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Latest In vitro and in vivo models of celiac disease

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Abstract

Introduction—Currently, the only treatment for celiac disease is a gluten free diet, and there is an increased desire for alternative therapies. In vitro and in vivo models of celiac disease have been generated in order to better understand the pathogenesis of celiac disease, and this review will discuss these models as well as the testing of alternative therapies using these models.

Areas Covered—The research discussed describes the different *in vitro* and *in vivo* models of celiac disease that currently exist and how they have contributed to our understanding of how gluten can stimulate both innate and adaptive immune responses in celiac patients. We also provide a summary on the alternative therapies that have been tested with these models and discuss whether subsequent clinical trials were done based on these tests done with these models of celiac disease.

Expert Opinion—Only a few of the alternative therapies that have been tested with animal models have gone on to clinical trials; however, those that did go on to clinical trial have provided promising results from a safety standpoint. Further trials are required to determine if some of these therapies may serve as an effective adjunct to a gluten free diet to alleviate the adverse affects associated with accidental gluten exposure. A “magic-bullet” approach may not be the answer to celiac disease, but possibly a future cocktail of these different therapeutics may allow celiac patients to consume an unrestricted diet.

Keywords

celiac; gliadin; gluten; in vitro; in vivo; model; monkey; mouse; nondietary; rat; T cell; therapy; treatment

1. Introduction

Celiac disease is prevalent in most of the industrialized world and is increasing with time[1]. Ingestion of wheat-derived gluten by celiac patients results in immune mediated injury of the intestines that is characterized by intestinal permeability, villous atrophy, and an inflammatory infiltration of the lamina propria that consists primarily of lymphocytes and plasma cells[2, 3]. Intestinal permeability in celiac disease can occur as a result of a number of reasons, one of which is the release of zonulin that disrupts the tight junctions in the epithelial layer[3]. The increased intestinal permeability in celiac disease allows for paracellular transfer of gluten derived peptides to the lamina propria, resulting in the

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presentation of gluten derived peptides to, and subsequent activation of, T cells. Previous publications have focused on the gluten-derived epitopes that are presented by the most predominant variant of DQ2 in celiac disease, DQ2.5,and have shown that there is a specific set of peptides derived from α-gliadin that are immunogenic[4]. These peptides are rendered more immunogenic by complexing with intestinally derived tissue transglutaminase (tTG), resulting in the deamidation of select epitopes[5]. Intestinal plasma cells produce autoantibodies directed against the self-protein, (tTG); these antibodies are used as a diagnostic marker for disease.

Gluten derived peptides can also stimulate non-T cells directly, including epithelial cells, monocytes, and dendritic cells [6–8]. These cells can produce a variety of inflammatory cytokines in response to stimulation with gluten[7, 8] including the expression of IL-15 by epithelial cells, which has been shown to play a crucial role in the activation of NK T cells and subsequent development of enteropathy in celiac disease [6, 9]. Thus, the stimulation of both non-T cells (innate immune response) and T cells (adaptive immune response) by gluten contribute to the development of intestinal inflammation in celiac disease.

In order to understand how both the innate immune response as well as the adaptive immune response to gluten can combine to manifest into celiac disease, models of the cellular responses as well as in vivo models of the disease have been generated (Figure 1). An increasing number of animal models are now being used to test novel therapies that target the different pathways induced by gluten stimulation. This review builds upon our previous review in 2009 [10] and summarizes the recent advances in *in vitro* and *in vivo* models of celiac disease and their application in clinical trials of potential therapies.

2. In Vitro Models of Celiac Disease

2.1 Cell Lines

Many studies demonstrate that gluten can induce inflammatory responses in the first line of defense in the intestine, the epithelial layer. This includes the release of zonulin, leading to disruption of tight junction proteins in the epithelial layer, and subsequent intestinal permeability [3]. In vitro models of an epithelial layer consistent with celiac disease include Caco-2 and IEC-6 epithelial cell lines. Exposure of these cells to gluten results in very diverse responses. These include physical changes, such as cytoskeletal rearrangement and disruption of the tight junction integrity, but also altered expression of inflammatory cytokines[11]. Recent studies have shown that a peptide derived from the α-gliadin molecule, called p31–p43 (a stimulator of innate responses in celiac disease), induces expression of cell surface IL-15 by Caco-2 cells, and thereby stimulates the proliferation of T cells [9]. This is in contrast to the p57–68 peptide, which does not induce cell surface expression of IL15 by Caco-2 cells, but does activate T cells when presented in the context of DQ2, which would be an adaptive immune response. This difference is due to the ability of p31–43 (also called the innate peptide) to disrupt endocytic vesicle trafficking in epithelial cells, whereas p57–68 does not [12–14]. Even more intriguing is that p31–43 (but not p57–68) also induces the expression of tissue transglutaminase 2 by Caco-2 cells, finally providing a potential cellular source for TG2 [15].

Caco-2 cells have also allowed for increased understanding of how the intestinal microbiome plays a role in the pathogenesis of celiac disease. Celiac patients have increased levels of Bacteroides in their microbiome in contrast to normals [16, 17]. When Caco-2 cells are exposed to Bacteroides fragilis and gliadin, there is a subsequent increase in intestinal permeability as well as increased production of TNFα and IL-1β[18]. These findings in Caco-2 cells demonstrate how intestinal microbiota in conjunction with gliadin may potentiate the inflammatory cascade associated with celiac disease.

Monocytes and dendritic cells (DCs) have also been used as models of celiac disease; they are unique in that they can respond to gluten directly as well as support inflammatory T helper cells that are gluten responsive. With respect to monocytes responding to gluten in an innate fashion, monocytes cultured with proteolytic fragments of gliadin (PT-gliadin) will produce the inflammatory cytokines TNFα and IL-8 [7]. Similarly, when DCs are cultured with PT-gliadin, they secrete the inflammatory cytokines IL-6, IL-8, and TNFα[8]. Culture with PT-gliadin also promotes migration of the DCs in response to CCL19 and CCL21 [8]. Both are chemokines important in trafficking to lymph nodes [19]. Similar to the studies done with bacteria and Caco-2 cells, a very recent publication demonstrated that co-culture of DCs with gliadin and different enterobacteria led to different cytokine profiles [20]. Culturing gliadin treated DCs with Shigella or E. Coli led to increased production of IL-12 and TNFα as compared to gliadin treated DCs incubated with B. Longum. Yet when cultured with IL-15 and PT-gliadin, monocytes can also shape the adaptive immune response to wheat by producing IL-1β, IL-6, IL-15, IL-23, TNFα and CCL20, and activating Th17 and Th1 responses to gliadin [21]. This last result introduces the concept of using a mix of antigen presenting cells (APC) and T cells to create an in vitro model of adaptive immunity to gluten and gliadin.

2.2 APC/T Cell Mixes

Mixes of APCs with intestinally derived T cells from celiac patients have been used extensively to model the presentation of gluten derived epitopes in celiac disease. This includes identifying the specific alleles of DQ2 and DQ8 that are capable of presenting gliadin derived immunogenic epitopes, as well as identifying the specific gliadin derived immunogenic epitopes that are presented by these alleles [22, 23]. They were also used to demonstrate that transglutaminase rendered certain epitopes more immunogenic through the deamidation process [5]. Gene dosage analyses and evaluation of the contributions of specific amino acids inside the peptide groove of the MHC II allele to the binding of different gliadin epitopes were also done with the APC/T cell *in vitro* model [24, 25]. Most recently, this model was used to determine that T cells from DQ2.2+ celiac patients recognize a glutenin derived epitope that is entirely different from the α-gliadin derived 33mer peptide found to be very immunogenic in DQ2.5 celiac patients [4]. Similarly, DQ9, which deviates from DQ8 by only one amino acid at position β57, will affect presentation of gliadin derived epitopes. Specifically, DQ9 restricted T cell responses will not respond to the DQ8 α-gliadin or γ-gliadin epitopes, only the DQ8 glutenin derived epitope, DQ8-glut-1[4]. Thus, the APC/T cellmodel is providing crucial data on the adaptive immune responses to gluten that can then be used to treat patients at an individual level.

2.3 Mucosal Biopsy Cultures

The previous models utilized cells that were isolated separately from one another; in contrast, intestinal biopsy cultures extracted from celiac patients incorporate all of the cell-cell interactions present in the small intestine. This model is most likely the oldest of all the models described in this review[26]. Studies done with this model range from analyzing the T cell stimulatory potential of different fractions of gluten and gliadin[27, 28] to understanding how gluten derived peptides are transported across the surface epithelium [29]. One recent study that utilized this model determined that regulatory T cells exist in the mucosa of untreated celiac patients, but are rendered dysfunctional by overexpression of IL-15 [30]. Another recent study that used duodenal biopsy culture demonstrated that Th17 cells are generated in celiac disease[31].

Mucosal biopsy cultures have also been used to determine the immunogenicity of wheat species other than the common Triticum Aestivum as well as other species used in cereals [32–34]. In one study, Triticum monococcum was found to stimulate the expression of

IFNγ, and with one cultivar (Monlis), the expression of IL15 [35]. In contrast, oat species (Avena Potenza and Avena genziana) did not increase expression of IL15 by enterocytes, nor proliferation of crypt epithelial cells, but did increase IFNγ expression in intestinally derived T cells [34]. Quinoa, of the genus Chenopodium, considered to be safe for celiac patients, also was capable of stimulating mucosal biopsy cultures from celiac patients to express IFNγ; albeit one cultivar, Pasankalla, did not as compared to untreated cultures [33]. Since IL-15 is derived from epithelial cells and IFNγ from T cells, these studies demonstrate that the mucosal biopsy culture model proves to be quite useful in determining both innate and adaptive responses to gluten derived from alternative cereals, and as such their potential toxicity for celiac disease patients. The potential toxicity for celiac patients can then be tested using a short term challenge, as was done in the study on Triticum monococcum[35].

3. In Vivo Models of Celiac Disease

Although *in vitro* models have provided great understanding and insight into the pathogenesis of celiac disease at a cellular level, they cannot fully model the systemic development of celiac disease. While the best *in vitro* model is probably the intestinal biopsy culture, which incorporates the effect of cell-cell interactions and signaling, this model may lack hormone and neurologic signals delivered by other organs or cell systems. To ensure such signals are not overlooked, animal models of celiac disease have been developed. This section describes the animal models of celiac disease that have been recently used.

3.1 Spontaneous Models

The spontaneous animal models are models in which no sensitization is required for development of disease. Most research on spontaneous animal models has been done on the dog and monkey models, although there has been one publication on a potential spontaneous horse model of celiac disease[36]. In the dog model, Irish setters develop partial villous atrophy and intraepithelial lymphocyte (IEL) infiltration in response to consumption of gluten [37–39]. Rhesus macaques will also develop similar pathology in response to gluten consumption; however, the MHC II is not associated with the gluten dependent pathology in either model [40, 41]. Of great interest is that one of the rhesus macaques developed dermatitis similar to dermatitis herpetiformis, the skin manifestation of celiac disease [42]. This monkey spontaneously developed antibodies specific for epidermal transglutaminase and tissue transglutaminase, similar to DH patients [43], and the dermatitis resolved after administration of a gluten free diet. However, in contrast to the mouse model of DH, IgA deposition at the dermal papillae was not associated with subepidermal splitting [44]. Also, the gluten dependent enteropathy that developed in other rhesus macaques did not present in the one monkey that developed the gluten dependent dermatitis[41, 45].

3.2 Induced Models

The rat model continues to be used today in a number of studies. In this model, germ-free Wistar AVN rats are administered gliadin immediately after birth[46]. This leads to shortening of villi, crypt hyperplasia, and increased numbers of intestinal CD8αβ+ IELs [46]. Recent papers that have utilized this model have evaluated the role of intestinal bacteria in the development of celiac disease[47, 48]. In Laparra et al., administration of Bifidobacterium longum was shown to protect against the effects of gliadin sensitization of the rats[47]. Specifically, TNFα was significantly decreased, but IL10 was significantly increased in jejunal tissue sections, thereby significantly decreasing the inflammation induced by gliadin alone in the jejunum. Olivares et al. used MALDITOF-TOF peptide fingerprinting analysis to confirm that feeding the rats *B. longum* results in the up-regulation of anti-inflammatory processes and that this is enough to partially ameliorate gliadin induced stress when both *B.*

longum and gliadin are administered to the rats [48]. In a separate study, it was determined that co-administration of Shigella or E. coli with gliadin resulted in an increase in the impairment of tight junctions and resulted in translocation of gliadin peptides into the lamina propria[49]. These results support the theory that the intestinal microbiome plays a crucial role in the development of celiac disease.

There are also mouse models where enteropathy is induced. In one mouse model by Freitag et al., this induction was achieved by transferring CD4+CD25-CD45RBlow cells from gliadin sensitized mice to recipient Rag 1−/− mice [50]. Another new model involved Balb/c mice that were bred for three generations on a gluten free diet; subsequent offspring were then weaned and raised on a gluten free chow for up to 10 weeks of age. The resultant mice were placed onto a standard chow for 30 days. These mice had increased numbers of infiltrating CD3+ IELs with a decreased (villous height)/(crypt depth) ratio [51].

3.3 Transgenic Models

In order to analyze specific pathways involved in the development of celiac disease, transgenic and knock out mice have been generated. The transgenes and knock out constructs utilized are many, but the most widely used are mice that express the HLA genes DQ8 or DQ2 that are tightly associated with celiac disease [44, 52–72]. These mice have resulted in the greatest number of publications on animal models of celiac disease and continue to be used in a number of studies addressing mechanism as well as novel therapies for celiac disease. Briefly, these mice have demonstrated that the HLA molecules DQ8 and DQ2 can contribute to the development of a potent inflammatory T cell response against gliadin, but that this alone is not sufficient for the development of gluten dependent enteropathy characterized by shortened villi. Other mitigating factors are necessary for this to occur, and the studies with transgenic mice have demonstrated (for the most part) that perturbations of the innate immune response in the intestine are necessary for features of gluten dependent enteropathy to develop. These include the administration of cholera toxin, which acts as an adjuvant, and indomethacin, which causes small intestinal pathology. Poly I:C stimulates the innate immune response through TLR3 [73], and was used in a mouse line in which TG2 was knocked out [74]. This latter manuscript by Dafik et el demonstrated that TG2 is not required for the development of poly I:C generated villous atrophy; instead, activation of TG2 is probably a consequence of villous atrophy and not a cause of villous atrophy. Of increasing interest are the transgenic mice that overexpress IL-15. One line of IL-15 mice has human IL15 inserted behind an enterocyte specific promoter (T3b), and this resulted in increased numbers of CD8+ cells infiltrating the small intestine [75]. Interestingly, these mice developed anti tTG IgA in the absence of a gluten-specific CD4+ T cell response[76]. A different line of IL-15 mice used a minimal MHC class I D^d promoter for the expression of IL-15; in a later study, these mice were then crossed with DQ8 transgenic mice [55]. The resultant IL-15 DQ8 transgenic mice developed increased numbers of CD3+ IELs in response to feeding with gliadin[55]. This latter manuscript demonstrates how combining an adaptive response to gluten with a chronic perturbation of the innate immune system (IL-15 overexpression) results in a model that is more similar to celiac disease than either alone.

4. Testing Novel Therapies

Ultimately the goal to any disease specific research is to find a cure or reliable treatment to suppress the disease process. A gluten free diet is currently the only therapy available for patients with celiac disease. While effective, it is difficult to maintain due to cost, availability of products, social acceptance, and patient knowledge of what defines gluten-free. In addition to these patient factors, there is the issue of "hidden gluten" which results from contamination during manufacturing [77]. The definition of gluten free also varies from

country to country and is due to the absence of an internationally safe threshold of gluten for patients with CD [78].

Given these obstacles, as many as 50% of patients with celiac disease who adhere to a gluten free diet do not achieve histologic remission[79]. Lack of histologic remission may be due to daily consumption of 50 mg of gluten, the equivalence of 1/100TH of a slice of bread[77, 79]. This amount is not insignificant, as it is estimated that patients adherent to a gluten free diet will inadvertently consume an average of 5 to 50 mg of gluten each day as a result of gluten contamination [77].

These issues have led to increased interest in therapeutics which may either replace or supplement a gluten free diet to diminish the adverse effects associated with accidental gluten exposure. Just as *in vitro* and *in vivo* models have helped advance understanding of the complex pathophysiology which leads to the development of celiac disease; such models are also paramount to testing new therapies. Of note, not all *in vivo* and *in vitro* models discussed in earlier portions of this paper have been utilized in testing new therapeutics. The following section will focus on recent developments in therapeutics and the models that have been utilized to ensure their safety and efficacy.

4.1 Wheat Alternatives and Alterations – Sorghum, C173, Hydrolyzed wheat, Transamidated wheat

Recognizing that immunogenic gluten is predominantly derived from wheat, other avenues of therapeutic research have investigated different alternatives to wheat as well as ways to alter wheat, rendering it less immunogenic to celiac patients.

Sorghum is a cereal grain related to maize that has been integral to the diet of people in Africa and Asia for thousands of years. Sorghum has recently been used to make multiple wheat-free products, including breads, tortillas, cookies, and flatbreads. In vitro organ culture studies of sorghum failed to show an increase in inflammatory markers. A five day challenge in two celiac patients did not result in gastrointestinal symptoms or serologic changes. [80]. Further studies in larger populations and which include assessment of histology are needed before further recommendations can be made on the safety of sorghum for celiac patients.

Quinoa has often been touted as a safe grain for celiac patients and while most cultivars of quinoa are safe consumption, this is not true for all. Incubation of duodenal organ cultures from celiac patients on a gluten free diet with prolamins from different quinoa cultivars demonstrated that four out of fifteen cultivars had celiac-toxic epitopes with values within the range considered acceptable for gluten free designation. However, two cultivars had epitopes capable of stimulating the adaptive and innate immune responses[33]. Quinoa is not necessarily off the list of acceptable items for celiac patients; however it is important to note that not all quinoa is equally gluten-free.

The creation of gluten-free wheat would seem to be a logical therapeutic avenue; however, this is a challenging endeavor as gluten plays a key role in determining the visco-elasticity and polymerization of bread [81]. Another approach to creating celiac-safe wheat is to breed and hybridize different wheat species to create a new species devoid of the immunogenic gluten derived proteins. Different wheat species and cultivars vary significantly in the levels of T-cell-stimulatory gluten epitopes [82].

C173 is an experimental wheat line bred from crossing two mutant plants with spontaneous deletions of several gliadins and glutenins; specifically Gli-A2, Gli-D1, and Gli-D3. Incubation of duodenal mucosal biopsies from treated and healed celiac patients with C173

did not decrease the villous to crypt ratio but was associated with increased levels of IFN-gamma, IL-2, IL-10, and anti-tTg antibodies in the collected supernatant [83]. Lack of histologic changes would suggest that this wheat is less toxic; however, the continued production of inflammatory cytokines is of great concern. With increased and continued exposure to such wheat, histologic changes could potentially develop over time; therefore C173 may not be appropriate for celiac patients.

Another mechanism to render wheat less toxic is to ferment the wheat with specific combinations of lactobacilli and fungal proteases with the goal of completely hydrolyzing gluten [84]. Hydrolyzed wheat flour still maintains its mechanical properties and therefore it can be used to make bread, pasta, and sweets [85, 86]. Incubation of duodenal mucosa from celiac patients on a gluten free diet and healthy controls with this fermented wheat flour did not increase IFN-gamma mRNA levels [87]. When consumed by celiac patients, this fermented bread does not change intestinal permeability [84]. In a randomized trial of 6 patients, those who ingested 200 grams per day of fully hydrolyzed baked goods (containing 8ppm residual gluten) for 60 days did not report symptoms, nor did they demonstrate changes in morphology or serology[86]. A similar study involving ingestion of 200 grams per day of fully hydrolyzed sweet baked goods did not show a change in clinical complaints, serology, or intestinal permeability [87].

Another method to create less immunogenic wheat involves transamidation of the α-gliadin derived peptides by incubating commercial wheat flour with microbial transglutaminase and lysine methyl ester. Tests to evaluate the safety and efficacy of this altered wheat include T cell lines derived from duodenal biopsy cultures and clinical trials. T cell lines derived from 12 celiac patients were incubated with this altered wheat for 48 hours. Some of these patients had been treated with a gluten free diet, some had not. As a result of this incubation, INF-gamma expression was inhibited and binding of immunogenic peptides to DQ2 was decreased, though not completely eliminated [32]. More recently, a randomized, single blinded, controlled 90 day trial examined the effect of such altered wheat (3.7 grams of transamidated gluten per day) in celiac patients previously maintained on a gluten free diet. Clinical relapse in the form of symptoms, changes in intestinal permeability, serology, histology, and intestinal IFN-gamma mRNA was diminished in the group receiving the deamidated wheat in comparison to the celiac patients receiving regular flour [88]. While results are promising, they are not sufficiently adequate to suggest that transamidated wheat is ready to replace a gluten free diet.

These studies demonstrate that celiac safe wheat that retains necessary mechanical properties may be feasible; though further studies are required in larger populations to ensure that continued and prolonged consumption of such wheat does not result in delayed serologic and histology changes. It is unlikely that these specialized wheat varieties would be a financially acceptable option to patients with celiac disease.

4.2 Changing the face of gluten – Glutenases and Gluten Binders

It is well recognized that gluten triggers the inflammatory cascade associated with celiac disease. One therapeutic approach is to either change the gluten so that it is no longer immunogenic or to change the body's ability to "see" and react to the gluten. This section will discuss the new developments in glutenases and gluten binders as they pertain to these therapeutic mechanisms since our last paper.

Glutenases are enzymes that splice glutamine and proline residues on gluten and thereby decrease the immunogencity of gluten. They are intended as supplements to a gluten free diet, with the aim of diminishing harmful effects associated with accidental gluten

contamination. We will be discussing recent advances in the more promising glutenases; ALV003, AN-PEP, and Stan-1.

ALV003 contains two glutenases – a glutamine-specific cysteine endoprotease derived from germinating barley seeds (EP-B2) and a proline-specific prolyl endoprotease from Sphingomonas capsulate (SC-PEP)[79]. When studied in rats, ALV003 retains enzymatic activity in acidic environments such as the stomach and duodenum [89]. Phase 1 clinical trials in healthy and celiac patients, have shown that 300 mg of ALV003 is safe and can degrade 88 ± 5% of 1 gram of gluten [90]. A phase 2A clinical trial showed that celiac patients who consumed 2 grams of gluten daily with ALV003 for 6 weeks experienced no serologic changes as well as fewer symptoms and morphological changes, in comparison to celiac patients receiving placebo [91]. Encouraging results from this study have prompted plans for a phase 2b study.

AN-PEP consists of a prolyl endopeptidase derived from Aspergillus Niger. *In vitro* studies have demonstrated that it retains activity at pHs comparable with the gastrointestinal lumen, is resistant to pepsin degradation, and is an effective glutenase [92]. Use of a multi-compartmental in-vitro system simulating the gastrointestinal tract (stomach, duodenum, jejunum, ileum) has shown that “ingestion” of bread with AN-PEP leads to effective degradation of gluten in an acidic environment during the time span typically required for mechanical and chemical digestion in the stomach. In vitro studies have also shown that ANPEP eliminates gluten’s ability to stimulate T cells [93].

Based on these *in vitro* findings, a couple of clinical trials are underway. A randomized double-blind control trial studying the efficacy of AN-PEP to detoxify 8 grams of gluten in a commercial food product via assessment of histology and serology has been completed and results have been accepted for publication [94]. Plans are underway for a randomized, double-blind crossover study to evaluate the effect of caloric density on AN-PEP’s enzymatic properties. This study is not yet recruiting participants[95].

Another glutenase cocktail combines aspergillopepsin (ASP) from Aspergillus Niger in conjunction with dipeptidyl peptidase IV (DPPIV), an exopeptidase. ASP is not specific for immunotoxic gluten epitopes, but may aid in degradation of larger proteins into smaller peptides, thereby exposing target residues faster to more specific enzymes. DPPIV enhances ASP’s ability to degrade gluten; however it is inactive at a pH < 4 and therefore it requires concomitant use of antacids[96]. ASP may have a role as an adjunct to endopeptidases such as ALV003 and EP-B2.

STAN 1 is another enzyme cocktail commonly used in food supplements and appears to be an effective glutenase. A randomized, double-blind placebo controlled study found that ingestion of STAN-1 and 1 gram of gluten per day for 12 weeks did not change serology in celiac patients who previously had persistent positive serology despite adherence to a gluten free diet [97].

Glutenases physically change gluten so it loses its immunogenicity. Another mechanism to prevent gluten related immune responses is to use a polymeric binder designed to bind gluten. In the process of binding gluten, degradation and absorption are prevented, thereby allowing gluten to pass through the intestinal tract seemingly unnoticed.

Poly (hydroxythylmethacrylate-*co*-styrene sodium sulfonate) [P(HEMA-*co*-SS)] is a polymeric binder which effectively binds α-gliadin in an acidic environment. Testing of this binder in HLA-DQ8 mice receiving a gluten rich diet has shown decreased number of CD3+ IELs and decreased barrier dysfunction [59, 60]. Incubation of duodenal biopsy cultures with

partially digested gliadin and P(HEMA-*co*-SS) led to decreased secretion of TNF-α. [60]. Clinical trials have not yet been started for this novel therapy.

4.3 Modifying the Microbiome – Probiotics and helminths

Knowing that microbiota and some parasites can affect intestinal permeability and/or exert an immunomodulatory effect, there is increased interest in identifying organisms which may reverse or block such key components of celiac disease pathogenesis. The use of probiotics and helminths are now being investigated as possible therapeutics for celiac disease.

Just as Caco-2 cell lines have shown that Bacteroides fragilis can potentiate the inflammatory response [18], this cell line has also been used to investigate bacteria which may ameliorate this response. Bifidobacterium longum is frequently diminished in the microbiome of celiac patients in comparison to normals. The addition of Bifidobacterium longum to Caco-2 cells exposed to gliadin is associated with decreased TNFα production and therefore may be associated with a decreased inflammatory potential [98].

Probiotics have also been studied extensively in HLA transgenic mice and rats[47, 48, 64, 65]. All of these studies indicate that probiotic therapy can ameliorate the inflammation induced by gluten and be useful as a supportive therapy for the gluten free diet; however, probiotics will probably not rise to the level of a monotherapy and will need to be administered indefinitely. For use as a monotherapy, further expansion on bioengineered bacteria would be necessary[53].

Building upon these *in vitro* and *in vivo* models, a randomized, double-blind placebo controlled study investigated the use of *Bifidobacterium infantis* as a probiotic in celiac patients. Results of this study have been accepted for publication and will be available in the near future. In this study, celiac patients who were recently diagnosed by serology received two capsules (4×10^9 colony forming units) of *Bifidobacterium infantis* with each meal while consuming 12 grams of gluten per day over a three week period. Discussion with the authors reveals that there was no change in intestinal permeability and post-trial duodenal biopsies showed villous atrophy. There was, however, a trend towards lower serologic values following treatment with this probiotic, as well as significant improvements in symptoms suggesting that this probiotic may be advantageous in celiac patients with more gastrointestinal complaints [99].

In addition to bacteria, helminths have also been considered as possible intestinal immunomodulators. Previous *in vivo* mouse models of peanut allergy have demonstrated that helminths are safe and are capable of suppressing the development of peanut specific IgE [100]. Pig whipworm (Trichuris suis) and human hookworm have also been studied in IBD patients.

Expanding this possible therapy to the celiac population, a Phase 1a/2b trial investigated the ability of human hookworm (Necator Americanus) to inhibit immune responsiveness of healthy celiac patients when exposed to gluten. Patients were inoculated with the helminth and 20 weeks later were given a five day gluten challenge. Infection with this helminth was well tolerated from a safety and side effect profile. This intervention did not appear to prevent histologic or systemic inflammatory responses (as indicated by the presence of gluten-specific INF-gamma producing peripheral blood mononuclear cells) when comparing cases to controls, though there appeared to be a trend favoring reduced histologic damage and inflammatory response [101].

Approximately one year following this initial trial, the control patients were invited to participate in a follow-up trial which examined the cytokine profile that follows helminth

infection and gluten challenge. Results of this study demonstrated decreased production of pro-inflammatory cytokines (IFN-gamma and IL-17A), suggesting that hookworm infection may shift the immune response towards a TH2 phenotype as opposed to a TH1/TH7 inflammatory response [102].

4.4 Protecting tight junctions and preventing changes in intestinal permeability – Larazotide acetate

Gluten is capable of inducing changes in tight junctions and intestinal permeability, a key step in the pathogenesis of celiac disease.TG2 inhibitors and Larazotide are therapeutics which aim to maintain the integrity of tight junctions as a way of preventing downstream inflammatory cascades [103, 104]. TG2 inhibitors inhibit changes in tight junction permeability in Caco-2 cells [103] and are also effective in reducing the production of anti-TG2 antibodies and corresponding crypt cell proliferation when studied in duodenal biopsy cultures from untreated (gluten containing diet) celiac patients[103].

Larazotide acetate is a synthetic peptide designed to prevent opening of tight junctions and has been shown to prevent gliadin associated changes in tight junctions and intestinal permeability in Caco-2 cells [104]. Similar results were obtained in HLA-DQ8 transgenic mice, with which administration of larazotide acetate inhibited gliadin induced infiltration of inflammatory cells and maintained tight junction integrity [104].

Larazotide Acetate (aka AT1001) has undergone multiple Phase 2 clinical studies. Twelve milligrams of AT1001 was safe and well tolerated by 21 celiac patients while consuming a gluten containing diet. Preliminary results from this trial suggested AT1001 can prevent changes in intestinal permeability, decrease stimulation of IFN-gamma, and decrease gastrointestinal symptoms[105]. Following this study, a dose-ranging, placebo-controlled study at 10 clinical sites investigated AT1001 in 86 celiac patients. Patients were randomized to receive either AT1001 (0.25 mg, 1 mg, 4 mg, or 8mg) or placebo three times a day. Intestinal permeability was tested, but was highly variable with no difference observed between those on a gluten free diet and those on a gluten containing diet. Like the previous study, AT1001 was well tolerated and appeared to be effective at reducing gastrointestinal symptoms. The latter effect appeared to be most potent at the 0.25 and 4 mg doses [106]. A Phase 2B study is currently recruiting patients to evaluate the efficacy and safety of different doses of AT1001 (0.5, 1 and 2 mg three times a day) in patients with celiac disease already on a gluten free diet, but with persistent symptoms[107].

4.5 Blocking the inflammatory cascade – Ascorbate and anti-IL15 therapy

Another method of treating celiac disease is to target different key molecules in the inflammatory cascade. The addition of ascorbate (vitamin C) to gliadin exposed duodenal biopsies from treated (GFD) celiac patients was associated with diminished production of IFNγ, TNFα, IL-6, IFNα, IL-15 and IL-17. [108] Antibodies targeting the IL-15 receptor or IL-15 itself were successful in inhibiting villous atrophy in one of the IL-15 overexpressing mouse lines[109, 110].

4.6 Vaccines - Nexvax2®

Stemming from experience with food and environmental allergies, another possible therapy is to create a vaccine that might induce gluten tolerance. Nexvax2® is a vaccine consisting of three deamidated gluten peptide sequences which are recognized by the majority of gluten-specific T cells. Nexvax2® has been studied in HLA DQ2-dependent mouse models of gluten immunity.[111] A recent Phase 1 study investigated the safety of weekly injections of Nexvax2® in celiac patients on a gluten free diet. The vaccine was l well tolerated, although some gluten related side effects appeared to be more common with the higher

doses. These gluten related side effects are likely due to the presence of deamidated gluten peptide sequences in the vaccine. Patients who received the vaccine also developed IFNγ producing anti-gluten T cells[112].

4.7 Immunomodulators (CCX282b, aka Traficet-EN®)

A hallmark feature of celiac disease is the lymphocytic infiltration of the intestinal epithelium. Lymphocytes are directed to the intestine via stimulation of chemokine receptor 9 (CCR9) by its only ligand, CCL25. CCR9 has been implicated in the development of inflammatory bowel disease, celiac disease, and primary sclerosing cholangitis. CCX282-B is an antagonist of CCR9 and has been studied as a therapeutic agent for inflammatory bowel disease in the settings of Molt-4 cells and $TNF\alpha^{\Delta ARE}$ mice (mouse model for Crohns disease)[113]. A recent phase 2 clinical trial investigated the efficacy of CCX282-B in celiac patients. Patients received either CCX282-B (250 mg by mouth twice daily) or placebo for 13 weeks while consuming a gluten rich diet. Endpoints included histologic response, degree of lymphocytic infiltration, serologic changes, and patient reported symptoms. This study has been completed, but results are not yet available [107].

5. Expert Opinion

While celiac disease is one of the better understood autoimmune diseases, there are still a number of questions regarding the role of genetics, the intestinal microbiome, inflammatory cascades, and how environmental factors contribute to the development of this disease and its variable spectrum of phenotypic expression. Currently, the only therapy available is a gluten free diet, which, while effective, can be quite difficult for patients to follow, resulting in significant expenses, frustration, and feelings of social isolation. Use of in vitro and in vivo models including cell lines (Caco-2 and IEC-6), intestinal biopsy cultures, spontaneous in vivo models (dog and monkey), induced models (germ-free Wister AVN rats and mice), and transgenic models (mice) have allowed for significant advances in the understanding of the complex pathophysiology of this disease and testing of new and alternative therapies for celiac disease.

The use of helminths and probiotics are intriguing new alternative therapies for celiac disease. Their use is based on the “hygiene hypothesis” as well as increased recognition that the intestinal microbiota plays an interactive role with the gut immune system. Increased recognition of the mutualistic relationship humans share with intestinal microbiota has also led to increased understanding of how they can exert an immunomodulatory effect on the gut and how in the absence of such immunomodulation, there is an increased predisposition towards autoimmunity[114]. Clinical trials evaluating the use of such organisms have been published; further studies will be needed to define the appropriate dose and to determine if these interventions can deliver histologic, serologic, and symptomatic protection for celiac patients in the face of a gluten challenge. Certainly this concept is exciting as it makes the leap to restore balance to the gut’s immune system.

The search for celiac-safe wheat has been making progress, though creating wheat which retains important mechanical properties and is palatable are significant food engineering tasks. Current experimental wheat lines may be safer than regular wheat, but recent studies do not support that this wheat is safe enough for celiac patients. Truly celiac-safe wheat may be developed over time, though we anticipate such wheat may be quite expensive and may be cost prohibitive.

Glutenases and gluten binders continue to have great promise as an adjunctive therapy to a gluten free diet with the goal of mitigating adverse effects associated with accidental gluten exposures. A major limitation to the use of such products is that they would need to

withstand the acidic environment of the stomach and also effectively degrade or bind all gluten-derived immunogenic peptides before the chyme enters the duodenum. These requirements are substantial and probably would prohibit the use of glutenases and gluten binders as a monotherapy in a gluten rich diet. Until a therapeutic agent is developed that would permit celiac patients to consume an unrestricted diet, glutenases and binders could play an important role as adjunctive therapy.

Therapies which target specific pathophysiologic processes (ie: changes in intestinal permeability (TG2 inhibitors, Larazotide acetetate), lymphocytic tracking to the intestinal epithelium (CCX282B), and inhibitors of inflammatory cascade markers (ascorbate, IL-15 antibodies), will likely hold the ultimate promise of treatment. It is also important to note that a “magic-bullet” may not be the solution to a complex disease such as celiac disease. Therapy may one day include a cocktail of medicines which addresses different aspects of the inflammatory cascade.

As we increase our knowledge of the complex pathophysiology underlying celiac disease, we will be able to create new disease targeted therapies. The *in vitro* and *in vivo* models discussed in this article will certainly remain as the first line of testing for all of these novel therapies. Ultimately, the goal would be to find a cure which reverses the immunologic cascade and thereby abolish the need for gluten avoidance.

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Declaration of Interest

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* of interest

** of high interest

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Article Highlights

1. Many more studies are using in vitro models as opposed to in vivo models to characterize the ability of gluten to stimulate and innate immune response.
2. A couple of new animal models of celiac disease have been generated, including one that uses horses.
3. HLA transgenic mice have been used in the majority of articles that utilize animal models of celiac disease.
4. A number of clinical trials have come to completion using the alternative therapies tested in the animal models. These have been (or soon will be) published.

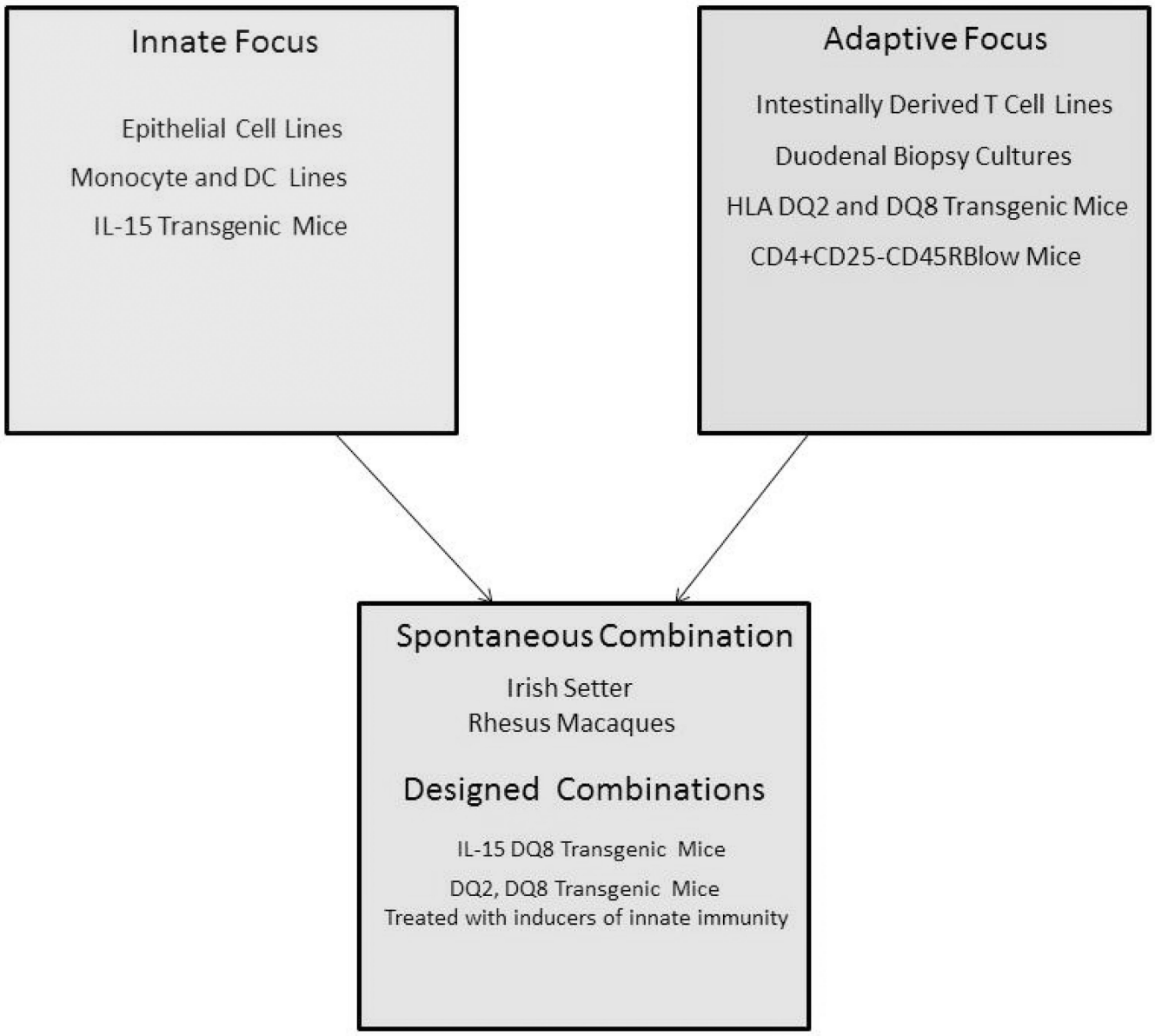

Figure 1.
Diagram of which models best address innate immunity associated with gluten, which adaptive, and which models have been used to address both simultaneously.