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REVIEW

Zonulin, a regulator of epithelial and endothelial barrier functions, and its involvement in chronic inflammatory diseases

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ABSTRACT

Beside digesting nutrients and absorbing solutes and electrolytes, the intestinal epithelium with its barrier function is in charge of a tightly controlled antigen trafficking from the intestinal lumen to the submucosa. This trafficking dictates the delicate balance between tolerance and immune response causing inflammation. Loss of barrier function secondary to upregulation of zonulin, the only known physiological modulator of intercellular tight junctions, leads to uncontrolled influx of dietary and microbial antigens. Additional insights on zonulin mechanism of action and the recent appreciation of the role that altered intestinal permeability can play in the development and progression of chronic inflammatory disorders has increased interest of both basic scientists and clinicians on the potential role of zonulin in the pathogenesis of these diseases.

This review focuses on the recent research implicating zonulin as a master regulator of intestinal permeability linked to the development of several chronic inflammatory disorders.

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Introduction

Increased intestinal permeability has been recently proposed to be an integral element, along with genetic makeup and environmental triggers, in the pathogenesis of chronic inflammatory diseases (CID), including allergic, autoimmune, and metabolic diseases.[1-3] The incidence of these conditions in industrialized countries has been on a steady rise since the 1950s,[4] leading to the formulation of the hygiene hypothesis.[4,5] The rate and timeline of these epidemics imply that genetic factors are necessary but not sufficient in determining which individuals will develop these diseases, pointing to a key role of environmental factors as driving forces to cause CID in genetically predisposed individuals. Increased hygiene in developing countries was not paralleled by similar epidemics of CID, questioning the validity of the hygiene hypothesis, while pointing out to a more complex dynamic of host-environment interaction centered on the possible epigenetic role of the microbial ecosystem with which we co-exist since birth. The appreciation of the microbiome composition as a key "transductor" of pre-, peri-, and post-natal environmental factors affecting clinical outcome has led to the formulation of the microbiota hypothesis, which postulates the key lifestyle changes, including modality of birth, overuse of antibiotics and, most importantly, dietary differences in industrialized countries causes changes in the microbiome composition and ultimately fuel the onset of CID (Fig 1).[6,7] It is now clear there is a symbiotic relationship between the microbiome and the host. As early as 2001, it was described that commensal bacteria have an effect on intestinal permeability.[8]

Evidence has shown the impaired gut barrier function is a key pathogenic component rather than the epiphenomenon of several CID.[9-11] In this review we will discuss the role of zonulin, the only physiologic modulator of intercellular on tight junctions discovered so far, in the development of several CID.[12]

Intestinal physiology and tight junctions

The human intestine is lined up by a single layer of epithelial cells that represents the largest interface between the environment and the host. The structural arrangement of the intestinal mucosa suggests an

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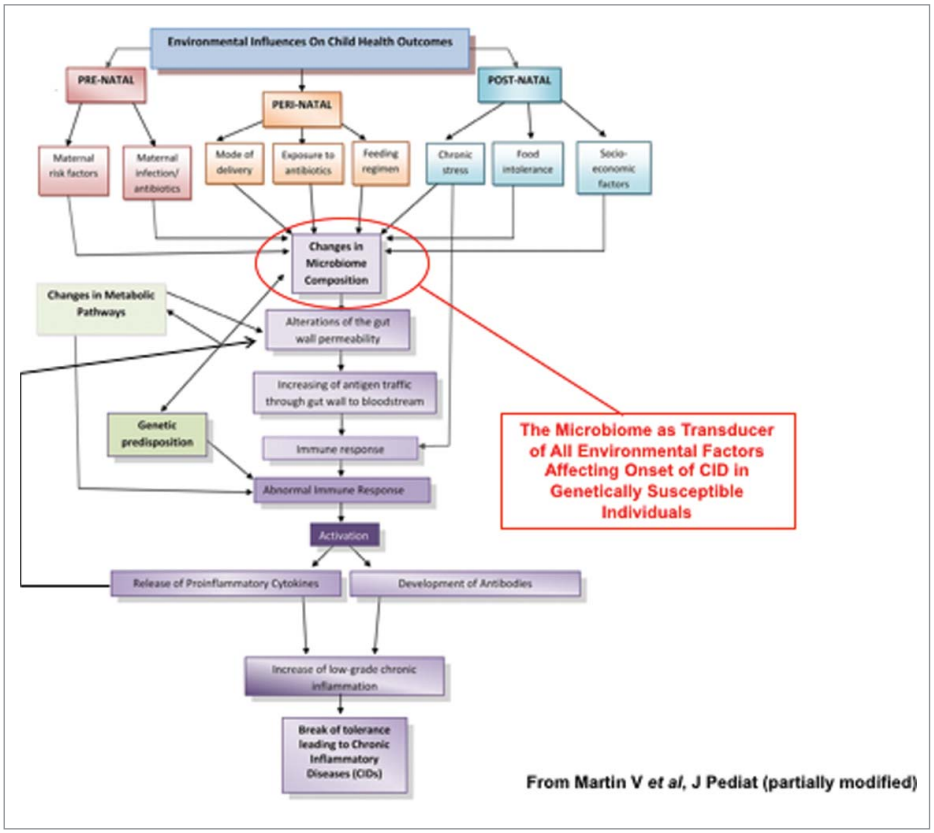

Figure 1. Proposed effect of environmental stimuli causing changes in microbiome composition leading to CIDs. Pre-, peri-, and/or post-natal environmental factor can affect microbiota composition causing loss of barrier function, increased antigen trafficking, and altered immune response in genetically susceptible individuals eventually leading to the onset of CID.

intimate cross talk between epithelial cells and the underneath immune system for the coordinated surveillance of the content of the intestinal lumen. The intestinal mucosa is charged with task of maintaining the balance between the absorption of nutrients and ions, the secretion of fluids, and the protection from microorganisms, toxins, and dietary antigens present in the lumen. The epithelial cells are held together by tight junctions, adherens junctions and desmosomes.[13] Historically, tight junctions were thought to be an impermeable barrier blocking paracellular passage of macromolecules. We now know that tight junctions are dynamic structures involved in both physiologic and pathologic regulation of intestinal epithelial antigen tafficking.[14]

Structure of tight junctions

Tight junctions are the most apical junctional complex connecting both neighboring epithelial and endothelial cells, first described in 1963 by Farquhar and Paladeand, and are comprised of transmembrane proteins including occludin,[15] claudins,[16] junctional adhesion molecules (JAM),[17] tricellulin,[18] and angulins.[19] These transmembrane proteins interact between themselves (both homophilic and heterophilic interactions) and with intracellular scaffolding proteins, including zonula occludins (ZOs), which are anchored to the actin cytoskeleton. The interaction of occludins, claudins, JAMs and tricellulin between cells and with ZOs maintain the integrity of the tight junction and control the passage of molecules through the paracellular space.

Regulation of intestinal tight junctions

Regulation of tight junctions is essential in maintaining barrier homeostasis, both in between body compartments and between body and external environment. Most of the research on intercellular tight junction regulation has been focused on cytokine-mediated

dysfunction in the context of established chronic inflammation, particularly those affecting the intestinal mucosa. TNF-α and IFN-γ have been extensively studied for their effects on the tight junction barrier in the gut. The effect of TNF-α on the intestinal barrier has been associated to IBD,[20] graft-versus-host disease,[21] and celiac disease (CD).[22] In patients with Crohn's disease (CrD) anti-TNF treatment is able to correct barrier disruption seen in the colon.[20]

The mechanism of TNF-α barrier disruption has been shown to be mediated by myosin-light-chain kinase (MLCK). MLCK activation alone has been shown to decrease tight junction permeability both *in vitro* and *in vivo*.[23,24] IFN-γ increases intestinal permeability through changes in expression and localization of tight junction proteins as well as rearrangement of the cytoskeleton.[25]

Pattern recognition receptors (PRRs) are important in the early innate immune response in the intestine. Toll-like receptors (TLRs) are a class of transmembrane PRRs that are important for microbial recognition and control of immune responses. TLR2 is one member of the TLR family, which recognizes conserved patterns on both gram-negative and gram-positive bacteria. TLR2 is expressed on many cell types through the intestine including epithelial cells.[26] Stimulation of TLR2 *in vitro* increased trans epithelial electrical resistance through PKC activation and translocation of ZO-1 to the tight junction complex.[27] ZO-1 is controlled by the PI3K/Akt pathway in a MYD88 dependent manner.[27] Additional studies provide evidence on the protective effect of TLR2 against DSS-induced colitis. The TLR2 stimulation did not cause increase in FITC-dextran passage or redistribution of ZO-1 away from the tight junction.[28]

Proteinase activated receptor (PARs) are a family of g-protein-couple-receptors that are activated by proteolytic cleavage of their N-terminus revealing a tether ligand. PAR2 is found on both the apical and baso-lateral side of enterocytes.[29] Stimulation of basolateral PAR2 results in increase permeability through redistribution of ZO-1, occludins, and F-actin.[30] In addition, Coelho *et al.* demonstrated that *in vivo* the apical stimulation with a PAR2 activating peptide (SLIGRL) causes a dose dependent increase in intestinal permeability.[31] Stimulation of PAR1 has also been shown to increase intestinal permeability.[32]

Role of intestinal permeability in disease

A large number of CID have been described to have alterations in intestinal permeability including IBD,[33] CD,[12,34-36] IBS,[37] multiple sclerosis (MS),[38] rheumatoid arthritis (RA),[33] type-1-diabetes (T1D),[39] asthma,[40,41] necrotizing enterocolitis[42-44] and autism spectrum disorders (ASD).[45] Interestingly, less than 10% of subjects with compatible genetic makeup advance to clinical disease, suggesting that environment stimuli play a key role in determining those that progress to disease. Since the intestinal epithelium is the largest mucosal surface that provides an interface between host and environment, inappropriate antigen trafficking through the intestinal mucosa may be involved.

Under normal physiological conditions, the majority (~90%) of antigens that pass through the intestinal epithelium travel through the transcellular pathway. The transcellular pathway is regulated and leads to lysosomal degradation of antigens into small non-immunogenic peptides. The remaining ~10% of proteins cross the epithelium through the paracellular pathway as full intact proteins or partially digested peptides as a tightly regulated antigen trafficking through intestinal tight junction modulation which leads to antigenic tolerance.[12]

The role of epithelial cells in maintaining mucosal homeostasis was postulated by Hermiston and Gordon using chimeric mice with a defective cadherin.[46] These mice developed profound epithelial defects including incomplete cell polarization, inappropriate actin cytoskeleton distribution, increased migration of enterocytes along crypt-villous axis and premature apoptosis.[46] These mice went on to develop an inflammatory bowel disease resembling CrD without additional external stimuli.[47] Additional studies show transgenic mice with constitutively active MLCK show increased intestinal permeability due to tight junction disassembly.[24] Although, these mice show increased permeability they do not manifest any signs of overt disease.[24] The increased permeability observed in these mice is considered similar to the barrier dysfunction seen in healthy relatives of patients with CrD, CD, and T1D. Experiments performed on JAM-A knockout mice revealed that these animals have increased intestinal permeability but only low grade colonic inflammation and normal epithelial architecture.[48] Similar

results were obtained with an intestinal specific non muscle myosin IIA heavy chain knockout mice (NM IIA cKO).[49] Both JAM-A−/− and NM IIA cKO mice also show increased susceptibility to DSS induced colitis. Together these data suggest intestinal permeability may contribute to the development of several CID, provided that additional genetic traits regulating immune response and exposure to an environmental trigger are present. This hypothesis is consistent with a case report of a healthy first degree relative of a CrD patient who displayed signs of increased intestinal permeability 8 y prior to her own development of CrD.[50]

Additional experiments on transgenic mice with constitutively active MLCK shed additional light on the role of increase permeability on disease development. The MLCK transgenic mice were crossed with recombination-activation gene (rag)-1 knockout mice (which lack mature B and T cells). The Rag-1−/− and constitutively active MLCK mice as well as their Rag1−/− with normal MLCK received $CD4^+CD45RB^{hi}$ naive T cells from wild-type mice. While both groups of mice went on to develop colitis, the colitis in mice with constitutively active MLCK was accelerated and more clinically severe.[24]

Intestinal barrier disruption has been shown to have a role in disease development, but it's also been shown not be sufficient for disease development. Another key piece of the puzzle seems to be the involvement of the mucosal immune system. It has been reported that barrier dysfunction can influence the immunoregulatory process of the mucosa.[51] Induction of mucosal erosion and barrier dysfunction through intrarectal ethanol was followed by increased IFN-γ and IL-10 producing mononuclear cells and $CD4^+CD25^+$, latency-associated peptide (LAP) expressing T cells in the lamina propria. The ethanol administration and subsequent presence of the LAP^+ T-cells protected against trinitrobenzene sulphonic acid (TNBS) acid induced colitis. The induction of LAP^+ T cells was dependent on $CD11c^+$DCs, TL2, and normal microbiome.[51] Interestingly, $CD11c^+$ DCs are able to interact with epithelial cells and increase their ability to induce T-regulatory cells.[52] These observations suggest a key role for the interaction between the epithelial cells, immune cells, and the luminal microenvironment in the maintenance of intestinal homeostasis.

Zonulin as a master regulator of intercellular tight junction in health and disease

Research while developing a vaccine for *Vibrio cholera*, led our group to the discovery of zonula occludens toxins (Zot), an enterotoxin which is able to reversibly open intracellular tight junctions.[53] Subsequent research led to the appreciation of the complexity of the signaling cascades triggered by Zot involved in its regulation of the paracellular pathway.

Zot causes polymerization of actin of targeted cells leading to disassembly of tight junction complexes through a protein kinase C (PKC)-dependent mechanism.[54] Immunofluorescent studies have shown that Zot is able to interact with epithelial cells along the GI tract with the highest binding in the jejunum and distal ileum and also decreasing along the villous to crypt axis.[55] These binding studies confirm data on the regional effect of Zot along the intestine.

Given the complexity of the intracellular signaling activated by Zot leading to tight junction modulation, it was hypothesized that the toxin may mimic an endogenous protein which is able to regulate the epithelial tight junctions. The combination of Ussing chamber experiments and anti-Zot antibodies led to the identification a ~47 kDa human analog to Zot, named zonulin.[35] *Ex vivo* studies show endogenous human zonulin is able to increase permeability in both the jejunum and ileum.[56]

Studies on human sera from CD patients, who have increased zonulin levels[35] as determined by ELISA measurement using polyclonal zonulin cross reacting anti-Zot antibodies,[57] revealed that zonulin is pre-haptoglobin(Hp)-2, the pro-protein of Hp2 before enzymatic cleavage into its mature form. Recombinant zonulin produced by expressing the *HP2* cDNA in a baculovirus system, was detected by the anti-Zot antibodies[57] and showed the expected permeating effect on gut mucosa when tested ex *vivo* in C57BL/6 small intestine. When mice were gavaged with recombinant zonulin and subjected to sucrose and lactulose/mannitol tests they showed increased gastroduodenal and small intestine permeability measured within 24 hours of zonulin exposure, which returned to baseline level after 48 hours.[57] To confirm the increase in permeability was specific to zonulin (pre-Hp2), recombinant zonulin was subject to proteolytic cleavage, resulting in the mature α and β chains of Hp. The effects of zonulin observed in both the *in vivo* and *ex vivo*

experiments failed to cause changes in permeability after proteolytic cleavage.[57] Together these results confirmed zonulin to be pre-Hp2 and when cleaved in its mature Hp2 form, loses its effect on paracellular permeability.

Haptoglobin

Haptoglobin is an ancient protein which has been genetically mapped to first appear ~450 million years ago in bony fish.[58] Wicher and Fries suggested that Hp evolved from the complement-associated protein mannose-binding-lectin-associated serine proteinase (MASP). Hp's primary function is to bind free hemoglobin (Hb) in order to prevent the oxidative stress caused by free intravascular Hb. The Hp-Hb complex is cleared through binding of the scavenger receptor CD163 on monocytes/macrophages.[59]

In humans, Hp is found in 2 genetic variants, *HP1* and *HP2*. The Human *HP1* gene is homologous to the *HP* gene found in other mammals. Genetically human *HP1* is made up of 5 exons and 4 introns. *HP2* arose from an uneven crossover that occurred ~2 million years ago causing the duplication of exons 3 and 4 of *HP1* giving rise to a gene with 7 exons and 6 introns.[60]

Hp is translated as a pro-protein before enzymatic cleavage into an α and β chain by C1r-like protein in the endoplasmic reticulum (ER).[61] The β chain (~35kDa) is conserved in Hp1 and Hp2 and contains an inactive chymotrypsin-like serine protease domain, while the α chain exists in 2 forms α-1 (9kDa) and α-2 (18kDa), corresponding to the *HP1* and *HP2* gene respectively, and contain a complement control domain.[62,63] These 2 α chains give rise to 3 different possible genetic combinations in humans, Hp1-1 (Hp1homozygous), Hp2-1(Hp2-1 heterozygous), and Hp2-2 (Hp2 homozygous). After pre-Hp is cleaved, the α and β chains form polymers which are the mature functional form of Hp. The α and β chains form disulfide bridges and heterodimerize. The heterodimer α and β pair is able to then form polymers with other α and β pairs.

Before the discovery of zonulin as pre-Hp2, no biological function had been described for either form of Hp precursors, as they are cleaved in the endoplasmic reticulum and minimal pre-Hp is found circulating in the plasma. Interestingly, Hp was historically used clinically as a marker of general inflammation, similar to C-reactive protein today, as it is an acute phase protein. The discovery of zonulin as pre-Hp2 added a mechanistic mean to the elevated Hp levels in the course of inflammation.

Additionally, the distribution of Hp genotypes in a wide variety of disease has been extensively studied. In 2007 Carter and Worwood reviewed all the disorders in which Hp was reported to be associated. Of the 23 disorders that were linked to Hp, 11 were shown to be more common in patients with the Hp2-1 or Hp2-2 genotype.[64] The Hp2-2 phenotype has also been associated with worse prognosis of infectious diseases such as HIV[65] and tuberculosis.[66] Hp2 has also been shown to been associated with autoimmune disorders, CD[57,67] and CrD,[68,69] neurological disorders, epilepsy[70] schizophrenia,[71] complications in diabetes (including diabetic nephropathy[72] and diabetic retinopathy[73]), and Chagas' disease.[74,75] This association of Hp2 with CID was postulated to be related to the less efficient capability of Hp2 to bind Hb, which will cause oxidative stress from free Hb, and therefore inflammation. While this could be a plausible hypothesis, increased hemolysis and subsequent oxidative tissue damage have never been reported in these diseases.

Zonulin signaling

Sequential and structural analysis of zonulin revealed an epidermal growth factor (EGF)-like motif. It was therefore hypothesized that zonulin may disassemble TJ through EGF activation, since it has been described EGF can modulate the actin cytoskeleton,[76,77] similar to the effects seen with zonulin.[56,78] *In vitro* studies in Caco-2 cells showed zonulin caused EGFR phosphorylation and subsequent increases in permeability which was blocked by an EGFR inhibitor.[57] To confirm the effect was due to zonulin and not mature Hp2, trypsin digested zonulin was tested and showed no EGFR activation.[57] Additionally, it was shown that EGFR activation was dependent on PAR2 as demonstrated both in Caco2 cells in which the receptor was silenced, and in PAR2$^{-/-}$ mice.[57] Zonulin contains a PAR2 activating peptide-like sequence in its β-chain (FCAGMS) very similar to the PAR2 Zot activating peptide AT1002 (FCIGRL). It had been reported previously that several GPCRs including PAR2 are able to transactivate EGFR.[79]

The signaling pathways triggered by Zot and zonulin leading to tight junction disassembly have been

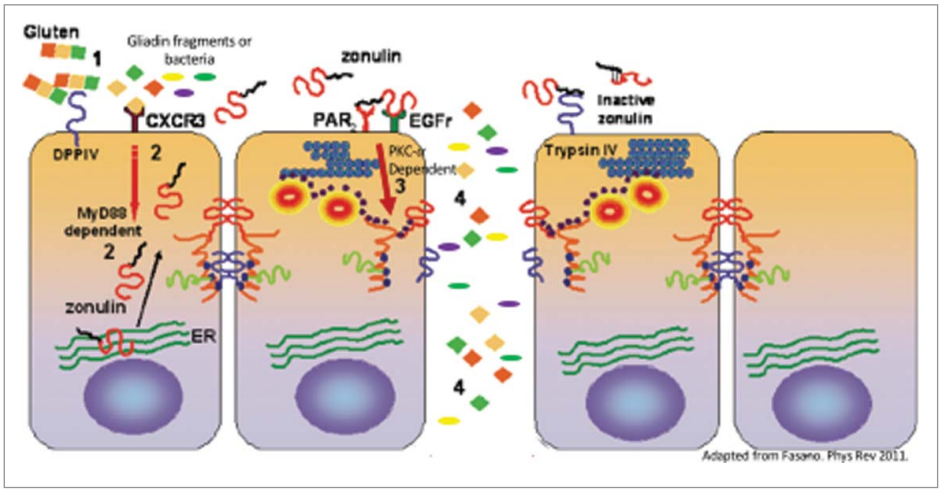

Figure 2. Mechanism of gliadin- and bacteria-induced zonulin release and subsequent increase in intestinal permeability. Gliadin specific peptides or bacteria (1) cause a CXCR-3-mediated, MyD88-dependent zonulin release (2). Zonulin transactivates EGFR through PAR2 leading to PCK-α dependent tight junction disassembly (3). Increased intestinal permeability leads to paracellular passage of non-self antigens (4) into the lamina propria where they are able to interact with the immune system.

extensively studied and resulted being similar (Fig 2).[80] Indeed, as shown with zonulin, Zot also binds to PAR2 through its AT1002 active domain generated during Zot trafficking in V. cholera.[81] AT1002 (FCIGRL) structurally resembles the PAR2 activating peptide tethering motif (SLIGRL) and causes increased permeability through displacement of ZO-1 and occludin from the cell junctions that occurred only if PAR2 was expressed in the target cells (Fig 2).[82] The displacement of ZO-1 and occludin was shown to be secondary to PCKα-dependent phosphorylation of ZO-1, causing decreased tight junction protein-protein interactions, and of myosin-1C that, together with the cytoskeletal rearrangement, temporarily removes ZO-1 and occludin from the junctional complex (Fig 2).[82] While ZO-1 displacement per se is not sufficient to cause a barrier defect,[83] the combination with other intracellular signaling events affecting TJ, including occludin displacement, actin polymerization, and myosin-1C phosphorylation[54,82] may contribute to a more profound rearrangement of the junctional complex that ultimately cause transient TJ disassembly.

Zonulin release

The two major triggers of zonulin release that have been described so far are bacteria and gliadin. It is well described that many enteric pathogens are able to produce enterotoxins that affect the intestinal tight junction of the host. In addition to enteroxins, several enteric pathogens, including commensal *Eschericha coli*, lab *E. coli*, virulent *E. coli*, and *Salmonella typhi* have been shown to cause a release of zonulin from the intestine when applied to the apical surface.[84] Following the release of zonulin, the intestine showed increased permeability and disassembly of ZO-1 from the tight junction complex.[84]

Gliadin is the other trigger that has been described to release zonulin.[85,86] Gliadin, only when applied to the apical surface, caused a release of zonulin, and subsequent increase in permeability, in both cell culture models and *ex vivo* studies of intestinal tissue.[87,88] The increase in permeability, but not the release of zonulin was blocked with pretreatment of the zonulin inhibitor AT-1001.[85] Lammers *et al.* described that specific non-digestible gliadin peptides are able to bind the CXCR3 receptor on the apical surface of enterocytes with subsequent MyD88-dependent zonulin release.[88] The CXCR3 receptor is also overexpressed on the apical surface of CD patients,[88] which may explain the increased levels of zonulin detected in intestinal explant obtained from CD patients when exposed to gliadin.[34] While the full signaling cascade following gliadin binding to CXCR3 leading to release of zonulin is not completely understood, it has been shown to be dependent on MyD88, a key adapter molecule in the TLR signaling pathway[88] (Fig 2). Gliadin is also able to cause a release of zonulin and pro-inflammatory cytokines from macrophages similar to the response seen after bacterial exposure.[86] The zonulin

Table 1. List of CIDs in which zonulin has been implicated.

Disease Category	Disease	Model	Disease Association	References
Autoimmune	Celiac Disease	Human	Specific role in pathogenesis	2,12,34,35,57,78,85,86,88,90-92
	Type-1-Diabetes	Human, Rat	Possible role in pathogenesis	3,11,103,106-108
	Inflammatory Bowel Disease/Colitis	Mouse	Possible role in pathogenesis	9
	Multiple sclerosis / EAE	Human, Mouse	Possible role in pathogenesis	12,114
Metabolic Disorders	Obesity/Insulin resistance	Human	Upregulated	119-121
	Type-2-Diabetes	Human	Upregulated	121,122
	Polycystic ovary syndrome	Human	Upregulated	125,126
Lung Disease	Acute Lung Injury	Mouse	Possible role in pathogenesis	129
	Asthma	Human	Possible role in pathogenesis	12
Heart Disease	Coronary artery disease	Human	Upregulated	133
Neurological Disease	Glioma	Human, Cell culture	Upregulated	134,135
Systemic Infectious Diseases	Septicemia	Human	Upregulated	137,138
	HIV	Human	Downregulated	142,143
Intestinal Diseases	Irritable Bowel Syndrome	Human	Upregulated	144
	Non-Celiac gluten sensitivity	Human	Upregulated	149
	Environmental Enteropathy	Human	Associated	150
	Necrotizing Enterocolitis	Rat, Cell Culture	Upregulated	153

release from macrophages is also MyD88 dependent, but TLR2 and TLR4 independent.[88]

Role of zonulin in specific diseases

Zonulin has been implicated in many CIDs (Table 1). Independent from the CID considered, the steps leading to break of tolerance and subsequent development of CID seem to be similar (Fig 3). Under physiological circumstances there is a tightly control of mucosal antigen trafficking (antigen sampling) that, in concert with specific immune cells and chemokine and cytokine mediators lead to anergy and, therefore, to mucosal tolerance (Fig 3). The inappropriate production of increased amount of zonulin causes a functional loss of barrier function, with subsequent inappropriate and uncontrolled antigen trafficking instigating an innate

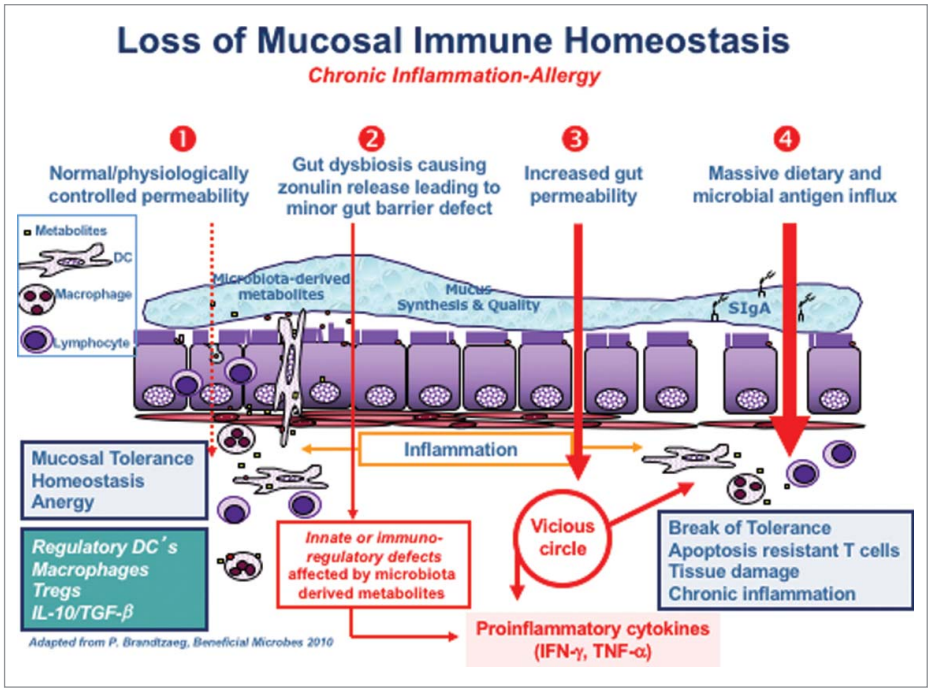

Figure 3. Proposed mechanism of zonulin in causing loss of barrier function leading to development of CID. Normal barrier trafficking of non-self antigens (antigen sampling), together with specific gut-associated lamina propria cells and cytokines micro milieu leads to mucosal tolerance (1). Environmental stimuli cause microbiome imbalance triggering zonulin release (2) leading to increased antigen influx from gut lumen to the lamina propria (3). Antigens in the lamina propria activate the immune system causing IFN-γ and TNF-α release further exacerbating the increased gut permeability and immune response (4). This leads to a vicious cycle which causes break of tolerance and ultimately, onset of CIDs in genetically predisposed individuals.

immune response by the submucosal immune compartment. If this process continues, an adaptive immune response is mounted causing production of pro-inflammatory cytokines, including IFN-γ and TNF-α that cause further opening of the paracellular pathway to the passage of antigens, creating a vicious cycle. Ultimately, these processes lead to break of tolerance with subsequent onset of CID (Fig 3) whose nature is influenced by the specific host genetic background that dictates which organ or tissue will be targeted by the inflammatory process. Below we will review the CID that have been associated with dysregulation of the zonulin pathway, with special emphasis on CD and T1D, the 2 conditions in which the role of zonulin in disease pathophysiology and gut barrier disruption has been demonstrated, while in many of the other CIDs zonulin has only been shown to be upregulated and the mechanism has not been described.

Autoimmune disorders

Celiac disease

CD is an autoimmune enteropathy triggered by the ingestion of gluten-containing grains in genetically susceptible individuals. CD is a complex genetic disorder in which many gene associations have been identified. The HLA status has been identified as accounting for up to 40% of the genetic load. The presence of either DQ2 (HLA-DQA1*05-DQB1*02) or DQ8 (HLA-DQA1*03-DQB1*0302) is necessary but not sufficient to develop CD as ~40% of the general population also has either DQ2 or DQ8. The ingestion of gluten in CD patients causes destruction of the intestinal villi through a mechanism only partially established. Diagnosis is based on serological screening showing presence of auto-antibodies to tissue transglutaminase enzyme, followed by an upper endoscopy with duodenal biopsy showing the typical celiac autoimmune enteropathy characterized by the presence of intraepithelial lymphocytes, crypt hyperplasia, and villous blunting. Current treatment options are limited to dietary restriction of gluten from the diet, gluten free diet (GFD). The GFD allows the intestinal mucosa to heal and villous architecture to return to normal in many cases. Although, diagnosis is based on an intestinal biopsy, CD is a systemic disease which can affect many different organs and cause several extra-intestinal symptoms.

Gluten is a complex molecule consisting of gliadin and glutenins. Gliadin is a subunit of the protein gluten which is found in wheat, rye, and barley. Through extensive research at least 50 toxic epitopes have been identified. Their effects include cytotoxicity, immunomodulatory, and barrier disruption. The α-gliadin fragment has been mapped with specific domains exerting different effects on the body. The 31-43 peptide exerts a cytotoxic effect, the 57-89 (referred to as 33mer) exerts an immunomodulatory effect, the 111-130 and 151-170 are able to bind CXCR3 and release zonulin[88] and the 261-277 causes interleukin (IL)-8 release.[89]

CD has been used as a model disorder to study the effect of zonulin since its involvement in the development and pathogenesis of the disease has been well documented.[2,12,34,35,57,78,85,86,88,90-92] In CD patients, zonulin is produced after gluten ingestion as shown by Drago *et al.*[34] Exposure of intestinal biopsies to pepsin-trypsin(PT)-digested gliadin fragments in the Ussing chamber caused an increase in zonulin release. Interesting, zonulin release could be measured in both CD in remission and healthy control patients, although in healthy controls the level of zonulin release was low and tightly regulated, as shown by the short-term release, with zonulin returning to baseline within 20 minutes.[34] Conversely, CD patients displayed a much more robust and prolonged zonulin release following gliadin stimulation,[34] followed by a significant increase in gut permeability. The release of zonulin and subsequent increase in intestinal permeability was blocked using the zonulin antagonist AT-1001.[34] Also, noteworthy was the observation that CD patients in full remission without PT-gliadin stimulation had constitutively increased zonulin produced by the intestine which correlated to an increase in permeability compared to healthy controls.[34]

AT1001 (now named Larazotide acetate), is a synthetic 8 amino acid peptide that antagonize the zonulin pathway.[93] Pre-clinical trials have shown larazotide acetate to be able to prevent the zonulin permeating activity.[11,94,95] Larazotide acetate was also able to block horseradish peroxidase passage and suppress the innate immune response following gliadin challenge in HLA-HCD4/DQ8 mice,[94] a double transgenic used to study responses to gliadin before severe inflammation and intestinal damage.[96-99]

Larazotide acetate is currently in clinical trials as a treatment for CD. Phase II clinical trial results have

shown that following a gluten challenge larazotide acetate is able to decrease permeability (only in inpatient setting),[100] decrease GI and extra-intestinal symptoms,[100-102] and block in increase of tTG antibodies.[101,102] Additionally, a study on patients who had persistent symptoms despite following a strict GFD showed larazotide was able to reduce their symptoms.[103] Larazotide acetate is now entering phase III clinical trials in CD patients.

Type-1-diabetes

T1D is an autoimmune condition caused by the destruction of the insulin producing β-cell of the pancreas.[104] The exact pathogenesis of T1D is not completely understood, however both genetic and environment factors seem to be at play. T1D shares a genetic association with CD with the HLA locus, specifically HLA DQ2 and DQ8. The trigger of T1D has not been discovered but many possible environmental factors have been scrutinized, although none have been confirmed as a clear causative agent of T1D. T1D has similar pathogenic challenges to other autoimmune disorders, as the environmental trigger must cross the intestinal barrier and interact with the immune system.

Gastrointestinal symptoms have been well documented to occur in T1D patients but thought to be due to altered intestinal motility secondary to autonomic neuropathy.[105] Recent studies have described increased intestinal permeability to prelude these GI symptoms and the development of T1D.[106,107] These studies, including those performed in BioBreeding diabetic-prone (BBDP) rats, which spontaneously develop T1D, suggest a possible pathogenic role for intestinal barrier defects in T1D. The BBDP rats have increase intestinal permeability in the small intestine (but not in the colon) which precedes the loss of tolerance to glucose by at least one month.[108] In addition, histological analysis of the pancreatic islets cells at the time of the loss of barrier function, was normal.[108] These studies show the loss of intestinal barrier function occurs before the histological damage or loss of glucose tolerance seen in T1Ds. Subsequent experiments confirmed these findings and reported the increased intestinal permeability was zonulin-dependent.[11] Furthermore, oral administration of the zonulin blocker AT1001 (larazotide acetate) in the BBDP rats corrected the gut barrier defect and reduced the incidence of diabetes.[11]

The involvement of zonulin in T1D seen in BBDP rats was confirmed in human studies showing ~50% of T1D patients have increased serum zonulin levels, some of them showing these changes in the pre-diabetic phase of the disease.[3] Interestingly, a subset (~25%) of first degree relatives of T1D patients also showed increased serum zonulin.[3] These data suggest zonulin may play a role in the pathogenesis of T1D in a subset of patients.

Inflammatory bowel disease

Increased intestinal permeability has been shown to play a crucial role in the pathogenesis of both CrD and UC.[109-113] Arrieta *et al.* used the IL-10 knockout (IL-10−/−) colitis model to show a direct relationship between increases in small intestinal permeability and development of colitis.[9] IL-10−/− mice were shown to have increased permeability in the small intestine that preluded the development of colitis. Treatment with the zonulin inhibitor AT-1001 caused a significant reduction in the severity of colitis.[9] Together, these experiments suggest a role for increased small intestinal permeability in causing aberrant antigen trafficking with subsequent activation of the gut immune cell that, ultimately migrate to the large intestine where they cause more severe colitis. Therefore, restoration of a normal small intestinal barrier function may be an effective treatment option for colitis.

Multiple sclerosis

Multiple sclerosis (MS) patients show increased permeability of both the blood-brain barrier (BBB) and the intestine. Interestingly, patients with progressive MS showed increased levels of serum zonulin, while those with relapsing-remitting MS who were in remission showed serum zonulin levels similar to controls.[12] A study focused on the experimental autoimmune encephalomyelitis (EAE) mouse model of MS has further described how zonulin is involved in MS.[114] Intestinal permeability and intestinal zonulin are increased during the pre-clinical phase of neurological symptoms, suggesting a role for zonulin in disease development.[114]

Metabolic disorders and obesity

Obesity

Obesity has recently been shown to be associated with chronic inflammation,.[115-117] In an obesity mouse model, increased intestinal permeability and

absorption of macromolecules were observed.[118] Additionally, obese patients are at risk for developing secondary complications to their obesity such as high cholesterol, type-2-diabets (T2D), coronary heart disease, high blood pressure, and stroke. Three studies have shown that serum zonulin level is increased in obese vs non obese subjects.[119-121] Zak-Golab *et al.* have shown correlation between total bacteria and serum zonulin levels. They suggest that the gut microbiota may cause increased zonulin levels, with subsequent abnormal gut permeability to endotoxin (lipopolysaccharide - LPS) and, ultimately micro-inflammation seen in obesity.[120]

Evidence has also been provided suggesting that zonulin not only is associated to obesity, but also with its metabolic complications. Serum zonulin has been shown to be increased in T2D patients,[121,122] and it has been suggested, through multivariate analysis, that the relationship between insulin sensitivity and serum zonulin may be modulated through IL-6.[119] In it interesting to note that zonulin promoter is under IL-6 control[123] and, therefore, zonulin modulation by IL-6 may be mechanistically related to its expression. In addition, serum zonulin was increased in obese children with non-alcoholic fatty liver disease (NAFLD) compared to obese children without NAFLD and correlated with the severity of steatosis.[124]

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is an endocrinopathy in women of reproductive age due to elevated levels of androgens. It has been reported that both genetic makeup and the environment contribute to the development of PCOS.[125] The majority of women with PCOS are overweight and insulin resistant. It is well described that PCOS is characterized by a chronic state of inflammation. Studies have suggested increased zonulin associated to altered gut permeability,[126] as key pathogenic elements, together with intestinal microbiota, for development of PCOS.[127]

Lung diseases

In addition to the GI tract, the lung is another mucosal surface where altered tight junctions function can play a role in a variety of diseases. The mechanisms of airway inflammation are still incompletely established.

Acute lung injury

Leakage of plasma contents into the lungs is observed in acute lung injury (ALI) and acute respiratory distress syndrome.[128] Zonulin has been implicated in the disassembly of lung tight junctions in ALI.[129] Blocking of the zonulin pathway by AT-1001 or by zonulin neutralizing antibodies reduced the severity of ALI.[129] Additionally, both zonulin and its agonist peptide, AT-1002, intensified ALI and increased lung permeability. The mechanism is thought to be through zonulin dependent complement activation in the lung.[129]

Asthma

In addition to increases in lung permeability, intestinal permeability has also been implicated in other lung diseases like asthma.[40,41] Preliminary data suggest that a subset of asthmatic patients have increased serum zonulin levels and ~40% have increased intestinal permeability.[12]

These data suggest that both the lung and intestinal mucosa may be routes through which specific antigens can gain access to the submucosa with subsequent exposure to the immune system leading to lung inflammation.

Heart diseases

Coronary artery disease

Coronary artery disease (CAD) is a major cause of death throughout the world. Several studies have shown a link between infectious pathogens and CAD.[130-132] Additionally, enterobacteria have been detected in atherosclerotic plaque biopsies. CAD patients have been shown to have increased serum zonulin levels and high levels of *Enterobacteriaceaes* in their blood.[133] These data suggest that zonulin-dependent bacterial translocation may cause increase levels of bacteria in the circulation, with subsequent onset of atherosclerotic plaque leading to CAD.

Neurological disorders

The BBB is formed by endothelial cells and separates the circulating blood from the brain. Since zonulin can also modulate endothelial tight junctions, it was hypothesized zonulin dysregulation may be involved in the pathogenesis of neurological disorders.

Zonulin has also been shown to be involved brain tumors, specifically gliomas.[134,135] Skardelly *et al.* showed there was increased expression of zonulin in

gliomas which correlated with the degree of malignancy and degradation of the BBB.[135] *In vitro* studies on a glioma cell line showed zonulin was expressed in high amounts compared to non-glioma control cells.[134] Additionally, zonulin has been shown to induce transmigration of neuronal progenitor cells across the BBB.[134]

Systemic infectious diseases

Septicemia

Intestinal barrier dysfunction has been implicated in the pathogenesis and progression of septicemia. Yoseph *et al.* have shown in an experimental model of sepsis that tight junction proteins expression is altered.[136] In patients with septicemia serum zonulin levels were found to be increased.[137] Post-surgical septicemia continues to be a common complication despite advances in surgical techniques and perioperative care. It was hypothesized that zonulin could be a key contributor to post-surgical septicemia.[138] Liu *et al.* described how treatments with probiotics can decrease post-surgical septicemia and is correlated with decreased serum zonulin levels.[138] These data suggest increased release of zonulin from enterocytes leads to the migration of bacteria across the epithelium which can lead to septicemia.

HIV

It is widely accepted that the intestine plays an integral role in the immunopathogenesis of human immunodeficiency virus (HIV).[139-141] Interestingly, it has been reported that decreased zonulin levels correlated with increased mortality in HIV patients.[142] Additionally, treatment with HIV treatment drugs, maraviroc and raltegracir (CCR5 receptor antagonist and integrase inhibitor), increased serum zonulin levels.[143] Combined, these data suggest that the zonulin pathway in its innate immunity function can be protective against HIV infection.

Intestinal diseases

Irritable bowel syndrome

The pathophysiology leading to the development of irritable bowel syndrome (IBS) is unknown, but it has been reported that patients with IBS have increased gut permeability.[37] Recent data show patients with diarrhea associated IBS (IBS-D) having increased serum zonulin levels.[144] Interestingly, PAR2 has been suggested to be involved in the increased permeability detected in IBS-D patients.[145] Serine-proteases, which activate PAR2, are found to be increased in the lumal contents of IBS-D patients but not in constipated or alternating IBS patients.[146] Diluted fecal supernatants of human IBS-D patients increased permeability of mouse mucosa when added to the apical surface. These changes were not detected in PAR2−/− mice.[147] Additionally, IBS-D patients who carry either the HLA-DQ2 or DQ8 genotype have increased gut permeability compared to IBS-D who do not carry one of those HLA genotypes.[148] Taken together, these data suggest zonulin signaling through PAR2 may be involved in the pathogenesis of IBS-D.

Non-celiac gluten sensitivity

Non-celiac gluten sensitivity (NCGS) is a newly described condition that clinically presents similar to CD but does not have the intestinal damage seen in CD.[149] It has been reported patients with NCGS may have an increase in intestinal permeability following gluten exposure.[36] Preliminary evidence also suggest increased serum zonulin levels in patients with NCGS.[144]

Environmental enteropathy

Environmental enteropathy (EE) is a disease of unknown etiology seen in developing countries. It is hypothesized that constant exposure to infectious agents in the proximal intestine causes increased gut permeability, excess of macromolecules and endotoxin trafficking that trigger chronic inflammation leading to an enteropathy that structurally resembles CD. Like in CD, the enteropathy causes decreased absorption of key nutrients eventually leading to stunted growth. We have recently shown that serum zonulin levels and other markers of barrier dysfunction are correlated with stunted growth in EE patients.[150] This data provided additional evidence that a functional loss of barrier function may play a key role in the pathophysiology and EE.

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is a devastating disease that affects premature infants resulting in bacteria translocation causing local infection, inflammation and eventually necrosis in portions of the intestine. Changes in tight junction protein expression and localization during NEC have been described in several

studies.[42-44,151,152] A recently published study implicated the role of zonulin in NEC. Pre-treatment of Caco2 cells with Bifidobacterium before LPS exposure decreased release of zonulin and preserves tight junction competency.[153] Ling *et al.* went further to confirm these results in an *in vivo* rat model of NEC.[153] This study highlights a model in which changes in the microbiome can influence the release of zonulin and, subsequently intestinal barrier function.

Conclusions

Zonulin is a protein involved in the functional regulation of both epithelial and endothelial barrier functions whose role is health and disease is still object of active research. In this review we have provided an overview for its role in the development and pathogenesis of several CIDs. While the specific pathophysiological role of zonulin in many of these diseases is poorly understood, we propose the loss of gut barrier function, through increased zonulin, as an essential step to initiate the inflammatory process. In CD and possibility T1D, research shows gliadin as the trigger of zonulin release leading to gut barrier dysfunction. In other CIDs the specific instigator causing increased zonulin release is not known, but an imbalanced microbiome or its inappropriate distribution along the gastrointestinal tract may be the triggering factors as described in Fig 3. Dysbiosis of the microbiome may cause the release of zonulin leading to the passage of luminal contents across the epithelial barrier causing the release of pro-inflammatory cytokines. The presence of cytokines eventually sustains the increased permeability causing massive influx of dietary and microbial antigens leading to the activation of T-cells. Depending on the genetic makeup of the host, these T-cells can remain within the GI tract causing CID of the gut (IBD, CD) or migrate to several different organs to cause systemic CID. Research using larazotide acetate, a zonulin antagonist, in animal models and now in clinical trials, not only confirmed the pathogenic role of zonulin in many CIDs, but also opens the possibility for its possible therapeutic use not only in CD, but also in other CIDs in which a pathogenic role for zonulin has been hypothesized or proved. Further research is needed to completely understand the mechanism zonulin plays in the development, pathogenesis, and progression of several CIDs.

Disclosure of potential conflicts of interest

CS has no potential conflict of interest. AF is co-founder and stock holder of Alba Therapeutics.

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