



B Lymphocytes Contribute to Celiac Disease Pathogenesis

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Celiac disease (CD) is a complex intestinal disorder with autoimmune features that develops in genetically susceptible individuals expressing HLA-DQ8 or HLA-DQ2 molecules.¹ The presence of anti-tissue transglutaminase 2 (TG2) and anti-deamidated gluten peptide antibodies represent strong disease markers.² Inflammation and villous blunting in the small intestine result from an abnormal immune response to gluten proteins. CD is characterized by the loss of oral tolerance to gluten manifested by HLA-DQ2- or HLA-DQ8-restricted antiglutin inflammatory CD4 T cells and a massive expansion of cytotoxic CD8⁺ intraepithelial lymphocytes (IELs) that mediate the killing of intestinal epithelial cells (IECs).³ These observations have led to the general idea that CD is primarily a T-cell-mediated immune disorder. However, studies in human suggest that B cells could play a central role in CD pathogenesis.⁴ We have recently engineered a mouse model of CD that develops villous atrophy (VA) in a gluten, HLA-DQ8, and TG2-dependent manner. Intestinal tissue destruction in this model is also dependent on CD4⁺ and CD8⁺ T cells and requires interferon- γ . We now demonstrate that B cells are required for the development of VA and the associated full licensing of cytotoxic IELs in this pathophysiologically relevant mouse model of CD, providing support for the exploration of B-cell-directed therapies for the treatment of CD.

Methods

DQ8-D^d-villin-IL-15tg mice developing the main features of CD upon ingestion of gluten³ and DQ8-D^d-villin-IL-15tg crossed with muMT mice lacking mature B cells were used. B-Cell depletion was achieved by treating DQ8-D^d-villin-IL-15tg mice with 250 μ g anti-mouse CD20 (clone 5D2, isotype IgG2a; Genentech). Villous height-to-crypt depth ratio was quantitatively determined on cross-sections of the terminal ileum, which is the primary site where VA develops in our mouse model.³ The acquisition of cytotoxic properties by IELs was assessed by measuring the expression of the activating natural killer cell (NK) receptor NKG2D, and cytolytic granules (granzyme B, perforin) in IELs with the use of flow cytometry and quantitative polymerase chain reaction.³ Detailed methods are described in the [Supplementary Methods](#).

Results

To determine whether B cells were required for the development of tissue damage, we analyzed the development of VA in our DQ8-D^d-villin-IL-15tg mouse model maintained on a gluten-free diet (sham), fed with gluten, or fed with gluten and treated with an anti-CD20 depleting

antibody ([Supplementary Figure 1](#) and [Figure 1A](#) and [B](#)). Despite B-cell depletion ([Supplementary Figure 1B–D](#)) preventing the development of antiglutin IgA and IgG antibodies upon introduction of gluten ([Figure 1E](#) and [F](#)), intestinal IgA-producing plasma cells ([Supplementary Figure 1G](#)) were still present after anti-CD20 treatment. Strikingly, the development of VA was impaired when B cells were depleted in gluten-fed mice ([Figure 1A](#) and [B](#)) demonstrating the requirement of gluten-specific humoral responses to promote a pathogenic response. Likewise, the development of VA was abrogated in DQ8-D^d-villin-IL-15tg-muMT mice lacking mature B cells ([Supplementary Figure 2A](#) and [B](#)).

Given that ablation of B cells significantly reduced the development of villous atrophy and that studies in humans and in DQ8-D^d-villin-IL-15tg mice³ have demonstrated that IELs infiltrating the celiac lesion are critically involved in tissue damage, we next assessed the impact of B-cell depletion on IELs. As shown in [Figure 1C](#), the reduction of intestinal tissue damage in the absence of B lymphocytes was associated with a decrease in the number of IELs. Intestinal tissue destruction arises from the killing of IECs by IELs that have acquired a cytolytic phenotype. We found that the amount of cytotoxic CD8⁺ IELs expressing the activating NK receptor NKG2D in the absence of inhibitory CD94/NKG2A receptors ([Figure 1D](#)) as well as the levels of the cytotoxic molecules granzyme B and perforin ([Figure 1E](#) and [F](#) and [Supplementary Figure 2C](#) and [D](#)) were decreased in mice lacking B cells, while the expression of the mouse NKG2D ligand, *rae1*, and the nonclassical major histocompatibility complex (MHC) class I molecule *qa1* remained unchanged ([Supplementary Figure 2E](#) and [2F](#)). Altogether, these results indicate that B cells play a role in CD pathogenesis by promoting the cytotoxic potential of IELs and the development of VA.

Discussion

B Cells as antigen-presenting cells have been involved in the development of organ-specific disorders.⁵ In the context of CD, *in vitro* assays have shown that such cooperation between B cells and gluten-specific CD4 T cells can take place.⁴ The strict dependence on gluten exposure for the

Abbreviations used in this paper: CD, celiac disease; IELs, intraepithelial lymphocytes; IECs, intestinal epithelial cells; TG2, tissue transglutaminase 2; VA, villous atrophy.

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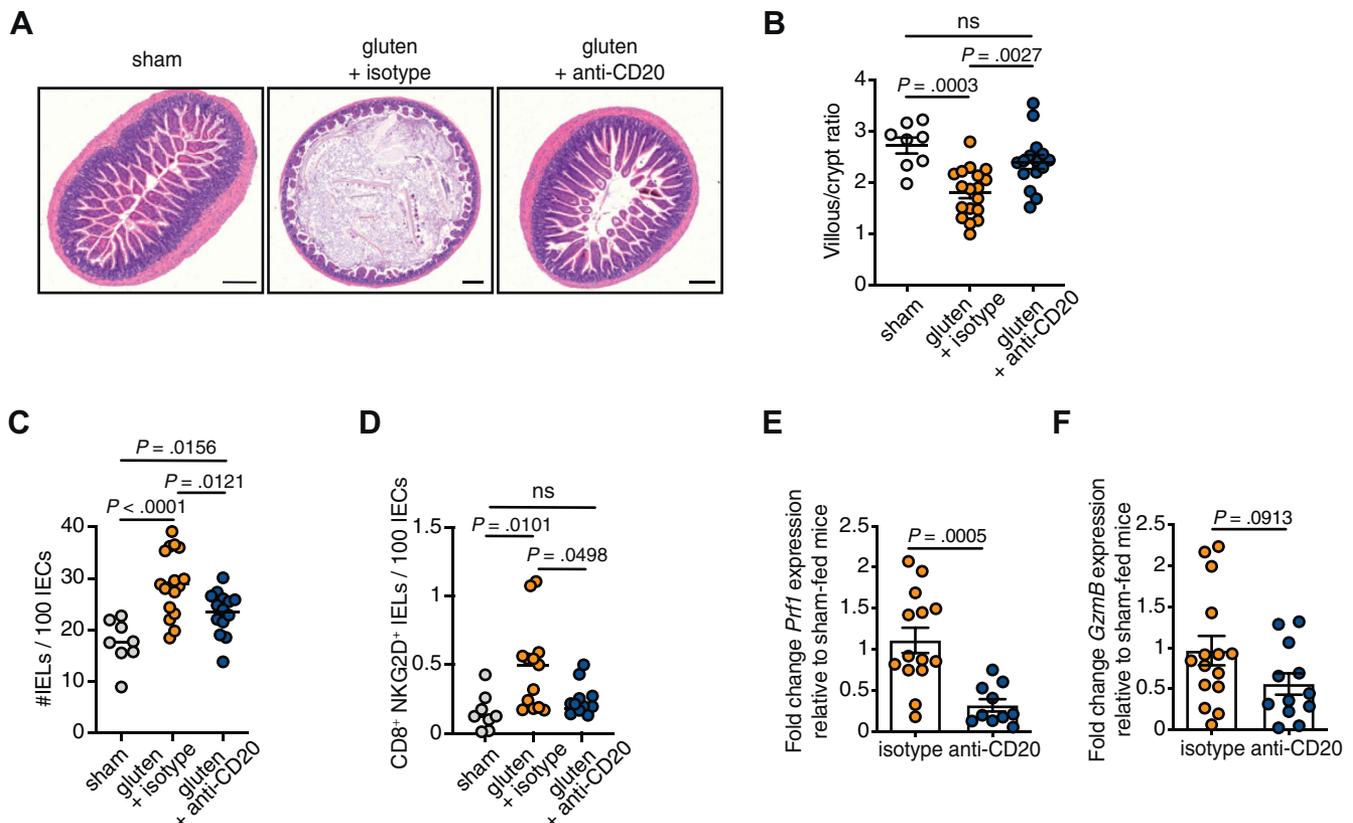


Figure 1. The development of villous atrophy and the acquisition of cytotoxic properties by intraepithelial CD8⁺ T cells in DQ8-D^d-villin-IL-15tg mice is impaired in the absence of B cells. DQ8-D^d-villin-IL-15tg mice were maintained on a gluten free diet (sham), or fed with gluten every other day for 30 days and treated with 250 μ g anti-CD20 antibody (gluten + anti-CD20) or its isotype control (gluten + isotype) every 2 weeks. (A) Hematoxylin-stained ileum sections showing villous atrophy—as evidenced by villous height-to-crypt depth ratio ≤ 2 —in the gluten-fed DQ8-D^d-villin-IL-15tg mice. Scale bar = 250 μ m. (B) Morphometric assessment of the villous height-to-crypt depth ratio demonstrating villous atrophy in gluten-fed mice (ratio ≤ 2) compared with sham-fed mice and gluten-fed mice treated with the anti-CD20 antibody. (C) Quantification of intraepithelial lymphocytes (IELs) among intestinal epithelial cells (IECs); mean. (D) The intestinal epithelium was isolated and analyzed by flow cytometry. IELs were identified as TCR β^+ CD4⁻ CD8⁺ cells, and the frequency of CD8⁺ NKG2D⁺ TCR $\alpha\beta^+$ cells were obtained. The total amount of IELs per 100 IECs was determined on hematoxylin and eosin–stained slides. NKG2D⁺ NKG2D⁻ IELs are indicated by absolute number per 100 IECs. (E) The expression of perforin (*prf1*) in the epithelial compartment was measured by quantitative polymerase chain reaction. Relative expression levels in gluten-fed mice were normalized to the expression levels observed in sham-fed mice. (F) The expression of granzyme B (*GzmB*) was measured as in E. Data are representative of 5 (B, C) or 4 (D–F) independent experiments, shown as mean \pm SEM; ANOVA/Tukey multiple comparison was performed for B, C, and D. Unpaired Student *t* test was used for E and F.

production of anti-TG2 antibodies suggests that an interaction between gluten-specific CD4⁺ T cells and TG2-specific B cells having internalized TG2-gluten complexes is required for their generation (as reviewed by Iversen and Sollid⁴). In addition, a transcriptional B-cell signature was associated with the extent of tissue damage in CD.⁶ Finally, plasma cells were found to be the most abundant gluten peptide MHC-expressing cells in the lamina propria of CD patients.⁷

The present study demonstrates that B cells play a role in the activation of IELs and tissue damage, both of which are key features of active CD.^{1,2} Although B-cell depletion did not restore the villous height-to-crypt depth ratio and the levels of cytotoxic IELs to steady-state level, our results demonstrate that B cells significantly contribute to both the development of VA and the acquisition of cytotoxic properties by CD8⁺ IELs, which are known to be responsible for

enterocytes cytolysis. These results in combination with our previous work highlighting a role for TG2 activation and CD4⁺ T cells in promoting tissue damage and IEL activation,³ suggest that B cells may be involved in the amplification of the CD4⁺ T-cell response to a magnitude sufficient to affect the activation of cytotoxic IELs and the ensuing tissue destruction. While providing evidence for a role for B cells in the activation of IELs and tissue damage, this study does not rule out that antibodies also could play a role in CD pathogenesis.⁸ Interestingly, the critical role of antigen presentation by B cells to CD4⁺ T cells for enhancing destructive immune reactions was also suggested in the context of experimental autoimmune encephalomyelitis and lupus.⁵ Future studies will determine the role of HLA-DQ2/DQ8 expression on B cells and assess their role as antigen-presenting cells in CD pathogenesis.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <https://doi.org/10.1053/j.gastro.2021.02.063>.

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Celine Meyer, PhD (Data curation: Supporting; Formal analysis: Supporting); Valérie Abadie, PhD (Conceptualization: Lead; Formal analysis: Lead; Funding acquisition: Lead; Methodology: Lead; Supervision: Lead; Writing – original draft: Lead; Writing – review & editing: Lead)

Conflicts of Interest

The authors declare no conflicts.

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Supplementary Methods

Mice

Mice used in these studies were on the C57BL/6 background. Mice were maintained under specific pathogen-free conditions at the University of Chicago and at the Sainte-Justine University Hospital Research Centre. DQ8-D^d-villin-IL-15tg mice were recently described.³ DQ8-D^d-villin-IL-15tg-muMT mice were obtained by crossing DQ8-D^d-villin-IL-15tg mice with muMT mice obtained from Jackson Laboratories (Bar Harbor, ME, USA). All strains were maintained from birth on a gluten-free chow (AIN76A; Envigo). For all experiments, mice were used at 10 weeks of age. All experiments were performed in accordance with the Institutional Biosafety Committee, the Institutional Care and Use Committee of the University of Chicago, the Canadian Council on Animal Care guidelines, and the Institutional Committee for Animal Care in Research of the Sainte-Justine University Hospital Research Centre.

Gluten feeding and B-cell depletion

To study the response to dietary gluten, mice were transferred from a gluten-free diet to a standard rodent chow at the beginning of each experiment and allowed to consume the gluten-containing chow ad libitum. In addition, supplemental gluten (20 mg crude gliadin; Sigma-Aldrich) was administered via intragastric gavage every other day for 30 days, with the use of a 22-gauge round-tipped needle (Cadence Science). To study the response to dietary gluten in the absence of mature B cells, DQ8-D^d-villin-IL-15tg mice were injected intraperitoneally every 2 weeks, starting at 7 days before the introduction of gluten, with 250 μ g of a CD20-specific monoclonal antibody (anti-CD20 antibody, clone 5D2, Genentech) or its isotype control (IgG2a; BioXCell), as shown in [Supplementary Figure 1A](#).

Histology

Hematoxylin and eosin staining was performed on 5 μ m thick sections of 10% formalin-fixed paraffin-embedded ileum. The segments of the distal ileum analyzed were consistently taken 0.5 cm from the cecum. Slides were analyzed with the use of a Leica DMi8 microscope with HC PL Fluotar L \times 20/0.40 and HC PL APO \times 40/0.75 objectives and equipped with the image processing and analysis software LasX (Leica). All assessments were performed blindly by 2 independent investigators. The villous height/crypt depth ratios were obtained from morphometric measurements of 5 well-oriented villi. The villous height-to-crypt ratio was calculated by dividing the villous height by the corresponding crypt depth. Villous height was measured from the tip to the shoulder of the villous or up to the top of the crypt of Lieberkuhn. The crypt depth was measured as the distance from the top of the crypt of Lieberkuhn to the deepest level of the crypt. If the poor orientation of a section prevented a correct morphometric assessment of the sample, additional tissue sections were cut and analyzed. The intraepithelial lymphocyte count was assessed by counting

the number of intraepithelial lymphocytes among at least 100 enterocytes.

Epithelial compartment, lamina propria, spleen, mesenteric lymph nodes, Peyer's patches, and blood cell isolation

Epithelial cells including intraepithelial lymphocytes (IELs) and lamina propria cells were isolated as previously described with the use of EDTA containing calcium-free medium and collagenase VIII, respectively. For the analysis of the natural killer cell (NK) receptors by means of flow cytometry, a cell purification step using a 40% Percoll (GE Healthcare) was used to enrich lymphocyte cell populations as previously described.³ Spleen, mesenteric lymph nodes, and Peyer's patches were dissected, made into a single-cell suspension by means of mechanical disruption, and passed through a 70- μ m nylon cell strainer (Corning). Blood was collected from the submandibular vein into heparinized tubes, and red blood cells were lysed with the use of the lysis buffer solution from BD Biosciences.

Flow cytometry

The following conjugated antibodies were purchased from eBioscience: TCR β APC (H57-597) CD8 α APC-eFluor 780 (53-6.7), CD8 β PE-Cy5 (eBio H35-17.2), and CD314 (NKG2D) PE (CX5). The following antibodies were purchased from BD Biosciences: CD4 PE-Cy7 (GK1.5), NKG2A/C/E FITC (20d5), IgA FITC (C10-3), CD16/CD32 (Fc Block) (2.4G2), CD3 V500 (UCHT1), and CD19 BUV661 (1D3). CD45 Pacific Blue (30-F11) was purchased from Biolegend. CD8 α APC-eFluor 780 (53-6.70), B220 PE-Cyanine7 (RA3-6B2), and Live/Dead Fixable Violet Dead Cell Stain Kit were purchased from Thermo Fisher Scientific. Flow cytometry was performed with a BD LSRFortessa II cell analyzer (BD Biosciences) and data were analyzed with the use of FlowJo software (Treestar).

Intraepithelial lymphocytes expressing NKG2D were identified as TCR $\alpha\beta$ ⁺ CD8⁺ NKG2D⁺ by means of flow cytometry. The quantification of NKG2D-expressing CD8⁺ IELs per 100 intestinal epithelial cells was determined as previously described.³

Anti-gliadin enzyme-linked immunosorbent assay

Serum was harvested 30 days after mice received the first gliadin feeding. High-binding 96-well plates enzyme-linked immunosorbent assay (Nunc; Thermo-Scientific) were coated with 50 μ L of 100 μ g/ml of pepsin-trypsin-digested gliadin in 100 mmol/L Na₂HPO₄ overnight at 4°C. Plates were washed 3 times with phosphate-buffered saline solution containing 0.05% Tween-20 (PBS-T) and blocked with 200 μ L of 2% bovine serum albumin in PBS-T for 2 hours at room temperature. Serum was assessed in duplicate and at 2 dilutions, typically 1:50 and 1:200. Sera were incubated overnight at 4°C, and plates were washed 3 times with PBS-T. Anti-mouse Ig-horseradish peroxidase (HRP; Southern Biotech) in blocking buffer was added to the plates and incubated for 1 hour at room temperature. The

plates were washed 5 times with PBS-T. Fifty μL HRP substrate TMB (Thermo-Scientific) was added and the reaction stopped by the addition of 50 μL 2N H_2SO_4 (Fluka Analytical). Absorbance was read at 450 nm on a Thermo Scientific Multiskan Go microplate reader. Levels of anti-gliadin IgG and IgA were expressed in optical density values.

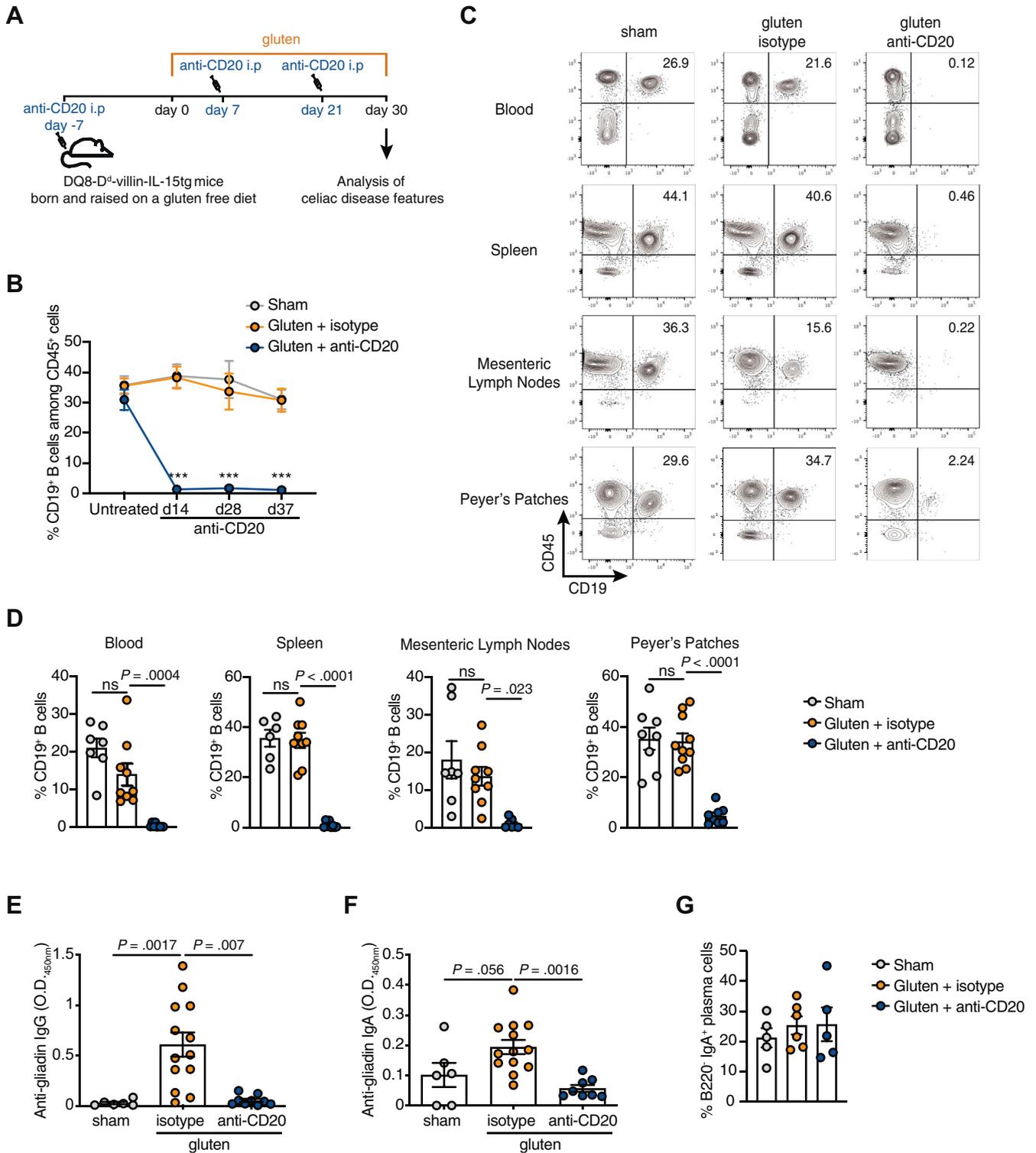
RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA isolation was performed on epithelial cells with the use of the RNeasy Mini Kit (Qiagen). RNA concentration and quality were determined by means of ultraviolet spectrophotometry (Epoch Microplate Spectrophotometer; BioTek). cDNA synthesis was

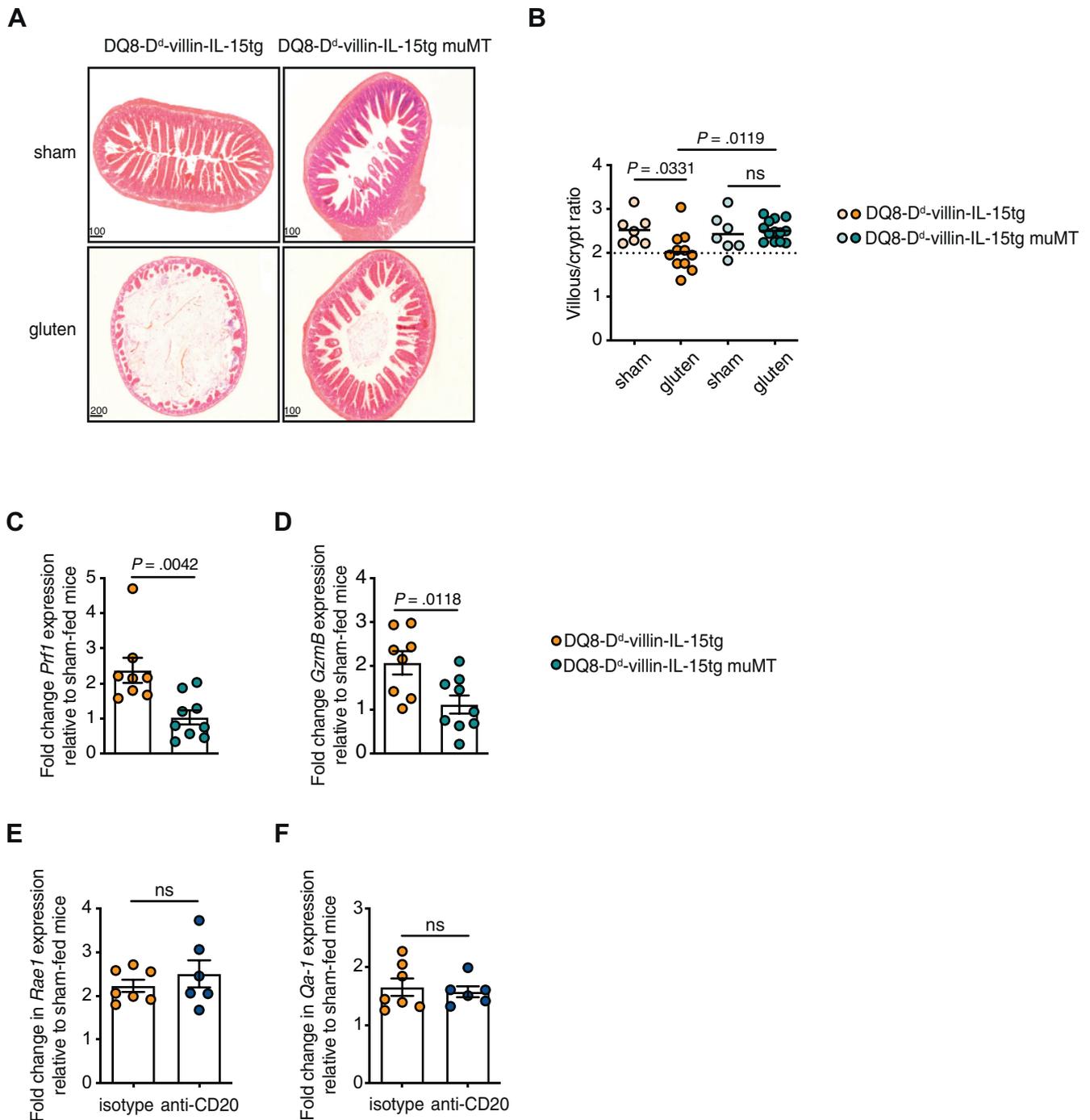
performed with the use of qScript cDNA SuperMix (QuantaBio) according to the manufacturer's instructions. Expression analysis for murine *gzm*, *prf1*, *rae1*, and *qa1* was performed with the use of TaqMan gene expression assays and normalized to *gapdh* (Thermo Fisher Scientific). Relative gene expression levels were determined using the $\Delta\Delta\text{Ct}$ method to calculate the relative changes in gene expression relative to sham-fed mice.

Statistical analysis

Tests were performed as indicated in the figure legends with the use of GraphPad Prism. Data are presented as mean \pm SEM. The statistical tests used and *P* values are indicated in each figure or figure legend. *P* values $< .05$ were considered to be statistically significant.



Supplementary Figure 1. Anti-CD20 treatment eliminates B cells and anti-gluten antibodies while preserving mucosal plasma cells. (A) Experimental scheme. (B) Frequency of blood CD19⁺ B220⁺ B lymphocytes, demonstrating the efficacy of B-cell depletion. ****P* < .001. (C) Representative dot-plots showing depletion efficacy in the blood, spleen, mesenteric lymph nodes, and Peyer's patches. (D) Frequency of CD19⁺ B lymphocytes in the blood, spleen, mesenteric lymph nodes, and Peyer's patches. (E, F) Serum anti-gliadin IgG (E) and IgA (F) levels were measured by means of enzyme-linked immunosorbent assay 30 days after gluten feeding. (G) Percentage of B220[−] IgA⁺ plasma cells among CD45⁺ CD3[−] cells found in the lamina propria of the different groups of mice. Results are representative of 3 (C–F) or 2 (G) independent experiments pooled together and shown as mean ± SEM. Analysis of variance/Tukey multiple comparison.



Supplementary Figure 2. Gluten-fed DQ8-D^d-villin-IL-15tg-muMT mice have decreased levels of cytotoxic molecules and intestinal tissue destruction. (A–D) DQ8-D^d-villin-IL-15tg mice and DQ8-D^d-villin-IL-15tg-muMT were maintained on a gluten-free diet (sham), or fed with gluten every other day for 30 days (gluten). (A) Hematoxylin-stained ileum sections. (B) Villous height-to-crypt depth ratio measured from ileum sections of sham and gluten-fed DQ8-D^d-villin-IL-15tg and DQ8-D^d-villin-IL-15tg-muMT mice. Four independent experiments are presented as mean \pm SEM. Analysis of variance/Tukey multiple comparison. (C) Expression of perforin (*prf1*) in the epithelial compartment was measured by means of quantitative polymerase chain reaction (qPCR). Relative expression levels in gluten-fed mice were normalized to the expression levels observed in sham-fed mice. (D) The expression of granzyme B (*gzmB*) was measured as in (C). (E, F) DQ8-D^d-villin-IL-15tg mice were maintained on a gluten-free diet (sham), or fed with gluten every other day for 30 days and treated with 250 μ g anti-CD20 antibody (gluten + anti-CD20) or its isotype control (gluten + isotype) every 2 weeks: (E) expression of retinoic acid early inducible gene 1 (*rae1*) was measured as in (C); (F) expression of major histocompatibility class Ib molecule *qa-1* was measured as in (C). qPCR results are representative of 3 independent experiments, shown as mean \pm SEM. Unpaired Student *t* test.