Immunopathogenesis of Celiac Disease

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1 Introduction

The mucosal associated lymphoid tissue is the largest component of the immune system of the body, due to its central role at the interface with the external environment and shows the ability to discriminate between infectious agents and commensal bacteria and food antigens. Under normal conditions, homeostasis at mucosal surfaces depends on mechanisms mediated by secretory IgA antibodies and tolerogenic T and B cells, limiting the entrance of pathogens by immune exclusion, and inducing responses of oral tolerance. After the absorption of food proteins through the intestinal epithelium, lamina propria (LP) antigen presenting cells (APC), particularly dendritic cells (DCs), transport these antigens to draining mesenteric lymph nodes, where they promote gut-homing T-cell responses. Regulatory T cells are responsible for the inhibition of inflammatory responses to food antigens during oral tolerance [1, 2]. In celiac disease (CD), however, dietary gluten drives a T-cell mediated immune response leading to the destruction of the epithelium in the small intestine.

Dietary Gluten and Proteolysis of Gluten Peptides

The wheat protein fraction can be classified, according to their structural properties and solubility, in α, γ, and ω-gliadins (monomeric, alcohol soluble), as well as low- and high-molecular weight glutenins (polymeric, soluble under stronger conditions), though the term gluten is currently used to identify proline- and

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glutamine-rich proteins contained in the storage fraction of wheat, barley, and rye grains, collectively referred as prolamines [3, 4]. Under normal conditions, proteins are mostly hydrolyzed by gastric, pancreatic and intestinal brush border proteases, resulting in the formation of smaller peptides or isolated amino acids, which may cross the epithelium more easily. However, the high proline content of gluten proteins makes them resistant to proteolysis, and long fragments containing from 15 to 50 residues are generated in the intestinal lumen [5, 6].

Partially digested gluten peptides, which include several copies of immunogenic epitopes, may cross the intestinal epithelium and translocate into the LP by different pathways, either paracellular, through the tight junctions between enterocytes [7, 8]; or transcellular, by a mechanism involving enterocyte endocytosis and lysosome degradation, which is altered in CD and may allow intact peptides to cross the epithelium [9–12]; but also by the mechanism of retrotranscytosis, which depends on peptide binding to secretory immunoglobulin A1 (SIgA1), a transferrin receptor (CD71) ligand, overexpressed at the apical epithelial membrane in CD mucosa [12, 13]; or following the interaction with C-X-C chemokine receptor type 3 (CXCR3) expressed by enterocytes, leading to the release of zonulin, a potent modulator of the intestinal barrier function [14]. Finally, gluten peptides can reach the LP by direct access through extensions of monocyte-derived DCs, which are sandwiched between epithelial cells [4].

Other Environmental Factors

Only a small percentage of individuals carrying CD-predisposing HLA.DQ alleles actually develops T cell mediated immunity and tissue damage after the ingestion of gluten, and this suggests that other non-MHC genetic variants (most of them shared with other autoimmune diseases) and/or other environmental factors may be also involved in the pathogenesis of CD [15]. Among these environmental factors, microbiota and/or intestinal infections are most cited. The microbiota has an important role in the maintenance of intestinal homeostasis, by promoting epithelial integrity and the generation of tolerogenic cells, and this is mainly mediated by bacterial metabolism of dietary susbstrates with immunomodulatory effects, such as small-chain fatty acids (SCFAs) [16–18]. In this context, the large gluten peptides generated by partial digestion in the small intestine of CD patients are a good substrate for bacterial metabolism.

The changes in the duodenal and faecal microbiota reported in patients with CD have been characterized by the decrease in beneficial species (*Lactobacillus* and *Bifidobacterium*), the expansion of *Proteobacteria*, and the proliferation of opportunistic pathogens, such as *Neisseria*, *E. coli*, or *Pseudomonas* [19–21]. These changes in the composition of gut microbiota could play a role in increasing the permeability of the epithelial barrier [22], but commensal bacteria may also affect the immunogenicity of peptides by modulating the metabolism of gluten and its proteolitic activity. It has been reported that *Lactobacillus*, which is depleted in CD patients, can degrade and detoxify partially-digested immunogenic peptides, whereas the opportunistic pathogen *Pseudomonas aeruginosa* metabolizes these large gluten fragments generating shorter highly-immunogenic peptides that cross the epithelium more easily [23]. Moreover, *Pseudomonas* also triggers a

pro-inflammatory response in intestinal epithelial cells, mediated by the upregulation of protein-activated-receptor-2 (PPAR-2) [24]. Finally, the immunogenicity of gluten peptides can be also reduced by the effect of transglutaminase from *Streptomyces mobaraensis* [25].

Enteral infections by viruses such as Adenovirus, Enterovirus, Hepatitis C virus and Rotavirus, have been associated with CD [26]. The effect of Rotavirus infection may be mediated by changes in the permeability of the small intestine, leading to an increased passage of dietary antigens through the epithelium [26, 27]. It has been also suggested that viral infections may determine the upregulation and release of transglutaminase [28]. Moreover, the probably shared effect of some viruses in promoting the loss of tolerance to gluten, and the development of the disease, can be explained by the induction of type 1 interferons. The presence of elevated IFN-α levels in the mucosa of patients with CD may be a critical factor for the proinflammatory differentiation of DCs [29], as suggested by the onset of the disease in patients with hepatitis C treated with interferon (IFN)-α [30, 31].

Reovirus infections are common during early childhood, normally without clinical manifestations [32], but, when the infection occurs at the same time of first dietary intake of the protein, the virus may impair the induction of tolerance to food antigens by suppressing the generation of tolerogenic regulatory T cells, and driving a Th1 response to dietary antigens. In two studies published by the same group, using a HLA-DQ8-transgenic mice model, it has been shown that reovirus, and similar enteric viruses, may promote the disruption of immune homeostasis at inductive sites of oral tolerance (ie. mesenteric lymph nodes) by imprinting a proinflammatory signature in DCs, which involves the upregulation of the transcription factor interferon regulatory factor (IRF)-1 [33, 34].

2 Gluten-Specific Immunity

The predisposition to CD is associated to certain HLA-DQ allotypes, and T-cell mediated immunity is restricted by HLA class II molecules, due to its role in the preferential presentation of gluten epitopes to gluten-reactive CD4 + T cells [28, 35]. This response is determined in the organized gut-associated lymphoid tissue, such as mesenteric lymph nodes or Peyer´s patches, which are the induction sites of the immune response to gluten. Several gliadin and glutenin-derived T-cell epitopes, with different immunogenicity, have been identified and listed according to the definition of an specific T cell clone, the corresponding HLA-restriction element, and the nine-amino acid core of the epitope [36, 37]. Most gluten proteins are proteolyzed by intestinal enzymes, but a few large fragments remain undigested, containing several gluten epitopes which can elicit T cell responses. The differences in the immunogenicity of gluten peptides, and the selection of gluten T-cell epitopes, as occur in HLA-DQ2.5 positive CD patients, is determined by the resistance to proteolytic degradation, the substrate affinity to tissue transglutaminase (TG2), and the binding specificity to HLA-DQ molecules [38].

2.1 Deamidation of Gluten Peptides and Binding Affinity to HLA-DQ Molecules

The resistance of gluten peptides to proteolytic degradation by intestinal digestive enzymes depends on the high proline content of gluten proteins, and the resulting large undigested fragments are also rich in glutamine residues, which make these peptides a very good substrate for the enzyme TG2 [39–41]. TG2 plays a fundamental pathogenic role in CD by catalyzing the deamidation (posttranscriptional modification) of gluten peptides, and the enzyme is also the main antigen of anti-TG2 antibodies [42]. The enzyme recognizes specific glutamine residues in protein sequences of the type glutamine-X-proline (G-X-P), converting them into negative-charged glutamate residues [41]. Deamidation highly increases the binding affinity of gluten peptides to HLA-DQ molecules and, therefore, facilitates the subsequent recognition of these T-cell epitopes by gluten-specific CD4 + T cells [43–45].

It has recently been reported that the source of pathogenic TG2 is luminal TG2 derived from the renewal and shedding of enterocytes [46]. Under normal conditions, TG2 is a cytosolic enzyme, highly expressed in lymphoid tissue [47], though studies in mice have suggested that TG2 is not constitutively active in vivo [48], and other inflammatory or viral stimuli are required for the transient activation of the enzyme [49, 50]. TG2 may also have a role in the mechanism of retrotranscytosis of gluten peptides across the epithelium, following its interaction with the transferrin receptor (CD71) and secretory IgA at the apical surface of enterocytes [51]. Moreover, in this same study it was confirmed that the use of TG2 inhibitors have also the effect of blocking the transport of gliadin peptides through this pathway.

The HLA-DQ2.5 and DQ8 molecules associated to CD show distinct peptide binding groove preferences, according to the different T cell repertoire found in each CD patient. These molecules have a high binding affinity for deamidated peptides with negatively charged anchor residues in specific positions, as well as a high ability of generating stable peptide-HLA complexes. For example, the core structure of the HLA-DQ2.5 peptide pocket shows a preference for bingeing negatively charged glutamate residues at positions P4, P6 and P7 [43, 52], whereas the HLA-DQ8 molecule shows preferences for more external residues, at positions P1 and P9 [53]. The positions of glutamate residues are related to those of proline in the peptide binding groove, particularly for the HLA-DQ2.5 molecule [54, 55]. In HLA-DQ2.5 individuals, the immunodominant epitopes eliciting specific T cell responses are mainly found in α and ω-gliadins, whereas responses to γ-gliadins are less relevant [56].

2.2 Gluten-Reactive CD4 + T Cells and Proinflammatory Cytokines

The recognition of gluten epitopes by gluten-specific CD4 + T cells with HLA-DQ restriction leads to the synthesis of a pro-inflammatory cytokine profile

characterized by interferon (IFN)-γ and interleukin [IL]-21, as well as low levels of the immunoregulatory interleukin [IL]-10 and transforming growth factor (TGF)-β [57–59]. The small intestine of untreated CD patients also contains other cytokines, such as interleukin [IL]-15, interleukin [IL]-18 and interferon (IFN)-α [29, 60, 61]. These cytokines contribute to the development of the enteropathy, by promoting the activation of cytotoxic intraepithelial lymphocytes (IEL) and the destruction of epithelial cells, and by providing help to B cells to produce antibodies to gluten and TG2 [62–64]. By using a (DQ-D-villin-IL-15tg transgenic) mouse model overexpressing IL-15 in both the epithelium and the LP, it has been found that IFN-γ is required for the expansion of intraepithelial lymphocytes (IELs) and the development of villous atrophy. Moreover, in this model, the administration of TG2 inhibitors with dietary gluten prevented the lesion [65].

Recent technical developments have allowed the characterization of the T and B cell immune responses elicited by gluten**.** Gluten-reactive CD4 + T cells are found in the small intestine and peripheral blood from both treated and untreated CD patients [35, 66]. Gluten-specific T cells induced by oral gluten challenge, or expanded from the intestine of untreated CD patients, share the specificity for deamidated, immunodominant T cell epitopes, in both children and adults [67, 68]. Moreover, by using a HLA-DQ-gluten peptide tetramers, it was found that these cells were specific to four immunodominant epitopes of α- and ω-gliadins [69]. After activation, gluten-specific CD4 + T cells were clonally expanded in both the intestine and the peripheral blood of untreated CD patients, and these clonotypes persist in low levels for decades as memory T cells, with the same specificity, even on a gluten-free diet. These gluten-specific T cells undergo a rapid expansion and dominate the subsequent recall response after gluten challenge [69].

The TCR repertoire of gluten-specific CD4 + T cells is polyclonal with a biased use of TCR-Vα chain segments, probably reflecting their preferential interaction with HLA-DQ molecules [70, 71]. By using tetramers constructed with five gluten peptides complexed to HLA-DQ2.5, a small cluster of small intestinal CD4 + T cells was defined, and characterized by a distinct phenotype, similar to that found in peripheral blood from untreated HLA-DQ2.5 + CD patients [72]. These cells, mostly effector memory T cells (CD45RA-, CD62L-), expressed a number of activation markers, such as C-X-C chemokine receptor type 3 (CXCR3), CD38, CD161 and HLA-DR, but also the stimulatory checkpoint molecules OX40 and CD28, CD39, and the programmed cell death protein 1 (PD-1). Moreover, by RNA sequencing analysis, these cells transcribed also markers of follicular B helper T cells, such as CD200, CD84, C-X-C chemokine ligand type 13 (CXCL13) and IL-21, which may indicate a possible role in the differentiation of plasma cells in the inflamed tissue [72].

2.3 *Role of B Cells and Production of Autoantibodies*

Untreated CD patients produce specific antibodies, and serum antibody levels disappear after gluten withdrawal from the diet. The number of plasma cells In the intestinal LP of patients with active CD is highly increased, and a great proportion of these cells are involved in the production of IgA antibodies specific for gluten or TG2 or both [73, 74]. In active CD, there is a two to threefold increase of these antibodies in the intestinal lesion, and subepithelial TG2-specific IgA deposits have been found in all disease stages, even before the onset of symptoms, or before the intestinal lesion is confirmed [75, 76]. B cells are not only antibody-secreting cells, receiving help from T cells when both share the same antigen specificity, but they are also very efficient APC. Plasma cells have been confirmed as the dominant APC of immunodominant gluten epitopes in the intestinal LP of CD patients [74].

Under normal conditions, the intracellular location of TG2 may be responsible of preventing the induction of B cell tolerance to TG2, and the generation of autor-reactive B cells, which produce auto-antibodies after receiving appropriate T cell help [38, 77]. The initial proliferation of these cells take place outside the CD lesion, and the interaction between B and T cells occur once they enter the intestine. This interaction between gluten-specific T cells and TG2-specific B cells has been confirmed, both in vitro and in vivo [77]. The production of anti-TG2 antibodies depends on the presence of dietary gluten, and these antibodies are only found in individuals expressing HLA-DQ2.5/DQ8, which suggests the involvement of glu-ten-specific CD4 + T cells in this process.

Both the uptake of gluten peptides, and deamidation by active TG2, may depend on the formation of covalent TG2-gluten complexes (acting as hapten-carrier complexes) following B-cell receptor (BCR)-mediated endocytosis by TG2-specific B cells. The internalization of these complexes is linked to the presentation of deamidated gluten epitopes, bound to membrane-linked HLA-DQ molecules, to gluten-specific T CD4 + cells, because the efficiency of B cells as APCs is based on the epitope recognized by the BCR and the activity of BCR-bound TG2 [46]. Activated gluten-specific CD4 + T cells provide cognate help to gluten-specific B cells to differentiate into antibody-producing plasma cells [77, 78], but these CD4 + T cells also control the activity of cytotoxic intraepithelial CD8 + T cells [65, 72, 74]. Therefore, the mutual activation of T and B cells is manifested by both the production of autoantibodies and the release of a pro-inflammatory cytokine profile, leading to the amplification of the immune response to gluten.

3 Epithelial Stress and Tissue Destruction

In CD, mucosal tissue injury is the result of both innate [not necessarily driven by gluten) and adaptive immunity (gluten specific), and the intestinal chronic inflammation permanently reconfigures the tissue-resident TCRγ + IEL

compartment. Several gluten peptides, whose paradigm is gliadin peptide p31-42 (but also p31-49 and p31-55), have been identified with pathogenic effects related to the induction of innate immunity and epithelial cell stress, irrespective of CD4 + T-cells and the restriction by HLA-DQ2/DQ8 molecules (revised by Chirdo et al. [79]). These peptides seem to have a direct effect on epithelial cells, though they are not recognized by gliadin-specific T CD4 + cells.

In CD, intestinal IELs lose the expression of inhibitory CD94/NKG2A receptors, while increasing the expression of the activating receptors NKG2D and CD94/NKG2C. At the same time, epithelial cells increase the expression of their ligands MIC and HLA-E [80, 81]. Epithelial damage leads to an increased gut permeability, which may allow the passage of larger, partly-digested gliadin peptides, to the LP mucosa, where they are the target of TG2 and may be presented to gluten-specific T CD4 + cells in the context of HLA-DQ molecules, thereby triggering a positive feedback loop that maintains inflammation and the development of the lesion [28]. The expression of NKG2D is driven by the upregulation of IL-15 by epithelial cells, and cytotoxic IELs stimulated by IL-15 can mediate TCR-independent cytotoxicity [80, 82]. However, in CD in contrast to the adaptive immune response to gluten, innate immunity does not require the presence of gluten peptides, and it may be activated by other intercurrent factors as intestinal infections, dysbiosis or other aggressive events taking place at the intestinal mucosa [83].

3.1 Inflammatory Mediators and Epithelial Stress

The epithelial cell stress induced by gluten peptides is consequence of the activation of the NFκB pathway, and the extracellular TG2, following the inhibition of the chloride channel CFTR [84], as well as the alteration of vesicular trafficking and the activation of the NLRP3 inflammasome, as shown in murine models [85]. The result is the upregulation of IL-15, type I IFNs and other inflammatory mediators, and the release of reactive oxygen species (ROS) and intracellular Ca2 + , due to mitochondrial malfunction, as well as the induction of crypt cell proliferative, probably by acting on the epidermal growth factor pathway [79].

The mechanism by which gluten peptides interact with epithelial cells is still elusive. Several gluten peptides may use the chemokine receptor CXCR3, activating the My88D and NFkB pathways on epithelial cells, and increasing permeability by the release of zonulin, a protein that rearranges the cell cytoskeleton and modifies tight junctions [14]. Furthermore, the interaction of peptide p31-43 with the chloride channel CFTR has recently been described [84]. This membrane channel is involved in the adaptation of enterocytes to oxidative stress, and the interaction with gluten peptides may affect the function of the endocytic pathway in the epithelial cells [86]. This interference results in the activation of extracellular TG2 and the inflammasome NRLP3, as well as in the upregulation of IL-15 by epithelial cells.

The induction of innate immunity and epithelial cell stress responses leads to the upregulation of IL-15, cyclooxygenase (COX)-2, and the expression of CD25 and CD83 activation markers by LP mononuclear cells [87]. IL-15 has become the cornerstone in eliciting the intestinal mucosal injury in CD. The production of this cytokine is not confined to the epithelium, but it is also secreted by DCs and other APCs from the intestinal LP mucosa [88]. However, whether the upregulation of IL-15 induced by gluten peptides in the intestine is a specific phenomenon of CD is still a controversial matter [89].

On the other hand, the source of the stimuli for IL-15 production in CD does not exclusively derive from gluten peptides, as DCs produce also IL-15 in response to type I IFNs. These IFNs (particularly, IFNα) are produced in response to enteroviral infections, and they may participate in the pathogenesis of several immunologically-based diseases, such as Systemic Lupus, Rheumatoid Arthritis or Diabetes Mellitus type 1 [90]. Type I IFNs may have a central role in DCs reprogramming and the loss of tolerance to harmless (dietary) antigens. Particularly, in the intestine, type I IFNs may stimulate the production of IL-15 and IFNγ by DCs, activating antigen presentation and the corresponding adaptive immune response to gluten in the LP, but also the cytotoxic function of CD8 + TCRα and TCRγ cells and innate lymphoid cells (ILC) [65].

IFNα may be involved in Th1 cell differentiation by enhancing IFNγ production. It has been observed that IFN-α administration in susceptible individuals can induce a Th1 response leading to hyperplastic lesions [29]. Although not yet confirmed, IFNα may be secreted by activated fibroblasts and macrophages, and even DCs, in the LP mucosa after an episode of intestinal infection [31]. Moreover, it could contribute to intestinal inflammation by rescuing activated T-cells from apoptosis, maintaining memory T-cells once the stimulus has disappeared, and increasing expression of co-stimulatory molecules in local APCs [29]. IL-18 is a cytokine produced by macrophages, DCs and epithelial cells, which enhances the expression of IL-12- or IFNα-dependent IFNγ on memory and effector cells. Under normal conditions, the intestine expresses IL-18, but this increases in CD at the expense of its mature form, which requires the involvement of the IL-1β converting enzyme (ICE) or local proteinases [61].

Using two in vitro culture models in gluten-sensitive macaques, it has been observed that the IFNγ secreted by activated T-cells in the LP increases gut permeability and promotes immunoreactive α-gliadin (p57-89) peptide 33-mer passage across the epithelium [9, 91, 92]. According to the degree of intestinal inflammation, the paracellular pathway may also affects peptide transport across the epithelium, after binding to the chemokine receptor CXCR3, the activation of the MyD88 adapter, and the release of zonulin [14]. An increased mRNA expression of CXCL10 and CXCL11 has been found in biopsies from patients with active CD, as well as high serum levels of CXCL10 [93]. The study confirmed that CXCL10 is produced by plasma cells and epithelial cells, and its expression increases when IL-15 is present. The expression of CXCR3 is also increased in cells infiltrating the epithelium and LP mucosa, T cells and plasma cells [79, 93].

3.2 Intraepithelial CD8 + T Lymphocytes and Activating NK Receptors

IELs are increased in active CD [94], but these cells are not gluten-specific [95]. Moreover, after NK cell reprogramming, the TCR repertoire of IEL is extremely restricted. Cytotoxic IELs express NK receptors that recognize stress-induced ligands on epithelial cells, leading to the destruction of the small intestinal mucosa, independently from their TCR specificity [96]. In CD, there is an expansion of IELs expressing the activating natural killer receptor NKG2D and the heterodimer NKG2C-CD94, in the absence of inhibitory CD94/NKG2A receptors [80, 81]. These cells recognize non-classical MHC molecules MICA/B and HLA-E expressed by intestinal epithelial cells under stress, which are the main ligands for NKG2D and NKG2C, respectively [80–82]. It has been observed that cytokines from CD4 + T cells control the activity of cytotoxic IELs, and tissue destruction [65].

4 Integrative Model of CD Pathogenesis

Celiac disease develops as the result of a complex interaction between several innate and adaptive immune pathways that culminates in tissue destruction. Several elements, including gluten, TG2, HLA-DQ2, CD4 + T cells, IL-15 and cytotoxic IELs, are cooperatively involved in promoting the destruction of the intestinal epithelium by up-regulating the IFNγ response and the expansion of IELs with a fully activated cytolytic phenotype [65]. The immunopathology of CD is the result of the activation of gluten-reactive CD4 + T cells in the LP, with B cells probably acting as antigen-presenting cells, and stress-induced changes in epithelial cells, which are associated with the upregulation of IL-15 and the expression of non-classical MHC-class I molecules [95]. Cytokines produced by gluten-reactive CD4 + T cells, such as IFNγ and IL-21, upregulate HLA-E expression and increase cytotoxicity on NK and other cytotoxic cells, but also provide help to antibody-producing B cells.

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