

Sensitization to Gliadin Induces Moderate Enteropathy and Insulinitis in Nonobese Diabetic-DQ8 Mice

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Celiac disease (CD) is frequently diagnosed in patients with type 1 diabetes (T1D), and T1D patients can exhibit Abs against tissue transglutaminase, the auto-antigen in CD. Thus, gliadin, the trigger in CD, has been suggested to have a role in T1D pathogenesis. The objective of this study was to investigate whether gliadin contributes to enteropathy and insulinitis in NOD-DQ8 mice, an animal model that does not spontaneously develop T1D. Gliadin-sensitized NOD-DQ8 mice developed moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction, but not insulinitis. Administration of anti-CD25 mAbs before gliadin-sensitization induced partial depletion of CD25⁺Foxp3⁺ T cells and led to severe insulinitis, but did not exacerbate mucosal dysfunction. CD4⁺ T cells isolated from pancreatic lymph nodes of mice that developed insulinitis showed increased proliferation and proinflammatory cytokines after incubation with gliadin but not with BSA. CD4⁺ T cells isolated from nonsensitized controls did not respond to gliadin or BSA. In conclusion, gliadin sensitization induced moderate enteropathy in NOD-DQ8 mice. However, insulinitis development required gliadin-sensitization and partial systemic depletion of CD25⁺Foxp3⁺ T cells. This humanized murine model provides a mechanistic link to explain how the mucosal intolerance to a dietary protein can lead to insulinitis in the presence of partial regulatory T cell deficiency. *The Journal of Immunology*, 2011, 187: 4338–4346.

The environmental factors that trigger most autoimmune diseases remain unknown. One exception is celiac disease (CD), which is one of the most common autoimmune disorders (1). CD is caused by the ingestion of gluten, a mixture of wheat proteins composed of gliadin and glutenin (1–4). Genetic factors also have a crucial role in CD pathogenesis (4). Almost all patients with CD carry HLA-DQ2 or HLA-DQ8, and these alleles contribute 40% of disease susceptibility (5). HLA-DQ8 and -DQ2 heterodimers confer susceptibility to CD by presenting a set of toxic gliadins and glutenins to specific CD4⁺ T lymphocytes. Adult CD diagnoses have dramatically increased in North America because of increased diagnostic efficiency and increased

prevalence. CD is now a public health concern with a prevalence of ~1% among North Americans (2, 6).

Type 1 diabetes (T1D) is an autoimmune disease that results from a T cell-mediated attack of β cells in pancreatic islets. As with CD, there is a strong genetic component to T1D, of which the HLA class II genes *DQ* and *DR* play a major role (7). These susceptibility genes are thought to be important regulators of immune responses (8). However, the declining proportion of newly diagnosed disease in children with high-risk genotypes suggests that environmental factors have an important pathogenic role in T1D, but the exact triggers remain largely unclear (9).

CD and T1D are comorbid conditions (10–12). CD is diagnosed in 3–11% of T1D patients, and ~10% of children and 2% of adults with T1D exhibit Abs against tissue transglutaminase (tTG), the autoantigen in CD (3, 10, 13–15). The association between CD and T1D has been attributed to their similar genetic basis (16–18). HLA haplotypes, such as DR3-DQ2, have been associated with defective oral tolerance and enhanced immune responses to dietary proteins (19, 20). Intriguingly, parallel birth cohort studies showed that infants at genetic risk for CD and T1D are at greater risk of either disease when exposed early to dietary cereals (21, 22). A genotyping study that enrolled 8064 T1D patients and 9339 control subjects showed that patients with T1D and CD express seven common alleles that regulate autoimmune responses (23, 24). Thus, genetic predisposition may confer susceptibility through DQ2/DQ8 HLA and non-HLA mechanisms that involve dysregulated immune responses to gut-encountered Ags (25). Studies in clinical and T1D animal models have proposed that exposure to gliadin in the diet contributes to diabetes in genetically susceptible hosts (22, 23, 26–29).

In this study, we used mice that lack all mouse endogenous class II molecules, but express the human *DQ8* gene, in the autoimmune predisposing background of NOD mice (30) to investigate the role of gliadin-sensitization in the development of enteropathy and insulinitis. In contrast to NOD mice, a validated animal model of

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Abbreviations used in this article: CD, celiac disease; IEL, intraepithelial lymphocyte; MLN, mesenteric lymph node; NOR, nonobese resistant; PBS-T, PBS-0.05% Tween-20; PLN, pancreatic lymph node; PT-gliadin, peptic-tryptic digest of gliadin; T1D, type 1 diabetes; Treg, regulatory T cell; tTG, tissue transglutaminase.

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diabetes (31, 32), NOD-DQ8 mice do not spontaneously develop diabetes (33, 34). Our findings demonstrate that gliadin-sensitization resulted in barrier dysfunction and moderate enteropathy, but no insulinitis. However, a partial depletion of regulatory T cells before gliadin sensitization induced severe insulinitis. The presence of gliadin-responsive T cells in the pancreatic lymph nodes (PLNs) of mice that develop insulinitis suggests that gliadin-specific T cell reactivity has a role in insulinitis development in this model.

Materials and Methods

Mice

Transgenic male mice that express HLA-DQ8 in an endogenous MHC class II-deficient background were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB⁺ DQ8 mice (33). Eight- to 10-wk-old male mice were used for experiments. Mice were weaned and maintained on a low-fat (4.4%), gluten-free diet, purchased from Harlan Laboratories and bred in a conventional, specific pathogen-free colony at McMaster University. Fourteen-week-old male NOD mice, 6-wk-old NOD mice, and 14-wk-old nonobese resistant (NOR) mice (obtained from Dr. J. Danska, Hospital for Sick Children, Toronto, ON, Canada) were used in additional experiments as positive and negative controls, respectively, for the development and evaluation of insulinitis. Glycemic status was monitored weekly using a glucometer (Abbott Diabetes Care) and was determined at the time of sacrifice using a Roche modular instrument. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Anti-CD25 Ab treatment

Prior to gliadin sensitization, mice received two i.p. injections of monoclonal anti-CD25 Abs (PC61, 250 μ g; Leinco Technologies, St. Louis, MO), 1 wk apart. This procedure induces a partial depletion of CD4⁺ CD25⁺ Foxp3⁺ T cells and induces an immune dysregulation. Control groups were pretreated with two i.p. injections of PBS (250 μ l) 1 wk apart.

Gliadin sensitization

One week after anti-CD25 Ab treatment, mice were sensitized with a peptic-tryptic digest of gliadin (PT-gliadin). PT-gliadin was prepared as described previously (35). Gliadin (Sigma-Aldrich) was dissolved in endotoxin-free 0.2 N HCl for 2 h in a 37°C water bath with 1 g pepsin (Sigma-Aldrich). After 2 h of digestion, the pH was adjusted to 7.4 using endotoxin-free 2 M NaOH. Trypsin (Sigma-Aldrich) was added, the solution was boiled vigorously for 30 min, and the PT-gliadin was stored at -20°C. To sensitize the mice, they were gavaged with 500 μ g PT-gliadin plus 25 μ g cholera toxin (Sigma-Aldrich) once per week for 3 wk. Gliadin-sensitized mice were switched to a gluten-containing diet at the time of sensitization. Another set of mice was sensitized with 500 μ g BSA (Sigma-Aldrich) plus 25 μ g cholera toxin once per week for 3 wk. Control groups were gavaged with 25 μ g cholera toxin only. Control groups were maintained on a gluten-free diet throughout the experiments.

Glucose tolerance test

To determine impaired glucose tolerance, mice were fasted for 6 h. Mice were then injected with glucose (Sigma-Aldrich) i.p. at a dose of 1 g/kg. Venous plasma glucose was checked prior to glucose injection, and at 20, 40, 60, 90, and 120 min after glucose injection (36). Blood glucose levels were tested using a glucometer (Abbott Diabetes Care). Glucose tolerance tests were conducted after the third gliadin-sensitization.

FACS analysis

One week after the second anti-CD25 Ab injections, cell suspensions of spleen, mesenteric lymph nodes (MLNs) and PLNs were prepared in RPMI 1640 (1% Penstrep, 10% FCS, 2 mM L-glutamine) by passing organs through a 100- μ m nylon mesh screen to dissociate the cells. Suspensions were depleted of RBCs by lysis and resuspended in FACS buffer (PBS containing 0.1% azide and 2% BSA). Cells were stained with fluorochrome-labeled cell-surface Abs including CD4-allophycocyanin (RM4-5), CD8a-PerCP (53-6.7), and CD25-PE (7D4) for 30 min at 4°C (BD Biosciences-Pharmingen). For intracellular staining, cells were permeabilized using the Foxp3 staining buffer set (eBioscience) and incubated with FITC-conjugated Abs toward Foxp3 (FJK-16s; eBioscience) for 90 min at 4°C. Stained cells were acquired using the LSR II (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Histologic evaluation

Cross-sections of the proximal small intestine were fixed in 10% formalin, embedded in paraffin, and stained with H&E for histologic evaluation by light microscopy. Two sections of the proximal small intestine were evaluated for evidence of inflammation, and villus-to-crypt ratios were determined. Twenty villus-to-crypt ratios were measured for each mouse in a blinded fashion. CD3⁺ intraepithelial lymphocytes (IELs) per 20 enterocytes in five randomly chosen villus tips were counted according to the previously described methods and expressed as IEL/100 enterocytes (37).

The pancreas was removed, fixed in 10% formalin, and embedded in paraffin. Sections of the pancreas were stained with H&E for histologic evaluation and assessment of insulinitis. As described previously, insulinitis was determined by evaluating islet infiltration from grade 0–4: 0, no infiltration; 1, perivascular or periductal infiltrates; 2, leukocyte penetration of up to 25% islet mass; 3, leukocytes penetration of up to 75% of islet mass; 4, end-stage insulinitis with <20% of islet mass remaining (38–40). An average insulinitis score was determined for each group of mice. The liver and lungs were also removed and fixed in formalin. Sections were embedded in paraffin and stained with H&E and evaluated for signs of inflammation. Slides were viewed by light microscopy (Olympus), and images were acquired and analyzed using Image Pro Plus.

Immunohistochemistry for CD3⁺ cells

Immunostaining for CD3⁺ cells was performed on paraffin sections to detect the presence of IELs in sections of the proximal small intestine as described previously (41). Paraffin sections were incubated overnight at 4°C with rabbit anti-mouse primary Abs to CD3 (1:2000; Dako); this was followed by incubation with HRP conjugated anti-rabbit Abs. The Abs were visualized using 3-amino-9-ethylcarbazole and counterstained with Mayer hematoxylin. Negative controls were performed in the absence of primary Ab. Immunostaining for CD3⁺ cells was performed on paraffin sections of the pancreas as described above. Slides were viewed by light microscopy (Olympus) under 40 \times magnification. Images were acquired and analyzed using Image Pro Plus.

Anti-tTg ELISA

tTg was derived from guinea pig liver (Sigma-Aldrich). Transglutaminase was diluted in PBS and 0.1 μ g was added to each well of a 96-well Nunc-Immuno plate (Nunc). Plates were incubated overnight at 4°C. Plates were washed five times with PBS and blocked with 1% BSA in PBS for 1 h at room temperature. Serum was added to the plates at 50 μ l per well and incubated for 1 h at room temperature. Plates were washed five times, and secondary anti-mouse IgA (Sigma-Aldrich) Abs were added and incubated an additional 2 h. Secondary Abs were HRP conjugated, and tetramethylbenzidine was the substrate (SurModics). Positive reactivity was determined using a positive cutoff value of ≥ 3 SD above the mean of the control group (42).

Anti-gliadin ELISA

Gliadin (Sigma-Aldrich) was dissolved in 70% ethanol and then diluted in PBS. Ninety-six-well Nunc-Immuno plates (Nunc) were coated with gliadin by adding 5 μ g to each well. The plate was incubated overnight at 4°C. Plates were washed with PBS-0.05% Tween-20 (PBS-T) and blocked using 1% BSA in PBS for 1 h at room temperature. Serum was diluted into 1% BSA in PBS either 1:10 for detection of IgA or 1:50 for detection of IgG and added to plates for 2 h at room temperature. Plates were washed with PBS-T, and HRP conjugated secondary anti-mouse IgA (Sigma-Aldrich) or anti-mouse IgG (Sigma-Aldrich) Abs were added to the wells for 1 h at room temperature. Plates were washed with PBS-T, and tetramethylbenzidine (SurModics) was used as the substrate.

Ussing chambers

Two sections of jejunum from each mouse were used for Ussing chamber experiments as described previously (41, 43). A 3–4-cm piece of jejunum was collected, cut into two sections, and placed in Krebs buffer aerated with 95% O₂ and 5% CO₂ (pH 7.3–7.4). Each segment of intestine was cut open along the mesenteric border to form a flat mucosal sheet and mounted into an Ussing chamber. The chamber exposed 0.6 cm² of tissue surface area to 8 ml of circulating oxygenated Krebs buffer containing 10 mM glucose (serosal side) and 10 mM mannitol (mucosal side) maintained at 37°C. Net active transport across the epithelium was measured via a short circuit current response (Isc, μ A) injected through the tissue under voltage-clamp conditions. Tissue conductance (the passive permeability to ions) was calculated using Ohm's law. Baseline Isc (μ A/cm²) and conductance (mS/cm²) were recorded at equilibrium, 20 min after mounting jejunum sections.

In vitro proliferation assay and cytokine analysis

Cell suspensions of PLNs were prepared in RPMI 1640 (1% penicillin/streptomycin, 10% FCS, 2 mM L-glutamine) by passing organs through a 100- μ m nylon mesh screen to dissociate cells. CD4⁺ T cells were isolated from PLNs through negative selection (EasySep Mouse CD4⁺ T Cell Enrichment Kit; Stemcell). Cell yield was assessed by FACS analysis, with an enriched population of 89–96% CD4⁺ T cells. Isolated CD4⁺ T cells were labeled with CFSE as previously described and washed three times with RPMI 1640 (44). Splenocytes were treated with mitomycin C (Sigma-Aldrich), and dendritic cells were isolated through positive selection (Easysep Mouse CD11c Selection Kit, Stemcell). Dendritic cells (5×10^4 cells/well) were cocultured with isolated CD4⁺ T cells (2×10^5 cells/well) in the presence of PT-gliadin (500 μ g/ml), BSA (500 μ g/ml), media alone in a round-bottom 96-well plate. For positive controls, cells were cultured with Con A. Cells were cultured for 4 d at 37°C, 5% CO₂. Cells were harvested on day 4 to assess proliferation by FACS analysis. Cells were resuspended in FACS buffer and stained with anti-CD4-allophycocyanin (BD Biosciences-Pharmingen) and 7-AAD (Sigma-Aldrich). CFSE-labeled cells were acquired using the LSR II (BD Biosciences). Viable cells, as determined by 7AAD exclusion, were gated on CD4⁺ T cells. CFSE intensity for this population was determined using FlowJo software (TreeStar).

Supernatants from cell cultures were collected at 48 and 96 h. The presence of proinflammatory cytokines in the supernatant was determined using a cytometric bead array inflammation kit (BD Biosciences) and analyzed using BD FACSSarray Bioanalyzer System (BD Biosciences). The presence of TGF- β was determined by ELISA (R&D Systems).

Statistical analysis

Statistical analysis was performed with GraphPad Prism software. For more than two treatment groups, an ANOVA with a Bonferroni post hoc test for multiple comparisons was used. When two groups were compared, an unpaired *t* test was used; $p < 0.05$ was considered significant. Data are displayed as mean \pm SEM.

Results

Gliadin-sensitized NOD-DQ8 mice developed barrier dysfunction and altered villus/crypt ratios

We have shown previously that HLA-DQ8 mice develop activation of the innate and adaptive arms of the immune system, and increased tissue conductance after gliadin sensitization (41, 43, 45). Despite this finding, gliadin-sensitized HLA-DQ8 mice develop only a mild enteropathy, characterized by increased IELs, similar to a Marsh I lesion in CD (46). To determine whether NOD-DQ8 mice develop gliadin-induced barrier dysfunction and enteropathy, mice were sensitized with a peptic-tryptic digested form of gliadin once per week for 3 wk. Cholera toxin was used as a mucosal adjuvant. A group of mice was pretreated with anti-CD25 mAbs to determine whether a preexisting immune dysregulation could exacerbate gliadin-induced mucosal dysfunction and enteropathy. Regulatory T cells (Tregs) are marked by the expression of CD4, CD25, and the transcription factor Foxp3. Therefore, we define a Treg as a CD4⁺CD25⁺Foxp3⁺ T cell, and will refer to CD4⁺CD25⁺Foxp3⁺ T cells as Tregs or CD4⁺CD25⁺Foxp3⁺ Tregs hereafter. Furthermore, CD25 (IL-2R α subunit) is essential for the generation, peripheral expansion, and maintenance of Tregs (47–52). Monoclonal anti-CD25 Abs have been used to deplete the Treg population (47, 49, 53). Therefore, NOD-DQ8 mice were treated with two i.p. doses of anti-CD25 mAbs, 1 wk apart, prior to gliadin sensitization. We used Ussing chambers to measure tissue conductance in sections of the small intestine. Enteropathy was evaluated by villus-to-crypt ratios and by immunostaining for CD3⁺ IELs (37, 41).

Gliadin-sensitized NOD-DQ8 mice had increased tissue conductance compared with untreated controls and to anti-CD25 treated mice (Fig. 1). No difference in conductance was seen between untreated controls and anti-CD25 mAb-treated plus BSA-sensitized mice. There was also no difference in tissue conductance between gliadin-sensitized mice and sensitized mice that

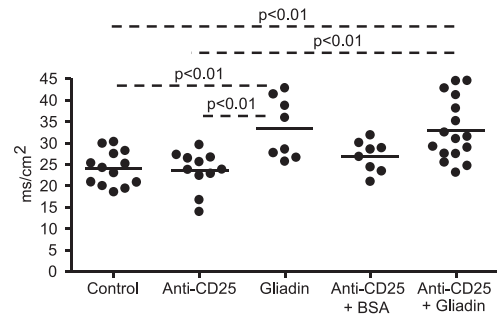


FIGURE 1. Impaired intestinal barrier function in gliadin-sensitized NOD-DQ8 mice. Sections of small intestine were mounted in Ussing chambers and tissue conductance (ms/cm²) was measured 24 h after the final gavage. Each dot represents an individual mouse; *p* values were computed using an ANOVA with a post hoc test for multiple comparisons.

were pretreated with anti-CD25 mAbs, suggesting that barrier dysfunction was gliadin-dependent and was not induced by anti-CD25 mAb treatment. Gliadin-sensitized mice developed lower villus-to-crypt ratios (Fig. 2) and increased IEL counts (Fig. 3), reminiscent of a Marsh II lesion, compared with untreated control mice, anti-CD25 mAb treated mice, and anti-CD25 mAb-treated plus BSA-sensitized mice. Previous Treg depletion did not further increase the severity of gliadin-induced enteropathy in NOD-DQ8 mice. These results suggest that, compared with HLA-DQ8 mice, DQ8 mice on the NOD background are more sensitive to gliadin-

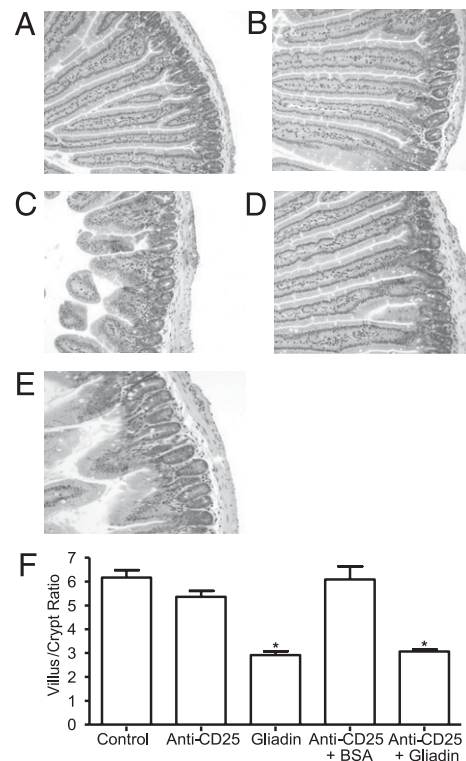


FIGURE 2. Histopathology of the small intestine showing decreased villus-to-crypt ratio in gliadin-sensitized NOD-DQ8 mice. H&E-stained sections of the proximal small intestine in untreated controls (A) and anti-CD25 mAb-treated (B), gliadin-sensitized (C), anti-CD25 mAb treated plus BSA-sensitized (D), and anti-CD25 mAb treated plus gliadin-sensitized mice (E). Original magnification $\times 10$. F, Quantification of villus/crypt ratios ($n = 8$ for each group). Data represented as mean \pm SEM; *p* values were computed using an ANOVA with a post hoc test for multiple comparisons. * $p < 0.05$ versus control mice, anti-CD25 mAb treated mice, and anti-CD25 + BSA-treated mice.

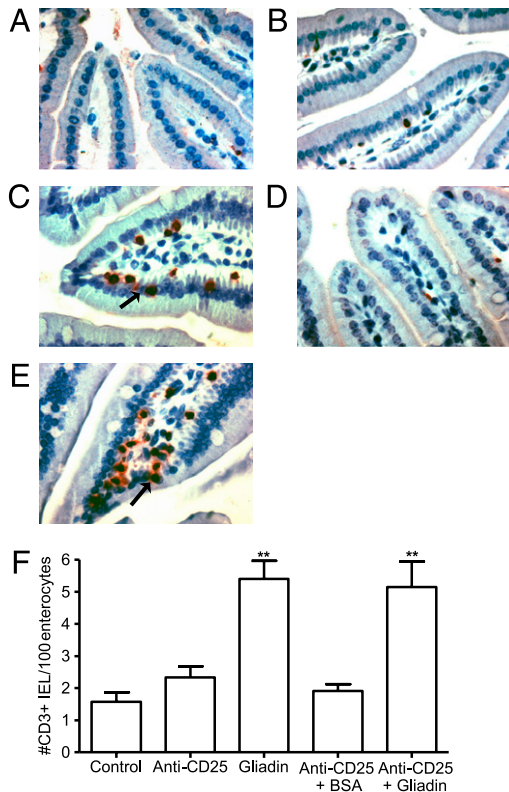


FIGURE 3. Immunohistochemistry showing increased number of CD3⁺ IELs in gliadin-sensitized mice. CD3⁺-stained sections of the proximal small intestine in untreated controls (A), anti-CD25 mAb-treated (B), gliadin-sensitized (C), anti-CD25 mAb-treated plus BSA-sensitized (D), and anti-CD25 mAb-treated plus gliadin-sensitized mice (E). Original magnification $\times 40$. Black arrows indicate IELs. F, Quantification of CD3⁺ cells in villi tips, expressed as IEL per 100 enterocytes ($n = 8$ for each group). Data are represented as mean \pm SEM; p values were computed using an ANOVA with a post hoc test for multiple comparisons. ** $p < 0.01$ versus control mice, anti-CD25 mAb treated mice, and anti-CD25 + BSA-treated mice.

induced enteropathy, and partial depletion of Tregs has no evident role in the induction of enteropathy in the model.

Gliadin-sensitized NOD-DQ8 mice develop anti-gliadin and anti-tTG Abs

In contrast to anti-gliadin IgA and IgG Abs, IgA autoantibodies toward tTG are highly specific for CD (54). HLA-DQ8 mice develop anti-gliadin IgG Abs after gliadin-sensitization (41, 45). We tested whether gliadin sensitization in NOD-DQ8 mice led to the production of Abs toward gliadin and tTG. Four of 11 gliadin-sensitized mice developed anti-gliadin IgG Abs in the serum and three of nine anti-CD25 plus gliadin-sensitized mice developed anti-gliadin IgG Abs. Anti-gliadin IgG Abs were not detected in the serum of nonsensitized mice (Fig. 4A). Anti-tTG IgA Abs were found in the serum of three gliadin-sensitized mice and two anti-CD25 mAb-treated plus gliadin-sensitized mice. No control, anti-CD25 mAb treated only, or BSA-sensitized mouse tested positive for anti-tTG Abs (Fig. 4B). The presence of anti-tTG Abs in a proportion of gliadin-sensitized mice is in accordance with the more moderate enteropathy observed in these mice compared with HLA-DQ8 mice (41).

Anti-CD25 Ab treatment partially depleted CD4⁺CD25⁺Foxp3⁺ T cells

To confirm that anti-CD25 mAb treatment depleted the regulatory population, NOD-DQ8 mice were treated with two i.p. doses of

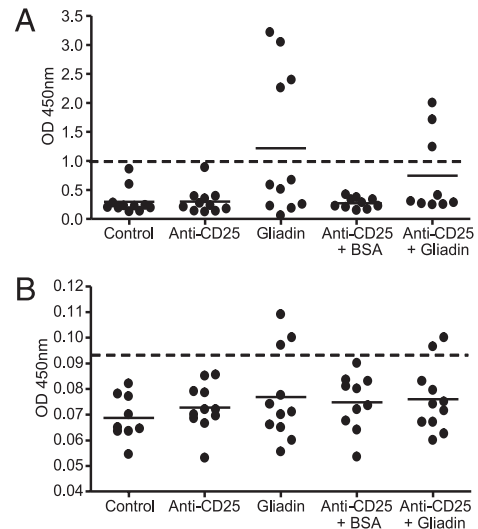


FIGURE 4. Anti-gliadin and anti-tTG ELISAs. Serum was collected from NOD-DQ8 mice, and the presence of anti-gliadin IgG Abs (A) and anti-tTG IgA Abs (B) was determined by ELISA. Anti-gliadin and anti-tTG positive reactivity was determined using a positive cutoff value of ≥ 3 SD above the mean of control mice (dotted line) (40). Each dot represents an individual mouse.

anti-CD25 mAbs, each 1 wk apart. Cells were collected 1 wk following the second Ab treatment, and the percentage of Tregs was determined. Similar to previous findings, we showed a partial depletion of CD4⁺CD25⁺Foxp3⁺ Tregs (Supplemental Fig. 1) (47, 49). Compared with PBS-treated controls, the CD25⁺Foxp3⁺ cells decreased from 10.57 to 2.32% of the total CD4⁺ lymphocyte population in the spleen of anti-CD25 mAb-treated mice (Supplemental Fig. 1A, left panels). The remaining Foxp3⁺ cells expressed low levels of CD25 or no CD25. In addition, anti-CD25 mAb treatment resulted in the partial depletion of Tregs in the MLN and PLN. In the MLN, the CD25⁺Foxp3⁺ cells decreased from 11.54 to 3.27% of the CD4⁺ T cell population (Supplemental Fig. 1A, center panels). In the PLN, the CD25⁺Foxp3⁺ cells decreased from 11.94 to 3.0% of the CD4⁺ T cell population (Supplemental Fig. 1A, right panels). Thus, anti-CD25 mAb treatment before gliadin sensitization led to a partial, but significant depletion of Tregs in the spleen, MLN, and PLN (Supplemental Fig. 1B).

Partial Treg depletion and gliadin sensitization was associated with insulinitis in NOD-DQ8 mice

NOR and NOD mice were used as negative and positive controls, respectively, to validate the insulinitis grading in NOD-DQ8 mice. At 6 wk of age, NOD mice had developed mild periinsulinitis (Supplemental Fig. 2A, 2D), while severe insulinitis developed at 14 wk of age (Supplemental Fig. 2B, 2D). NOR mice developed mild periinsulinitis at 14 wk of age (Supplemental Fig. 2C, 2D), as previously reported (55). Intestinal tissue conductance was significantly higher in 14-wk-old NOD mice, but not in 6-wk-old NOD mice, compared with NOR controls. NOR mice displayed normal barrier function (Supplemental Fig. 3). Thus, although NOD mice displayed higher tissue conductance values at the preinsulinitis stage, only at 14 wk of age (insulinitis stage) did this difference become statistically significant.

We investigated whether gliadin-induced mucosal changes were linked to insulinitis in NOD-DQ8 mice. Previous studies have shown that NOD-DQ8 mice on a gluten-containing diet develop a gliadin-dependent blistering of the skin, but do not develop diabetes (33). In addition, gliadin-sensitized HLA-DQ8 mice have shown

increased IL-10 production and increased recruitment of regulatory Foxp3⁺ cells within the lamina propria. It has been hypothesized that this regulatory immune response to gliadin may protect mice from overt autoimmunity (41, 45). Thus, we used anti-CD25 mAbs to partially deplete the Tregs, which we defined as CD4⁺CD25⁺Foxp3⁺ T cells (47, 56) prior to gliadin sensitization. Pancreatic islets were analyzed for infiltration of immune cells 24 h after the final gavage. Untreated control mice had normal islets (Fig. 5A). Anti-CD25 treated mice also had normal islets with no significant infiltration (Fig. 5B). Similar to the findings of Marietta et al. (33), gliadin-sensitized mice did not develop severe insulinitis (Fig. 5C). However, mice that were pretreated with anti-CD25 mAbs and subsequently sensitized with gliadin developed severe insulinitis (Fig. 5E). The insulinitis scores of anti-CD25 plus gliadin-sensitized mice were similar to those of 14-wk-old NOD mice (Supplemental Fig. 2). Treatment of anti-CD25 mAbs plus BSA sensitization did not lead to insulinitis, suggesting that the response was not induced by an unrelated Ag and was gliadin-dependent (Fig. 5D). The insulinitis scores of anti-CD25 mAb treated plus

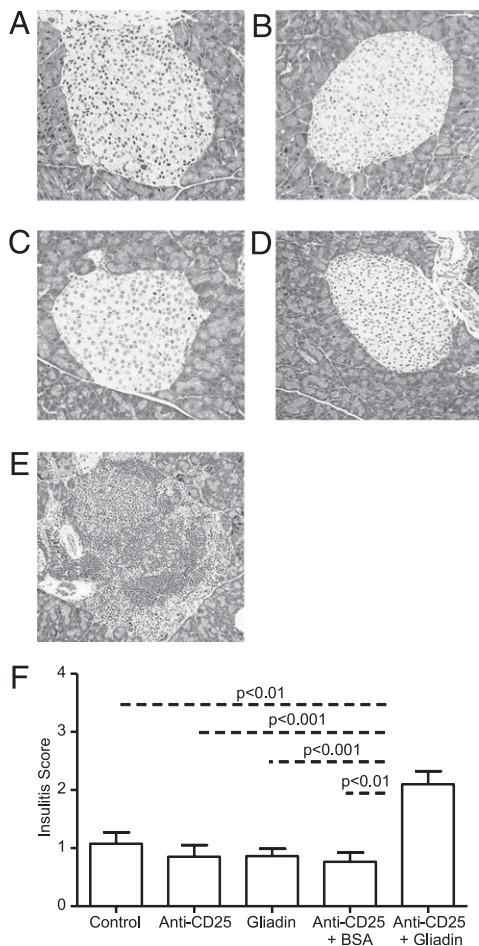


FIGURE 5. Gliadin-sensitized NOD-DQ8 mice develop severe insulinitis in the presence of an immune dysregulation. Insulinitis was determined by evaluating H&E-stained sections of the pancreas for islet infiltration, scoring each islet from grade 0 (no infiltration) to grade 4 (end-stage insulinitis with <20% of islet mass remaining) (38–40). An average insulinitis score was determined for each group of mice: untreated controls (A), anti-CD25 mAb treated mice (B), gliadin-sensitized mice (C), anti-CD25 mAb-treated plus BSA-sensitized mice (D), and anti-CD25 mAb treated plus gliadin-sensitized mice (E). Original magnification $\times 10$. F, Quantification of insulinitis scores for multiple mice ($n = 9$ per group). Data are represented as mean \pm SEM; p values were computed using an ANOVA with a post hoc test for multiple comparisons.

gliadin-sensitized mice were greater than those in untreated controls, anti-CD25 mAb treated mice, gliadin-sensitized, and to anti-CD25 mAb treated plus BSA-sensitized mice (Fig. 5F). The infiltrates were composed of CD3⁺ lymphocytes (Fig. 6). No differences were seen in blood glucose levels (data not shown) or glucose tolerance between groups (data not shown). These results suggest that NOD-DQ8 mice are susceptible to developing an inflammatory response in β -cell islets when systemic partial depletion of CD4⁺CD25⁺Foxp3⁺ cells is induced prior to gliadin sensitization.

Partial CD4⁺CD25⁺Foxp3⁺ cell depletion and gliadin sensitization did not lead to widespread autoimmunity

To determine whether inflammation was present in other organs, H&E-stained sections of the liver and lungs were examined for signs of infiltration and inflammation. There was no significant infiltration observed in the liver of nonsensitized controls, anti-CD25 mAb treated mice, gliadin-sensitized mice, anti-CD25 mAb treated plus BSA-sensitized mice, or in anti-CD25 mAb plus gliadin-sensitized mice (data not shown). Similarly, none of the groups exhibited inflammation within the lungs (data not shown). Thus, the infiltration observed in anti-CD25 mAb-treated plus gliadin-sensitized mice was restricted to the pancreas, indicating an absence of generalized autoimmunity.

T cells from PLNs of NOD-DQ8 mice that developed insulinitis exhibit increased proliferation and proinflammatory cytokine production when incubated with gliadin

HLA-DQ8 mice have shown increased proliferative responses toward gliadin peptides in both the spleen and MLN (45, 57). Evidence suggests that the gut and the pancreas are immunologically linked. In NOD mice, islet-infiltrating lymphocytes express the $\alpha 4\beta 7$ integrin, a gut-homing receptor (58). The ligand for $\alpha 4\beta 7$, MadCAM-1, is expressed within the pancreas and is

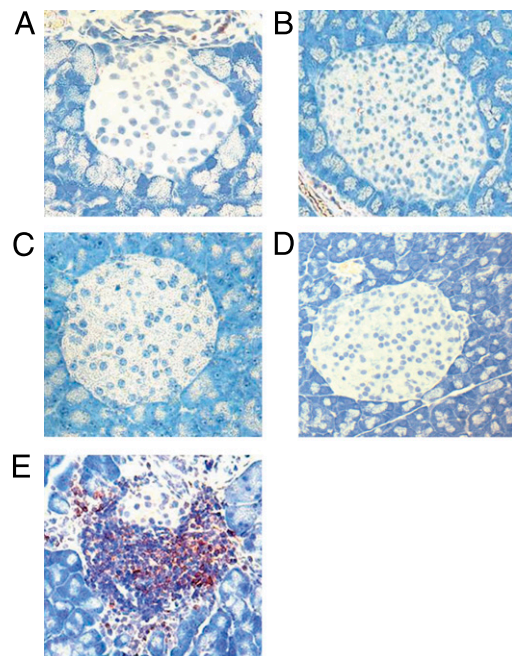


FIGURE 6. Immunohistochemistry showing the presence of CD3⁺ lymphocytes in pancreatic islets in NOD-DQ8 mice. Representative CD3⁺-stained sections of the pancreas in untreated controls (A), anti-CD25 mAb-treated (B), gliadin-sensitized mice (C), anti-CD25 mAb plus BSA-sensitized (D), and anti-CD25 mAb-treated plus gliadin-sensitized mice (E). Original magnification $\times 20$.

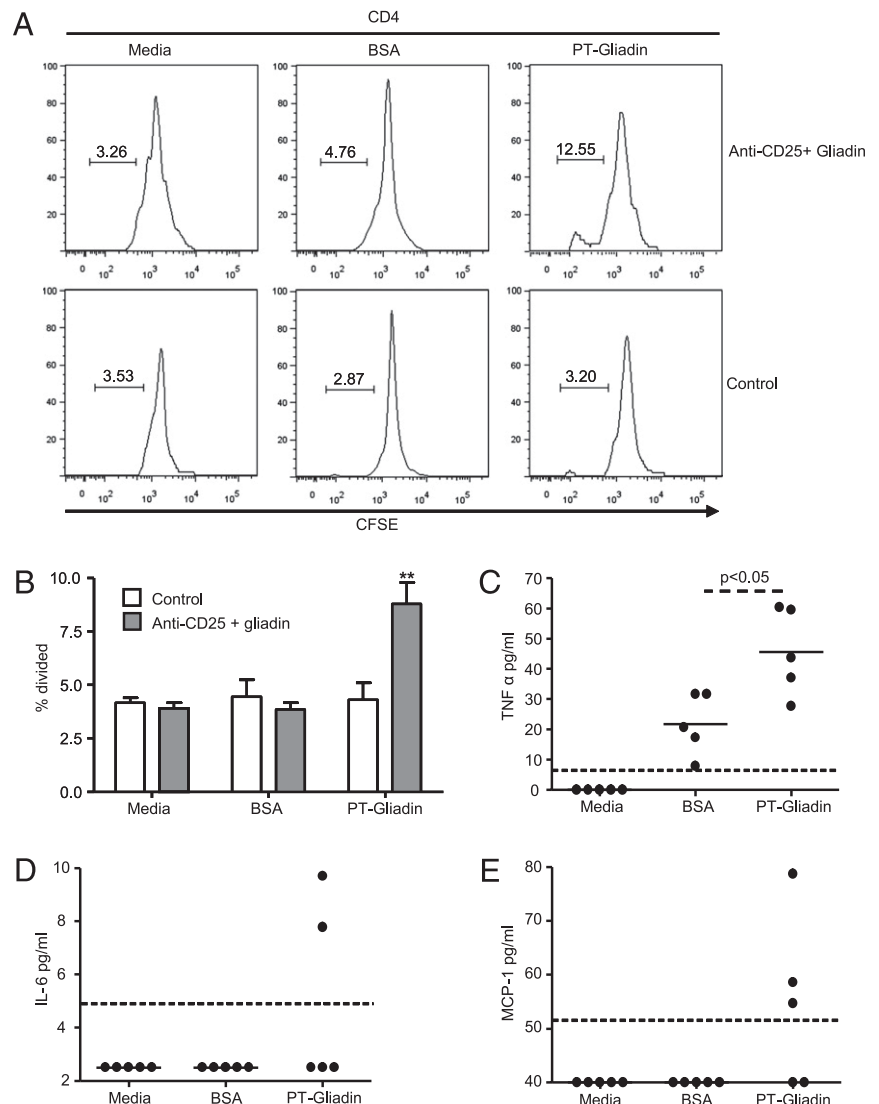
upregulated during insulinitis (59). These findings suggest that lymphocytes displaying gut homing markers are able to circulate between the gut and the pancreas, and that immune responses to dietary Ags may modulate insulinitis. We therefore determined whether cells in the PLNs of gliadin-sensitized NOD-DQ8 mice responded to gliadin by measuring proliferation and cytokine production. CD4⁺ T cells were isolated from the PLNs of control mice and anti-CD25 mAb plus gliadin-sensitized mice and incubated with gliadin, BSA, or media alone. In anti-CD25 mAb plus gliadin-sensitized mice, gliadin stimulation led to increased proliferation compared with BSA and media alone. In control mice, no increase in proliferation was observed in gliadin-stimulated cultures (Fig. 7A, 7B). Compared with untreated controls, gliadin stimulation increased proliferation in anti-CD25 plus gliadin-sensitized mice. Cytokine production was measured in the PLN cell culture supernatants. There was a significant increase in production of the proinflammatory cytokine TNF- α in the cell cultures from mice that developed insulinitis and were incubated with gliadin compared with BSA (Fig. 7C). In cultures from mice that developed insulinitis and were cultured with media or BSA, the production of MCP-1 and IL-6 was below the limit of detection, whereas incubation with gliadin resulted in detectable levels of both MCP-1 and IL-6 in some of the mice (Fig. 7D, 7E). No differences were found in IL-10 or TGF- β production (data not

shown). In PLN cell cultures from control mice, all cytokine levels were below the limit of detection (data not shown). These results suggest that gliadin-specific T-cells are found within the PLNs of mice that develop insulinitis and that these cells produce a Th1 cytokine response.

Discussion

The main objective of this study was to investigate the role of gliadin in the development of enteropathy and insulinitis in NOD-DQ8 mice. T cells in NOD-DQ8 mice are selected in the thymus in a DQ8-restricted manner. The presence of the *DQ8* gene ensures efficient gliadin Ag presentation by APCs to CD4⁺ T cells (45, 57, 60). We found that gliadin sensitization in NOD-DQ8 mice induced moderate enteropathy, characterized by decreased villus-to-crypt ratios and increased IEL counts in the proximal small intestine. Similar to other DQ8 transgenic mouse models, NOD-DQ8 mice developed gliadin-induced intestinal barrier dysfunction. These changes were induced by gliadin, as the immune and functional changes were not observed after sensitization with an unrelated Ag—BSA. Development of severe insulinitis, however, required partial depletion of CD25⁺Foxp3⁺ T cells prior to gliadin sensitization. T cells isolated from the PLNs of mice that developed insulinitis exhibited enhanced proinflammatory cytokine production and proliferation when cultured with gliadin,

FIGURE 7. T cells isolated from the PLNs of mice that develop insulinitis respond to gliadin stimulation. **A**, CD4⁺ T cells were isolated from the PLNs of control and anti-CD25 mAb-treated plus gliadin-sensitized mice and labeled with CFSE. CD4⁺ T cells were incubated with APCs with PT gliadin (*right panels*), BSA (*center panels*), or media alone (*left panels*) for 4 d. Proliferation was assessed with FACS analysis. Gated population represents the percentage of proliferated cells. **B**, Quantification of proliferation in **A** for control mice (*n* = 5) and anti-CD25⁺ gliadin mice (*n* = 5). Data are represented as mean \pm SEM; *p* values were computed using an ANOVA for multiple comparisons. ***p* < 0.01 versus other groups. TNF- α (**C**), IL-6 (**D**), and MCP-1 (**E**) production was assessed in cell culture supernatants from mice that developed insulinitis, using a cytometric bead array inflammation kit. The dotted line represents the limit of detection; *p* values were computed using an unpaired *t* test.



compared with culture with BSA or media alone. CD4⁺ T cells isolated from the PLNs of control mice did not respond to gliadin or BSA. These data suggest that a combination of mucosal damage and breakdown in tolerance to gliadin led to insulinitis in NOD-DQ8 mice.

There is mounting evidence that a defect in the intestinal barrier can promote T1D (61, 62). Several animal models of T1D have demonstrated that increased intestinal permeability can be detected prior to the clinical onset of diabetes (63–65). Barrier dysfunction has also been observed in patients with T1D and in their relatives (66, 67). A recent study in NOD mice has shown that infection with *Citrobacter rodentium* resulted in increased intestinal permeability and accelerated onset of insulinitis and diabetes (68). The results suggest an adjuvant role of intestinal barrier dysfunction in diabetes development. In our study, 14-wk-old NOD mice used as positive controls for insulinitis exhibited barrier dysfunction and severe insulinitis. Because gliadin is known to affect intestinal permeability in animal models of gluten sensitivity and in humans with CD (41, 65, 69–71), we investigated whether NOD-DQ8 mice developed gliadin-induced barrier dysfunction and insulinitis. It has been suggested that the patients with CD and the greatest risk of developing extraintestinal autoimmunity are those who have had a longer duration of gluten exposure (72). Studies in animal models and humans have shown a reduction in T1D risk following implementation of a gluten-free diet (29, 73, 74). We found that gliadin sensitization led to increased tissue conductance and an enteropathy, characterized by decreased villus-to-crypt ratios and increased IELs, that is reminiscent of moderate gluten sensitive enteropathy (75). In addition, a proportion of gliadin-sensitized mice developed both anti-gliadin IgG and anti-tTG IgA Abs. Similarly, a recent article using DQ8 transgenic mice that overexpress IL-15 in the lamina propria found that mice fed gliadin developed anti-gliadin and anti-tTG Abs, intraepithelial lymphocytosis, and IFN- γ -producing T cells, but lacked overt villous atrophy (76). Innate immune responses toward gliadin can directly cause mucosal damage, a process that involves production of IL-15. tTg also plays a key role in modifying the anti-gliadin immune response in CD. Whether these key innate immune responses have critical roles in the development of mucosal damage in NOD-DQ8 still needs to be elucidated. Despite these mucosal abnormalities in gliadin-sensitized NOD-DQ8 mice, insulinitis did not develop. These findings suggest that loss of barrier function and moderate enteropathy induced by gliadin are not sufficient, on their own, to induce insulinitis.

Recent studies have shown that inducible Tregs have a crucial role in maintaining oral tolerance (77). In the MLNs, IL-2 and TGF- β are required to induce production of CD4⁺CD25⁺Foxp3⁺ inducible Tregs from naive CD4⁺CD25⁻ T cells (78). Migration to the gut and expansion of these cells in the lamina propria by intestinal dendritic cells is crucial for the generation of oral tolerance (77, 78). In accordance with previous results using this technique, we showed that treatment with anti-CD25 mAbs led to partial depletion of Foxp3⁺ Tregs (47, 49, 52). Importantly, this includes the inducible Treg population, which is critical for controlling the immune response in the intestinal mucosa against oral Ags (52, 77). The partial depletion was likely sufficient to disrupt the immunologic balance between proinflammatory and regulatory mediators in NOD-DQ8 mice because insulinitis developed after sensitization with the relevant Ag in this model—gliadin. However, anti-CD25 mAb-treated plus gliadin-sensitized mice did not develop hyperglycemia or impaired glucose tolerance and were not, therefore, clinically diabetic at the time they were analyzed. Similarly, a study using anti-CD25 mAbs demonstrated

severe autoimmune gastritis only after administration of autoimmune gastritis target Ag plus anti-CD25 mAb treatment (53). Although insulinitis was evident in anti-CD25 mAb plus gliadin-sensitized mice, a longer follow-up time after gliadin sensitization may be needed for development of hyperglycemia and progression to diabetes. However, the degree of insulinitis in anti-CD25 plus gliadin-sensitized mice was similar to the insulinitis seen in 14-wk-old NOD mice in our study and reported elsewhere (79). Importantly, several studies suggest that a proinflammatory environment within the pancreatic islets can promote the development of diabetes. NOD-DQ8 mice have been shown to develop diabetes if TNF- α is overexpressed in β islets (34). Other studies have shown that the expression of the costimulatory molecule, B7-1, or IFN- α within the islets can promote diabetes development, even in a diabetes-resistant background (80, 81). Our results indicate that partial CD25⁺Foxp3⁺ T cell depletion prior to gliadin sensitization was associated with islet infiltration in NOD-DQ8 mice. Extraintestinal inflammation in gliadin-sensitized NOD-DQ8 mice with prior CD25⁺Foxp3⁺ cell depletion was localized to the pancreas, as liver and lung tissues were normal.

CD4⁺ T cells isolated from the PLNs of mice that developed insulinitis responded to gliadin, and not to BSA. In NOD mice and patients with T1D, diabetogenic T cells within the islets display the gut homing marker α 4 β 7 (82). Furthermore, the ligand for the α 4 β 7 integrin, MadCam-1, is expressed on the endothelium in the pancreas and is upregulated during insulinitis (83). It has been shown that T cells isolated from the MLNs of 3-wk-old NOD mice are able to induce the development of diabetes in NOD.SCID mice more efficiently than lymphocytes isolated from peripheral lymph nodes, pancreatic lymph nodes, and the spleen (84). This finding suggests the initial priming of diabetogenic T cells may take place in the gut and that activated T cells can home to the pancreas to cause insulinitis (84). In our model, oral sensitization to gliadin in mice with partially depleted Tregs led to the production of gliadin-responding T cells in the PLN. This gliadin-associated insulinitis could be permissive of T1D development in a genetically predisposed host. Gliadin predominantly activates innate mechanisms in animal models, but the adaptive immune system has also been shown to be activated in transgenic HLA DQ8 mice (41, 45). We demonstrate the presence of gliadin-responsive T cells in the PLNs of NOD-DQ8 mice, providing an experimental setting where both innate and adaptive immune mechanisms can be studied within and outside the gut.

The current prevalence of T1D is estimated to double by 2020 in children younger than 5 y, and ~10% of children with T1D have documented CD (10, 85). CD is common worldwide, with a dramatic increase in prevalence from just 0.2–0.9% during the last five decades (6). With 95% of CD cases estimated to be currently undiagnosed in North America, and the increasing incidence of both diseases, the potential effects of gliadin intolerance on T1D are alarming. Diabetes imposes a substantial cost burden on society, in part because of its complications and comorbidities (86, 87). Our results provide a model to elucidate the mechanisms through which a common dietary intolerance may increase T1D risk and test innovative dietary approaches to prevent and improve metabolic control in T1D (88).

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Disclosures

The authors have no financial conflicts of interest.

References

- Green, P. H. R., and B. Jabri. 2003. Coeliac disease. *Lancet* 362: 383–391.
- Fasano, A., I. Berti, T. Gerarduzzi, T. Not, R. B. Colletti, S. Drago, Y. Elitsur, P. H. Green, S. Guandalini, I. D. Hill, et al. 2003. Prevalence of celiac disease in at-risk and not-at-risk groups in the United States: a large multicenter study. *Arch. Intern. Med.* 163: 286–292.
- Not, T., K. Horvath, I. D. Hill, J. Partanen, A. Hamed, G. Magazzu, and A. Fasano. 1998. Celiac disease risk in the USA: high prevalence of anti-endomysium antibodies in healthy blood donors. *Scand. J. Gastroenterol.* 33: 494–498.
- Schuppan, D., Y. Junker, and D. Barisani. 2009. Celiac disease: from pathogenesis to novel therapies. *Gastroenterology* 137: 1912–1933.
- Kagnoff, M. F. 2007. Celiac disease: pathogenesis of a model immunogenetic disease. *J. Clin. Invest.* 117: 41–49.
- Rubio-Tapia, A., R. A. Kyle, E. L. Kaplan, D. R. Johnson, W. Page, F. Erdtmann, T. L. Brantner, W. R. Kim, T. K. Phelps, B. D. Lahr, et al. 2009. Increased prevalence and mortality in undiagnosed celiac disease. *Gastroenterology* 137: 88–93.
- Lambert, A. P., K. M. Gillespie, G. Thomson, H. J. Cordell, J. A. Todd, E. A. M. Gale, and P. J. Bingley. 2004. Absolute risk of childhood-onset type 1 diabetes defined by human leukocyte antigen class II genotype: a population-based study in the United Kingdom. *J. Clin. Endocrinol. Metab.* 89: 4037–4043.
- Daneman, D. 2006. Type 1 diabetes. *Lancet* 367: 847–858.
- Borchers, A. T., R. Uibo, and M. E. Gershwin. 2010. The geoepidemiology of type 1 diabetes. *Autoimmun. Rev.* 9: A355–A365.
- Larsson, K., A. Carlsson, E. Cederwall, B. Jönsson, J. Neiderud, B. Jonsson, Å. Lernmark, and S. A. Ivarsson; Skåne Study Group. 2008. Annual screening detects celiac disease in children with type 1 diabetes. *Pediatr. Diabetes* 9: 354–359.
- Güvenç, S., S. Kaymakoğlu, N. Gürel, K. Karşıdağ, K. Demir, D. Dincer, C. Kekik, S. Salman, T. Yilmaz, F. Beşşik, and Y. Cakaloğlu. 2002. The prevalence of manifest and latent celiac disease in type 1 diabetes mellitus. *Turk. J. Gastroenterol.* 13: 103–107.
- Spiekeroetter, U., J. Seissler, and U. Wendel. 2002. General screening for celiac disease is advisable in children with type 1 diabetes. *Horm. Metab. Res.* 34: 192–195.
- Collin, P., J. Salmi, O. Hällström, H. Oksa, H. Oksala, M. Mäki, and T. Reunala. 1989. High frequency of coeliac disease in adult patients with type-I diabetes. *Scand. J. Gastroenterol.* 24: 81–84.
- Mahmud, F. H., J. A. Murray, Y. C. Kudva, A. R. Zinsmeister, R. A. Dierkhising, B. D. Lahr, P. J. Dyck, R. A. Kyle, M. El-Youssef, and L. J. Burgart. 2005. Celiac disease in type 1 diabetes mellitus in a North American community: prevalence, serologic screening, and clinical features. *Mayo Clin. Proc.* 80: 1429–1434.
- Dieterich, W., T. Ehnis, M. Bauer, P. Donner, U. Volta, E. O. Riecken, and D. Schuppan. 1997. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* 3: 797–801.
- Doolan, A., K. Donaghue, J. Fairchild, M. Wong, and A. J. Williams. 2005. Use of HLA typing in diagnosing celiac disease in patients with type 1 diabetes. *Diabetes Care* 28: 806–809.
- Saukkonen, T., J. Honen, H. K. Åkerblom, and E. Savilahti. 2001. Prevalence of coeliac disease in siblings of patients with Type I diabetes is related to the prevalence of DQB1*02 allele. *Diabetologia* 44: 1051–1053.
- Shanahan, F., R. McKenna, C. F. McCarthy, and M. I. Drury. 1982. Coeliac disease and diabetes mellitus: a study of 24 patients with HLA typing. *Q. J. Med.* 51: 329–335.
- Harrison, L. C., and M. C. Honeyman. 1999. Cow's milk and type 1 diabetes: the real debate is about mucosal immune function. *Diabetes* 48: 1501–1507.
- Mojibian, M., H. Chakir, D. E. Lefebvre, J. A. Crookshank, B. Sonier, E. Keely, and F. W. Scott. 2009. Diabetes-specific HLA-DR-restricted proinflammatory T-cell response to wheat polypeptides in tissue transglutaminase antibody-negative patients with type 1 diabetes. *Diabetes* 58: 1789–1796.
- Norris, J. M., K. Barriga, E. J. Hoffenberg, I. Taki, D. Miao, J. E. Haas, L. M. Emery, R. J. Sokol, H. A. Erlich, G. S. Eisenbarth, and M. Rewers. 2005. Risk of celiac disease autoimmunity and timing of gluten introduction in the diet of infants at increased risk of disease. *JAMA* 293: 2343–2351.
- Norris, J. M., K. Barriga, G. Klingensmith, M. Hoffman, G. S. Eisenbarth, H. A. Erlich, and M. Rewers. 2003. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *JAMA* 290: 1713–1720.
- Smyth, D. J., V. Plagnol, N. M. Walker, J. D. Cooper, K. Downes, J. H. Yang, J. M. Howson, H. Stevens, R. McManus, C. Wijmenga, et al. 2008. Shared and distinct genetic variants in type 1 diabetes and celiac disease. *N. Engl. J. Med.* 359: 2767–2777.
- Plenge, R. M. 2008. Shared genetic risk factors for type 1 diabetes and celiac disease. *N. Engl. J. Med.* 359: 2837–2838.
- Mackay, I. R. 2009. Clustering and commonalities among autoimmune diseases. *J. Autoimmun.* 33: 170–177.
- Ventura, A., E. Neri, C. Ughi, A. Leopaldi, A. Città, and T. Not. 2000. Gluten-dependent diabetes-related and thyroid-related autoantibodies in patients with celiac disease. *J. Pediatr.* 137: 263–265.
- Füchtenbusch, M., A. G. Ziegler, and M. Hummel. 2004. Elimination of dietary gluten and development of type 1 diabetes in high risk subjects. *Rev. Diabet. Stud.* 1: 39–41.
- Bosi, E., M. R. Pastore, L. Molteni, E. Bazzigaluppi, E. Bonifacio, and L. Piemonti. 2005. Gluten-free diet in subjects at risk for type 1 diabetes: a tool for delaying progression to clinical disease? *Adv. Exp. Med. Biol.* 569: 157–158.
- Funda, D. P., A. Kaas, T. Bock, H. Tlaskalová-Hogenová, and K. Buschard. 1999. Gluten-free diet prevents diabetes in NOD mice. *Diabetes Metab. Res. Rev.* 15: 323–327.
- Kudva, Y. C., G. Rajagopalan, R. Raju, R. S. Abraham, M. Smart, J. Hanson, and C. S. David. 2002. Modulation of insulinitis and type 1 diabetes by transgenic HLA-DR3 and DQ8 in NOD mice lacking endogenous MHC class II. *Hum. Immunol.* 63: 987–999.
- Giarratana, N., G. Penna, and L. Adorini. 2007. Animal models of spontaneous autoimmune disease: type 1 diabetes in the nonobese diabetic mouse. *Methods Mol. Biol.* 380: 285–311.
- Cheŕa, D. 1998. Animal models of type I (insulin-dependent) diabetes mellitus. *J. Pediatr. Endocrinol. Metab.* 11: 11–19.
- Marietta, E., K. Black, M. Camilleri, P. Krause, R. S. Rogers, III, C. David, M. R. Pittelkow, and J. A. Murray. 2004. A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. *J. Clin. Invest.* 114: 1090–1097.
- Rajagopalan, G., Y. C. Kudva, R. A. Flavell, and C. S. David. 2003. Accelerated diabetes in rat insulin promoter-tumor necrosis factor- α transgenic nonobese diabetic mice lacking major histocompatibility class II molecules. *Diabetes* 52: 342–347.
- Thomas, K. E., A. Sapone, A. Fasano, and S. N. Vogel. 2006. Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in Celiac disease. *J. Immunol.* 176: 2512–2521.
- Bollyky, P. L., J. B. Bice, I. R. Sweet, B. A. Falk, J. A. Gebe, A. E. Clark, V. H. Gersuk, A. Aderem, T. R. Hawn, and G. T. Nepom. 2009. The toll-like receptor signaling molecule Myd88 contributes to pancreatic beta-cell homeostasis in response to injury. *PLoS ONE* 4: e5063.
- Biagi, F., O. Luinetti, J. Campanella, C. Klersy, C. Zambelli, V. Villanacci, A. Lanzini, and G. R. Corazza. 2004. Intraepithelial lymphocytes in the villous tip: do they indicate potential coeliac disease? *J. Clin. Pathol.* 57: 835–839.
- Goudy, K. S., B. R. Burkhardt, C. Wasserfall, S. Song, M. L. Campbell-Thompson, T. Brusko, M. A. Powers, M. J. Clare-Salzler, E. S. Sobel, T. M. Ellis, et al. 2003. Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. *J. Immunol.* 171: 2270–2278.
- Leiter, E. H. 2001. The NOD mouse: a model for insulin-dependent diabetes mellitus. *Curr. Protoc. Immunol.* Chapter 15: Unit 15.9.
- Esensten, J. H., M. R. Lee, L. H. Glimcher, and J. A. Bluestone. 2009. T-bet-deficient NOD mice are protected from diabetes due to defects in both T cell and innate immune system function. *J. Immunol.* 183: 75–82.
- Verdu, E. F., X. Huang, J. Natividad, J. Lu, P. A. Blennerhassett, C. S. David, D. M. McKay, and J. A. Murray. 2008. Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294: G217–G225.
- de Kauwe, A. L., Z. Chen, R. P. Anderson, C. L. Keech, J. D. Price, O. Wijburg, D. C. Jackson, J. Ladhams, J. Allison, and J. McCluskey. 2009. Resistance to celiac disease in humanized HLA-DR3-DQ2-transgenic mice expressing specific anti-gliadin CD4+ T cells. *J. Immunol.* 182: 7440–7450.
- Natividad, J. M., X. Huang, E. Slack, J. Jury, Y. Sanz, C. David, E. Denou, P. Yang, J. Murray, K. D. McCoy, and E. F. Verdú. 2009. Host responses to intestinal microbial antigens in gluten-sensitive mice. *PLoS ONE* 4: e6472.
- Parish, C. R., M. H. Glidden, B. J. Quah, and H. S. Warren. 2001. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. *Curr. Protoc. Immunol.* 4: 4.9.
- Black, K. E., J. A. Murray, and C. S. David. 2002. HLA-DQ determines the response to exogenous wheat proteins: a model of gluten sensitivity in transgenic knockout mice. *J. Immunol.* 169: 5595–5600.
- Green, P. H. R., K. Rostami, and M. N. Marsh. 2005. Diagnosis of coeliac disease. *Best Pract. Res. Clin. Gastroenterol.* 19: 389–400.
- McNeill, A., E. Spittle, and B. T. Bäckström. 2007. Partial depletion of CD69low-expressing natural regulatory T cells with the anti-CD25 monoclonal antibody PC61. *Scand. J. Immunol.* 65: 63–69.
- Morris, G. P., N. K. Brown, and Y. C. Kong. 2009. Naturally-existing CD4(+) CD25(+)Foxp3(+) regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. *J. Autoimmun.* 33: 68–76.
- Setiady, Y. Y., J. A. Coccia, and P. U. Park. 2009. In vivo depletion of CD4+FOXP3+ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by Fc γ RIII+ phagocytes. *Eur. J. Immunol.* 40: 780–786.
- Sakaguchi, S., M. Ono, R. Setoguchi, H. Yagi, S. Hori, Z. Fehervari, J. Shimizu, T. Takahashi, and T. Nomura. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212: 8–27.
- Piccirillo, C. A., and A. M. Thornton. 2004. Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. *Trends Immunol.* 25: 374–380.
- Horwitz, D. A., S. G. Zheng, and J. D. Gray. 2008. Natural and TGF- β -induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol.* 29: 429–435.
- McHugh, R. S., and E. M. Shevach. 2002. Cutting edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J. Immunol.* 168: 5979–5983.

54. Hadjivassiliou, M., D. S. Sanders, R. A. Grünewald, N. Woodroffe, S. Boscolo, and D. Aeschlimann. 2010. Gluten sensitivity: from gut to brain. *Lancet Neurol.* 9: 318–330.
55. Fox, C. J., and J. S. Danska. 1998. Independent genetic regulation of T-cell and antigen-presenting cell participation in autoimmune islet inflammation. *Diabetes* 47: 331–338.
56. Montero, E., G. Nussbaum, J. F. Kaye, R. Perez, A. Lage, A. Ben-Nun, and I. R. Cohen. 2004. Regulation of experimental autoimmune encephalomyelitis by CD4+, CD25+ and CD8+ T cells: analysis using depleting antibodies. *J. Autoimmun.* 23: 1–7.
57. Senger, S., F. Maurano, M. F. Mazzeo, M. Gaita, O. Fierro, C. S. David, R. Troncone, S. Auricchio, R. A. Siciliano, and M. Rossi. 2005. Identification of immunodominant epitopes of alpha-gliadin in HLA-DQ8 transgenic mice following oral immunization. *J. Immunol.* 175: 8087–8095.
58. Hänninen, A., M. Salmi, O. Simell, and S. Jalkanen. 1996. Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior. *Diabetes* 45: 1173–1180.
59. Hänninen, A., I. Jaakkola, and S. Jalkanen. 1998. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J. Immunol.* 160: 6018–6025.
60. Taneja, V., M. Behrens, L. T. Cooper, S. Yamada, H. Kita, M. M. Redfield, A. Terzic, and C. David. 2007. Spontaneous myocarditis mimicking human disease occurs in the presence of an appropriate MHC and non-MHC background in transgenic mice. *J. Mol. Cell. Cardiol.* 42: 1054–1064.
61. Vaarala, O. 2008. Leaking gut in type 1 diabetes. *Curr. Opin. Gastroenterol.* 24: 701–706.
62. Visser, J., J. Rozing, A. Sapone, K. Lammers, and A. Fasano. 2009. Tight junctions, intestinal permeability, and autoimmunity: celiac disease and type 1 diabetes paradigms. *Ann. N. Y. Acad. Sci.* 1165: 195–205.
63. Neu, J., C. M. Reverte, A. D. Mackey, K. Liboni, L. M. Tuhacek-Tenace, M. Hatch, N. Li, R. A. Caicedo, D. A. Schatz, and M. Atkinson. 2005. Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes. *J. Pediatr. Gastroenterol. Nutr.* 40: 589–595.
64. Graham, S., P. Courtois, W. J. Malaisse, J. Rozing, F. W. Scott, and A. M. Mowat. 2004. Enteropathy precedes type 1 diabetes in the BB rat. *Gut* 53: 1437–1444.
65. Watts, T., I. Berti, A. Sapone, T. Gerarduzzi, T. Not, R. Zielke, and A. Fasano. 2005. Role of the intestinal tight junction modulator zonulin in the pathogenesis of type 1 diabetes in BB diabetic-prone rats. *Proc. Natl. Acad. Sci. USA* 102: 2916–2921.
66. Sapone, A., L. de Magistris, M. Pietzak, M. G. Clemente, A. Tripathi, F. Cucca, R. Lampis, D. Kryszak, M. Carteni, M. Generoso, et al. 2006. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 55: 1443–1449.
67. Bosi, E., L. Molteni, M. G. Radaelli, L. Folini, I. Fermo, E. Bazzigaluppi, L. Piemonti, M. R. Pastore, and R. Paroni. 2006. Increased intestinal permeability precedes clinical onset of type 1 diabetes. *Diabetologia* 49: 2824–2827.
68. Lee, A. S., D. L. Gibson, Y. Zhang, H. P. Sham, B. A. Vallance, and J. P. Dutz. 2010. Gut barrier disruption by an enteric bacterial pathogen accelerates insulinitis in NOD mice. *Diabetologia* 53: 741–748.
69. Vogelsang, H., M. Schwarzenhofer, and G. Oberhuber. 1998. Changes in gastrointestinal permeability in celiac disease. *Dig. Dis.* 16: 333–336.
70. Drago, S., R. El Asmar, M. Di Pierro, M. Grazia Clemente, A. Tripathi, A. Sapone, M. Thakar, G. Iacono, A. Carroccio, C. D'Agate, et al. 2006. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand. J. Gastroenterol.* 41: 408–419.
71. Clemente, M. G., S. De Virgiliis, J. S. Kang, R. Macatagney, M. P. Musu, M. R. Di Pierro, S. Drago, M. Congia, and A. Fasano. 2003. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. *Gut* 52: 218–223.
72. Ventura, A., G. Magazzù, L. Greco; SIGEP Study Group for Autoimmune Disorders in Celiac Disease. 1999. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. *Gastroenterology* 117: 297–303.
73. Pastore, M. R., E. Bazzigaluppi, C. Belloni, C. Arcovio, E. Bonifacio, and E. Bosi. 2003. Six months of gluten-free diet do not influence autoantibody titers, but improve insulin secretion in subjects at high risk for type 1 diabetes. *J. Clin. Endocrinol. Metab.* 88: 162–165.
74. Toscano, V., F. G. Conti, E. Anastasi, P. Mariani, C. Tiberti, M. Poggi, M. Montuori, S. Monti, S. Laureti, E. Cipolletta, et al. 2000. Importance of gluten in the induction of endocrine autoantibodies and organ dysfunction in adolescent celiac patients. *Am. J. Gastroenterol.* 95: 1742–1748.
75. Marsh, M. N. 1992. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 102: 330–354.
76. DePaolo, R. W., V. Abadie, F. Tang, H. Fehlnher-Peach, J. A. Hall, W. Wang, E. V. Marietta, D. D. Kasarda, T. A. Waldmann, J. A. Murray, et al. 2011. Co-adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. *Nature* 471: 220–224.
77. Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Müller, T. Sparwasser, R. Förster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34: 237–246.
78. Zheng, S. G., J. Wang, P. Wang, J. D. Gray, and D. A. Horwitz. 2007. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J. Immunol.* 178: 2018–2027.
79. Planas, R., A. Alba, J. Carrillo, M. C. Puertas, R. Ampudia, X. Pastor, H. Okamoto, S. Takasawa, W. Gurr, R. Pujol-Borrell, et al. 2006. Reg (regenerating) gene overexpression in islets from non-obese diabetic mice with accelerated diabetes: role of IFNbeta. *Diabetologia* 49: 2379–2387.
80. Wen, L., F. S. Wong, J. Tang, N. Y. Chen, M. Altieri, C. David, R. Flavell, and R. Sherwin. 2000. In vivo evidence for the contribution of human histocompatibility leukocyte antigen (HLA)-DQ molecules to the development of diabetes. *J. Exp. Med.* 191: 97–104.
81. Stewart, T. A., B. Hultgren, X. Huang, S. Pitts-Meek, J. Hully, and N. J. MacLachlan. 1993. Induction of type 1 diabetes by interferon-alpha in transgenic mice. *Science* 260: 1942–1946.
82. Hänninen, A., M. Salmi, O. Simell, D. Andrew, and S. Jalkanen. 1996. Recirculation and homing of lymphocyte subsets: dual homing specificity of beta 7-integrin(high)-lymphocytes in nonobese diabetic mice. *Blood* 88: 934–944.
83. Hänninen, A., R. Nurmela, M. Maksimov, J. Heino, S. Jalkanen, and C. Kurts. 2007. Islet beta-cell-specific T cells can use different homing mechanisms to infiltrate and destroy pancreatic islets. *Am. J. Pathol.* 170: 240–250.
84. Jaakkola, I., S. Jalkanen, and A. Hänninen. 2003. Diabetogenic T cells are primed both in pancreatic and gut-associated lymph nodes in NOD mice. *Eur. J. Immunol.* 33: 3255–3264.
85. Patterson, C. C., G. G. Dahlquist, E. Gyürüs, A. Green, and G. Soltész; EURODIAB Study Group. 2009. Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. *Lancet* 373: 2027–2033.
86. Green, P. H. 2007. Where are all those patients with Celiac disease? *Am. J. Gastroenterol.* 102: 1461–1463.
87. Johnson, J. A., S. L. Pohar, and S. R. Majumdar. 2006. Health care use and costs in the decade after identification of type 1 and type 2 diabetes: a population-based study. *Diabetes Care* 29: 2403–2408.
88. Pinier, M., E. F. Verdu, M. Nasser-Eddine, C. S. David, A. Vézina, N. Rivard, and J. C. Leroux. 2009. Polymeric binders suppress gliadin-induced toxicity in the intestinal epithelium. *Gastroenterology* 136: 288–298.