years of age.

As CD is strongly associated with HLA genes - almost 100% individuals with CD are carriers of alleles encoding HLA-DQ2/DQ8 molecules - these findings suggest that children with the CD risk genotype have a different microbiota profile than those without genetic predisposition. However, it must be emphasized that about 25%-30% of the general population exhibits the same HLA genotypes as CD patients[121]. In addition, there are also non-HLA genes associated with CD.

CONCLUDING REMARKS AND FUTURE STRATEGIES

Although gluten is necessary in order to activate the processes leading to CD, there is evidence that an imbalance in the gut microbiota and intestinal epithelium can precede the specific gluten-dependent immune response. Under certain conditions affecting the intestinal microbiota, *e.g.,* after infections or antibiotic therapy, an increased translocation of dietary macromolecules (including gluten peptides) *via* the opening of epithelial junctions triggers a cascade of events in genetically susceptible individuals, leading to overt CD. Microbiota disturbances are observed not only in untreated CD patients, but also in potential CD patients and those following a GFD as well as in infants at high genetic risk of CD. The microbial fingerprint associated with CD is likely dependent on specific genetic factors, including (but not exclusively) the HLA-DQ2/-DQ8 genotype. Future strategies should include prospective, birth cohort studies involving comprehensive genome, microbiome and metabolome analyses. Such an approach could help identify a "CD-specific" microbial/metabolic fingerprint, which would

become the target for both primary prevention and management of CD.

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