Resistance to Celiac Disease in Humanized HLA-DR3-DQ2-Transgenic Mice Expressing Specific Anti-Gliadin CD4+ T Cells

Andrea L. de Kauwe, Zhenjun Chen, Robert P. Anderson, Catherine L. Keech, Jason D. Price, Odilia Wijburg, David C. Jackson, Jodi Ladhams, Janette Allison and James McCluskey

*J Immunol* 2009; 182:7440-7450; doi: 10.4049/jimmunol.0900233

http://www.jimmunol.org/content/182/12/7440

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article **cites 56 articles**, 27 of which you can access for free at:

http://www.jimmunol.org/content/182/12/7440.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Celiac disease (CD) is a common chronic inflammatory disorder of the small intestine affecting ~1% of the world’s population (1). It is precipitated by an inappropriate immune response against gluten proteins from ingested wheat, rye, barley, or sometimes oats. These dietary Ags are normally tolerated by the immune system, but in patients with CD the gluten-induced immunity characteristically results in villous atrophy (partial or total), crypt hyperplasia, and the accumulation of various leukocyte populations in both the lamina propria and epithelium (2). IgA against the autoantigen tissue transglutaminase (tTG) is frequently associated with untreated CD but disappears with gluten exclusion (3).

More than 90% of patients possess HLA-DQ2, encoded by DQA1*05 and DQB1*02 (4), while gluten-specific intestinal CD4+ T cells from HLA-DQ2+ patients are almost exclusively restricted by DQ2 (5). The epitopes recognized by patient-derived intestinal T cell clones and lines raised against gluten often correspond to gluten sequences selectively deamidated by tTG, rich in proline and glutamine, and which are relatively resistant to human intestinal proteases (6–10).

Despite these major advances and recent discovery of several non-HLA genetic associations in CD (11, 12), it remains unclear why >90% of HLA-DQ2+ individuals establish and maintain immune tolerance to gluten. The absence of an animal model of CD dependent on HLA-DQ2-restricted, gluten-specific CD4+ T cells has limited the opportunities to study early events in disease development.

Although only 5% of patients with CD possess HLA-DQ8 without having HLA-DQ2 (4), gluten immunopathology has only been reported in HLA-DQ8-transgenic (Tg) mice. Immunization of such animals elicits gluten-specific T cells with a regulatory profile (producing IL-6, TGF-β, and IL-10), with no autoantibody production and no enteropathy (13). Interestingly, breeding to the autoimmune-prone NOD background appears to render the HLA-DQ8 Tg mouse susceptible to gluten sensitivity since immunization with gluten and coadministration of pertussis toxin provoked a lesion similar to the cutaneous manifestation of CD, dermatitis herpetiformis, in 17% of the mice (14). But unlike dermatitis herpetiformis, neither enteropathy nor Tg-specific autoantibody production was detected in any of the animals.

Our laboratory has previously generated Tg mice that express the HLA-DR3-DQ2 haplotype (spanning HLA-DRA to DQB2) on a partial murine class II knockout background (Aβ+/−) (15). These mice demonstrate cell-specific regulation of the HLA-DR3 and DQ2 gene products, with fully functional CD4+ and CD8+ T cells
In the present study, we have bred mice with the HLA-DR3-DQ2 transgene to a complete class II knockout background (MHCIIαβ), introduced human CD4 (hCD4) (17), and also generated a HLA-DQ2-restricted, gliadin-specific CD4+ TCR Tg mouse to examine the pathological effect of dietary gluten on these mice.

**Materials and Methods**

*Generation and use of hCD4.DR3.DQ2.MHCIIαβ Tg mice*

Human CD4 Tg mice (hCD4 Tg; B6 × DBA/2 background) (17) and mice lacking the murine MHC class II locus (MHCIIαβ; B6 × 129 background) (16) were individually backcrossed to B6 for six to eight generations. MHCIIαβ mice were subsequently crossed with hCD4 Tg mice or hLA-DR3-DQ2 haploptote Tg mice (15), creating hCD4.MHCIIαβ and DR3-DQ2.MHCIIαβ mice. These double transgenic lines were mated to generate F1 mice expressing hLA-DR3, DQ2, and hCD4 in the complete absence of endogenous MHCII (hCD4.DR3.DQ2.MHCIIαβ Tg). The hLA-DR3-DQ2 haploptote was bred to homozygosity by intercrossing the hCD4.DR3.DQ2.MHCIIαβ Tg mice; all mice were hCD4+. Generation of hCD4.DR3-DQ2.MHCIIαβ Tg mice on a NOD background was undertaken in the same manner after each of the initial founder lines were backcrossed to NOD for eight generations.

*Mice housing and diet*

Mice were housed under specific pathogen-free conditions and after weaning were maintained on gluten-free food pellets (Pig Research Institute, Victorian Institute of Animal Health, Werribee, Australia). Gluten-containing food was standard laboratory mouse chow (The Walter and Eliza Hall Institute breeder cubes; Barastoc, Ridley Agriproducts) that contained 5% B cells. Enriched T cells were used as controls described above, but with smaller group sizes when using TCR Tg animals. Where Salmonella typhimurium infections were used to induce intestinal inflammation, mice were infected 14 days after immunization as previously described (20); dietary gluten was introduced 5 days later and all animals were culled after 25 wk. Serum was collected from all experimental animals at regular intervals after exposure to dietary gluten, with the final sample taken at death.

*In vitro T cell proliferation assays*

C57BL/6 mice or hCD4.DR3-DQ2.MHCIIαβ Tg mice (n = 4) were immunized with 80 μg of synthetic peptide emulsified 1:1 (v/v) in CFA (Difco) administered s.c. into the tail base and hind foot. Eight days post-immunization, spleen and draining lymph nodes were harvested and the T cell population was enriched by panning with anti-IgM plus anti-IgG (M; Molecular Probes) for 10 min at 37°C. Cells, cultured for 1 ha t4 ° C( 1 hr) in the T cell population was enriched by panning with anti-IgM plus anti-IgG (M; Molecular Probes) for 10 min at 37°C. Cells, cultured for 1 hr (107 cells/mouse). Recipient mice were injected i.p. with 0.5–1 × 107 CD4+ Tg and C57BL/6 mice were injected i.p.

*Generation of gliadin-specific TCR Tg mice*

Genomic DNA encoding the TCR β-chain and cDNA encoding the TCR α-chain were prepared from HH8.2 hybridomas and cloned into p3α/6-JTCR (23) and pES4 (24) plasmids, respectively. Vector sequences were removed by restriction enzyme digestion and constructs were co-infected into fertilized hCD4.DR3-DQ2.MHCIIαβ oocytes. Oocytes were transferred into surrogate mothers (F1 of CBA × B6) and pups were screened by PCR with primers for the transgenes. Two Tg founders were identified by PCR and confirmed by flow cytometry and two Tg Tg lines were established by backcrossing to hCD4.DR3-DQ2.MHCIIαβ Tg mice.

*In vivo proliferation using adoptive transfer of CFSE-labeled cells*

B cell-depleted splenocytes from unimmunized HH8.TCR Tg mice were labeled with CFSE (2.5 μM; Molecular Probes) for 10 min at 37°C. Cells were washed and adoptively transferred (i.v.) into hCD4.DR3-DQ2.MHCIIαβ Tg mice (0.5–1 × 107 cells/mouse). Recipient mice were described (19). Crude gluten (G-5004; Sigma-Aldrich) was used for oral gavage and immunization, where ~20 mg of crude gluten was suspended in 100 μl of peanut oil for gavage and 100 μg was emulsified in 50 μl of CFA for immunization.

**Gluten-sensitization protocols**

To assess the effect of immunization, 6-wk-old hCD4.DR3-DQ2.MHCIIαβ Tg mice were immunized s.c. with 80 μg of A-gliadin37–73 Q65E (n = 7), 80 μg of chymotrypsin-digested gliadin treated with TGT (n = 6) or 20 nmol of A-gliadin37–73 lipoepitope administered intranasally (n = 6). Following immunization, the diet was switched to gluten-containing food. At 8 wk, mice were boosted with the same immunogen in IFA. At 30 wk of age, all animals were killed and intestinal tissue was examined. To assess the effect of prior administration of a proinflammatory mediator, hCD4.DR3-DQ2.MHCIIαβ Tg and C57BL/6 mice were injected i.p. with pertussis toxin (200 ng/mouse; Sigma-Aldrich) and immunized 7 days later with 80 μg of chymotrypsin-digested gliadin or OVA in the presence of 200 ng of pertussis toxin (emulsified in CFA, delivered i.p.). On day 10, gliadin-immunized Tg mice (n = 14), gliadin-immunized B6 mice (n = 19), and OVA-immunized Tg mice (n = 11) had their diet switched to gluten-containing food. A separate group of gliadin-immunized Tg mice (n = 13) were maintained on gluten-free food. Eighteen weeks later, all mice were killed and intestinal tissue was examined. Experiments with hCD4.DR3-DQ2.MHCIIαβ Tg mice on a NOD background and HH8.TCR Tg mice were performed in a similar manner to those described above, but with smaller group sizes when using TCR Tg animals. Where Salmonella typhimurium infections were used to induce intestinal inflammation, mice were infected 14 days after immunization as previously described (20); dietary gluten was introduced 5 days later and all animals were culled after 25 wk. Serum was collected from all experimental animals at regular intervals after exposure to dietary gluten, with the final sample taken at death.
subsequently either immunized with 40 μg of A-gliadin_{57-73} Q65E or TPO peptide in the hind footpad or gavaged daily for 3 days with A-gliadin_{57-73} Q65E (2 mg/day) or vehicle alone. Three days following adoptive transfer and immunization/gavage, cells from the draining and non-draining lymph nodes were stained with Abs specific for TCRV8.3, TCRβ8, and murine CD4 and analyzed by flow cytometry.

**Cytokine detection**

Cells from the spleen, mesenteric lymph nodes (MLNs), and Peyer’s patches of unimmunized C57BL/6 and H88TgCR Tg mice were stimulated with 2 μM A-gliadin_{57-73} peptide (native or modified Q65E), 2 μg/ml Con A, or medium alone. After 48 h, supernatants were collected and incubated with cytokine detection reagents from a BD Biosciences Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit (no. 551287) according to the manufacturer’s instructions. Samples were examined by flow cytometry and data were analyzed using FCP Array software version 1.0 (BD Biosciences) and FlowJo software.

**ELISAs**

Anti-gliadin IgA ELISA. Crude gliadin (G-3375; Sigma-Aldrich) was dissolved in 8 M urea, 0.1 M NaH₂PO₄, and 10 mM Tris (pH 8.0) at 5 mg/ml and filtered. Nunc Maxisorp immunoplates were coated overnight with 1 μg/well dissolved gliadin in carbonate-bicarbonate coating buffer (pH 9.6) at 4°C. Plates were washed (five times) with PBST (PBS and 0.05% Tween 20); the last wash was incubated for 10 min to block plate. Mouse sera or human sera from celiac patients were diluted in PBST and added to plates in duplicate. Plates were washed (five times) before addition of alkaline phosphatase-labeled anti-mouse IgA (A-4937; Sigma-Aldrich) or anti-human IgA (A-3063; Sigma-Aldrich). Following washing, bound Ab was detected with 1 mg/ml p-nitrophenyl phosphate substrate (S0942; Sigma-Aldrich) in diethanolamine buffer (pH 9.8).

Anti-tTG IgA ELISA. Plates were coated with 0.5 μg/well guinea pig liver tTG (T-5398; Sigma-Aldrich) in 50 mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl₂ (pH 7.5) for 2 h at 37°C. Other steps are identical to the anti-gliadin IgA ELISA, except that the wash buffer contained 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, and 0.1% Tween 20. All plates were read at OD_{405 nm} and reactivity of mouse sera was determined using a positive cutoff value of ±3 SD above the mean obtained from pretreatment sera.

**Histology**

The proximal 9–10 cm of mouse small intestine (duodenum and jejunum) was dissected into 3-cm segments, cut longitudinally, and fixed in 10% neutral-buffered formalin; distal intestine (ileum, 3 cm) was also collected from many animals. Paraformaldehyde-fixed sections (4 μm) were H&E stained and assessed by two histological parameters: number of intraepithelial lymphocytes (IELs) per 100 enterocytes and the ratio of villus height compared with crypt depth. Both parameters were measured from ≥5 well-oriented villi and the median was graphed. Assessment occurred in a blinded manner.

**Statistical analysis**

The Kruskal-Wallis test was used to compare the histological data between more than two treatment groups. The comparison of only two treatment groups was performed using the Mann-Whitney U test. Tests were conducted using the median value of the data set.

**Results**

HLA-DQ2 molecules are expressed and functional in Tg mice

Human class II expression was first confirmed on various splenic populations of leukocytes from hCD4.DR3-DQ2.MHCII^{A/L} Tg mice and B6 controls. DQ2 expression was demonstrated on B cells, macrophages, and dendritic cells from hCD4.DR3-DQ2.MHCII^{A/L} Tg mice, but not on the same cells of B6 mice (Fig. 1A). Unlike activated human T cells, expression of DQ2 was not seen on either naive (Fig. 1A) or activated (data not shown) T cells from Tg or non-Tg mice. Murine class II was not expressed on Tg leukocytes (data not shown).

To demonstrate functionality of hCD4, the proportion of peripheral CD4^{+} T cells was compared in DR3-DQ2.MHCII^{A/L} Tg mice with and without coexpression of hCD4 (hCD4.DR3-DQ2.MHCII^{A/L} Tg vs DR3-DQ2.MHCII^{A/L} Tg). The proportion of normal B6 splenic lymphocytes expressing mCD4 was 20% (Fig 1B). In contrast, in HLA class II Tg mice lacking hCD4 (DR3-DQ2.MHCII^{A/L} Tg), only 13% of lymphocytes expressed mCD4 (Fig 1B), similar to the numbers in Tg mice homozygous for the DR3-DQ2 haplotype on an Ab^{s} background (15). However, expression of the hCD4 transgene boosted CD4^{+} T cell numbers to 18% of splenic lymphocytes in the hCD4.DR3-DQ2.MHCII^{A/L} Tg mice (Fig 1B), indicating the importance of the specific interaction between human CD4 and the human class II molecules. Interestingly, TCR Vβ usage was not significantly different between Tg mice with or without hCD4 (data not shown).
To confirm that the HLA molecules and CD4+ T cells were functional on the MHCI\textsuperscript{\textalpha}/\textbeta and hCD4 Tg background, CD4+ T cell responses were examined in the hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice. Two well-characterized human T cell epitopes were used to immunize Tg and non-Tg animals (n = 5 mice/epitope): MT65kDa\textalpha,13 is a M. tuberculosis heat shock protein-derived peptide that binds exclusively to HLA-DR3 (DRB1*0301) and is recognized by T cells from tubercloid leprosy patients (25); A-gliadin\textalpha,73 Q65E is a deamidated gluten peptide which includes two overlapping HLA-DQ2-restricted epitopes, DQ2-\alpha-I’ (PF PQPELPYQ) and DQ2-\alpha-II’ (PQPELPYQQ), that account for approximately one-half the gluten-specific T cells present in blood after in vivo wheat challenge in patients with CD (19, 26, 27). Eight days after immunization, T cells from draining lymph nodes were examined for their ability to proliferate in vitro in the presence of MT65kDa\textalpha,13, A-gliadin\textalpha,73 Q65E, or a panel of irrelevant HLA-DR- or DQ-restricted peptide controls. A specific proliferative response to MT65kDa\textalpha,13 was observed in T cells isolated from hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice previously immunized with this peptide, but not by T cells from B6 mice similarly immunized (data not shown). Likewise, T cells from hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice responded specifically to in vitro recall with A-gliadin\textalpha,73 Q65E in a dose-dependent manner (Fig. 1C). The native version of the A-gliadin\textalpha,73 peptide triggered a very weak T cell response at high concentrations of peptide (>5 \muM; Fig. 1C), but irrelevant HLA-DQ-binding peptides were nonstimulatory (Fig. 1D). Together, the data indicate that both the human class II molecules and the CD4+ T cells support Ag-specific immunity in hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice.

Low penetrance of celiac pathology in gluten-immunized DR3-DQ2 Tg mice

Given that T cells from hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice are reactive to the immunodominant gluten peptide in HLA-DQ2+ patients with CD (19), we hypothesized that these animals might develop hypersensitivity to gluten. The hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice are healthy while consuming gluten: histology of the small and large intestine, body weight, and stools were normal; Abs against gliadin or tTG were not detectable. However, prior activation of T cells by immunizing the hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice with gluten, chymotrypsin-digested gliadin, or various preparations of A-gliadin\textalpha,73 Q65E did not induce hypersensitivity to dietary gluten (data not shown).

Pertussis toxin has been used for many years as an adjuvant to promote the induction of organ-specific autoimmune when codeivered with self-Ags (28). Therefore, non-Tg B6 and hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta Tg mice were injected with pertussis toxin i.p. (day 0) and immunized 7 days later with chymotrypsin-digested gliadin or OVA i.p. in the presence of pertussis toxin and CFA. On day 10, one group of gliadin-immunized Tg mice (n = 14) had their diet switched to gluten-containing food, while a second group of gliadin-immunized Tg mice (n = 13) were maintained on gluten-free food. A group of OVA-immunized Tg mice (n = 11) and a group of gliadin-immunized non-Tg controls (n = 19) were also introduced to dietary gluten on day 10. Eighteen weeks after commencement of gluten-containing food, all mice were killed and intestinal samples were collected for histological analysis. Serum was collected at six time points throughout the course of the experiment and tested for anti-gliadin and anti-tTG Abs.

As shown in Fig. 2A, 2 of 14 animals from the gliadin-immunized plus gluten-fed group developed Tg-specific IgA Abs; Fig. 2A shows only data from day 61 but both animals also had Tg-specific IgA in their serum from day 38. End-point titer analysis revealed that one of these animals was producing high-tier anti-tTG IgA (1:1200), while

FIGURE 2. Gluten exposure in a proinflammatory context triggers mild low-penetrance gluten-induced pathology in HLA-DR3-DQ2 Tg mice. C57BL/6 or hCD4.DR3-DQ2.MHCI\textsuperscript{\textalpha}/\textbeta mice were immunized in the presence of pertussis toxin and then transferred to a gluten-containing diet. A and B, Serum was collected before treatment and gluten challenge, and again at various intervals after commencement of dietary gluten, and tested for IgA reactivity to (A) tTG or (B) crude gliadin by ELISA. Day 61 serum is representative of all intervals after day 38. Reactivity was determined using a positive cutoff value of \pm 3 SD above mean of pretreatment sera (dotted line). Sera from untreated patients with CD were used as a positive control for ELISA, obtaining OD of >0.9. C, Selection of H&E-stained small intestinal sections showing healthy tissue and mild enteropathy observed in a small proportion of gluten challenged mice; original magnification, \times 100 (left) and \times 200 (right).
the IgA autoantibodies for the second mouse titrated out at 1:300 (data not shown). High-titer anti-Tg IgA autoantibodies were not detected in any other animal (Fig. 2A) at any time point (data not shown). Analysis of sera for the presence of anti-gliadin IgA demonstrated consistent anti-gliadin reactivity in only a single Tg mouse (from the gliadin-immunized-gluten-free group), but interestingly in 10 of 19 gliadin-immunized B6 controls (Fig. 2B). Histological examination of small intestinal tissue found no evidence of intraepithelial lymphocytosis or architectural changes; however, all three groups of Tg mice demonstrated significantly higher IEL counts compared with non-Tg mice irrespective of exposure to dietary gluten (data not shown). In addition, the Tg mouse producing high-titer Tg-specific IgA autoantibodies had an elevated IEL count of 32 IELs/100 enterocytes compared with the determined upper limit of normal (20 IELs/100 enterocytes; data not shown). Mucosa from the jejunum of this mouse also demonstrated elongated crypts (suggestive of increased proliferative activity) and increased cellular content in the lamina propria, but villous structure remained intact (Fig. 2C). A 3-cm piece of distal intestine (ileum) was also collected from many animals for examination and no signs of enteropathy were observed in these tissues (data not shown). Another Tg animal from the gliadin-immunized/gluten-free group also demonstrated an elevated IEL count, elongated crypts, and a higher cellular density in the lamina propria (data not shown); both of these Tg mice were clinically scored as having mild enteropathy. A second experiment conducted in a similar manner (but without priming of the system with pertussis toxin before immunization) found that coinjecting with pertussis toxin resulted in one of eight gliadin-immunized plus gluten-fed Tg mice developing high-titer Tg-specific IgA autoantibodies and mild enteropathy (data not shown).

To increase the incidence and severity of enteropathy in the hCD4.DR3-DQ2.MHCII/−/− Tg mice, the hCD4 transgene, the HLA-DR3-DQ2 haplotype, and the MHCII/−/− knockout were individually backcrossed to the autoimmune-prone NOD strain for eight generations. The resulting Tg NOD strains were subsequently intercrossed to produce congenic hCD4.DR3-DQ2.MHCII/−/− Tg mice on a NOD background. Nevertheless, this genetic background did not accentuate the gluten-induced phenotype in the hCD4.DR3-DQ2.MHCII/−/− Tg mice (data not shown). Similarly, infection with Salmonella typhimurium, a natural enteric bacterial pathogen for rodents, coincidentally with gluten exposure, did not enhance the incidence or severity of gluten hypersensitivity in the hCD4.DR3-DQ2.MHCII/−/− Tg mice. A small number of animals (−10%) were found to be producing Tg-specific IgA and also had an elevated IEL count, but villous structure, body weight, and stools remained normal (data not shown).

Isolation of a murine HLA-DR-DQ-restricted, gliadin-specific CD4+ T cell hybridoma

One obstacle to the development of gluten-induced disease in the hCD4.DR3-DQ2.MHCII/−/− Tg mice may be a low frequency of gluten-specific T cells. We generated gliadin-specific CD4+ T cell hybridomas from hCD4.DR3-DQ2.MHCII/−/− Tg mice: animals were immunized with A-gliadin57–73 Q65E and peptide-specific CD4+ T cell clones were fused to a TCR-negative murine thymoma cell line, BW5147, which does not express CD4 or CD8. A proportion of the actively growing hybridomas were found to be mCD4+, hCD4+, and TCR+ (CD3+); Fig. 3A shows representative flow cytometric data of one such fusion (DB4). Homogeneous cell populations that demonstrated the highest expression of CD3 and CD4 were tested for gliadin specificity by stimulation with irradiated hCD4.DR3-DQ2.MHCII/−/− Tg splenocytes and A-gliadin57–73 Q65E or an irrelevant HLA-DQ2-binding peptide (29). A number of hybridoma lines were found to respond specifically to A-gliadin57–73 Q65E, producing IL-2 in a dose-dependent and...
HLA-DQ-dependent manner (Fig. 3, B and C, shows the response of a representative hybridoma line).

The HLA-DQ2 molecule carried by the majority of CD patients is DQα1*05/DQβ1*02 (DQ2.5). This heterodimer can be encoded in cis- or in trans. A second DQ2 molecule (DQ2.2), comprised of DQα1*0201/DQβ1*02, is associated with CD without DQ2.5 (4). Although possession of the DQA1*05 and DQB1*02 alleles confers the primary increased risk of CD, studies have shown that this risk is increased four to six times by homozygosity for the cis-haplotype or a second DQB1*02 allele on the other haplotype (30, 31). To verify the functional effect of DQ2 gene dosage on T cell recognition and further confirm the MHC restriction element for the hybridomas, one T hybridoma line (DB4) was cultured with A-gliadin<sub>57–73</sub> Q65E and an irradiated panel of B-lymphoblastoid cell lines (B-LCLs) expressing different DQα and DQβ chains (Fig. 3D). Hybridomas incubated with DQ2.5 homozygous B-LCLs (9022 cells) or B-LCLs expressing DQA1*05 and DQB1*02 in trans (T177 cells) responded to gliadin peptide in a dose-dependent manner, whereas neither DQ2.2, DQ7, nor DQ8 homozygous B-LCLs activated the T hybridomas (Fig. 3D). Importantly, the hybridomas were most efficiently activated by DQ2.5 homozygous B-LCLs (see EC<sub>50</sub> for the dose-response curves; Fig. 3D). This finding is consistent with human T cell clones isolated from intestinal lines raised against deamidated gliadin (32).

A second T hybridoma (HH8; with specificity similar to DB4) coexpressed TCR V<sub>α</sub>8.3 and TCR V<sub>β</sub>8.2 (data not shown). T cells using this combination of TCR<sub>α</sub>ß chains can be tracked using mAbs; therefore, the HH8 TCR was selected to generate TCR Tg mice. To identify which of the two epitopes in A-gliadin<sub>57–73</sub> Q65E (DQ2-α-I or DQ2-α-II) was recognized by the HH8 TCR, the hybridoma was cultured with irradiated hCD4.DR3.DQ2.MHCII<sup>α2ß2</sup> Tg splenocytes and 15-mer peptides spanning a nine-residue core sequence of the DQ2-α-II epitope (Fig. 4A). This finding is consistent with human CD4<sup>+</sup> T cells expressing the Tg TCR. Representative dot plots of FACS costaining for mCD4 and hCD4 is also shown on lymphocytes from thymus, spleen, and MLN of TCR Tg mice. Top and middle rows are gated on lymphocytes; bottom row is gated on mouse and human CD4<sup>+</sup> lymphocytes.

**FIGURE 4.** The fine specificity of murine gliadin-specific CD4<sup>+</sup> T cell hybridoma. HH8 resembles the human T cell response toward the dominant DQ2-α-II epitope in patients with CD. HH8 T hybridoma cells were cultured with irradiated hCD4.DR3.DQ2.MHCII<sup>α2ß2</sup> Tg splenocytes and (A) A-gliadin<sub>57–73</sub> Q65E or 15-mers spanning a nine-residue core sequence of the DQ2-α-II peptide or (B) lysine substitutions of each amino acid in A-gliadin<sub>57–73</sub> Q65E. Supernatants were added to IL-2-dependent CTLs in a 24-h proliferation assay. Bars represent the mean value obtained in a triplicate experiment; error bars represent the SD.

**Gliadin-specific Tg CD4<sup>+</sup> T cells are functional in vitro and in vivo**

To confirm the specificity of the CD4<sup>+</sup> T cells in the TCR Tg mouse and determine whether they were functionally responsive to Ag, splenocytes from these animals were depleted of B cells and examined for their ability to proliferate in vitro in the presence of A-gliadin<sub>57–73</sub> Q65E. Ag dose-dependent proliferation of the T cells occurred in response to the DQ2-α-II epitope (Fig. 6A) and fine mapping revealed dependence on the same core residues as observed with the parental HH8 hybridoma (Fig. 4B and data not shown).

**FIGURE 5.** HH8 TCR Tg mice develop single-positive CD4<sup>+</sup> T cells expressing the Tg TCR. Representative dot plots of FACS costaining for Vß8 and Vß8.3 expressed by HH8. Staining for mCD4 and hCD4 is also shown on lymphocytes from thymus, spleen, and MLN of TCR Tg mice. Top and middle rows are gated on lymphocytes; bottom row is gated on mouse and human CD4<sup>+</sup> lymphocytes.

A gliadin-specific TCR Tg line was generated on the hCD4.DR3.DQ2.MHCII<sup>α2ß2</sup> background using αβTCR genes of the HH8 T hybridoma. The TCR<sub>α</sub> and TCR<sub>β</sub> transgenes cosegregated and flow cytometric analysis indicated that ~93% of the thymocytes were CD4<sup>+</sup> and 39% were mature CD4<sup>+</sup>CD8<sup>−</sup> cells (Fig. 5). In the periphery of the TCR Tg mice, 24% of splenic lymphocytes and 53% of MLN lymphocytes were CD4<sup>+</sup> (both human and mouse; Fig. 5). Importantly, 97% of peripheral CD4<sup>+</sup> T cells expressed the transgene-encoded TCR (Fig. 5). These data indicate normal thymic development and peripheral export of T cells expressing the TCR<sub>α</sub> and β transgenes on the hCD4.DR3.DQ2.MHCII<sup>α2ß2</sup> background. In contrast, development of the CD8<sup>+</sup> T cell compartment is affected by the TCR transgenes as the CD8<sup>+</sup>CD4<sup>−</sup> subset is dramatically reduced in both the periphery and thymus (Fig. 5).
To assess whether the Tg CD4⁺ T cells were functional in vivo, purified T cells were labeled with the cytoplasmic dye CFSE and transferred into hCD4.DR3-DQ2.MHCII/H9004/Tg mice that were then immunized with A-gliadin$_{57-73}$ Q65E or control TPO peptide in CFA. Three days after transfer and immunization, draining and nondraining lymph nodes were removed and cell proliferation was analyzed by flow cytometry. The transferred T cells proliferated in the draining lymph nodes of mice immunized with A-gliadin$_{57-73}$ Q65E, with seven rounds of division observed; proliferation was not seen in cells from the nondraining lymph nodes of these mice (Fig. 6B). Similarly, transgene-encoded T cells proliferated in the draining nodes of mice immunized with native A-gliadin$_{57-73}$, chymotrypsin-digested gliadin, or whole crude gluten, but the proportion of cells dividing was lower than that observed with synthetic A-gliadin$_{57-73}$ Q65E (data not shown). T cell proliferation was not observed in TPO-immunized mice (data not shown). Furthermore, CD4⁺ T cells from the TCR Tg possessed a Th1-type effector phenotype. On stimulation of CD4⁺ T cells from the TCR Tg mice with A-gliadin$_{57-73}$ Q65E (and Con A), there was prominent secretion of IFN-γ and TNF-α and to a lesser extent IL-2, but relatively much less IL-4 or IL-5 (Fig. 7).

Taken together, these results demonstrate that CD4⁺ T cells in the TCR Tg mice are gliadin peptide-specific and functionally capable of responding to Ag in vitro and in vivo. Importantly, the gliadin-specific T cells secrete Th1-type cytokines upon encounter with gliadin peptide; such cytokines are proposed to be instrumental in the small intestinal damage associated with CD (34).

Gliadin peptide is detectable by T cells in draining lymphoid tissue following mucosal delivery

To determine whether immunogenic gliadin peptide is absorbed from the healthy murine gut, TCR Tg splenocytes were labeled with CFSE and transferred as reporter cells into hCD4.DR3-DQ2.MHCII/H9004/Tg mice. Following transfer, hCD4.DR3-DQ2.MHCII/H9004/Tg mice on
gliadin peptide, we investigated whether regulatory T cells might be induced by gluten ingestion. However, the proportion of T cells expressing CD4+CD25+Foxp3+ was no different in the spleen, MLN, Peyer’s patch, and auxiliary lymph node (peripheral lymph node) from B6 mice, TCR Tg mice on gluten-free food, and TCR Tg mice given gluten-containing food for 10 days (Fig. 8B). In addition, distinguishing gliadin-specific CD4+ T cells from the small proportion of CD4+ T cells expressing an endogenously encoded TCR (non-Tg T cells) revealed no significant difference (Fig. 8B).

Tg mice expressing gliadin-specific T cells remain healthy while consuming gluten

Experimental protocols used earlier in the hCD4.DR3-DQ2.MHCIIΔ/Δ mice to induce enteropathy, including immunization with gliadin peptide/protein before gluten gavage with coadministration of pertussis toxin or coincident infection with S. typhimurium, did not induce tTG-specific IgA or any discernable signs of intestinal pathology in the TCR Tg mice (data not shown). Notably, an increase in IEL numbers was not observed in the TCR Tg animals following any intervention. In fact, these animals appeared to have a lower IEL count than observed in B6 controls and “healthy” hCD4.DR3-DQ2.MHCIIΔ/Δ Tg mice. The lower number of IELs in the TCR Tg mice is probably attributable to the impact of the prearranged TCRα and TCRβ transgenes on CD8 T cell development and possibly also γδ T cell development, as these populations are reduced in the periphery and are known to comprise a significant proportion of intestinal IELs.

Discussion

HLA-DQ2 or DQ8 is necessary but not sufficient for development of CD (2). Early introduction of gluten while infants are not breast-fed, frequent episodes of gastroenteritis before age 6 mo, homogyosity for HLA-DQ2, and a first-degree relative having CD are among the strongest factors predicting development of CD (2, 31, 35, 36). Presumably, these factors influence the priming, expansion and/or maintenance of HLA-DQ2-restricted CD4+ Th1 cells specific for deamidated gluten found in established CD.

In human CD, enteroapthy probably evolves gradually from intraepithelial lymphocytosis to subtotal villous atrophy, and in clinical practice serum tTG IgA may precede intestinal damage (37, 38). Autoantibodies specific for tTG typically appear in serum for the first time between the ages of 1 and 7 years, but they can be transient or even reappear later in childhood (39). These IgA class autoantibodies disappear with gluten exclusion and are widely considered to be dependent on uptake of tTG-gluten peptide complexes by tTG-specific B cells receiving help from gluten-specific CD4 T cells (40, 41). Hence, contemporary hypotheses accounting for the development of CD invoke gene dose of HLA-DQ2, various genes regulating the activation threshold for CD4+ T cells, costimulation and danger signals provided by intestinal infection and/or innate immunity (triggered by gluten itself), mucosal transglutaminase activity, intestinal permeability, the dose of gluten ingested, and the unusual chemical properties of gluten (12, 42, 43).

Although this model of CD induction is plausible, supporting evidence is observational; many aspects would be testable in a valid animal model. To model the CD4+ T cell-mediated pathology of CD, we introduced the HLA-DR3-DQ2 haplotype and the hCD4 gene into mice lacking murine MHCII genes (MHCIIΔ/Δ). These mice Tg for hCD4.DR3-DQ2.MHCIIΔ/Δ express functional
HLA-DR3 and DQ2 molecules that mediate thymic selection generating Ag-specific CD4+ T cell immunity. Although about one in five Europeans homozygous for HLA-DQ2 develops CD (31), homozygous and heterozygous hCD4.DR3-DQ2.MHCIIαβ Tg mice do not spontaneously develop hypersensitivity to gluten. However, standard immunization protocols do produce HLA-DQ2-restricted CD4+ T cells in hCD4.DR3-DQ2.MHCIIαβ Tg mice that preferentially recognize the deamidated α-gliadin 17-mer immunodominant in human CD (19). Nevertheless, we have found that preexisting DQ2-restricted CD4+ T cell immunity to deamidated α-gliadin 17-mer in hCD4.DR3-DQ2.MHCIIαβ Tg mice is not sufficient for gluten ingestion to cause measurable intestinal pathology.

Induction of celiac pathology might require activation of local intestinal innate immunity at the time of initial exposure to dietary gluten. Conditions designed to create an inflammatory context, such as infecting with S. typhimurium (an enteric bacterial pathogen for rodents (44)) or prior exposure to a Rhesus strain of rotavirus (data not shown), did not result in consistent signs of celiac pathology, such as increased numbers of IELs or villous changes on histological examination. However, systemic administration of pertussis toxoid before immunization with the immunogenic gluten peptide lead to IgA autoantibody production and mild histological changes in the small intestine of a small proportion of hCD4.DR3-DQ2.MHCIIαβ Tg mice.

To explore the effect of other genetic factors, hCD4.DR3-DQ2.MHCIIαβ Tg mice were bred to the autoimmune-prone NOD background, which in DQ8 Tg mice predisposes to gluten-sensitive cutaneous pathology (14). However, hCD4.DR3-DQ2.MHCIIαβ Tg NOD mice immunized with gliadin and coinfected with pertussis toxoid remained healthy.

Next, we considered whether the magnitude of the CD4+ T cell response to gluten might be inadequate in hCD4.DR3-DQ2.MHCIIαβ Tg mice immunized with gliadin or the α-gliadin 17-mer to cause celiac-like intestinal pathology. Autoantigen-specific CD4+ TCR Tg mice have been used to generate various models of HLA-linked autoimmunity (45–48). Therefore, we generated gliadin-specific CD4+ TCR Tg mice on the hCD4.DR3-DQ2.MHCIIαβ background. The transgene-encoded TCR was derived from a DQ2-restricted CD4+ T cell hybridoma specific for the DQ2-α-II epitope commonly recognized by intestinal T cell clones from DQ2-αTg celiac patients (26, 33). Gliadin-specific CD4+ T cells were thymically selected and exported to the periphery of the TCR Tg mice, where they constituted the vast majority of T cells. By comparison, the maximum frequency of T cells specific for the immunodominant DQ2-α1 and DQ2-α-II epitopes, enumerated by MHC-peptide tetramers, is reported to be no more than 0.4% of the total peripheral blood CD4+ T cell population (49).

TCR Tg T cells proliferated following systemic administration of α-gliadin27–73 Q65E or chymotrypsin-digested gliadin in vivo and secreted Th1-type cytokines implicated in CD (34) in vitro. But ingestion of gluten or α-gliadin27–73 Q65E did not trigger intestinal pathology in TCR Tg mice, either spontaneously or induced by immunization strategies that have produced autoimmune disease in other TCR Tg models (45, 46, 48).

The resistance of these mouse models to development of celiac enteropathy is surprising given the increased precursor frequency of gliadin-specific CD4+ T cells engendered in the TCR Tg system. One possibility is that T cells were not exposed to sufficient levels of the gluten peptides to trigger tissue damage. Gavage with deamidated A-gliadin27–73 (2 mg daily) did stimulate modest T cell proliferation in gut-draining lymph nodes, but this was substantially less than observed in local draining lymph nodes following s.c. administration with one-fiftieth of the dose (0.04 mg). In gluten challenge experiments, mice were fed a substantial amount of gluten (~1 g/mouse, three times per week) in the form of standard gluten-containing bread. For comparison, spiking the gluten-free diet with 50 mg of gluten daily causes 20% reduction in the villous height:crypt depth ratio in patients with established CD (50). Even though the dose of gluten ingested was substantial, only a modest amount of peptide may have been absorbed due to relatively low permeability or luminal proteolysis; yet permeability would be expected to increase with coexistent enteritis and immunogenic gluten peptides are unusually resistant to rodent and human digestive proteases (10). Mucosal transglutaminase may have been the other factor limiting exposure of T cells to cognate (deamidated) gluten peptides with enhanced binding to HLA-DQ2. Although not directly measured, transglutaminase activity is up-regulated with inflammation (51) and would also be expected to increase with coincident enteric infection, an experimental condition that did not trigger gluten enteropathy. In summary, the experimental conditions assessed would have been expected to result in adequate presentation of immunogenic gluten peptides to reanimate primed T cells. Additionally, both hCD4.DR3-DQ2.MHCIIαβ Tg mice that received 5–10 million cognate T cells (by adoptive transfer) and unmanipulated TCR Tg mice would be expected to have substantially greater frequencies of T cells specific for immunodominant gluten peptide than observed in human CD (49).

Although mice may have been “tolerized” by accidental exposure to gluten before deliberate immunization with gluten, we think this possibility is unlikely. A more plausible explanation for our findings is that in the absence of mucosal damage, the gastrointestinal mucosa of the mouse is innately resistant to inflammation. Mice deficient in IL-10 or TGF-β are predisposed to intestinal inflammation (52, 53), indicating the importance of cytokine balance in mediating steady-state immunological hyporesponsiveness. PGE2 may be a crucial contributor to intestinal hyporesponsiveness because it is highly expressed by lamina propria mononuclear cells and is known to suppress immune responses (54). Notably too, GALT is a preferential site for the peripheral induction of Foxp3+ regulatory T cells (55). Such regulatory T cells may contribute to immunological homeostasis in the intestine with respect to ingested gluten, but our study found no evidence for an expanded population of Foxp3+ regulatory T cells in the gut-draining lymph nodes of TCR Tg mice following exposure to dietary gluten.

Given the potential for multiple quantitative susceptibility traits to contribute to disease penetrance in DQ2+ individuals, it may be necessary to make backcrosses of the DQ2 and TCR transgenes onto multiple mouse genetic backgrounds to screen for higher disease penetrance. Alternatively, if there were known gene polymorphisms with significant impact on susceptibility to CD, these could be targeted individually as components of an improved animal model of CD.

Taken together, our findings indicate that gluten ingestion in humanized mice expressing functional HLA-DQ2 and possessing a substantial population of CD4+ T cells specific for gluten is not sufficient to cause celiac-like enteropathy. These observations are particularly relevant in the context of an evolving viewpoint that CD may be better defined as a systemic immune response to gluten with fluctuating or occasionally absent gut pathology (56). Accordingly, the model provides a unique opportunity to study both the systemic immune response to gluten, as well as the local gut-associated regulation of gluten-specific T cells relevant to HLA-DQ2-associated CD.
Acknowledgments
We thank Anthony Purcell and Nick Williamson for quality control of synthesized peptides, Prithi Bhatia for assessment of mouse pathology, Maria Kaparakis for assistance with histology, Dianna Starczak for assistance with statistics, and Tom Gordon and Jenny Roland for providing patient sera.

Disclosure
Robert P. Anderson is the inventor of patents relating to diagnostics, therapeutics, and nontoxic gluten based upon knowledge of peptides recognized by T cells in celiac disease. Robert P. Anderson is also involved in the commercialization of these patents as Director, Chief Executive Officer, and is a substantial shareholder in Nexepet Pty Ltd and Nexgrain Pty Ltd.

References


