Chapter 6
Using Animal Models of Celiac Disease to Understand the Role of MHC II

Eric V. Marietta, Alberto Rubio-Tapia, and Joseph A. Murray

Untreated CD patients develop increased numbers of intraepithelial lymphocytes, inflammatory infiltration of the lamina propria, intestinal permeability, and in a majority of cases, total or subtotal villous atrophy [1]. The trigger for the development of CD is gluten, typically derived from wheat, barley, and rye [2]. Gluten is a group of storage proteins found in wheat, rye, and barley. In wheat, gluten consists of two smaller subgroups of proteins, gliadins, and glutenins. In established CD patients, gliadin-specific T cells are more prevalent than glutenin-specific T cells [3]; however, there may be an initial stage of development for all CD patients in which their T cells respond to a greater number of epitopes [4]. A focusing phenomenon occurs later in which T cells in well-established CD patients are predominantly specific for epitopes that are a result of deamidation of gliadin by tissue transglutaminase [5]. CD is also strongly associated with HLA-DQ2 and HLA-DQ8, wherein 95 % are DQ2+ and most of the remaining are DQ8+ [6]. This contribution of MHC II is only 40 % of the familial risk, though, leaving 60 % of the genetic risk due to non-HLA genes [7]. This latter point is of great interest to the researchers that use animal models of CD for a number of reasons.

The first reason is that none of the spontaneous animal models seem to have a clear association with specific MHC II alleles, as is seen in human CD [8]. This may be interpreted in a number of ways. One interpretation is that MHC II does not play a vital role in the development of gluten-dependent enteropathy; however, years of clinical research have shown that the HLA molecules are necessary, though not sufficient, for CD to occur [9]. Indeed, crucial information has come from T-cell lines derived from the jejunum of CD patients. Studies with these in vitro models using DQ2 and/or DQ8 restricted T cells determined that these intestinally derived T cells

E.V. Marietta, Ph.D. • A. Rubio-Tapia, M.D.
Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, MN, USA

J.A. Murray, M.D. (✉)
Division of Gastroenterology and Hepatology, Mayo Clinic, 200 1st Street SW, Rochester, MN 55906, USA
e-mail: Murray.Joseph@mayo.edu

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are responsive to specific epitopes and that the process of deamidation causes some of these epitopes to be more immunogenic [10]. Therefore, one has to conclude that MHC II is necessary, but insufficient by itself to develop gluten-dependent enteropathy. This chapter will therefore highlight what we have learned from animal models of celiac disease about the interplay between MHC II molecules and non-MHC II molecules that results in gluten-dependent enteropathy.

Non-transgenic Animal Models

Spontaneous

Of the non-transgenic animal models there are two categories, spontaneous and induced. All of the spontaneous animal models are large animals and include nonhuman primates, dogs, and horses [11–18]. Of these three models, the role of MHC II has been clearly defined in only the dog model, and Polvi et al. determined that the gluten-dependent enteropathy in the Irish Setter pups was not associated with specific MHC II alleles [16]. The role of MHC II in the rhesus macaque (nonhuman primate) model of CD is not clear. In the first two publications on the rhesus model of CD, the authors stated that "the two gluten-sensitive macaques studied extensively herein and in the accompanying report, FH09 and FH45, are of genotype DRB1*0303(12), DRB*1007 at the Mamu class II DRB(1) locus" [11, 12]. This statement suggests that there may be an association of the gluten-dependent enteropathy in the rhesus macaques, but in their third publication, no reference was made to MHC II [13]. Therefore, it is still possible that specific MHC II alleles may be associated with the gluten-dependent enteropathy in the monkey model, but with time this possibility becomes less likely. The recent publication that characterized a single horse association with MHC is uncertain [18]. Of the three spontaneous animal models, then, MHC II molecules may be necessary for the development of gluten-dependent enteropathy, but specific alleles that exclusively contribute to the development of inflammatory gluten-responsive T cells do not appear to be present as is found in CD (humans).

Induced

The induction of enteropathy has been done with the rodent models of CD, but not the large animal models of CD. In one rat model, gliadin is administered by gavage immediately after birth to germ-free Wistar AVN rats, resulting in shortened villi, crypt hyperplasia, and increased numbers of intestinal CD8αβ+IELs [19]. A similar model was developed in 2012 using Balb/c mice [20]. In this model, Balb/c mice were fed a gluten-free chow for three generations, and then the third-generation

mice were fed a gluten-containing chow [20]. The gluten-challenged mice (G+) developed increased IELs, villous atrophy, and crypt hyperplasia as compared to untreated mice [G−].

There is also a mouse model of induction that uses the transfer of CD4+CD25^{-} CD45RB^{lo} cells from gliadin-sensitized mice to recipient Rag 1−/− mice [21]. The recipient Rag1−/− mice developed infiltration of the basal lamina propria, cryptitis, crypt abscesses, lymphocytic infiltration of the lamina propria, crypt hyperplasia, and villous atrophy.

Transgenic Animal Models

MHC II Molecules

Transgenic animal models have been used extensively to address specific questions about the pathogenesis of CD. So far, the large-animal models, especially the rhesus macaque model, have recreated most of the disease, but is lacking in the tight association of specific alleles of MHC II with the development of gluten-dependent enteropathy. Thus, transgenic mice have been used to determine the role of MHC II in the development of CD.

Transgenic mice that express HLA-DQ8 were the first HLA transgenic mice to be evaluated for gluten sensitivity [22]. These mice did not spontaneously develop gluten-dependent enteropathy, but did generate a strong T-cell response to gluten after intraperitoneal injection of gluten with Complete Freund's Adjuvant [22]. In another study, sensitization of these mice to DQ8-α-I, a gliadin-derived epitope that is DQ8-restricted, demonstrated that the T-cell receptor (TCR) repertoire induced by sensitization with native peptides had a heteroclitic (stronger) response to deamidated peptides [4]. This result suggested that the focusing of the immune response in CD over time against deamidated gliadin epitopes may be a consequence of the T-cell response to gliadin-derived peptides that are presented by DQ8 and presumably DQ2. These DQ8 transgenic mice also did not develop enteropathy after gliadin sensitization [4].

In a later study, these mice were sensitized parenterally to gliadin and later administered gliadin orally; they subsequently developed increased numbers of intra-epithelial cell numbers (IELs) [23]. This would demonstrate that DQ8 is necessary for the development of gluten-specific T cells, as well as generating T-cell responses against deamidated gliadin, but is not sufficient for the development of gluten-dependent enteropathy. Indeed, the sensitization protocols demonstrated that environmental factors have to also contribute in order for the development of mild enteropathy (increased IELs, villous shortening) to occur.

Other studies with transgenic mice that express DQ2 and DR3 provided the same results [24–26]. Therefore, initial studies with HLA transgenic mice demonstrated that CD is the result of a combination of contributing factors, including (but not

exclusive to) predisposing HLA genes, environmental factors, and non-HLA genes. Other studies reinforced the conclusion that strong T-cell responses to gluten or gliadin were not sufficient to generate gluten-dependent enteropathy and, in fact, further demonstrated that environmental and/or non-HLA genes are significant contributing factors. In one study, a transgenic mouse was designed to express a TCR that was specific for HLA-DQ2 presenting an immunodominant epitope of gliadin, and then this was bred with HLA-DQ2/DR3 mice [26]. Even these mice did not develop gluten (gliadin)-dependent enteropathy after gliadin sensitization and oral feeding of gliadin [26].

Since the above HLA transgenic models did not fully develop gluten-dependent enteropathy, a number of studies induced enteropathy through the administration of drugs or chemicals. This method does have precedence in the human system. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), causes enteropathy in humans, resulting in increased intestinal permeability and increased numbers of IELs [27]. Other drugs and chemicals have been used to induce enteropathy in the different mouse models described above to address the interaction of gluten-specific T cells with the IELs that mediate enteropathy.

Reagents used to induce mild enteropathy in rodents include polyinosinic/polycytidylic acid (poly I:C), indomethacin, and methotrexate. Cholera toxin is also used, but it breaks oral tolerance, as opposed to generating enteropathy [28]. Poly I:C has been used in a number of rodent studies of CD. The first instance was where (CBA×BALB/c)F1 mice were injected intraperitoneally with poly I:C diluted in 0.01 M NaOH. These mice developed significant villous atrophy and crypt hyperplasia by 1 day postinjection that began to resolve 3 days postinjection [29]. Because poly I:C induces the production of IFN-α/β in vivo, poly I:C administration also resulted in a significant increase in natural killer (NK) cell activation [29]. A later study found that the intestinal epithelial cells treated with poly I:C expressed higher levels of IL-15, allowing for the expansion of cytotoxic IELs (CD8αα+) that express NK1.1 [30]. A different study found that the increased IL-15 production by poly I:C treated epithelial cells induced the expression of NKG2D by CD8αα IELs [31].

NSAIDs include diclofenac and indomethacin, both of which increase intestinal permeability in rats [32]. So far, the only NSAID used in the mouse models of CD has been indomethacin. In two studies, intestinal paracellular permeability was found to increase with the administration of indomethacin to HLA-DQ8 transgenic mice [33, 34]. Sensitization to gluten, consisting of an injection of gluten intraperitoneally, followed by gavage with gluten, in addition to indomethacin treatment, resulted in increased intestinal permeability [33].

In a different study, HLA-DQ8 transgenic mice were administered indomethacin orally for 13 days after oral sensitization to gliadin using cholera toxin [35]. Villous height was decreased in the mice that were sensitized to gliadin and injected with indomethacin, but was not when the mice were only injected with indomethacin or only sensitized to gliadin. IFN-γ production was also increased with the combined treatment of indomethacin and gliadin sensitization.

Methotrexate, which is an anti-folate drug, induces mucositis for 3–5 days after treatment [36]. This intestinal inflammation is characterized by increased production

of the inflammatory cytokines TNF-α and IFN-γ, by lamina propria lymphocytes in the absence of any shortening of villi. A later study used methotrexate in combination with gliadin feeding of DQ2 transgenic mice [37]; however, no gliadin-specific T cells were generated using this protocol, suggesting that methotrexate treatment does not break oral tolerance established for gliadin in these mice.

Non-MHC II Molecules

There have been a number of GWAS studies that have identified non-HLA genes as contributing to the development of CD [38, 39]. None of these genes have been evaluated in animal models of CD. Instead, genes encoding other molecules that have been demonstrated to play a role in the development of CD have been used in animal models. These include TTG and IL-15.

Tissue transglutaminase type 2 (TTG) is the self-protein to which there is a clear autoimmune response in CD [40]. However, it is unclear as to whether it has a pathogenic role or is simply a marker of enteropathy [41]. One study determined that CD patients do have anti-TTG antibodies depositing in the small intestine of untreated celiac patients [42]. To determine if anti-TTG antibodies by themselves can mediate damage to the small intestine, a number of studies utilizing mice have addressed the roles of TTG and anti-TTG antibodies. Our group observed that significant increases in anti-TTG IgA developed with age, but that this did not result in significant changes in enteropathy (Fig. 6.1 and unpublished data).

One group analyzed the pathogenicity of anti-TTG antibodies by overexpressing in mice anti-TTG antibodies that had been isolated from intestinal lymphocytes of a CD patient [43]. To do this, they used recombinant adeno-associated virus (rAAV) and a sequence for an antihuman TTG antibody identified with a phage display library. The final miniantibody (MB) replaced the human Fc domains with those of mouse, and 10^{10} anti-TTG MB rAAV virions were injected into C57BL6 mice [44]. Four weeks after injection, anti-TTG antibodies were detected in the sera, and deposits were found in the muscle tissue that was injected; however, no deposits were detected in the intestine and no increases in intestinal permeability were detected. These results demonstrated that anti-TTG antibodies by themselves cannot mediate the development of enteropathy.

Two other studies used mice that had TTG knocked out [45, 46]. The untreated TTG−/− mouse had no major developmental abnormalities [45]. A later study using the same line of mice and poly I:C treatment demonstrated that the temporary enteropathy (villous atrophy) induced by poly I:C still developed in the TTG knockout construct [46]. This latter result further supports the hypothesis that anti-TTG antibodies are not a cause of, but are instead a consequence of, enteropathy.

In in vitro studies, gliadin has been found to generate the production of cytokines by cell types other than T cells. The monocyte is one of these cell types, although the receptor is not fully elucidated [47]. In this in vitro study, THP cells, a monocytic cell line, produced IL-8 and TNF-α in response to stimulation with

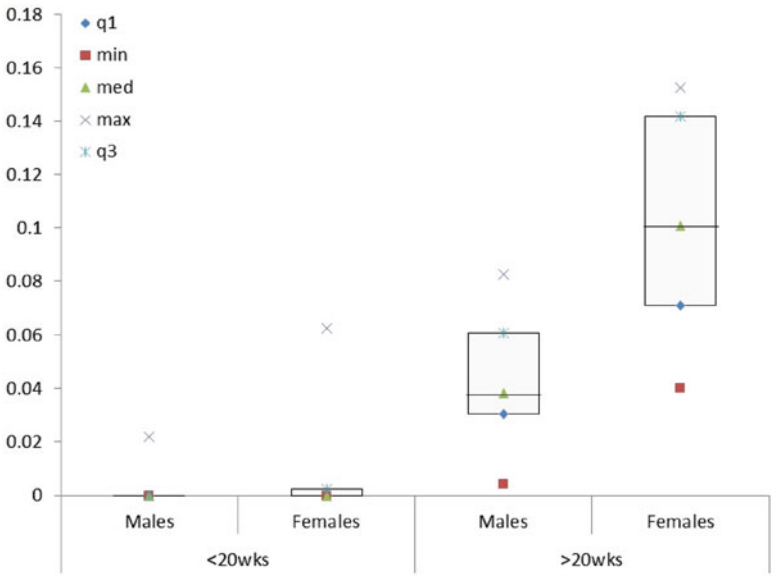

Fig. 6.1 Effect of age upon anti-TTG IgA serum levels in NOD Abo DQ8 mice. Forty-four NOD Abo DQ8 mice were evaluated for serum anti-TTG IgA using a guinea pig TTG-based ELISA. The units on the *y*-axis are Optical Density (OD) values

pepsin-digested gliadin in a manner that was independent of CD14 [47]. Our own studies have shown that gliadin-treated THP cells also express CXCL10 (T-cell recruiting chemokine) transcripts 2 h after stimulation with a peptic tryptic digest of gliadin, but not CXCL11 (Fig. 6.2a). This results in a significantly increased secretion of CXCL10 protein 24 h after stimulation with PTD gliadin (Fig. 6.2b).

Another cytokine, IL-15, has also been shown to play a significant role in the development of CD. CD patients have a higher expression of IL-15 by epithelial cells than normal controls [48]. This aberrant expression of IL-15 is thought to contribute to the expansion of cytotoxic CD8+ IELs that subsequently kill enterocytes [49]. To prove this, transgenic mice that overexpress IL-15 were used. So far, two different lines of IL-15 transgenic mice have been generated by two different groups [50, 51]. With the first IL-15 transgenic mouse that was generated, the mouse IL-15 gene was placed behind the promoter of a minimal MHC class I D^d gene (D gene of H-2^d haplotype) for expression throughout the mouse [51]. For the second IL-15 transgenic mouse generated, human IL-15 was placed behind an enterocyte-specific promoter (T3b) [50]. The phenotype of the D^d-IL-15 mouse without sensitization was the expansion of NK cells and memory CD8+ cells that resulted in a fatal leukemia that had an NK T-cell phenotype [50]. The T3b-IL-15 mouse developed increased numbers of CD8+ cells that infiltrated the small intestine as well as the development of anti-TTG IgA [52]. Neither of these models proved to develop enteropathy in a gluten-dependent manner; albeit they did develop enteropathy similar to that found in CD [53].

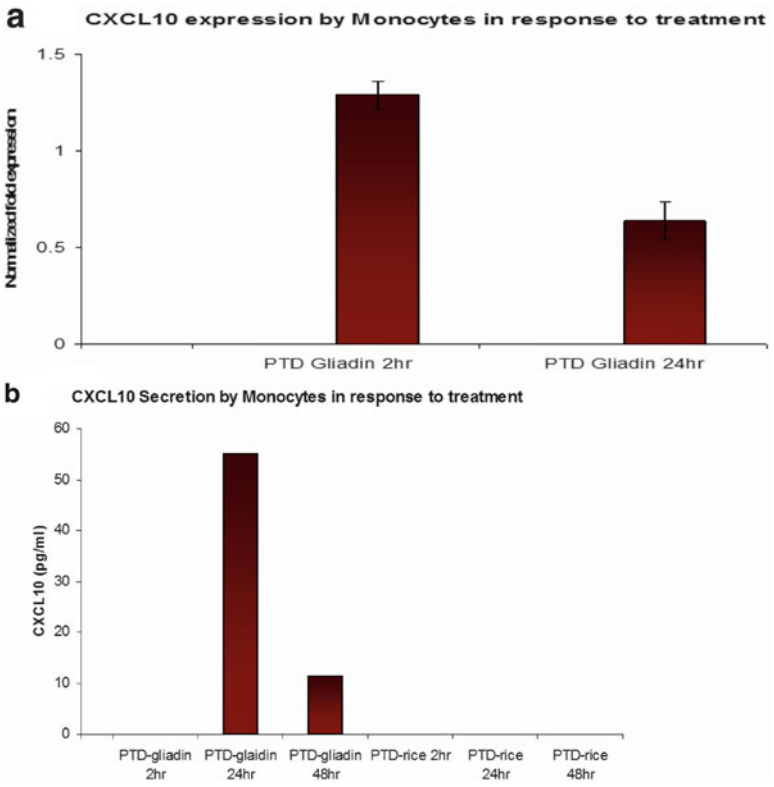

Fig. 6.2 CXCL10 expression and secretion. (**a**) CXCL 10 mRNA transcripts were present at 2 h post-gliadin treatment and significantly decreased 24 h after treatment with gliadin. (**b**) A significant increase in CXCL10 secretion occurred 24 h after THP monocytes were treated with a peptic tryptic digest of gliadin. Treatment with PTD rice did not result in detectable levels of secreted CXCL10

The above two IL-15 transgenic animal models demonstrate, then, that alterations to certain innate immune pathways, such as the expansion of activated IELs via IL-15, can by itself lead to autoimmune enteropathy. Indeed, autoimmune enteropathy in humans has many features that are similar with gluten-induced enteropathy (CD). These include almost exclusive small bowel involvement, villous blunting, and atrophy. Differences, though, are that only CD has increased numbers of IELs, and that only CD has a clear association with MHC II [54]. This, therefore, demonstrates that CD is a unique intertwining between gluten (gliadin)-reactive T cells and alterations to the innate immune system that lead to the development of enteropathy. For a true animal model of CD, these two phenomena need to not only be intertwined but essentially propagate each other.

Combining MHC II with Celiac Associated Non-MHC II Genes

One study approached this by crossing the D^d -IL-15 transgenic mouse with the DQ8 transgenic mouse, thereby combining gluten sensitivity with chronic enteropathy. The resultant IL-15 DQ8 transgenic mice developed increased numbers of CD3+ IELs in the absence of villous atrophy after feeding with gliadin [55]. Thus, this is the first animal model where enteropathy is gluten dependent (albeit without villous atrophy), and that is because both the genetic element and the perturbation to the innate system are chronic and not transient.

Testing MHC II-Based Novel Therapies

Because HLA transgenic mice are models that incorporate the celiac-associated MHC II molecules, any novel therapies for CD that are generated to target MHC II can be tested in these models. For example, these would include therapies that are based upon generating tolerance to alpha-gliadin and gliadin-derived peptides. In one study, recombinant alpha-gliadin was administered intranasally to Abo DQ8 mice before parenteral immunization with gliadin [56]. This resulted in a diminished T-cell response to gliadin. In another study, the probiotic-like bacteria *Lactococcus lactis* was bioengineered to secrete immunogenic gliadin-derived peptides in order to generate tolerance to those gliadin peptides [57]. In this study, DQ8 transgenic mice that were given these bacteria had a diminished delayed type hypersensitivity (DTH) response to the gliadin peptides but not control peptides, demonstrating that the administration of these bioengineered bacteria did suppress the immune response to the gliadin peptides in an antigen-specific manner.

Not Yet Evaluated by Animal Models

Although the animal models described above have provided extensive knowledge as to the pathogenesis of CD, there are some fundamental gaps that have not been addressed with animal models. These would include the roles of other molecules that have not been identified with GWAS studies but have been shown with in vitro models to clearly be playing a role in the pathogenesis of CD.

Probably, the most important set of molecules not yet addressed by animal models is zonulin and CXCR3. In a number of in vitro model studies, zonulin has been shown to be released by epithelial cells in response to gliadin binding to CXCR3, and that in turn loosens the tight junction proteins between the epithelial cells [58, 59]. This increased permeability then allows for the paracellular passage of even more gluten-derived peptides to enter the lamina propria and further propagate the

T-cell response to gluten-derived epitopes. The one animal model that has come the closest to addressing the roles of zonulin and CXCR3 in CD is the rhesus macaque model, in which gluten-dependent intestinal permeability was shown to be increased in the monkeys that developed celiac-like symptoms and was not seen in the monkeys that did not develop anti-TTG antibodies [11]. As of yet, no animal model has been generated that either overexpresses zonulin or knocks it out. There is a knockout construct of CXCR3, though [60, 61]. In a model of lung inflammation (short-term exposure to cigarette smoke), the numbers of infiltrating cytotoxic CD8+ cells were significantly decreased in the CXCR3 knockout mice as compared to the wild-type mice that were subjected to smoking [61]. The intestines of these mice were used in *ex vivo* experiments to determine that CXCR3 is the receptor for gliadin [59]. Neither paper explicitly addressed the architecture of the small intestines of the CXCR3−/− mice, so presumably these would have displayed normal villous height, etc. No models of overexpression of CXCR3 systemically or in the intestine have yet been generated.

Another molecule that has been shown to play a crucial role in the development of CD using in vitro models is CD71. This molecule is the receptor for transferrin and also binds to IgA antibodies at the cell surface. Once bound, it then transports the IgA with any bound ligand across the epithelial barrier into the lamina propria (transcellular transport). Previous studies have shown that gliadin-specific and/or TTG-specific antibodies bound to gliadin or TTG can then be transported to the lamina propria, where the proteins are then phagocytosed and presented to T cells by phagocytic antigen presenting cells. In the Balb/c model of gluten-dependent enteropathy, the CD71/IgA/gluten/tTG transcellular pathway was determined to be active [20]. In the Balb/c third-generation mice that were fed a gluten-containing chow (G+), CD71 expression was increased on the enterocytes as compared to the enterocytes of the (G−) mice, and IgA+ cells were increased in the lamina propria of the G+ mice as compared to the (G−) mice. No determination of the specificity of the IgA bound to the lamina propria cells was done, but CD71 and IgA did co-localize at both the apical and basal poles of the epithelial cells. This latter result strongly suggests that transcellular transport of IgA and bound proteins is occurring in the G+ mice. Because CD71 plays such a crucial role in the transport of iron, CD71 knockout constructs are embryonic lethal [62]. Still, constructs that overexpress CD71, especially a conditional expression, could provide more insight into the pathogenic role of this pathway in gluten-dependent enteropathy.

Conclusion

All of the animal models of CD have provided results that support the theory that the generation of gluten-specific T cells can arise independently of enteropathy (Table 6.1). The results from all of the animal models also suggest that there are a number of different MHC II alleles in all the species evaluated (dog, monkey, horse, rat, mouse) that can recognize gliadin-derived epitopes, but that in humans, only two,

Table 6.1 Animal Models of Celiac Disease

Animal models	Original characterization as celiac model
Horse	Van der Kolk, Vet Q, 2012
Dog	Batt, Res Vet Sci, 1984
Primate	Bethune et al., Plos One, 2008
Germ-free Wistar AVN rat	Stepankova, Scand J. Gastroenterol, 1996
Balb/c mice	Papista, Laboratory Investigation, 2012
Rag1−/− mice and cell transfer	Freitag, Gut, 2009
HLA-DQ2 transgenic mice	DeKauwe, J Immunol, 2009
HLA-DQ8 transgenic mice	Black, J Immunol, 2002
TTG mini antibody mice	Di Niro, Molecular Immunol, 2008
TTG −/− mice	De Laurenzi, Mol Cell Biol, 2001
IL-15 transgenic mice	Yokoyama, J Clin Immunol, 2011
IL-15-DQ8+ transgenic mice	DePaolo, Nature, 2012

DQ2 and DQ8, can give rise to inflammatory T cells that respond to deamidated gliadin epitopes. The animal models have also demonstrated that CD is a unique intertwining of the adaptive and innate immune responses to gliadin that gives rise to a self-propagating immune response in the presence of gliadin. What needs to be further examined (and determined) in CD using animal models is what factors are necessary for this intertwining. The need to use poly I:C, indomethacin, methotrexate, and cholera toxin in the animal models to induce intestinal inflammation along with gluten-specific T cells to generate enteropathy similar to CD, all point towards environmental factors as triggers of this intertwining. It should be noted that although CD is the autoimmune disease that is most closely associated with specific MHC class II alleles (DQ2 and DQ8), only 3–4 % of the DQ2+ population actually develops CD [63]; therefore, a relatively rare combination of genetics and environmental factors are required to develop well-established CD in DQ2+ and/or DQ8+ individuals.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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