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Leaky Gut and Autoimmune Diseases

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Abstract Autoimmune diseases are characterized by tissue damage and loss of function due to an immune response that is directed against specific organs. This review is focused on the role of impaired intestinal barrier function on autoimmune pathogenesis. Together with the gut-associated lymphoid tissue and the neuroendocrine network, the intestinal epithelial barrier, with its intercellular tight junctions, controls the equilibrium between tolerance and immunity to non-self antigens. Zonulin is the only physiologic modulator of intercellular tight junctions described so far that is involved in trafficking of macromolecules and, therefore, in tolerance/immune response balance. When the zonulin pathway is deregulated in genetically susceptible individuals, autoimmune disorders can occur. This new paradigm subverts traditional theories underlying the development of these diseases and suggests that these processes can be arrested if the interplay between genes and environmental triggers is prevented by re-establishing the zonulin-dependent intestinal barrier function. Both animal models and recent clinical evidence support this new paradigm and provide the rationale for innovative approaches to prevent and treat autoimmune diseases.

Keywords Antigens · Autoimmunity · Gut permeability · Immune response · Tight junctions · Zonulin

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Introduction

The intestinal epithelium is the largest mucosal surface providing an interface between the external environment and the mammalian host. Its exquisite anatomical and functional arrangements and the finely-tuned coordination of digestive, absorptive, motility, neuroendocrine, and immunological functions are testimonial of the complexity of the gastrointestinal (GI) system. Also pivotal is the regulation of molecular trafficking between the intestinal lumen and the submucosa via the paracellular space. The dimensions of the paracellular space are estimated to be between 10 and 15 Å, suggesting that under physiological circumstances, solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route. Macromolecule trafficking is dictated mainly by intestinal paracellular permeability, whose regulation depends on the modulation of intercellular tight junctions (TJ). A fast growing number of diseases, including autoimmune diseases, are recognized to involve alterations in intestinal permeability related to changes in TJ competency.

Classical Theories on the Pathogenesis of Autoimmune Diseases

Soon after autoimmune diseases were first recognized more than a century ago, it was believed that their development was associated with viral and bacterial infections. The connection between infection and autoimmune disease is often explained by a mechanism known as "molecular mimicry," whereby microbial antigens are postulated to resemble self-antigens [1]. The induction of an immune response to the microbial antigens results in a cross-reaction with the self-antigens and the induction of autoimmunity.

According to this theory, once the autoimmune process is activated, it becomes independent of continuous exposure to the environmental trigger and is therefore self-perpetuating and irreversible. Epitope-specific cross-reactivity between microbial antigens and self-antigens has been shown in some animal models to initiate autoimmunity [2]. Conversely, in most human autoimmune diseases, molecular mimicry seems to be a factor in the progression of a pre-existing subclinical autoimmune response, rather than in the initiation of autoimmunity [2]. Another theory suggests that microorganisms expose self-antigens to the immune system by directly damaging tissues during active infection, and that this leads to the development of autoimmunity. This mechanism has been referred to as the "bystander effect," and it occurs only when the new antigen is presented with the orally administered triggering antigen. Whether pathogens mimic self-antigens, release sequestered self-antigens, or both, however, remains to be elucidated.

New Proposed Hypothesis: the Leaky Gut as Third Element in Autoimmune Pathogenesis

A common denominator in autoimmune diseases is the presence of several pre-existing conditions that lead to an autoimmune process [3]. The first of these conditions is the genetic susceptibility of the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. The second is that the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system following its paracellular passage from the intestinal lumen to the gut submucosa; this process is normally prevented by competent TJ [3–5]. In many cases, increased intestinal permeability seems to precede disease and causes an abnormality in antigen delivery that triggers the multiorgan process leading to the autoimmune response [3–5]. Taking the above information into consideration, we propose that the pathogenesis of autoimmune diseases can be described by three key points [6]:

1. Autoimmune diseases involve a miscommunication between innate and adaptive immunity;
2. Molecular mimicry or bystander effects alone might not explain entirely the complex events involved in the pathogenesis of autoimmune diseases. Rather, the continuous stimulation by nonself-antigens (environmental triggers) seems to be necessary to perpetuate the process. Contrary to general belief, this concept implies that the autoimmune response can theoretically be stopped and perhaps reversed if the interplay between genes predisposing individuals to the development of autoimmunity and environmental triggers is prevented or eliminated;
3. In addition to genetic predisposition and exposure to triggering nonself-antigens, the loss of the protective function of mucosal barriers that interact with the environment (mainly the gastrointestinal and lung mucosa) is necessary for autoimmunity to develop.

Evidence Supporting This New Theory

Celiac disease (CD) is the best testimonial of the validity to the accuracy of the new paradigm for the pathogenesis of autoimmunity proposed above. Celiac disease is an autoimmune condition triggered by the ingestion of gluten-containing grains in genetically susceptible individuals (for more details, see CD section below).

Given the undisputable role of gluten in causing inflammation and immune-mediated tissue damage, CD is a unique model of autoimmunity in which, in contrast to most other autoimmune diseases, a close genetic association with HLA genes, a highly specific humoral autoimmune response against tissue transglutaminase auto-antigen, and, most importantly, the triggering environmental factor (gliadin), are all known. It is the interplay between genes (both HLA and non-HLA associated) and environment (i.e., gluten) that leads to the intestinal damage typical of the disease [7]. Under physiological circumstances, this interplay is prevented by competent intercellular TJ. Early in CD, TJs are opened [8–12]. Combined, this information provides the rationale for the treatment of the disease based on complete avoidance of gluten-containing grains from the patients' diet. Following gluten withdrawal, the symptoms resolve, the biomarkers of the autoimmune process return within normal limits, and the intestinal autoimmune insult heals. These outcomes support the notion that the autoimmune process can be reverted provided that the interplay between genes and environmental trigger(s) can be prevented.

Besides celiac disease, several other autoimmune diseases, including type 1 diabetes [13, 14], multiple sclerosis [15, 16], and rheumatoid arthritis [17], are characterized by increased intestinal permeability secondary to non-competent TJs that allow the passage of antigens from the intestinal flora, challenging the immune system to produce an immune response that can target any organ or tissue in genetically predisposed individuals [18–21].

Intestinal Barrier Function and Its Regulation

A century ago, TJs were conceptualized as a secreted extracellular cement forming an absolute and unregulated barrier within the paracellular space [22]. Biological studies of the past several decades have shown that TJs are dynamic structures subjected to structural changes that

dictate their functional status under a variety of developmental scenarios. To meet the many diverse physiological challenges to which the epithelial and endothelial barriers are subjected, TJs must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the TJ multiprotein network (Fig. 1). While our knowledge on TJ ultrastructure and intracellular signaling events have significantly progressed during the past decade, relatively little is known about their pathophysiological regulation secondary to extracellular stimuli. Therefore, the intimate pathogenic mechanisms of diseases in which TJs are affected have remained unexplored owing to limited understanding of the extracellular signaling involved in TJ regulation.

The Zonulin Pathway

The discovery of zonula occludens toxin (Zot), an enterotoxin elaborated by *Vibrio cholerae* that reversibly opens TJ [23], increased our understanding of the intricate mechanisms that regulate the intestinal epithelial paracellular pathway and led to the discovery of its eukaryotic counterpart zonulin [24, 25]. The physiological role(s) of the zonulin system remains to be established. This pathway appears to be involved in several functions, including TJ regulation responsible for the movement of fluid, macromolecules, and leukocytes between the bloodstream and the intestinal lumen and vice versa. Another possible physiological role of intestinal zonulin is the protection against microorganism colonization of the proximal intestine (innate immunity) [26].

Since zonulin is overexpressed in tissues and sera of subjects affected by autoimmune diseases, we elected to use sera from zonulin-positive and zonulin negative type 1 diabetes (T1D) and CD subjects to characterize further the molecular nature of zonulin. Through proteomic analysis of human sera, we have recently identified zonulin as pre-haptoglobin (HP)2 [11], a molecule that, to date, has only been regarded as the inactive precursor for HP2, one of the two genetic variants (together with HP1) of human HPs. Mature human HPs are heterodimeric plasma glycoproteins

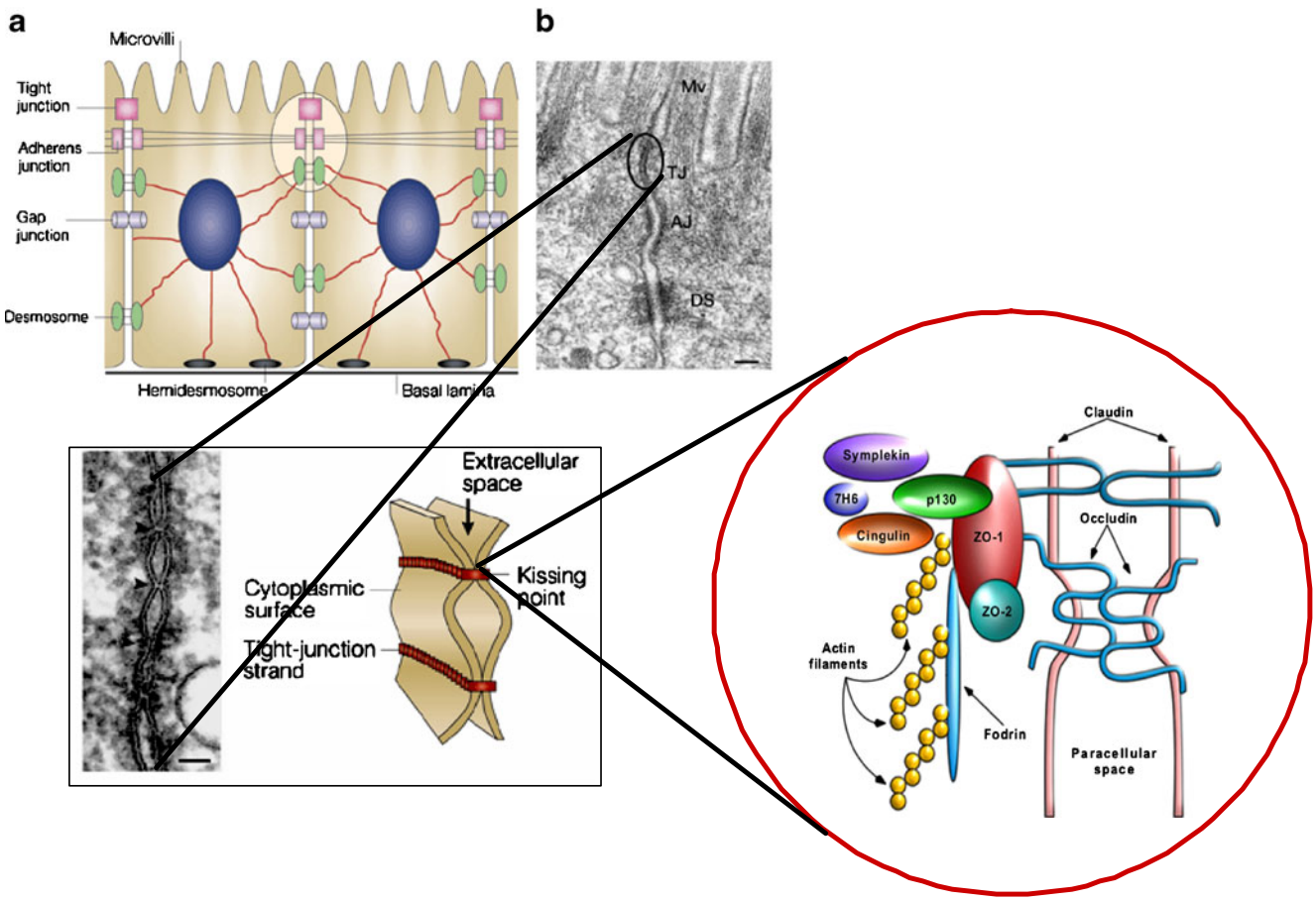

Fig. 1 Composition of intercellular tight junctions. The structural components of intercellular tight junctions can be classified in integral membrane proteins (occludin, claudins, and JAM), junctional complex proteins (ZO-1, ZO-2, p130 or ZO-3, 7H6, symplekin, cingulin), and the cell cytoskeleton structures (microtubules, intermediate filaments, and microfilaments)

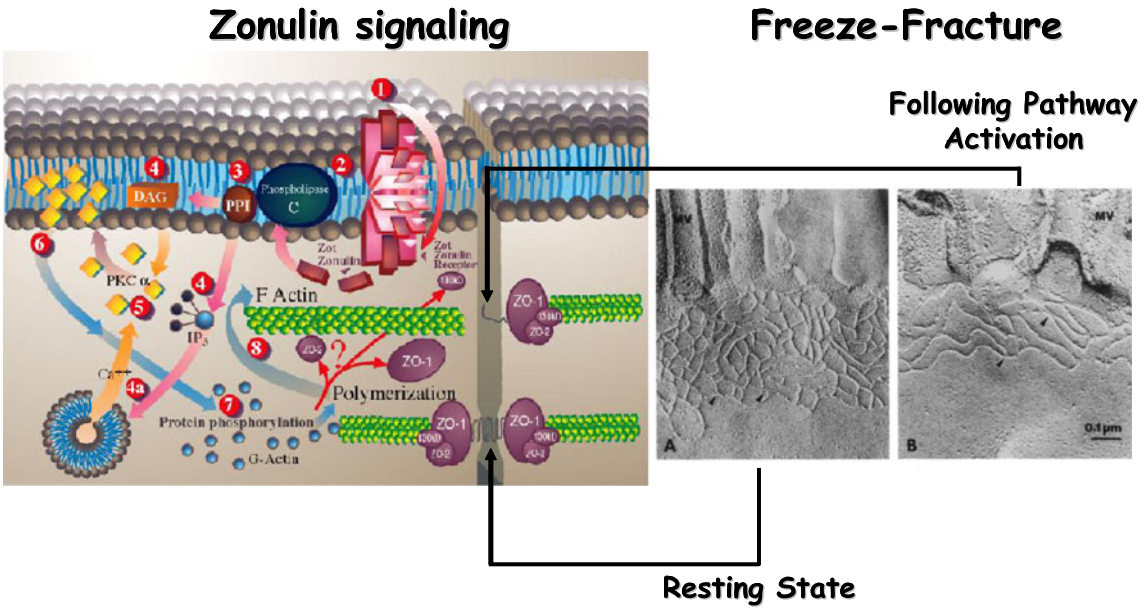

Fig. 2 Proposed zonulin intracellular signaling leading to the opening of intestinal TJ. Zonulin interacts with a specific surface receptor [1] whose distribution within the intestine varies. The protein then activates phospholipase C [2] that hydrolyzes phosphatidyl inositol [3] to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG) [4]. PKCα is then activated [5], either directly (via DAG) [4] or through the release of intracellular Ca^{2+} (via PPI-3) (*4a*). Membrane-associated, activated PKCα [6] catalyzes the phosphorylation of target protein(s), with subsequent polymerization of soluble G-actin in F-actin [7]. This polymerization causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO-1) from the junctional complex [8]. As a result, intestinal TJ becomes looser (see freeze fracture electron microscopy). Once the zonulin signaling is over, the TJs resume their baseline steady state

composed of α- and β-polypeptide chains. While the β chain (36 kDa) is constant, the α chain exists in two forms, i.e., α1 (~9 kDa) and α2 (~18 kDa). The presence of one or both of the α chains results in the three human HP phenotypes, i.e., HP1-1 homozygote, HP2-1 heterozygote, and HP2-2 homozygote. The zonulin pathway modulating TJ permeability is described in Fig. 2.

Zonulin-Dependent Impaired Intestinal Barrier Function and Autoimmune Diseases

Celiac Disease

CD is an immune-mediated chronic enteropathy with a wide range of presenting manifestations of variable severity. It is triggered by the ingestion of gliadin fraction of wheat gluten and similar alcohol-soluble proteins (prolamines) of barley and rye in genetically susceptible subjects with subsequent immune reaction leading to small bowel inflammation and normalization of the villous architecture in response to a gluten-free diet [27]. CD not only affects the gut, but it is a systemic disease that may cause injury to any organ. It is a complex genetic disorder, and HLA status appears to be the strongest genetic determinant of risk for celiac autoimmunity.

Gluten is a complex molecule made of gliadin and glutenins, both toxic for CD patients. The repertoire of gluten peptides involved in the disease pathogenesis is greater than appreciated previously, with at least 50 toxic epitopes in gluten peptides exerting cytotoxic, immunomodulatory, and gut permeating activities. These activities have been partially mapped to specific domains in α-gliadin: the cytotoxic peptide 31–43, the immunomodulatory peptide 57–89 (33-mer), the CXCR3 binding, zonulin releasing (gut permeating) peptides 111–130 and 151–170, and the IL8-releasing peptide 261–277 [21]. The effect of the permeating gliadin peptides in vivo was confirmed by the analysis of intestinal tissues from patients with active CD and non-CD controls probed for zonulin expression [8]. Quantitative immunoblotting of intestinal tissue lysates from active CD patients confirmed the increase in zonulin protein compared to control tissues [8]. Zonulin upregulation during the acute phase of CD was confirmed by measuring zonulin concentration in sera of 189 CD patients using a sandwich ELISA. Compared to healthy controls, CD subjects had higher zonulin serum concentrations ($p<0.000001$) during the acute phase of the disease that decreased following a gluten-free diet [25].

Current data suggest that altered processing by intraluminal enzymes, changes in intestinal permeability, and activation of innate immunity mechanisms seem to precede the activation of the adaptive immune response [28]. Based on these data and on the gliadin epitope mapping described above, it is conceivable to hypothesize the following sequence of events: after oral ingestion, gliadin interacts

with the small intestinal mucosa causing interleukin (IL)-8 release from enterocytes (peptide 261–277), so leading to immediate recruitment of neutrophils in the lamina propria. At the same time, gliadin permeating peptides 111–130 and 151–170 initiate intestinal permeability through a MyD88-dependent release of zonulin (as we have recently confirmed by identifying CXCR3 as the receptor that releases zonulin in a MyD88-dependent manner, see ref. [29]) that enables paracellular translocation of gliadin and its subsequent interaction with macrophages (through 33-mer and other immunomodulatory peptides) within the intestinal submucosa [30]. This interaction initiates signalling through a MyD88-dependent, but TLR4 and TLR2-independent pathway, resulting in the establishment of a proinflammatory (Th1-type) cytokine milieu [30] that results in mononuclear cell infiltration into the submucosa. The persistent presence of inflammatory mediators such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ causes further increase in permeability across the endothelial and epithelial layers [31], suggesting that the initial breach of the intestinal barrier function caused by zonulin can be perpetuated by the inflammatory process after the access of gliadin to the submucosa. In genetically predisposed individuals this, in turn, may permit the interaction of T cells with antigen presenting cells, including macrophages, leading ultimately to the antigen-specific adaptive immune response causing the autoimmune insult of the intestinal mucosa seen in patients with CD [32].

Once gluten is removed from the diet, serum zonulin levels decrease, the intestine resumes its baseline barrier function, the autoantibody titers are normalized, the autoimmune process shuts off and, consequently, the intestinal damage (that represents the biological outcome of the autoimmune process) heals completely.

Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune condition, sometimes associated to diseases that are characterized by marked immunologic features, such as CD and thyroiditis [33, 34]. GI symptoms in diabetes mellitus have been generally ascribed to altered intestinal motility secondary to autonomic neuropathy [35].However, other studies suggest that an increased permeability of intestinal TJ is responsible for both the onset of the disease and the GI symptoms that these patients often experience [36]. This hypothesis is supported by a study performed on a spontaneously diabetic animal model [37]. The authors of this study showed an increased permeability of the small intestine of Bio Breeding Diabetes Prone (BBDP)/Wor diabetic-prone rats that precedes at least a month the onset of diabetes. Further, histological evidence of pancreatic islet destruction was absent at the time of increased permeability but clearly present at a later time [37]. Therefore, the authors presented evidence that increased permeability occurred before either histological or overt manifestation of diabetes in this animal model. We confirmed these data by reporting in the same rat model that zonulin-dependent increase in intestinal permeability precedes the onset of T1D by 2–3 weeks [38]. Oral administration of the zonulin inhibitor, AT1001 (now called Larazotide acetate), to BBDP rats blocked autoantibody formation and zonulin-induced increases in intestinal permeability, so reducing the incidence of diabetes [38]. These studies suggest that the zonulin-dependent loss of intestinal barrier function is one of the initial steps in the pathogenesis of T1D in the BBDP animal model of the disease. The involvement of zonulin in T1D pathogenesis was corroborated by our studies in humans showing that ~50% of T1D patients has elevated serum zonulin levels that correlated with increased intestinal permeability [39]. We also provided preliminary evidence suggesting that, as in the BBDP rat model of the disease, zonulin upregulation precedes the onset of diabetes in T1D patients [39]. Interestingly, a smaller percentage (~25%) of unaffected family members of probands with T1D have also been found to have increased serum zonulin levels and increased gut permeability [39], suggesting that loss of intestinal barrier function is necessary but not sufficient for the onset of the autoimmune process.

Several reports have linked gliadin (the environmental trigger of CD autoimmunity that also causes zonulin release from the gut, see refs. [8] and [26]) to T1D autoimmunity both in animal models and in human studies [40–42]. More recently, we reported a direct link between antibodies to Glo-3a (a wheat-related protein), zonulin upregulation, and islet autoimmunity in children at increased risk for T1D [43]. Glo-3A antibody levels were inversely associated with breast-feeding duration and directly associated with current intake of foods containing gluten in islet autoimmunity cases but not in controls [43]. Further, zonulin was directly associated with Glo-3A antibody levels in cases but not in controls, suggesting that the presence of Glo-3A antibodies and zonulin upregulation in islet autoimmunity cases are related to an underlying difference in mucosal immune response as compared to controls.

Asthma

Asthma is a complex clinical syndrome characterized by airflow obstruction, airway hyperresponsiveness, and inflammation. The mechanisms by which airway inflammation and alterations in airway function are maintained are incompletely understood. Because wheezing can also be triggered by food challenges in some asthmatic children, increased intestinal permeability of asthmatics [44] may play a role in susceptibility to environmental allergens. We

have generated preliminary data suggesting that serum zonulin levels are high in a subset of subjects affected by asthma and that approximately 40% of asthmatic patients have an increased intestinal permeability [21]. This preliminary observation suggests that, besides inhalation, an alternative route for the presentation of specific antigens or irritants may occur through the GI mucosal immune system following their paracellular passage (normally prevented by the intercellular TJ).

Multiple Sclerosis

Besides an increase in blood–brain barrier permeability, multiple sclerosis (MS) patients may also experience an increased permeability of intestinal TJ. Yacyshyn and coworkers have demonstrated that 25% of MS patients studied had an increased intestinal permeability [45]. The fact that patients with MS [45] and Crohn's disease [46] both present an increased number of peripheral B cells exhibiting CD45RO, a marker of antigen exposure, further support the concept of preexisting, genetically determined small intestinal permeability abnormalities with subsequent altered antigen exposure as a pathogenic factor common to these diseases.

To challenge this hypothesis, we measured serum levels of zonulin in MS patients with different subtypes—relapsing–remitting [RRMS] vs. secondary–progressive [SPMS]—and activities to ascertain whether expression of zonulin into peripheral circulation can differentiate these two groups. Approximately 29% of patients with either RRMS or SPMS had elevated serum zonulin levels (a percentage similar to increased intestinal permeability in MS patients reported by Yacyshyn et al., see ref. [45]), with overall average serum levels ~2.0-fold higher than in controls. Interestingly, patients with RRMS in remission showed serum zonulin levels comparable to controls [21].

Inflammatory Bowel Diseases

Crohn's disease and ulcerative colitis are inflammatory diseases involving the GI tract in which abnormal paracellular permeability defects precede the development of both syndromes and, therefore, appear to play an important role in disease pathogenesis [46, 47]. The pathogenesis of inflammatory bowel disease (IBD) remains unknown, although in recent years there is convincing evidence to implicate genetic, immunological, and environmental factors in initiating the autoimmune process. Several lines of evidence, however, suggest that an increased intestinal permeability plays a central role in the pathogenesis of IBD. In clinically asymptomatic Crohn's disease patients, increased intestinal epithelial permeability precedes clinical relapse by as much as 1 year, suggesting that a permeability defect is an early event in disease exacerbation [48]. The hypothesis that abnormal intestinal barrier function is a genetic trait involved in the pathogenesis of IBD is further supported by the observation that clinically asymptomatic first-degree relatives of Crohn's disease patients may have increased intestinal permeability [48]. We have recently generated evidence suggesting that zonulin upregulation is detectable in the acute phase of IBD and that its serum levels decrease (but still are higher than normal) once the inflammatory process subsides following specific treatment [21]. While a primary defect of the intestinal barrier function (possibly secondary to activation of the zonulin pathway) may be involved in the early steps of the pathogenesis of IBD, the production of cytokines, including IFN-γ and TNF α secondary to the inflammatory process serve to perpetuate the increased intestinal permeability by reorganizing TJ proteins ZO-1, junctional adhesion molecule 1, occludin, claudin-1, and claudin-4 [49]. In this manner, a vicious cycle is created in which barrier dysfunction allows further leakage of luminal contents, thereby triggering an immune response that in turn promotes further leakiness.

Ankylosing Spondylitis

Ankylosing spondylitis (AS) is a common and highly familial rheumatic disorder that typically affects young and middle-aged adults and is characterized by stiffness and pain in the back. The link between increased intestinal permeability and AS has been clearly established [50]. Using different markers of TJ permeability, two independent studies [51, 52] found an increased intestinal permeability in both AS patients and their relatives. These changes precede the clinical manifestations of the disease, suggesting a pathogenic role of TJ dysfunction in AS.

Using a proteomic approach, Liu et al. have identified HP as an AS biomarker [53]. The authors investigated the serum protein profiles of AS patients and healthy controls from a large Chinese AS family using two-dimensional electrophoresis analysis. A group of four highly expressed protein spots was observed in all ankylosing spondylitis patients' profiles and subsequently identified as isoforms of HP by ESI-Q-TOF MS/MS [53].

Proof of Pathogenic Role of Zonulin-Mediated Intestinal Barrier Defect in Autoimmunity: the Celiac Disease and Type 1 Diabetes Paradigms

CD and type 1 diabetes autoimmune models suggest that, when the finely tuned trafficking of macromolecules is deregulated due to a leaky gut, autoimmune disorders can occur in genetically susceptible individuals [21]. This theory implies that removing any of the three key elements

(genes, environmental trigger(s), or impaired barrier function) should block the autoimmune process. To challenge this hypothesis, zonulin inhibitor Larazotide acetate was used with encouraging results in the BBDP rat model of autoimmunity [38]. Besides preventing the loss of intestinal barrier function, the appearance of autoantibodies, and the onset of disease, pretreatment with Larazotide acetate protected against the insult of pancreatic islets and, therefore, of the insulitis responsible for the onset of type 1 diabetes [21].

This proof-of-concept in an animal model of autoimmunity provided the rationale to design human clinical trials in which Larazotide acetate was initially tested in an inpatient, double-blind, randomized placebo controlled trial to determine its safety, tolerability, and preliminary efficacy [54]. No increase in adverse events was recorded among patients exposed to Larazotide as compared to placebo. Following acute gluten exposure, a 70% increase in intestinal permeability was detected in the placebo group, while no changes were seen in the Larazotide acetate group [54]. Gastrointestinal symptoms were significantly more frequent among patients of the placebo group as compared to the Larazotide acetate group [54]. Larazotide acetate has now been tested in approximately 500 subjects with excellent safety profile and promising efficacy as concern protection against symptoms caused by gluten exposure in CD patients [55].

Conclusions

The classical paradigm of autoimmune pathogenesis involving specific gene makeup and exposure to environmental triggers has been recently challenged by the addition of a third element, the loss of intestinal barrier function. Genetic predisposition, miscommunication between innate and adaptive immunity, exposure to environmental triggers, and loss of the intestinal barrier function secondary to dysfunction of intercellular TJ seem to be all key ingredients involved in the pathogenesis of autoimmune diseases. Both in CD and T1D gliadin may play a role in causing loss of intestinal barrier function and/or inducing the autoimmune response in genetically predisposed individuals. This new theory implies that once the autoimmune process is activated, it is not autoperpetuating, rather can be modulated or even reversed by preventing the continuous interplay between genes and environment. Since TJ dysfunction allows this interaction, new therapeutic strategies aimed at reestablishing the intestinal barrier function offer innovative, unexplored approaches for the treatment of these devastating diseases.

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