Chapter 7

Immunopathogenesis of Celiac Disease

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Celiac disease is a chronic inflammatory process of the small intestine mediated by the immune system which affects genetically susceptible individuals following the ingestion of prolamins from wheat and other cereals. The interaction between genetic and environmental factors determines the loss of tolerance to gluten and the development of the intestinal lesion, with variable clinical and functional repercussions, characterized by an increased number of lymphocytes within the epithelium and the lamina propria, enterocyte apoptosis, the mucosal transformation, and the presence of anti-transglutaminase antibodies. The most accepted pathogenesis model for Celiac disease includes changes in digestion and in the transepithelial transport of gluten, and it is focused on the mechanisms of adaptive immunity triggered by the stimulation of CD4+ T lymphocytes after recognition of gluten peptides deaminated by the tissue transglutaminase (tTG) enzyme in the context of HLA-DQ2/DQ8 molecules, and the production of proinflammatory cytokines, specially IFNγ. Furthermore, gluten has also a direct toxic effect on the epithelium, which depends on innate immunity with IL15 as the central mediator, manifested by the epithelial expression of stress molecules and the activation of cytotoxic functions by intraepithelial lymphocytes. The interaction between IL15 and its receptor, expressed by epithelial cells, may be also relevant for the induction of adaptive immunity to gluten. Further clarification is needed on several issues, like the passage of gluten into the lamina propria, the activation of free tTG, or the mechanisms regulating the activity of IL15, among others.
1. Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine caused by an improper immune response to wheat gluten and related proteins from other cereals affecting genetically predisposed individuals at any stage of life. It is a common disorder with an estimated prevalence of nearly 1% in most of the populations studied, although only 1 of every 7-10 cases has been diagnosed. Unfavorable interactions between susceptibility genes and environmental factors trigger this response against gluten in the intestinal mucosa, including an innate component responsible for epithelial injury and other adaptive mediated by CD4+ T lymphocytes specific to the lamina propria, and determines the remodeling of the mucosa. Along with the loss of oral tolerance to gluten, it generates other alterations which affect intraluminal digestion; direct action of the gluten peptides on the epithelium and the transepithelial transport of lamina propria mucosa have also been identified in CD.

The activation of CD4+ T lymphocytes from the mucosa on the lamina propria after the recognition of gliadin peptides modified by the transglutaminase 2 (TG2) enzyme, in the context of HLA-DQ2/DQ8 molecules, triggers an inflammatory response dominated by a Th1 cytokine profile, in which IFNγ predominates along with other proinflammatory cytokines (TNFα, IL 15 and IL 18), but with absence of IL 12 and a proportional decrease of immunoregulatory cytokine expression such as IL 10-14 and TGFβ. Accordingly, a lesion of the small intestine mucosa occurs, which affects the absorption and utilization of nutrients and whose clinical and functional impact varies with the degree of atrophy or mucosal remodeling (Figure 1).

![Figure 1. Duodenal mucosa from a non-celiac control patient (A) and from a celiac patient at the time of diagnosis (B) where the optical microscope reveals a lesion with villous atrophy and crypt hyperplasia.](image)

In CD, the characteristic small intestine lesion can be recognized in several interrelated phases, described by Marsh. Type 0, preinfiltrative, is characterized by mucosa with normal morphology, although local humoral immunity is altered; Type I, or infiltrative lesion, shows normal architecture in the mucosa, but with an increased IEL count (>25/100 enterocytes); Type II, hyperplastic lesion, is characterized by elongated or hyperplastic crypts with normal...
villusous height and IEL infiltration; Type III, destructive lesion, may be partial (3a), subtotal (3b) or full (3c); this is the typical lesion diagnosis, with villi loss and tissue reorganization; Type IV, hypoplastic lesion is a true atrophic lesion with collagen deposits, observed in a small group of patients who do not respond to the gluten-free diet (Refractory CD). 

Gluten sensitization and activation of a specific response against gluten in the intestinal mucosa is an invariable feature of CD, however, the precipitant may be another factor which would be responsible for the full expression of the mucosal lesion; for example, in the form of a destructive lesion with loss of intestinal villi. According to the hypothesis proposed by Anne Ferguson a few years ago, candidate factors may include an increase in intestinal permeability, nutritional defects, an increase in the amount of dietary gluten, alterations or defects in the intraluminal digestion of ingested gluten, adjuvant effects of a gastrointestinal infection, or some as yet unidentified gene not linked to HLA.

2. Pathogenic Theories of Celiac Disease

The metabolic theory held that CD was the result of an enzyme defect or some other mechanism that ultimately meant an incomplete gluten, or wheat gliadin digestion. Among the studies conducted to confirm this hypothesis, one in particular reported that homogenates from the small intestinal mucosa of untreated celiac patients were less efficient in degrading the product of gliadin digestion with pepsin and trypsin (PT), when compared with homogenates from non- celiac patients. These results led to propose that the incomplete digestion of gliadin was the trigger for the immune response, by means of the “lost peptidase hypothesis” or “metabolic hypothesis”. 

This hypothesis, based on the incomplete digestion of gluten proteins in the intestinal mucosa of celiac patients, was subsequently confirmed using PT digests from alpha, beta, and gamma-gliadin, as well as many other immunodominant peptides. It is striking that none of these studies found qualitative differences between the peptides generated in the mucosa of celiac patients and those from non-celiac patients; the only difference seemed to be their quantity, since in both cases the same peptides were generated, though in different amounts. Other studies did not find differences; instead, the enterocytc brush border enzymes in celiac patients, hydrolyzed PT-gliadin with the same effectiveness as those in non-celiacs.

Today, the enzyme-based hypothesis, formerly considered as a possible contributing factor in the pathogenesis of CD, has been virtually forgotten, due to a better molecular understanding of the pathophysiology of this disease. This has allowed the unraveling of many of the immunological mechanisms involved in the development of intestinal lesion, as well as to the discovery of the HLA-DQ2/DQ8 haplotype as a key factor in genetic predisposition.
3. The Immunological Theory of Celiac Disease

3.1. Immunity Against Dietary Antigens. Oral Tolerance

Under normal conditions, the response to the dietary proteins is oral tolerance, which is defined as the lack of a systemic immune response to certain antigens ingested after their systemic administration\(^\text{21}\). However, in CD there is a loss of tolerance to gluten and similar proteins. The capacity of the digestive tract’s immune system to distinguish between dietary antigens and pathogenic microorganisms could be due to the fact that these furnish a persistent stimulus, associate other danger signals or else invade lymphoid tissues distant from the mucosa. Several mechanisms responsible for oral tolerance have been described: deletion (apoptosis), clonal anergy (functional inactivation of effector cells) and induction of regulatory T lymphocytes, which act through cytokines (IL 10 or TGF).\(^\text{22,23}\)

The regulation of the response to dietary antigens, is determined by the way in which T lymphocytes recognize these antigens and the type and functional state of antigen-presenting cells (APCs) such as dendritic cells (DCs). Data from animal models and from observations in humans have led to explain oral tolerance as the result of immunoregulatory bowel conditions that lead to the differentiation of regulatory T lymphocytes (Treg)\(^\text{24,25}\) and from other cells with a homeostatic function such as Tγδ+ cells and invariant NKT (iNKT). Another possibility is that the normal gut may respond with an IFNγ-dominated Th1 profile, even against dietary antigens, which would be the result of a balance between various factors (epithelial integrity, T lymphocytes development, immunoregulation, etc.). Th1 differentiation would not associate with tissue damage due to the control exerted by immature APCs upon effector lymphocytes, the former of which have a short half-life, and to the elimination induced by regulatory T lymphocytes.\(^\text{25}\)

Dendritic cells (DCs) are the major APCs, especially for naive T lymphocytes and play a key role in intestinal homeostasis\(^\text{26-28}\), as well as serving as a link between the innate and adaptive immune responses.\(^\text{29,30}\) In the absence of other co-stimulatory signals, antigen presentation by these cells favors oral tolerance by decreasing its stimulatory capacity and/or promoting regulatory T lymphocyte differentiation\(^\text{31}\), characterized by the CD4+ CD25\text{high} phenotype,\(^\text{24,32}\) and by the FoxP3 transcription factor, crucial for the functional development and maturation of these cells.\(^\text{33}\) However, recent studies suggest that although FoxP3 is a transcription factor linked to the regulatory phenotype, it is not unique to a single cell type and would not be the best marker to identify regulatory T lymphocytes.\(^\text{34}\) DCs also appear to have an important immunopathogenic role in CD due to their capacity to mature in response to danger signals from the innate immunity and to foster the induction of adaptive immune responses.\(^\text{22,35}\)

Regulatory T lymphocytes (Treg) are the main homeostatic immune cells and have a key role in controlling of local inflammation. These cells perform their function by blocking T lymphocyte clonal expansion, both CD4+ and CD8+, as well as by inhibiting IL 2 production. By means of producing cytokines such as IL 10 and TGFβ, Treg cells can modulate local inflammation by inhibiting Th1 responses and IFNγ production through cooperation with B cells in the IgA synthesis.\(^\text{36,37}\) Other cells involved in gut homeostasis are CD4+ T3, which perform their function through TGFβ production.\(^\text{38}\) Recently, it has been determined that this cell population, at some point depends on the presence of FoxP3; it has been suggested, therefore, that Th3 and Treg cells could be the same cell population.\(^\text{38}\)
There is another CD4+ population which expresses neither the FoxP3 transcription factor nor CD25 molecules on its surface and which has central role in controlling the inflammatory response to dietary antigens\textsuperscript{39}, such as the Tr1 lymphocytes, the leading IL10 producers in the intestine. Under certain conditions, Th1, Th2 or Th17 lymphocytes can become IL10 producers, so Tr1 cells could not be other that CD4+ lymphocytes which have been chronically stimulated to reduce the production of pro-inflammatory cytokines and maintain IL10 levels.\textsuperscript{40} Within the gut, the activity of these non-Treg cells is more important than that of Treg in oral tolerance, since their number is far higher than that of CD4+CD25+FoxP3+ cells.

In addition to Treg cells, other cells that may be involved in the maintenance and regulation of intestinal homeostasis and oral tolerance are Tγδ+ intraepithelial lymphocytes (IEL), which significantly contribute to the circulating TCR+population\textsuperscript{41}, and their number is increased in the intestine of patients with CD.\textsuperscript{42} Following their interaction with the antigen via TCR, these Tγδ+ cells quickly and transiently express the CCR7 receptor, which allows their migration to the lymph nodes where they may act as APCs and induce specific Treg cell differentiation.\textsuperscript{43}

Invariant NKT (iNKT) cells display NK cell markers such as CD161 (NK1.1), and an invariant Va24β11 TCR that recognizes antigens along with CD1d molecules (MHC-1), highly expressed in the intestinal epithelium\textsuperscript{44} and which represent 0.5-20\% of the total cells.\textsuperscript{45,46} A population with CD3-NK-like phenotype has also been described in the epithelium, which drastically decreases in CD patients.\textsuperscript{47,48} Activated iNKT lymphocytes have a dual role, the iNKT CD4-CD8 subpopulation produces cytokines with a Th1 (IFNγ, TNFα) profile, while iNKTs CD4+ cells synthesize both Th1 and Th2 (IL 4, IL 13) cytokines.\textsuperscript{32,49,50} The acquisition of a Th1 or Th2 profile depends on the strength of the interaction between the antigen and the CD1d molecule, the predominant cytokines in the local microenvironment and other co-stimulatory signals.\textsuperscript{45} This ability to rapidly produce large amounts of Th1/Th2 cytokines, confers iNKT lymphocytes a significant role in oral tolerance, by modulating DC maturation towards the tolerogenic pathway, which is involved in the differentiation of Treg (IL 10 and TGFβ) cells\textsuperscript{44,51}, as well as inducing clonal of antigen-specific T lymphocytes.\textsuperscript{32}

The origin of immune cells in the duodenal mucosa is not clear at all. Under physiological conditions, during its activation, lymphocytes acquire recirculation properties which depend on the expression of adhesion molecules and chemokine receptors to direct their migration to specific tissues and microenvironments.\textsuperscript{52,53} Activated lymphocytes in the intestinal lymphoid tissue tend to return to the intestine. This selective migration is directed by the α4β7 integrin, whose ligand is mucosal addressing (MAdCAM-1) from the high endothelial venules, Peyer’s patches and mesenteric lymph nodes in the intestine.\textsuperscript{53} The CCR9 chemokine receptor intervenes in the effector T lymphocyte recruitment for the intestine via interaction with its ligand CCL25 (TECK), selectively expressed in part of the intestine.\textsuperscript{54} Conversely, selectin carbohydrate ligands P and E, are collectively referred to as human leukocyte antigens (CLA).\textsuperscript{55} Other chemokine receptors such as CCR4, CCR8, and CCR10 have also been implicated in the selective migration to the skin.\textsuperscript{55,56}

Therefore, in diseases where the pathogenic involvement of immune response mediated by antigen-specific lymphocytes is known, as in the case of CD\textsuperscript{2,57}, an increase in the selective migration profile markers in the circulating cell populations in celiac patients is expected. However, little information is available on the expression of these cell markers not only in CD patients, but also in the general population. Preliminary results\textsuperscript{31,58} from healthy adult volunteers without known autoimmune or malignant diseases, suggest that circulating blood CDs are double positive for migration markers to the intestine and skin while circulating monocytes
3.2. The Two Signal Pathogenic Model

The immunological theory is the one that currently best explains the pathogenesis of CD. Formerly, it was thought that what happens in the lamina propria mucosa, in the context of a CD4+ T lymphocyte mediated response, with HLA-DQ2/8 restriction and IFNγ release, was fundamental in the development of this enteropathy. Recently, it has been observed that the innate immunity, which acts primarily in the intraepithelial compartment, is also critical to the immune response to gluten. The most accepted immunopathogenic model states that gluten has a double effect involving innate immunity (direct toxic effect of gluten on the epithelium) and adaptive or specific immunity (through T CD4+ lymphocytes of the lamina propria and underlying tissue).59

This immunopathogenic model integrates several necessary elements in the intestinal mucosa, such as the presence of gluten peptides (toxic and immunogenic), the effect of some of these peptides on the epithelium, TG2 enzyme activity, the presence APCs which express HLA-DQ molecules and CD4+ T lymphocytes reactive to gluten. Toxic peptides not recognized by T lymphocytes have a rapid and unspecific effect on the epithelium, while the response to immunogenic peptides is slower, after passing through the epithelium to reach the mucosal lamina propria there to undergo TG2 deamidation, after which to bind with high affinity to HLA-DQ2 or DQ8 molecules. Gluten specific T lymphocytes recognize these T epitopes modified in the context of membrane DQ2 or DQ8 molecules in local APCs, such as DCs. These immune responses (innate and adaptive) trigger different mechanisms which cause damage through epithelial cytotoxicity and restructuring of the extracellular matrix (the so called mucosal transformation).

Wheat gluten contains two families of proteins, gliadin and glutenin (insoluble in alcohol), with fragments harmful for CD patients and which are also found in the proteins in rye (secalins), barley (hordeins) and oats (avenines). Gliadin proteins can be subdivided into α-, γ- and ω-gliadins and into subunits of high molecular weight (HMW), medium molecular weight (MMW) and low molecular weight (LMW) for glutenines.62 All these proteins are designated by the generic name of prolamins since they share a very similar amino acid sequence and a high content of the hydrophobic amino acids glutamine and proline.63,64 Peptides considered toxic induce damage in cultured intestinal duodenal biopsies, or after being administered in vivo on the proximal or distal intestine; those which are immunogenic stimulate T lymphocyte lines with DQ2/DQ8 restriction, obtained from the intestine or peripheral blood from CD patients.67

3.3. Innate Immune Response to Gluten

Some gluten fragments, such as p31-49 or 31-43 from the α-gliadin, induce an immediate innate immune response, associated neither with T lymphocytes nor with HLA-DQ2/8 dependent antigen presentation, although these mechanisms are not yet fully understood.68 In an ex vivo culture model from biopsies from CD patients, it has been observed that the immediate response induced by the 31-49 peptide is associated with IL 15 expression, cyclooxygenase (COX-2) and CD25 and CD83 activation markers by mononuclear cells from the lamina propria.69 Furthermore, oxidative stress appears mediated by the formation of nitric oxide, which comes primarily from...
iNOS induction in enterocytes\textsuperscript{70,71}, which, in turn, induces the expression in these cells of ligands like MICA.\textsuperscript{72} Gliadin is also able to weaken tight-junction type bonds located between intestinal epithelial cells.\textsuperscript{9}

Intraepithelial lymphocytes (IELs) are found in the basolateral area of epithelial cells and have a crucial role in the immune surveillance of the intestinal epithelium. The population of IELs in the small intestine is a mixture of TCRαβ+ T lymphocytes, TCRγδ+ T lymphocytes and NK cells, although most of them are TCRαβ+CD8+ lymphocytes.\textsuperscript{2} Furthermore, most TCR+ IELs express diverse NK receptors different from those expressed by T lymphocytes in the circulating peripheral blood.\textsuperscript{73} These NK receptors act not only as costimulatory molecules, but also as T lymphocyte activators in stress situations.\textsuperscript{74} In active CD, the number of CD8+ TCRαβ+ and TCRγδ+ IELs is very high. It is unclear whether this situation depends on epithelial homeostasis changes or if it is a consequence of the proinflammatory environment created by the CD4+ T lymphocyte mediated response in the lamina propria mucosa.

![Diagram](image.png)

**Figure 2.** Gluten has a dual effect on the small intestine mucosa. Toxic peptides, such as the 19-mer, induce a nonspecific innate immune response characterized by the presence of IL 15, produced by enterocytes. IL 15, in turn, activates the NF-κB transcription factor in the adjacent cells, which enhances IL 15 production and iNOS induction, responsible for an oxidative stress and innate feedback situation. The expression of molecules such as MICA and/or HLA-E is increased in enterocytes and IL 15 triggers cytotoxicity (apoptosis) on these cells by inducing the expression of NKG2D and NKG2C molecules (ligands MICA and HLA-E respectively) in intraepithelial lymphocytes. Finally, IL 15 may weaken the tight-junctions between enterocytes. The adaptive response is facilitated by increased intestinal permeability allowing the passage of immunogenic peptides like 33-mer to the lamina propria, which are deaminated by the tissue transglutaminase (TG2) enzyme. Besides, IL 15 activates dendritic cells, which increases the surface expression of the costimulatory molecules necessary for an effective antigen presentation and restricted by HLA-DQ2/8, to T lymphocytes. These cells trigger an IFNγ predominant Th1 response with IL 10 absence, and the release by stromal cells of keratocinic growth factor and metalloproteinases. The Innate and Adaptive Immune Responses are responsible for the intestinal damage.
The principal mechanism that prompts the innate response depends on the release of IL-15 by enterocytes.\textsuperscript{75} In CD, IL-15 expression is observed both in the epithelium surface enterocytes as well as on the mononuclear cells in the lamina propria mucosa.\textsuperscript{76,77} IL-15 promotes the survival, activation and proliferation of IELs, independently of TCR interaction, besides controlling the clonal expansion TCR\textgreek{y}\delta IELs and of cells bearing NKG2D receptors\textsuperscript{78,79}, whose ligands are MICA molecules (MHC-I-non-classical) expressed by enterocytes.\textsuperscript{76,77,80} In addition, IL-15 favors a NK-type reprogramming of IELs by activating intracellular perforin/granzyme signaling cascades as well as and Fas/FasL, which contribute to trigger inflammation and cytotoxicity on the enterocytes.\textsuperscript{75,78,81} IL-15 favors immune response feedback by inducing the secretion of mediators of non-specific inflammation, such as arachidonic acid and leukotrienes, by the IEL. It also induces the formation of the inducible Nitric Oxide Synthase enzyme (iNOS)\textsuperscript{67, 71} by stromal cells from the lamina propria by a means of a mechanism dependent on the NF-\textkappa B transcription factor, which favors the presence of oxygen-reactive species and oxidative stress. Finally, IL-15 contributes to the weakening of the tight junctions\textsuperscript{9}, with increased intestinal permeability and the passage of gluten the lamina propria mucosa. In CD pathogenesis, IL-15 acts as a mediator between the innate response and the epithelial lesion besides promoting the survival of specific T lymphocytes and the maintenance of the inflammatory response\textsuperscript{82} (Figure 2).

In Refractory CD (RCD), the survival, expansion and acquisition of the NK phenotype by IELs is much more pronounced than in classical CD, possibly resulting from the presence of large amounts of IL-15. In type II RCD, patients have an aberrant clonal population of IELs that lose surface expression of TCR CD3. In studies using lines of aberrant IELs from patients with type II RCD, it has been observed that, under stimulation with IL-15, these cells express granzyme B and are capable of lysing the HT29 epithelial cell line, suggesting a role for aberrant IELs in the continuous epithelial damage in seen in RCD II.\textsuperscript{83} Therefore, the NK transformation suffered by IELs via IL-15 is an essential step in the immunopathogenesis of RCD.

Gliadin might have a direct toxic effect on the intestine and the induction of a gliadin dependent innate immune response in the duodenum would not be unique to patients with CD. In Caco-2 cell lines, gliadin stimulation induces an apoptosis increase and transepithelial permeability.\textsuperscript{84} Gliadin-induced CD maturation has been described in mice, as well as quimiocin release.\textsuperscript{85} In enterocyte cell lines, gliadin and the derived peptides 13-and 33-mer increase zonulin dependent intestinal permeability\textsuperscript{9} and also the expression of proinflammatory genes and cytokine secretion in macrophage lines.\textsuperscript{86} Unlike other dietary proteins, gliadin can also induce expression of maturation markers and the release of cytokines and chemokines in DCs, through an NFkB-dependent mechanism.\textsuperscript{85} In this context, it has been suggested that gliadin may be a nonspecific IL-15 inductor in the duodenum in both CD patients and non-CD individuals.\textsuperscript{87} Recent studies indicate that the enterocyte apical membrane can recognize gluten fragments through the CXCR3 chemokine receptor.\textsuperscript{88} Furthermore, some APCs such as monocytes, macrophages and DCs, can recognize gluten through the TLR4 pattern recognition receptor.\textsuperscript{86,89} Remarkably, the intracellular signaling cascade in both mechanisms (CXCR3 and TLR4) converges on the myeloid differentiation factor (MyD88).\textsuperscript{88} The role of these receptors in the context of the intestinal innate immune response and whether they are the only receptors involved in this response remain open questions.
3.4. Beyond the Innate Immune Response: IL 15/IL 15Rα Interaction

Although the effects of IL 15 are traditionally considered to be associated with innate immunity, they are also important in the induction of adaptive immunity, which is particularly evident in CD, where, besides the innate effects like NK-like reprogramming of IELs\textsuperscript{78,79,81} or stress molecule/MICA induction enterocytes\textsuperscript{72}, it can also act as a clear nexus between both types of immune responses being a potent DC activator\textsuperscript{90,91}, and so also to the specific CD4+ T lymphocytes. IL 15 thus becomes the initiator of the clonal expansion and the Th1-type immune response manifested by intraepithelial lymphocytosis, crypt hyperplasia and villous flattening.

The IL-15 receptor shares two subunits with the IL-2 receptor: the common γ chain and the IL 2Rβ subunit.\textsuperscript{91,92} The former is also shared with other cytokines (IL 4, IL 7, IL 9 and IL 21) each of which have other specific sub-units responsible for the binding specificity and, thus for posterior signaling.\textsuperscript{93} However, despite this similarity in the receptor, IL 15 and IL 2 have very different roles. Thus IL 2 appears to be a key modulator of the T lymphocyte dependent adaptive immune processes, while IL 15 has a much wider range of action, although focused primarily on the innate response.\textsuperscript{90} The receptor’s subunit, IL 15Rα, is responsible for bestowing ligand specificity. In fact, IL 15 has a high binding specificity to the IL 15Rα receptor, to type I transmembrane protein, even in the absence of IL 15Rβ and IL 15Rγ/γc subunits.\textsuperscript{92} Messenger RNA levels from IL 15Rα have been detected on a wide variety of cellular systems, immunological as well as non-immunological\textsuperscript{92,94}, suggesting a complex regulatory mechanism as well as that IL 15/IL 15Rα signaling can interrelate various cell systems.\textsuperscript{9,5} Furthermore, IL 15 is able to positively modulate IL 21, another cytokine involved in CD.\textsuperscript{96}

Recent studies have found that the duodenum of CD patients exhibits increased levels of the IL 15 receptor (IL 15R) compared to the intestine of non-CD patients. The fact that higher levels of IL 15R are maintained even after complete normalization of mucosal histology in patients treated with GFD suggests that it is a pre-disposing factor in the development of this pathology. Such high levels of IL 15R confer CD patients a lower immune response threshold to IL 15.\textsuperscript{97,98} This immune mechanism based on a lower threshold for IL 15 in CD patients may be key in the pathogenesis, as it facilitates the connection between the establishment of an innate immune response to gluten, and an adaptive immune response against this protein, which prevents the development of oral tolerance mechanisms.

3.5. Adaptive Immune response to gluten

Tissue transglutaminase (TG2) is a widely distributed enzyme in the body, whose main function is to catalyze the modification of proteins by transamination or deamination. In CD, TG2 has a fundamental role in the pathogenic mechanism through the enzymatic modification of immunodominant gliadin peptides, which increases their affinity for the HLA-DQ\textsuperscript{99} molecule but is also the main (auto) antigen for specific serum antibodies, which are of great value for diagnosis.\textsuperscript{100} In patients with active CD, TG2 is expressed in the epithelial brush border and in the subepithelial zone of the lamina propria area mucosa.\textsuperscript{101} The main TG2 exogenous substrate is gliadin, which contains positively charged amino acids. TG2 induces ordered and specific glutamine residue substitution for negatively-charged glutamic acid residues\textsuperscript{100}, which promotes interaction with other basic amino acids located in anchoring positions to the HLA-DQ2 and DQ8 molecules, and increases their ability to stimulate CD4 T lymphocytes+.\textsuperscript{101,102} The enzymatic
Celiac Disease and Non-Celiac Gluten Sensitivity

Modification which unMASKS the most Immunogenic epitopes to gliadin and other prolaminS, OR gives rise to new ones through interaction with extracellular matrix proteins, could be responsible for the loss of tolerance and the appearance of autoimmune diseases. 103,104

Gliadins are a heterogeneous mixture of more than 40 components which contain multiple immunogenic peptides against which patients show different sensitivity and even a single patient may respond to more than one. Immunodominant peptides, such as those of from the α-gliadin region 57,75, induce specific immune responses in virtually all patients. 105-107 The major epitopes on α- and γ-gliadins, as well as on glutenins, have been identified; many bind to HLA-DQ2 and DQ8 others and, in most cases, TG2 deamidation shows an increased antigenicity, except for glutenin derivatives. 101,102,108 The richness of glutamine and proline, and their location in the primary structure influences peptide immunogenicity by determining the molecular structure and acting as preferential binding residue in the HLA-DQ molecule motifs as well as controlling TG2 specificity, which acts on glutamine residues at positions adjacent to those of proline in QXP-type sequences but not in QP or QXXP (Q=glutamine, P=proline, X=other). 1,107,108 By means of algorithms based on the separation these residues and through the presence/absence of other amino acids, it has been possible to predict more than the existence of more than 50 immunogenic peptides in wheat gluten, hordeins and secalins and which are nearly absent in avenins. 108

In active CD, there has been an increase in the passage, through the epithelium, of both toxic as well as immunogenic fragments. 109 Incomplete intraluminal gluten digestion can generate residual fragments, such as the 33 amino acid peptide of α-gliadin 71,110, whose glutamine and proline content confers resistance to proteolysis by digestive enzymes, favoring the formation of large fragments with several immunodominant T epitopes, which are the preferred substrates of TG2. 111 The bacterial enzyme prolyl-endopeptidase (PEP) induces the rapid degradation of this fragment and prevents the formation of T epitopes able to activate the immune response harmful to the intestine. 110

Adaptive immunity mediated by specific T lymphocytes requires that the antigen presentation to T lymphocytes of the lamina propria be performed by APCs bearing the HLA-DQ2/DQ8 restriction element. The HLA-DQ2 and DQ8 molecules confer susceptibility through their main function, that is to say, to present small gluten peptides to the intestinal CD4+ T lymphocytes in the APCs membrane, but which could also modulate the development of the repertoire of T lymphocytes in the thymus. 112 The CD4+ T lymphocytes recognize gliadin peptides in the DQ2/DQ8 molecule context that bind peptide fragments with negatively charged amino acids in certain positions of the structural binding motifs, located in the central position (4th, 6th, 7th) to HLA-DQ2 and outermost (1st, 4th, 9th) for HLA-DQ8. 104,112 The fact that, in each peptide, the residues are deaminated in different positions suggests that the specific immune response to gluten could be generated against various pathogenic causes.

The main APCs from the lamina propria mucosa are the macrophages (20%) and, especially, the DCs (80%). The DCs come mostly from extravasated monocytes recruited to the inflamed mucosa, where they differentiate in situ. 30,35 In active CD, there is an increase of APCs, mainly DCs expressing activation markers on the surface. These DCs, with a HLA-DQ2+ CD11c+ CD68-CD1c-BDCA3-phenotype play a central role in the activation of memory T lymphocytes reactive to gluten that accumulate in the small intestine of CD patients and which are ultimately responsible for tissular injury. 35,113 APCs can also be activated as a consequence of IL 15 released during the innate response induced by gluten. 114,115 In an animal model, digested wheat gluten was observed to induce maturation of DCs, along with the expression of costimulatory molecules and
chemokine secretion. In CD, a rapid accumulation of CD14+CD11c+ DCs can be seen preceding structural changes, indicating that this subtype is directly related to the immunopathology of the disease. The expression of CCR2 and CD14 in these cells may indicate that they are monocytes extravasated from peripheral blood.

CD4+ T lymphocytes in the lamina propria mucosa recognize gliadin peptides such as the 33-mer (fragments 56-88 of α-gliadin), modified by TG2 and presented by HLA-DQ2 or DQ8 molecules by DCs, leading to response dominated by Th1 profile cytokines with IFNγ predominance and other pro-inflammatory cytokines (TNF, IL18, among others) and a proportional decrease of regulatory or anti-inflammatory cytokines (IL 10 and TGFβ). This pro-inflammatory profile will be ultimately involved in the tissue remodeling mechanisms.

The presence of gluten specific CD4+ T lymphocytes has been confirmed in the lamina propria of the small intestine mucosa in CD patients, from which gluten specific cell clones were obtained. These cells express the αβ T lymphocyte receptor (TCR) and a CD45RO+ memory cell phenotype and, after stimulation, they produce Th0/Th1 type cytokines, predominantly IFNγ but with absence of interleukin-12 (IL 12), a pattern which disappears under remission. The increased production of Th1 cytokines is related to with delayed hypersensitivity reactions and autoimmune phenomena and, in functional studies, it has been shown that activation of these cells is associated with extracellular matrix alterations in the lamina propria and the epithelial proliferation.

The differentiation of CD4+ T lymphocytes predominantly towards a Th1 or Th2 phenotype of cytokine production depends on the nature and concentration of the antigen, APC type and the local cytokine concentration. An alteration in the cytokine balance could explain findings in the celiac intestine where an abnormal or uncontrolled Th response against gluten could lead to intestinal inflammation and damage. However, the absence of the main Th1-inducing factor (IL 12) suggests that the differentiation of Th1 effector cells could be related to other cytokines, among them, interferon-α (IFNα) or interleukin-18 (IL 18), which share some of their functions with it. Additionally, other Th1-and IL 12-mediated enteropathies, such as Crohn's disease, show more severe damage with tissue loss and the degree of injury is related to the levels of Tumor Necrosis Factor-α (TNFα).

In the celiac intestine there could be an IFNγ increase along with an altered pro-and anti-inflammatory cytokine balance, such as the one between IFNγ and TGFβ. The epithelium and the lamina propria in a healthy intestine express TGFβ, but in CD, it decreases in the epithelium surface and disappears from the crypts, increasing in the lamina propria around macrophages and activated T lymphocytes, where there is no tissue destruction. IFNα can intervene in the differentiation of Th1 cells, promoting the production of IFNγ, and it has been observed that administration of IFNα in susceptible individuals may promote Th1 responses associated with hyperplastic injuries. Although yet to be confirmed, IFNα could be secreted by fibroblasts and activated macrophages or even by lamina propria DCs after an intestinal infection episode, which would contribute to inflammation rescuing apoptosis activated T cells, maintaining memory T cells after stimulus disappearance and increasing the expression of costimulatory molecules in local APCs. In contrast to IL 12, IL 18, produced by macrophages, DCs and epithelial cells, does not act on naive cells but on memory and effector cells, enhancing the IL 12 or IFNα dependent IFNγ expression. Under normal conditions, the intestine expresses IL 18, however, it increases in CD at the expense of its mature form requiring intervention of the Converter Enzyme IL 1β (ICE) or local proteases.
In active CD, there is an increase in plasma cells in the lamina propria, with a density two to three times higher in the celiac lesion\textsuperscript{121}, and CD is characterized by the presence of a variety of serum antibodies against self and foreign molecules.\textsuperscript{122} In 1997, TG2 was identified as the main autoantigen to antiendomysium antibodies.\textsuperscript{122} There have also been other different autoantibodies, including antibodies against actin-type proteins, different types of collagen and several members of the transglutaminase family: TG3, TG6, and Factor XII.\textsuperscript{124} It ought to be mentioned that complexes formed by IgA/TG3 have been found in the skin of patients with dermatitis herpetiformes\textsuperscript{57,123}, and the presence of antibodies against the TG6 neuronal enzyme has been related to ataxia.\textsuperscript{124} These findings could explain the development of extraintestinal manifestations in CD.

B lymphocytes are also professional APCs via BCR receptor. There are few virgin or memory B lymphocytes and most are plasma blasts or plasma cells from the lamina propria with low HLA class II expression.\textsuperscript{125} It is likely that B lymphocytes have a more important role as a APCs in the mesenteric lymph nodes in order to amplify of the T cell response to the gluten. B lymphocytes specific for TG2 would preferentially stimulate reactive T lymphocytes against peptides specific to deaminated gliadin, which would explain why antibodies against these peptides are good CD predictors.

### 4. Interaction between Innate and Adaptive Immunity to Gluten

Induction of the adaptive response in CD is tightly controlled by innate immunity. DCs not only recognize invading pathogens but decide what kind of effector response must be deployed. Clearly, with no signals from intestinal DCs, the gluten specific T lymphocyte response could not be triggered. Recently, using the THP-1126 human macrophage cell line, it has been demonstrated that gliadin is able to stimulate cytokine production and induce monocyte derived DC maturation.\textsuperscript{127} In other studies with \textit{ex vivo} tissue cultures it was observed that gliadin and the p31-43 gliadin derived fragment can induce IL 15 secretion\textsuperscript{67} and increase IEL cytotoxicity.\textsuperscript{78,79} IL 15 is particularly produced by activated DCs and other APCs, in such a way that DCs simultaneously intervene in two effector responses: adaptive (mediated by gluten specific CD4+ T lymphocytes) and innate (mediated by IELs).\textsuperscript{128,129}

IL 15 production by DCs dependent on the specific T lymphocyte response may explain why the innate response to gliadin is produced only in the duodenum of celiac patients and not in other individuals. A pro-inflammatory state of the mucosa would be an essential prerequisite for gliadin triggering the innate immunity. It is still unknown by what means the mechanism by which gliadin, and especially the p31-43 fragment, is able to directly stimulate IL 15 production, although recent studies suggest that TG2 may play an important role in this process.\textsuperscript{68}
5. Gluten Transport across the Epithelium

Under normal conditions, protein peptides are hydrolyzed in the intestinal lumen leading to smaller peptides or isolated ones isolated amino acids by means of gastric, pancreatic and intestinal peptidases and also from the brush border before transepithelial transport to the lamina propria mucosa. Incomplete intraluminal gluten digestion originates residual fragments such as the one at the 57-75 position of α-gliadin, resistant to enzymatic proteolysis due to its content of glutamine and proline, which includes several immunodominant T epitopes. Due to their large size, gluten peptides like 33mer are not readily absorbed through the normal mechanisms followed by dietary proteins. The principal theories state that gliadin could reach the lamina propria where the adaptive immune response takes place through two main routes: the transcellular route through enterocytes and the paracellular route through the Tight Junctions (TJs) between enterocytes. A third possibility involves direct gluten access to the lamina propria gluten due to direct uptake by DCs. However, lack of studies that address this issue in model human biopsies make it difficult to elucidate the subject.

The great majority of dietary proteins are absorbed, as simple amino acids or small peptides, through the intestinal epithelium by transcellular transport. This process involves endocytosis mechanisms in the apical membrane and, in transit to the basal membrane, endosomes are generally conjugated with lysosomes carrying, in turn, more proteases, which facilitates complete peptide degradation. However, the antigenic structure of gliadin favors differential transport within the enterocytes which may associate lysosome evasion, reaching the lamina propria in an immunogenic context. Several studies support this possibility and it has been observed that, in CD patients, there is a high level of transport from the enterocyte apical membrane to the basal membrane by an IFNγ dependent mechanism. IFNγ weakens the intestinal barrier, promoting the internalization of TJs, and in a Caco-2 cells model, it has been observed that stimulation with IFNγ is associated with increased translocation of the 33mer peptide.

Recently, another transepithelial gliadin transport mechanism has been identified which would be mediated by the CD71 transferrin receptor. This CD71 receptor is overexpressed on the apical surface of the enterocytes in active CD and it binds to the secreted IgA. Transcytosis experiments performed ex vivo suggest that CD71 can mediate the transport of IgA-gliadin complexes, and in patients with active CD, IgG-gliadin complexes have also been found. Given the fact that the neonatal Fc receptor (FcRn) is expressed in epithelial cells of the human intestine and that it can mediate apical to basolateral transcytosis of IgG-antigen immunocomplexes, FcRn could also transport antigens across the epithelial barrier by transcytosis of immunocomplexes formed by IgG anti-gliadin and gliadin.

The P31-43 peptide can produce two major effects on the alteration of intracellular vesicular traffic: it modifies recycling of the IL 15/IL 15Rα complex, which favors innate immunity overexpression and activation, it also increases enterocyte proliferation in the crypts through cooperation between the IL 15 receptor and the epidermal growth factor (EGFR), with consequent remodeling of the duodenal mucosa. Moreover, peptide accumulation in the enterocyte lysosomes activates the innate response via ROS-TG2, TG2 then acting as an activator of proteasomic ubiquitination of degradation leading to mucosal inflammation, decreasing the expression of the PPARγ molecule.
Celiac Disease and Non-Celiac Gluten Sensitivity

The intestinal lumen proteins can pass inside by means of paracellular transport between enterocytes. Intestinal permeability is increased in celiac patients due to alteration of TJs between enterocytes, compared to non-celiac control subjects. This finding appears to have a genetic component as also seen in non-affected relatives of celiac patients. This, however, by itself does not explain the massive peptides traffic produced in active CD. Another possibility involves active gliadin effect on intestinal permeability favoring its weakening. Gluten is recognized in the apical membrane of enterocytes through the CXCR3 chemokine receptor, which promotes the paracrine secretion of the zonulin protein. When it is recognized by adjacent enterocytes, zonulin triggers an intracellular signaling cascade which implies the reorganization of its cytoskeleton favoring TJ joints decoupling between enterocytes. Therefore, gliadin, besides acting indirectly through IL 15, may also induce the opening the TJs, which destroys the integrity of the epithelial barrier and makes it possible that the larger peptides have easier access to the lamina propria.

6. Inflammatory Mechanisms in Celiac Disease

The presence of inflammatory mechanisms in the lamina propria is not enough to trigger tissue damage. None of the known cytokines involved in CD is ultimately responsible for the injury mechanisms, as mediator molecules are released as a consequence of the innate and adaptive responses, or as it seems likelier, as a result of the interaction between these two. Intestinal inflammation and damage are usually the result of the interaction between lymphoid and non-lymphoid cells that release different mediators, many of which are non-specific, able to interact and amplify signals culminating in intestinal mucosa tissue damage. Non-specific mechanisms are mediated by an innate immune response that does not require antigen presentation and therefore T lymphocyte intervention. Transcription factor NF-κB plays a major role in such responses, and among its many effects, enterocyctic IL 15 secretion is included, as in the case of CD. The IL 15 cytokine, the main innate immunological response, is itself a factor of positive feedback for the signal that induces the expression of NF-κB in adjacent cells. Another NFκβ effect is the induction of the iNOS (inducible nitric oxide synthase) enzyme, whose presence in the lamina propria is an oxidative stress factor that affects NF-κB re-induction and the maintenance of an inflammatory response.

NF-κB also has a key role in the connection between innate immunity and adaptive immunity. The DCs, which initiate adaptive immunity in the lamina propria by antigen presentation to gluten-reactive CD4+ T lymphocytes, need the activation of this transcription factor in order to increase the membrane expression of HLA (DQ2/8) molecules and of costimulatory ones (CD80/B7.1, CD86/B7.2, CD83) and thus, carrying out the antigen presentation function. Furthermore, these cells can be activated by innate immunity cell populations, such as NK, iNKT and/or Tγδ, which in turn are activated by stress signals induced in an innate immunity context. Thus, DCs act as a sensor capable of uniting both innate and adaptive responses which, once activated, would also stimulate growth and function of these innate immunity and the swift production of perforins and granzymes, besides being an IFNγ source. Both feedback loops formed by the interaction between innate lymphocytes/DCs and the NFκB/IL 15-iNOS system activation, would help maintain the state of stress in the intestinal mucosa. Stromal fibroblasts are also susceptible to the local stress microenvironment (presence of nitric oxide, IFNγ, IL 15, etc.). As a result, these cells secrete the keratinocyte growth factor (KGF) to the
lamina propria, which seems to be involved in crypt hyperplasia, characteristic of a type II Marsh lesion. There is also an increase in the expression of adhesion molecules on the vascular endothelium and chemokines synthesis, which contribute to the recruitment of inflammatory cells, and the synthesis of metalloproteinases (MMPs) along with the blocking of their tissue inhibitors (TIMP-1). MMPs are an endopeptidase family whose primary role is the degradation of extracellular matrix components (such as proteoglycans and glycoproteins) and mucosal destruction, which is manifested, according to their severity, as type III forms of destructive Marsh lesion. In the inflamed gut the expression of some MMPs increases, and in CD there has been described a correlation between non-specific inflammation mechanisms, as the levels of MMP-12 expression, and the presence of IFNγ, with the degree of mucosal injury.

7. Celiac Disease and Intestinal Microbiota

In patients with CD alterations in the intestinal microbiota have been detected, which are characterized by an increase of Gram-negative bacteria and reduced bifidobacteria. Recent studies have found differences in the fecal microbiota of patients with untreated CD, which are partially restored after GFD. Specific components of the intestinal microbiota can influence maturation of dendritic cells in terms of phenotype and function, as well as their interactions with epithelial cells. This would define the role of dendritic cells in the disease’s progression. However, further studies are required to explain how these changes in the intestinal flora may affect the pathogenesis and prognosis of CD.

Preliminary results from our group suggest the presence, in the intestinal protein extract, of 7 bands with specific gliadinase activity which are metalloproteasic in nature and can involve microbial activity. This could be a differentiating factor would allow to identify, with a confidence of more than 90%, whether the duodenal explant originated from a celiac patients (active or in remission), or from a non-EC control patient. Available data do not allow us to affirm that the different bacterial populations recently described in the duodenum of celiac patients are the carriers of these gliadinases. However, the fact that this enzymatic activity has not been found virtually on no non-celiac individuals seems to indicate that the bacterial population and activity may participate in the pathogenesis of CD.

8. Some Unresolved Issues

First, elucidation is still needed on how immunogenic gliadin peptides pass from the intestinal lumen to the lamina propria in the early stages of CD. It has been suggested that peptides can be transported over an increased intestinal permeability secondary to a viral intestinal infection, or by means of IgA-mediated retrotranscitosis.

Second, the p31-49 peptide of the α-gliadin has a direct effect on the intestinal epithelium. However, although this seems clear, it is still unknown how it is produced and how it contributes to the development of CD.

Third, the TG2 is a crucial factor in antigen presentation of gluten derived peptides. In basal conditions, TG2 is expressed intracellularly in an inactive form or on the cell surface. It still not known how TG2 is activated and released in CD. It has been proposed that TG2 is released after
tissue damage induced by the initial response of T lymphocytes to unprocessed gluten peptides. Another non-exclusive possibility is TLR3 activation by its ligands during an enteroviral infection which can result in TG2 activation.²⁴⁸

Fourth, in active CD, the breakdown in IL 15 regulation leads to massive overexpression of IL 15, although it is unknown how this occurs. GFD has a direct effect on the decreased expression of IL 15 together with a decrease of the adaptive response mediated by CD4+ T lymphocytes, therefore, these cells may have a direct effect on IL 15 expression. Another possibility is that the signals derived from the innate immune response through TLRs may be responsible for the elevated IL 15 levels.⁸³
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