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*J Immunol* 2009; 182:7440-7450; doi: 10.4049/jimmunol.0900233
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Resistance to Celiac Disease in Humanized HLA-DR3-DQ2-Transgenic Mice Expressing Specific Anti-Gliadin CD4+ T Cells1

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Celiac disease is a chronic inflammatory enteropathy caused by cellular immunity to dietary gluten. More than 90% of patients carry HLA-DQ2 encoded by HLA-DQA1*05 and DQB1*02, and gluten-specific CD4+ T cells from intestinal biopsies of these patients are HLA-DQ2-restricted, produce Th1 cytokines and preferentially recognize gluten peptides deamidated by tissue transglutaminase. We generated mice lacking murine MHC class II genes that are transgenic for human CD4 and the autoimmune and celiac disease-associated HLA-DR3-DQ2 haplotype. Immunization with the α-gliadin 17-mer that incorporates the overlapping DQ2-α-I and DQ2-α-II epitopes immunodominant in human celiac disease generates peptide-specific HLA-DQ2-restricted CD4+ T cells. When exposed to dietary gluten, naive or gliadin-primed mice do not develop pathology. Coincident introduction of dietary gluten and intestinal inflammation resulted in low-penetration enteropathy and tissue transglutaminase-specific IgA. Two further strains of transgenic mice expressing HLA-DR3-DQ2 and human CD4, one with a NOD background and another TCR transgenic having over 90% of CD4+ T cells specific for the DQ2-α-II epitope with a Th1 phenotype, were also healthy when consuming gluten. These humanized mouse models indicate that gluten ingestion can be tolerated without intestinal pathology even when HLA-DQ2-restricted CD4+ T cell immunity to gluten is established, thereby implicating additional factors in controlling the penetrance of celiac disease. The Journal of Immunology, 2009, 182: 7440–7450.

1 Abbreviations used in this paper: CD, celiac disease; tTG, tissue transglutaminase; B-LCL, B lymphoblastoid cell line.
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3 This work was supported by a National Health and Medical Research Council of Australia Program Grant.
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5 Received for publication January 22, 2009. Accepted for publication April 17, 2009.
6 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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(15). In the present study, we have bred mice with the HLA-DR3-DQ2 transgene to a complete class II knockout background (MHCIIαβ) (16), 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 haplotype Tg mice (15), creating hCD4.DR3.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 haplotype 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.

Mouse 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 wheat. Upon introduction of dietary gluten, mouse cages were also supplemented three weekly with commercial white bread (~12 g/mouse or ~1 g of gluten/mouse). Female breeders were maintained on standard (gluten-containing) food. Unless otherwise specified, mice were used at 6–10 wk of age.

Abs and flow cytometry

mAbs specific for HLA class II β-chain (RM5.112), HLA-DQ (SPV-L3), and TCR Vβ8.2 (F23.2) were prepared in our laboratory and conjugated with FITC or biotin. Fluorochrome-labelled Abs that recognize B220 (RA3-6B2), CD11c (HL3), F4/80 (BM8), mouse CD4 (mCD4; GK1.5), hCD4 (SK3), CD64 (53-6.7), I-Aβ (AF6-120.1), TCR Vβ2 (B20.6), TCR Vβ3 (KT25), TCR Vβ4 (KT14), TCR Vβ5 (KT7), TCR Vβ6 (p95-7), TCR Vβ7 (TR310), TCR Vβ8.1/8.2 (MR5-2), TCR Vβ8.3 (1B3.3), TCR Vβ9 (MR10), TCR Vβ10 (B21.5), TCR Vβ11 (RR3-15), TCR Vβ12 (MR11-1), TCR Vβ13 (MR12-3), TCR Vβ14 (14-2), TCR Vβ17* (KJ23), mouse CD3 (145-2C11), TCR Vα8.3 (KT50), TCR Vγ8 (F23.1), and CD25 (PC61) were purchased from BD Biosciences Pharmingen. Intracellular Foxp3 staining was performed using an alpholycoycin anti-mouse/ rat Foxp3 staining set (clone FJK-16s; eBioscience) according to the manufacturer’s instructions. Stained cells were acquired using a FACsCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Antigens

Synthetic peptides were purchased from Mimmotopes. They were as follows: α-gliadin 17-mer A-gliadin1-73 (5QLQFPFPQQLYPQPSQ5L), deamidated α-gliadin 17-mer A-gliadin1-73 Q65E (5QLQFP FPQPELPYPQPSQ5L), LBA2 (KSP40FPFPQQLYPQPSQ5L) from EBV, TPO (632IDVWLGGLAENFLP645) from human thyroid peroxidase, and MT65DSa1-12 (JKTAYDEEAAK) from the Mycobacterium tuberculosis 65-kDa heat shock protein. The combitope (QPQFPFPQFPQLELPYPQPSQ5L), deamidated α-gliadin 33-mer (aLQQFP FPQPELPYPQPSQ5LPQFPQPQPSQ5L), deamidated gda09 (aSOGEP GQPSQPSQ5L) from α-gliadin), deamidated DQ2-γ1 (5PQFPFPQFP FPQFPFPQPSQ5L) from γ-gliadin), deamidated α-gliadin peptide (QPQFPFPQFPQLELPYPQPSQ5L) was scanned from A-gliadin, Q65E (17 peptides in total), and six overlapping 15-mer containing Gly-Ser-Gly-flanked 9-mer sequences from A-gliadin, Q65E (i.e., GSGLQPFPFPQELSG through to GSGLQPFPFPQELSG, underscores indicate the core epitope) were purchased from Research Genetics and Pepscan Mass. Spectroscopy and HPLC were used to confirm the authenticity and >70% purity of the peptide. The A-gliadin, Q65E peptide was synthesized as previously described (18). Gliadin (G-3375; Sigma-Aldrich) was digested with α-chymotrypsin (G-3142; Sigma-Aldrich) and deamidated with guinea pig liver transglutaminase (T-5398; Sigma-Aldrich) as previously 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-gliadin1-73 Q65E (n = 7), 80 µg of chymotrypsin-digested gliadin treated with TGT (n = 6) or 20 nmol of A-gliadin1-73 lipopeptide 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 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.

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 footpad. Eight days postimmunization, spleen and draining lymph nodes were harvested and the T cell population was enriched by panning with anti-IgM plus anti-IgG (100 µg/ml; M684–14M, M685–14M; Sigma-Aldrich). Cells were incubated for 1 h at 4°C (1 × 10⁷ cells/ml) and unbound cells were removed. T cell-enriched lymphocytes typically contained 80–85% T cells. Enriched T cell suspensions were cultured with syngeneic APCs (splenocytes) and medium alone, synthetic peptide or Con A as previously described (15). Proliferation was assessed by [3H]thymidine incorporation as described elsewhere (15). Results are expressed in cpm or as a stimulation index, with the mean of each triplicate plotted and error bars representing the SD.

T cell hybridomas

CD4+ T cells were isolated and enriched from the spleen and draining lymph nodes of hCD4.DR3-DQ2.MHCIIαβ Tg mice (n = 5) following immunization with 80 µg of A-gliadin1-73 Q65E peptide and boosting 2 wk later (same peptide in IFA, s.c.). These cells were used to generate BS5147-T cell hybridomas as previously described (21). Activation of T hybridomas was assessed by IL-2 bioassay as previously reported (22). The IL-2-dependent cell line ßTLL-2 (ATCC TIB-214) was maintained with 20–100 U/ml recombinant human IL-2.

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 the p3A4(βTCCR) (23) and pE4S (24) plasmids, respectively. Vector sequences were removed by restriction enzyme digestion and constructs were co-introduced into fertilized hCD4.DR3-DQ2.MHCIIαβ oocytes. Oocytes were transferred into surrogate mothers (F1 of CBA × C57BL/6) and pups were screened by PCR with primers for the transgenes. Two TCR Tg founders were identified by PCR and confirmed by flow cytometry and two TCR 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

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subsequently either immunized with 40 \( \mu \)g 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 TCRV\(\alpha\), TCR\(\beta\), 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 H8STCR Tg mice were stimulated with 2 \( \mu \)M A-gliadin\(_{57-73}\) peptide (native or modified Q65E), 2 \( \mu \)g/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 FCAP 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 NaH\(_2\)PO\(_4\), and 10 mM Tris (pH 8.0) at 5 mg/ml and filtered. Nunc Maxisorp immunoplates were coated overnight with 1 \( \mu \)g/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-TG IgA ELISA. Plates were coated with 0.5 \( \mu \)g/well guinea pig liver TG (T-5398; Sigma-Aldrich) in 50 mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl\(_2\) (pH 7.5) for 2 h at 37°C. 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).

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. Paraffin-embedded sections (4 \( \mu \)m) were stained with Abs specific for TCRV\(\alpha\) and immunization/gavage, cells from the draining and non-draining lymph nodes were stained with Abs specific for TCRV\(\alpha\), TCR\(\beta\), and murine CD4 and analyzed by flow cytometry.

**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\(^{\alpha\alpha}\) Tg mice and B6 controls. DQ2 expression was demonstrated on B cells, macrophages, and dendritic cells from hCD4.DR3-DQ2.MHCII\(^{\alpha\alpha}\) 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 for the DR3-DQ2 haplotype on an Abo/o background (15). However, expression of the hCD4 transgene boosted CD4\(^+\) T cell numbers to 18% of splenic lymphocytes in the hCD4.DR3-DQ2.MHCII\(^{\alpha\alpha}\) Tg mice (Fig. 1B), indicating the importance of the specific interaction between human CD4 and the human class II molecules. Interestingly, TCR V\(\beta\) usage was not significantly different between Tg mice with or without hCD4 (data not shown).

In contrast, in HLA class II Tg mice lacking hCD4 (DR3-DQ2.MHCII\(^{\alpha\alpha}\) 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\(^{\text{a/bv}}\) background (15). However, expression of the hCD4 transgene boosted CD4\(^+\) T cell numbers to 18% of splenic lymphocytes in the hCD4.DR3-DQ2.MHCII\(^{\alpha\alpha}\) Tg mice (Fig. 1B), indicating the importance of the specific interaction between human CD4 and the human class II molecules.
To confirm that the HLA molecules and CD4+ T cells were functional on the MHCII$^{\alpha/\alpha}$ and hCD4 Tg background, CD4+ T cell responses were examined in the hCD4.DR3-DQ2.MHCII$^{\alpha/\alpha}$ Tg mice. Two well-characterized human T cell epitopes were used to immunize Tg and non-Tg animals ($n \geq 5$ mice/epitope): MT65kDa$_{13}$ is a $M. tuberculosis$ heat shock protein-derived peptide that binds exclusively to HLA-DR3 (DRB1*0301) and is recognized by T cells from tuberculous leprosy patients (25); A-gliadin$_{57-73}$ Q65E is a deamidated gluten peptide which includes two overlapping HLA-DQ2-restricted epitopes, DQ2-α-I (PF PQPELPY) and DQ2-α-II (PQPELPYQP), 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$_{13}$, A-gliadin$_{57-73}$ Q65E, or a panel of irrelevant HLA-DR- or DQ-restricted peptide controls. A specific proliferative response to MT65kDa$_{13}$ was observed in T cells isolated from hCD4.DR3-DQ2.MHCII$^{\alpha/\alpha}$ 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.MHCII$^{\alpha/\alpha}$ Tg mice responded specifically to in vitro recall with A-gliadin$_{57-73}$ Q65E in a dose-dependent manner (Fig. 1C). The native version of the A-gliadin$_{57-73}$ peptide triggered a very weak T cell response at high concentrations of peptide (>5 μM; 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.MHCII$^{\alpha/\alpha}$ Tg mice.

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

Given that T cells from hCD4.DR3-DQ2.MHCII$^{\alpha/\alpha}$ 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.MHCII$^{\alpha/\alpha}$ Tg mice are healthy while consuming gluten: histology of the small and large intestine, body weight, and stools were normal; Abs against gliadin or rTG were not detectable. However, prior activation of T cells by immunizing the hCD4.DR3-DQ2.MHCII$^{\alpha/\alpha}$ Tg mice with gluten, chymotrypsindigested gliadin, or various preparations of A-gliadin$_{57-73}$ 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 autoimmunity when codelevered with self-Ags (28). Therefore, non-Tg B6 and hCD4.DR3-DQ2.MHCII$^{\alpha/\alpha}$ 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-rTG Abs.

As shown in Fig. 2A, 2 of 14 animals from the gliadin-immunized plus gluten-fed group developed rTG-specific IgA Abs; Fig. 2A shows only data from day 61 but both animals also had rTG-specific IgA in their serum from day 38. End-point titer analysis revealed that one of these animals was producing high-titer anti-rTG 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.MHCII$^{\alpha/\alpha}$ Tg 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) rTG 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 $\geq$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 titered 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 increased 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 did not enhance the incidence or severity of gluten hypersensitivity in the hCD4.DR3.DQ2.MHCIIΔΔ 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 CD4⁺ T hybridoma specific for the immunodominant CD-associated DQ2-α-II epitope

One obstacle to the development of gluten-induced disease in the hCD4.DR3.DQ2.MHCIIΔΔ Tg mice was that HLA-DQ2 haplotypes, and the MHCIIΔΔ 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.MHCIIΔΔ Tg mice on a NOD background. Nevertheless, this genetic background did not accentuate the gluten-induced phenotype in the hCD4.DR3.DQ2.MHCIIΔΔ 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.MHCIIΔΔ 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 and characterization of a murine HLA-DQ2-restricted, gliadin-specific CD4⁺ T cell hybridoma.

Following fusion of immune T cells, hybridomas were phenotyped and assayed for their Ag specificity. A, FACS histograms of cell surface mouse CD3 (mCD3), mCD4, and hCD4 expression on the BW5147 fusion partner and the representative T hybridoma DB4. B, DB4 T hybridoma cells were cultured with irradiated hCD4.DR3.DQ2.MHCIIΔΔ Tg splenocytes and A-gliadin57–73 Q65E or TPO; after 24 h, supernatants were added to IL-2-dependent CTLLs and their proliferation was assayed after 24 h. C, Activation of DB4 cells incubated with A-gliadin57–73 Q65E or gluten at 5 µg/ml assayed as above. D, Activation of the DB4 hybridomas cultured with A-gliadin57–73 Q65E and a panel of irradiated B-LCLs expressing DQ2 (α1*0501, β1*0201/2) encoded in cis (○) or trans (▲) or DQ8 (α1*0301, β1*0302). Dotted line indicates the EC₅₀ for the two observed responses measured by IL-2 bioassay as above.
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 peptides (61–69, 66–69, 71–75) and an irradiated panel of B-lymphoblastoid cell lines (B-LCLs) expressing different DQA and DQB 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 EC50 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 Vα8.3 and TCR Vβ8.2 (data not shown). T cells using this combination of TCRαβ 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-gliadin57–73 Q65E (DQ2-α-I or DQ2-α-II) was recognized by the HH8 TCR, the hybridoma was cultured with irradiated hCD4.DR3-DQ2.MHCII+ transgenic splenocytes and 15-mer peptides spanning a nine-residue core sequence of the DQ2-α-II peptide or (A) A-gliadin57–73 Q65E or (B) lysine substitutions of each amino acid in A-gliadin57–73 Q65E. Supernatants were added to IL-2-dependent CTLLs 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+ T cells are present and populate the periphery in TCR Tg mice**

A gliadin-specific TCR Tg line was generated on the hCD4.DR3-DQ2.MHCIIαβ background using α/βTCR genes of the HH8 T hybridoma. The TCRα and TCRβ genes cosegregated and flow cytometric analysis indicated that ~93% of the thymocytes were CD4+ and 39% were mature CD4+CD8+ cells (Fig. 5). In the periphery of the TCR Tg mice, 24% of splenic lymphocytes and 53% of MLN lymphocytes were CD4+ (both human and mouse; Fig. 5). Importantly, 97% of peripheral CD4+ T cells expressed the transgene-encoded TCR (Fig. 5). These data indicate normal thymic development and peripheral export of T cells expressing the TCRα and β transgenes on the hCD4.DR3-DQ2.MHCIIαβ background. In contrast, development of the CD8+ T cell compartment is affected by the TCR transgenes as the CD8+CD4+ subset is dramatically reduced in both the periphery and thymus (Fig. 5).

**FIGURE 4.** The fine specificity of murine gliadin-specific CD4+ 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+ α/β Tg splenocytes and (A) A-gliadin57–73 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-gliadin57–73 Q65E. Supernatants were added to IL-2-dependent CTLLs in a 24-h proliferation assay. Bars represent the mean value obtained in a triplicate experiment; error bars represent the SD.

**FIGURE 5.** HH8 TCR Tg mice develop single-positive CD4+ T cells expressing the Tg TCR. Representative dot plots of FACS costaining for Vβ8 and Vα8.3 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+ lymphocytes.
To assess whether the Tg CD4⁺/H11001 T cells were functional in vivo, purified T cells were labeled with the cytoplasmic dye CFSE and transferred into hCD4.DR3-DQ2.MHCII/H9004/H9004/H9004 Tg mice that were then immunized with A-gliadin₅₇₋₇₃ 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-gliadin₅₇₋₇₃ 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-gliadin₅₇₋₇₃, chymotrypsin-digested gliadin, or whole crude gluten, but the proportion of cells dividing was lower than that observed with synthetic A-gliadin₅₇₋₇₃ 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-gliadin₅₇₋₇₃ 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/H9004/H9004 mice. Following transfer, hCD4.DR3-DQ2.MHCII/H9004/H9004/H9004 Tg mice on

![FIGURE 6.](http://www.jimmunol.org/) CD4⁺ T cells from naive TCR Tg mice respond to the DQ2-α-II gliadin peptide both in vitro and in vivo. A, Naive splenocytes from TCR Tg mice were depleted of B cells and cultured with irradiated hCD4.DR3-DQ2.MHCII/H9004/H9004/H9004 splenocytes and A-gliadin₅₇₋₇₃ Q65E; cells were assessed for proliferation by incorporation of [³H]thymidine during the last 24 h of a 72-h culture. B, Naive B cell-depleted splenocytes from TCR Tg mice were CFSE labeled and transferred into hCD4.DR3-DQ2.MHCII/H9004/H9004/H9004 mice immunized with 40 µg of A-gliadin₅₇₋₇₃ Q65E emulsified in CFA. On day 3, cells from draining and nondraining lymph nodes were analyzed by FACS for mCD4, TCR Vβ8.3, and TCR Vβ8. Dot plots are gated on lymphocytes.

![FIGURE 7.](http://www.jimmunol.org/) Gliadin-specific T cells from HH8 TCR Tg mice produce Th1-type cytokines upon encounter with cognate peptide containing the DQ2-α-II epitopes. Cells from the spleen, MLNs, and Peyer's patches (PP) of (A) C57BL/6 mice or (B) TCR Tg mice were cultured with medium alone, 2 µM A-gliadin₅₇₋₇₃ Q65E, or 10 µg/ml Con A. After 48 h, supernatants were added to a BD Biosciences Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit and analyzed by FACS. Dot clusters in each plot represent IL-2, IL-4, IL-5, IFN-γ, and TNF-α (top to bottom). Clusters to the right of the dotted line indicate cytokine production above background levels.
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 axillary 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.MHCIIA/Δ mice to induce enteropathy, including immunization with gliadin peptide/protein before gluten gavage with coadministration of pertussis toxin or coincidental 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.MHCIIA/Δ Tg mice. The lower number of IELs in the TCR Tg mice is probably attributable to the impact of the prearanged 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, homozygosity 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, enteropathy 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 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.MHCIIA/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.MHCIIKb 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.MHCIIKb 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.MHCIIKb 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 ofIELs or villous changes on histological examination. However, systemic administration of pertussis toxin 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.MHCIIKb Tg mice.

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

Next, we considered whether the magnitude of the CD4+ T cell response to gluten might be inadequate in hCD4.DR3-DQ2.MHCIIKb 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.MHCIIKb 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+ 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-α-1 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 A-α-gliadin72–73 Q65E or chymotrypsin-digested gliadin in vivo and secreted Th1-type cytokines implicated in CD (34) in vitro. But ingestion of gluten or A-α-gliadin72–73 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-α-gliadin72–73 (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 and 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.MHCIIKb 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 Bhalal 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.

Disclosures
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 Nexpep Pty Ltd and Nexeain Pty Ltd.

References


