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Regulatory T Cells Control Th2-Dominant Murine Autoimmune Gastritis

Jessica Harakal,^{*,†} Claudia Rival,^{*,†,‡} Hui Qiao,^{†,‡} and Kenneth S. Tung^{*,†,‡}

Pernicious anemia and gastric carcinoma are serious sequelae of autoimmune gastritis (AIG). Our study indicates that in adult C57BL/6-DEREG mice expressing a transgenic diphtheria toxin receptor under the *Foxp3* promoter, transient regulatory T cell (Treg) depletion results in long-lasting AIG associated with both H⁺K⁺ATPase and intrinsic factor autoantibody responses. Although functional Tregs emerge over time during AIG occurrence, the effector T cells rapidly become less susceptible to Treg-mediated suppression. Whereas previous studies have implicated dysregulated Th1 cell responses in AIG pathogenesis, eosinophils have been detected in gastric biopsy specimens from patients with AIG. Indeed, AIG in DEREG mice is associated with strong Th2 cell responses, including dominant IgG1 autoantibodies, elevated serum IgE, increased Th2 cytokine production, and eosinophil infiltration in the stomach-draining lymph nodes. In addition, the stomachs exhibit severe mucosal and muscular hypertrophy, parietal cell loss, mucinous epithelial cell metaplasia, and massive eosinophilic inflammation. Notably, the Th2 responses and gastritis severity are significantly ameliorated in IL-4⁻ or eosinophil-deficient mice. Furthermore, expansion of both Th2-promoting IFN regulatory factor 4⁺ programmed death ligand 2⁺ dendritic cells and ILT3⁺ rebounded Tregs was detected after transient Treg depletion. Collectively, these data suggest that Tregs maintain physiological tolerance to clinically relevant gastric autoantigens, and Th2 responses can be a pathogenic mechanism in AIG. *The Journal of Immunology*, 2016, 197: 27–41.

An essential function of the immune system is to distinguish foreign from self-antigens, allowing for protection from invading pathogens while preventing autoimmune responses. T cell tolerance, initiated in the thymus, is maintained by the peripheral lymphoid organs in communication with local tissue and environmental factors, such as those existing in the skin and mucosal sites (1). The cellular mechanisms include T cell deletion, anergy, and suppression by regulatory T cells (Tregs), which likely operate in concert to fully maintain the tolerance state. CD4⁺ Foxp3⁺ Tregs play a critical role in maintaining immune homeostasis and tolerance to self-antigens by suppressing effector T cell and innate cell activities (2). The transcription factor Foxp3 is required for Treg development and function (3–5). Loss-of-function mutations in *Foxp3* lead to scurfy syndrome in mice

that exhibit progressive fatal multiorgan autoinflammation (6, 7) and to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome in patients (8, 9).

Autoimmune gastritis (AIG) is a common disease of the stomach associated with autoantibodies that target intrinsic factor (IF), which supports vitamin B12 absorption, and the gastric H⁺K⁺ATPase, the proton pump expressed by acid-secreting parietal cells in gastric glands (10–15). Accordingly, AIG patients are predisposed to the development of gastric cancer (16–18) and pernicious anemia, the most common sequela of vitamin B12 deficiency, which has an estimated prevalence of ~1.9% among the elderly Western population (19, 20). The histological characterization of active human AIG includes immune cell infiltration in the corpus and body regions of the stomach and loss of gastric zymogenic and parietal cells (21). Because of their strong resemblance to the human disease, murine AIG models have been frequently used for research on tolerance and mechanisms of organ-specific autoimmune disease.

Experimental AIG research has focused on addressing whether a defect in tolerance mechanisms, such as Tregs, is the underpinning of human autoimmune diseases and the rationale behind Treg-based therapies. For many years, this question has been investigated in the day 3 thymectomy (d3tx) model of BALB/c mice (22–25). It was thought that Tregs exit the thymus after the non-Treg T cells and should be preferentially depleted by thymectomy between neonatal days 1 and 5 (26–29). This idea was supported by the blockade of AIG by transfer of normal Tregs soon after thymectomy (22, 24, 30, 31). However, more recent studies have yielded new findings inconsistent with this concept: 1) Tregs with the capacity to suppress autoimmune disease were detected in the lymph nodes and spleen before day 3 (32); 2) d3tx led to an increase, rather than a reduction, of functional Treg fractions (33, 34); 3) Treg depletion by anti-CD25 Ab (PC61) in d3tx mice greatly enhanced the AIG immunopathology (34, 35); and 4) d3tx

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Abbreviations used in this article: AIG, autoimmune gastritis; CTLA-4, CTL-associated protein-4; d3tx, day 3 thymectomy; DC, dendritic cell; DT, diphtheria toxin; DTR, DT receptor; GTR, glucocorticoid-induced TNF receptor; IF, intrinsic factor; IRF4, IFN regulatory factor 4; NDLN, non-draining lymph node; PD-L2, programmed death ligand 2; SDLN, stomach-draining lymph node; Tfh, T follicular helper; Treg, regulatory T cell; WT, wild-type.

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mice developed severe lymphopenia, and the attendant homeostatic expansion of the autoreactive effector T cell compartment, including gastritogenic T cell clones, could also contribute to disease (26, 34, 36–38).

To more directly address Treg depletion without the confounding lymphopenic state, recent studies have turned to genetically modified mouse lines expressing the diphtheria toxin (DT) receptor (DTR) under the control of a *Foxp3* promoter, from which Tregs can be depleted by DT treatment. In both neonatal and adult *Foxp3*^{DTR} knock-in mice, continuous DT treatment led to dramatic expansion and activation of adaptive and innate cells, a scurfy-like phenotype, and death of unknown cause by 3–4 wk (39). Adult BALB/c *Foxp3*^{DTR} mice with transient Treg depletion also suffered from death within 4–5 wk. Moreover, despite the re-emergence of Tregs, the mice exhibited rapidly increased cytokine production, enhanced Ag-specific T cell activation, development of AIG with mononuclear cell infiltration, and parietal cell autoantibody responses (40). These findings raise the critical questions of whether transient Treg deficiency is sufficient to induce AIG, and why the restored Treg population fails to maintain tolerance (41).

In addition to the *Foxp3*^{DTR} knock-in mice, recent studies were conducted with the DEREg (Depletion of REGulatory T cells) mice that express a bacterial artificial chromosome containing a DTR and enhanced GFP fusion protein. Whereas DT-treated newborn C57BL/6-DEREg mice develop scurfy-like syndrome, adult C57BL/6 and BALB/c-DEREg mice were reportedly free of disease after transient Treg depletion (42, 43). This lack of pathological findings was attributed to the maintenance of tolerance by a minor population of residual DT-insensitive Tregs. Adult BALB/c-DEREg mice crossed with the *Foxp3*^{GFP} mice also failed to induce AIG, but blepharitis and scurfy-like autoinflammation were detected (43). However, unlike the *Foxp3*^{DTR} knock-in mice, the adult DEREg mice with transient Treg depletion did not succumb to early fatality (42, 44).

To develop an AIG model that is more suitable for detailed mechanistic analyses, we have conducted studies using the C57BL/6-DEREg mice, kindly donated by Drs. Lahl and Sparwasser, to systematically examine autoimmune disease development after transient Treg depletion. Contrary to previous findings, the study in our laboratory revealed that adult DT-treated C57BL/6-DEREg mice developed frequent and severe AIG that persisted for ≥ 16 wk. We found that after Treg depletion, the non-Treg T cells rapidly became less susceptible to Treg-mediated suppression. This finding provides a tangible explanation for the failure of the functional rebounded Treg population to control AIG. Moreover, AIG that develops in the adult DEREg model exhibited a unique Ab response and immunopathological features indicative of a robust Th2-dominant autoimmune response dependent on IL-4 and eosinophils.

Materials and Methods

Mice and *Foxp3*⁺ Treg depletion

C57BL/6-DEREg mice were kindly provided by Drs. Lahl and Sparwasser (Twin Core). A/J mice, C57BL/6 *Rag1*^{-/-} mice, and C57BL/6 *IL-4*^{-/-} mice were purchased from The Jackson Laboratory. C57BL/6 PHIL mice were generously provided by James Lee (Mayo Clinic). *Rag1*^{-/-} DEREg mice were generated by crossing *Rag1*^{-/-} mice with DEREg mice. *IL-4*^{-/-} DEREg and DEREg PHIL mice were generated by crossing the DEREg mice with the *IL-4*^{-/-} or the PHIL mice, respectively. To obtain B6AF1-DEREg mice, C57BL/6-DEREg mice were crossed with A/J mice. To deplete Tregs, 40 μ g/kg body weight of DT (Lot 322326; Calbiochem) was injected i.p. into 6- to 12-wk-old DEREg mice on days 0, 2, and 5. All experimental days were counted from day 0. As a control, we studied wild-type (WT) mice injected with DT and DEREg mice injected with PBS. All mice were bred in house in a specific pathogen-free facility and tested

negative for fur mites, pinworm infection, and *Helicobacter* spp. infections. Experiments were conducted following the guidelines of the Animal Care and Use Committee of the University of Virginia.

Histopathology and AIG severity grading

Mice were euthanized from 1 to 16 wk after Treg depletion. The stomachs were weighed after the removal of gastric contents, then fixed in Bouin's solution, embedded in paraffin wax, and cut into 5- μ m-thick sections. The presence of inflammation and parietal cells were examined by H&E stain, and mucin-positive cells were identified by periodic acid–Schiff/hematoxylin stain. AIG was graded as unknown samples. The AIG severity score was determined by the summation of the extent of inflammation, parietal cell loss, and mucinous cell hyperplasia. Each change was assigned a grade of 0 (none), 1 (mild), 2 (moderate), 3 (moderate and diffuse or severe but focal), or 4 (severe and diffuse). Because parietal cell loss and mucinous cell hyperplasia are structural changes and likely associated with loss of gastric function, we multiplied their scores by a factor of 1.5. Therefore, the total AIG scores ranged from 0 to 16.

Immunofluorescence microscopy

Serum gastric autoantibodies were detected and semiquantified by indirect immunofluorescence staining of 6- μ m-thick frozen sections of normal mouse stomach prefixed in 1% periodate-lysine-paraformaldehyde. After the sections were blocked with PBS containing normal goat serum and 3% BSA, they were incubated with serum from control or Treg-depleted mice, followed by incubation with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), anti-mouse IgG1 (Southern Biotech), or anti-mouse IgG2a (Southern Biotech). Staining intensity was scored as 0 (negative) to 3 (most positive). To study IF autoantibodies, mouse sera were costained with IF Ab, a generous gift from David Alpers (Washington University, St. Louis, MO), on normal stomach sections. Mouse sera staining (described above) was detected with Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories) followed by blockade with a biotin-avidin blocking kit (Vector Laboratories) and costained with goat anti-human IF Ab followed by biotinylated horse anti-goat IgG (Vector Laboratories) and neutralite avidin–Texas Red (Southern Biotech). To detect eosinophils by direct immunofluorescence, 6- μ m frozen stomach sections were fixed in cold 1:1 acetone:ethanol and blocked with Tris–NaCl blocking buffer (PerkinElmer), 3% H₂O₂ (EMD Chemicals), 0.1% NaN₃ (ICN Biomedicals), followed by a biotin-avidin blocking kit. The sections were then stained with rat anti-mouse SiglecF Ab (BD Pharmingen) and biotinylated goat anti-rat IgG (Southern Biotech). Then, staining was enhanced by the biotin tyramide kit according to the manufacturer's instructions (PerkinElmer), followed by neutralite avidin–Texas Red. To detect parietal cells on the same sections, the slides were then costained with DEREg mouse serum Ab to parietal cell Ags, as described above, and mounted with Vectashield containing DAPI (Vector Laboratories). Unless noted otherwise, all stains were performed at room temperature, and the slides were examined and photographed with a Nikon Microphot-FXA fluorescent microscope and Nikon HB-10101AF Mercury Lamp and Olympus Q-Color5 camera. Alternatively, they were examined with a Zeiss LSM 700 confocal microscope or a Zeiss Axio Observer microscope with Qioptiq OptiGrid.

ELISA detection of gastric H⁺K⁺ATPase Ab and IF Ab

To detect H⁺K⁺ATPase Ab, 96-well plates were incubated with 0.5 μ g/ml H⁺K⁺ATPase (Arotec Diagnostics) in 10 mM Tris–HCL (pH 8)/0.15 M NaCl/0.1% sodium desoxycholate for 16 h at 4°C. The wells were blocked with PBS containing 1% BSA for 1 h at 37°C. Experimental and control mouse sera were added to the wells at 1:50 and 1:100 dilutions in duplicate and incubated for 1 h at room temperature. The wells were then incubated with HRP-conjugated goat anti-mouse IgG (Southern Biotech) diluted 1:2000 for 1 h at room temperature. After rigorous washing, each well was reacted with OPD substrate solution (Sigma-Aldrich). The reaction was terminated with 2.5N H₂SO₄, and absorbance at 490 nm was determined with a microplate reader. H⁺K⁺ATPase concentrations were expressed as arbitrary units relative to a standard curve.

To detect IF Ab, 96-well plates were coated with 5 μ g/ml recombinant rat IF (Cloud-Clone) in 0.5M Na₂HCO₃ for 4 h at room temperature and blocked with PBS containing 0.05% Tween 20 overnight. Experimental and control mouse sera were added to the wells at 1:50 dilutions for 1 h. The wells were then incubated with HRP-conjugated goat anti-mouse IgG (Southern Biotech) diluted 1:2000 for 1 h. As a positive control, a goat anti-human IF Ab (provided by David Alpers, Washington University, St. Louis, MO) was added for 1 h, followed by biotinylated horse anti-goat IgG (Vector Laboratories) diluted 1:200 for 1 h, and streptavidin-

conjugated HRP (PerkinElmer Life Sciences) diluted 1:1000 for 1 h. All incubations were performed at room temperature. After rigorous washing, each well was reacted with OPD substrate solution. The reaction was terminated with 2.5N H₂SO₄, and absorbance at 490 nm was determined with a microplate reader.

Serum IgE determination by ELISA

The 96-well plates were coated overnight with 5 µg/ml unlabeled goat anti-mouse IgE (Southern Biotech) in PBS. After blocking and 2-h incubation with serum samples at 1:200 and 1:400 dilutions in duplicate, the wells were incubated with HRP-conjugated goat anti-mouse IgE (Southern Biotech) at room temperature. After rigorous washing, each well was reacted with OPD substrate solution, the reaction was terminated with 2.5N H₂SO₄, and absorbance at 490 nm was determined with a microplate reader. IgE concentration (in micrograms per milliliter) was determined from the standard curve of known IgE concentration.

In vivo CD4 T cell depletion, IFN-γ neutralization, and IL-17 neutralization

For CD4 T cell depletion, mice were injected with 100 µg anti-CD4 mAb (clone GK1.5, generated at the Lymphocyte Culture Center of University of Virginia) in PBS starting at the same time as the first DT injection, then every 3–4 d for 3 wk. Mice were euthanized at 8 wk. For IFN-γ neutralization, mice were injected with 500 µg anti-IFN-γ Ab (clone XMG1.2; BioXCell) in PBS starting at the same time as the first DT injection, then every 3–4 d until they were euthanized at 3 wk. Control mice were injected with corresponding doses of polyclonal IgG from normal rat sera (Thermo Scientific). As a positive control, we used the same IFN-γ Ab to block the development of neonatal autoimmune ovarian disease (45). Neonatal autoimmune ovarian disease was induced by i.p. injection of 100 µg mAb (1G2 clone) to zona pellucida 3 peptide 336–342 on days 3 and 5 after birth (46), and anti-IFN-γ Ab was injected i.p. every 3–4 d starting from postnatal day 3. Ovarian disease was scored at 2 wk of age, as previously described (45). For IL-17 neutralization, mice were injected with 250 µg anti-IL-17A Ab (clone 17F3; BioXCell) in PBS starting at the same time as the first DT injection, then every 2–3 d until they were euthanized at 3 wk. This dosing regimen was based on previous publications (47). Control mice were injected with corresponding doses of polyclonal IgG from normal rat sera (Thermo Scientific).

Antibiotic treatment

Mice were treated with water or antibiotics beginning 7 d prior to the first dose of DT treatment and lasting for 2.5 wk total. Mice were subjected to daily oral gavage with 200 µL autoclaved water or autoclaved water supplemented with neomycin (1 mg/ml; Sigma-Aldrich), vancomycin (0.5 mg/ml; Sigma-Aldrich), metronidazole (1 mg/ml; Sigma-Aldrich), gentamicin (1 mg/ml; Sigma-Aldrich), and ampicillin (1 mg/ml; Sigma-Aldrich) and filtered through a 0.2-µm syringe filter, as previously described (48). WT mice treated with DT and antibiotics were used as controls. Mice were harvested 3 wk after the first dose of DT.

Cell isolation from lymph nodes, spleen, and gastric mucosa

At euthanization, the spleen, stomach-draining lymph nodes (SDLN), axillary and brachial nondraining lymph nodes (NDLN), and/or pooled (paragastric, renal, cervical) lymph nodes were removed and prepared into single-cell suspensions in RPMI 1640 (Life Technologies) by mechanical disruption and filtration through a 70-µm cell strainer. Splenic erythrocytes were lysed using NH₄Cl buffer. The stomach infiltrating cells were isolated as described (49). Briefly, after the stomach contents were removed by several washes in ice cold PBS, the stomach mucosa was injected with 20 ml cold PBS containing 5% FBS, using a 5-ml syringe and 26-gauge needle. The stomach was gently massaged to release the infiltrating cells that were then filtered through a 70-µm nylon mesh, centrifuged at 400 × g for 5 min, counted, and stained for flow cytometry.

Flow cytometric analysis

Single-cell suspensions were stained with Abs to CD4 (RM4-5), CD45 (30-F11), PD1 (RMP1-30), glucocorticoid-induced TNF receptor [(GITR) (DTA-1)], CD11b (M1/70), programmed death ligand 2 [(PD-L2) (CD273, 122)], and ILT3 (gp49 receptor, H1.1) purchased from eBioscience; and CD25 (PC61), CD69 (H1.2F3), CXCR5 (2G8), SiglecF (E50-2440), Ly6G (1A8), CD44 (IM7), CD62L (MEL-14), CD11c (HL3), and MHCII (25-9-17 and 10-3.6) purchased from BD Pharmingen. For cell surface staining, FcγRII/III were blocked with 2.4G2 Ab, and cells were stained for 20 min at 4°C with Abs to the appropriate surface

markers. Dead cells were excluded by the LIVE/DEAD Red Dead Cell Stain Kit (Life Technologies), and samples were fixed with 2% paraformaldehyde. For intracellular staining, cells were fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences) or the Foxp3 Staining Kit (eBioscience) and then stained with Abs to IFN regulatory factor 4 (IRF4) (3E4; eBioscience), Foxp3 (FJK-16s; eBioscience), or CTL-associated protein-4 (CTLA-4) (UC10-4F10-11; BD Pharmingen). For intracellular cytokine staining, the isolated lymph node cells were restimulated in vitro with 50 ng/ml PMA (Sigma-Aldrich) and 2 µg/ml ionomycin (Sigma-Aldrich) at 1 × 10⁶ cells per milliliter in RPMI 1640 (Life Technologies), supplemented with 10% (v/v) heat-inactivated FCS (HyClone), penicillin and streptomycin (Life Technologies), L-glutamine (Life Technologies), nonessential amino acids (Life Technologies), sodium pyruvate (Life Technologies), HEPES (Life Technologies), and 2-ME (Sigma-Aldrich). After 2 h, 10 µg/ml brefeldin A (BD Biosciences) was added, and 4 h later, the cells were stained with anti-CD4 Ab. The cells were then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained for intracellular IL-4 (11B11; eBioscience), IL-5 (TRFK5; BD Pharmingen), IFN-γ (XMG1.2; BD Pharmingen), or IL-17 (ebioTC11-18H10.1, eBioscience). Flow cytometry data were acquired on a five-color FACScan or six-color FACSCanto I flow cytometer (BD Biosciences) using BD FACSDiva Software (Version 6.0) and analyzed with FlowJo software (TreeStar).

In vitro CD4⁺CD25⁺ Treg suppression assay

A total of 4 × 10⁵/ml CD4⁺CD25[−] naive T cells were purified from the lymph nodes of untreated WT mice using CD4⁺CD25⁺ MicroBeads (Miltenyi Biotec) and AutoMACS (Miltenyi Biotec) according to the manufacturer's instructions. They were cultured with graded numbers of CD4⁺CD25⁺ Tregs isolated from the lymph nodes of naive or Treg-depleted DERE mice. Alternatively, 4 × 10⁵/ml CD4⁺CD25[−] T cells were purified from the lymph nodes of WT mice or Treg-depleted DERE mice and were cultured with graded numbers of CD4⁺CD25⁺ Tregs isolated from the pooled lymph nodes of untreated WT mice. The purity of the isolated CD4⁺CD25⁺ cells was 91.4 ± 5.1%. In each experiment, cell mixtures were cultured in the presence of 4 × 10⁶/ml irradiated splenocytes as APCs and anti-CD3 mAb diluted 1:1000 (Cedarlane) in a 96-well round-bottom plate for 80 h. Cell proliferation was assessed by [³H] thymidine incorporation during the final 8 h of culture. Percentage of suppression was calculated as described previously (50).

In vivo Treg reconstitution

CD4⁺CD25⁺ Tregs were isolated from pooled lymph nodes and spleens of WT donors. Erythrocyte-lysed spleen cells were enriched for the T cell fraction on a mouse T cell enrichment column (R&D Systems). CD4⁺CD25[−] T cells were isolated using CD4⁺CD25⁺ Microbeads (Miltenyi Biotec) and AutoMACS (Miltenyi Biotec). Treg-depleted B6AF1-DERE mice were injected i.v. with 2 × 10⁶ viable Tregs, either at the time of the first dose of DT treatment or 7 d later. The purity of the isolated CD4⁺CD25⁺ cells was 97.5 ± 2.2%.

Statistical analyses

Statistical differences were determined by the nonparametric Mann-Whitney *U* test, Kruskal-Wallis test with Dunn's posttests, or Spearman correlation test using GraphPad Prism Version 5.0. The one-tailed Fisher exact test was used to assess differences in disease incidences. Analyses of in vitro suppression assays were performed after transforming the percent suppression data to the log10 scale. Three-way ANOVA was used, with the experiment factor taken as a random effect. *F*-tests were used to compare differences between groups. A *p* value <0.05 was considered statistically significant.

Results

CD4⁺ T cell-dependent AIG occurs in DERE mice following CD4⁺ Foxp3⁺ Treg depletion

To address the role of Tregs in physiological self-tolerance, we investigated the emergence of spontaneous autoimmune diseases in adult mice with Treg depletion. Depletion of ~60% of Tregs in WT C57BL/6 or (C57BL/6 × A/J)F₁ (B6AF1) mice by anti-CD25 Ab (PC61) treatment did not result in autoimmune disease (51, 52). However, AIG was observed in adult DERE mice after ~90% of Tregs were ablated by three DT injections (days 0, 2, and 5). A

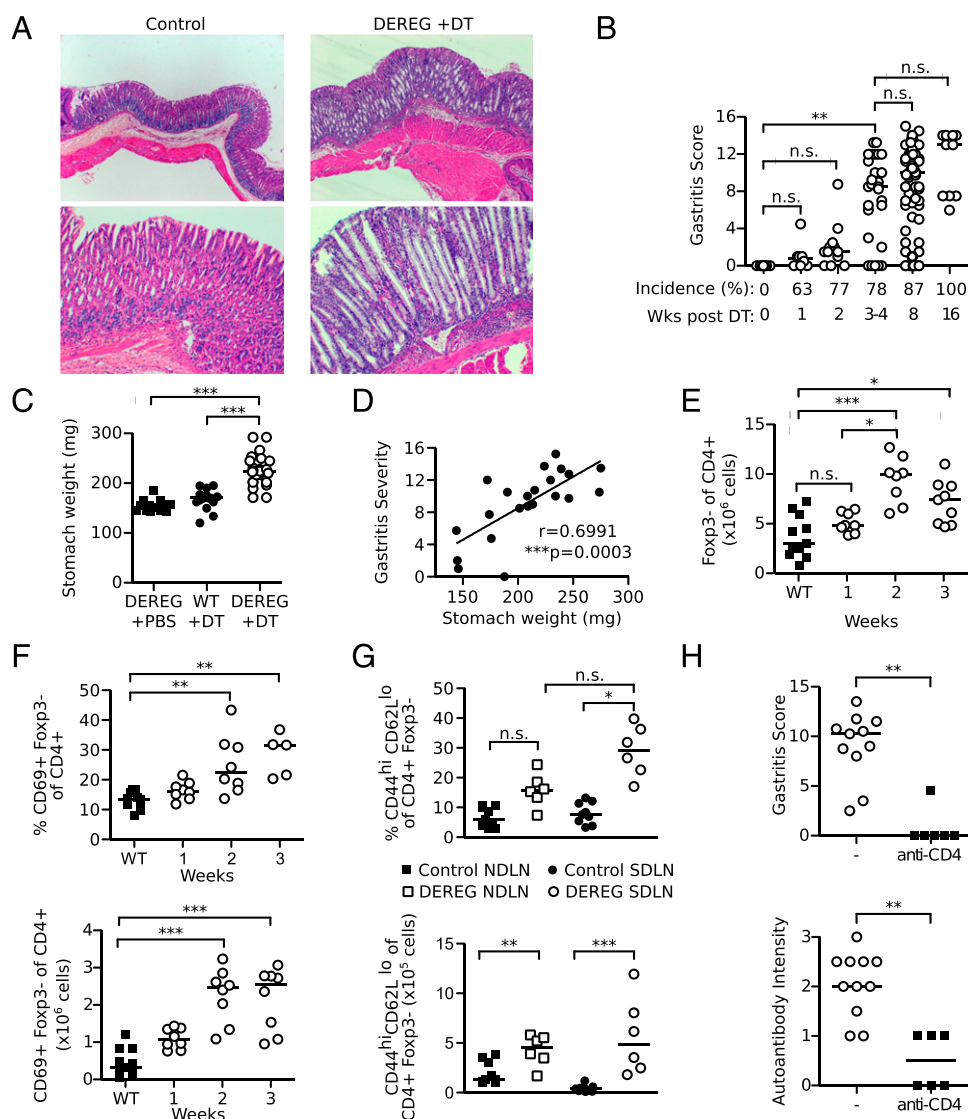
complete autopsy revealed severe autoimmune immunopathology in the stomach of Treg-depleted C57BL/6-DEREG and B6AF1-DEREG mice; they had comparable disease incidences and severity and showed no gender bias (Supplemental Fig. 1A, 1B). Mild and focal monocytic inflammatory infiltrates were observed in the kidney, pancreas, liver, salivary gland, lung, and lacrimal gland of some Treg-depleted mice, whereas the eye, skin, small bowel, and large bowel were devoid of inflammation (data not shown).

Inflammatory cell infiltration appeared in the gastric mucosa of DT-treated DERE mice by 1 wk after the first dose of DT treatment. Disease incidence was significantly elevated by week 2 ($p = 0.0303$) (Fig. 1B). By 3–4 wk, there was significant escalation of the severity of gastric inflammation, loss of parietal cells, mucosal epithelial cell hyperplasia with mucinous metaplasia, and hypertrophy of the mucosa and muscularis externa (Fig. 1A, 1B). Maximal AIG was found in the gastric wall of the proximal stomach and extended to the squamous epithelium of the adjacent cardia (the mouse “abdominal” esophagus), but spared the gastric antrum and the thoracic esophagus (data not shown). Notably, severe AIG persisted for ≥ 16 wk after Treg depletion (Fig. 1B). These pathological changes account for the increased stomach weight (Fig. 1C), providing a quantitative assessment of the overall AIG pathological condition that correlates with the gas-

tritis severity (Fig. 1D). We observed no difference in the stomach weights between the C57BL/6-DEREG and B6AF1-DEREG mice (Supplemental Fig. 1C). The control DERE mice treated with PBS showed no evidence of AIG (Fig. 1A–C). Moreover, gastric inflammation was not due to DT toxicity or off-target expression of the DTR transgene, because the stomachs of DT-treated WT mice (Fig. 1A–C) and DT-treated Rag1^{-/-} DERE mice were free of disease ($n = 7$; data not shown).

Treg-depleted DERE mice exhibited an increase in CD4⁺ Foxp3⁻ T cell numbers in the pooled lymph nodes (Fig. 1E). Their activation status was evidenced by an increase in percentages and numbers of CD69⁺CD4⁺Foxp3⁻ cells at 2 wk after Treg depletion (Fig. 1F), and by an elevation of CD44^{hi}CD62L^{lo} effector/memory CD4⁺Foxp3⁻ T cell frequency and numbers at 3 wk, most pronounced in SDLN (Fig. 1G). To investigate the pathogenic role of the activated CD4⁺Foxp3⁻ T cells in AIG, we depleted CD4⁺ cells by injecting a monoclonal anti-CD4 Ab for 3 wk, starting at the time of the first dose of DT. This approach resulted in a significant reduction of gastritis at 8 wk (Fig. 1H). The robust gastric autoantibody response detected in DERE mice with AIG was also significantly reduced after CD4⁺ T cell depletion (Fig. 1H). Together, our findings indicate that Treg depletion alone results in severe CD4⁺ T cell-dependent AIG in DERE mice.

FIGURE 1. Transient Treg depletion results in AIG dependent on CD4⁺ T cells. **(A)** Representative stomach histological specimens showing well-organized mucosa (control mice, left) or hypertrophy, abundant mucin-positive cells, and reduction in parietal cells (Treg-depleted DERE mice, right) (H&E stain; original magnification $\times 40$ [top]; original magnification $\times 100$ [bottom]). **(B)** Kinetics of gastritis incidences and severity after Treg depletion. **(C)** Stomach weights at 3 wk. **(D)** Correlation of gastritis score and stomach weight at 3 wk. **(E)** Kinetics of absolute numbers of Foxp3⁻ of CD4⁺ T cells and **(F)** percentages (top) and absolute numbers (bottom) of CD69⁺Foxp3⁻ of CD4⁺ T cells in pooled lymph nodes after Treg depletion. **(G)** Percentages (top) and absolute numbers (bottom) of CD44^{hi}CD62L^{lo} of CD4⁺Foxp3⁻ cells in the NDLN and SDLN at 3 wk in control and Treg-depleted DERE mice. **(H)** Gastritis score and gastric autoantibody intensity of mice at 8 wk after Treg depletion with or without anti-CD4-depleting Ab treatment. Data were pooled from 2–11 independent experiments per time point. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis tests with Dunn’s posttests (B, C, E, F, and G [top]), Mann–Whitney U test (G [bottom] and H), Spearman correlation test (D).



DEREG mice with AIG develop serum autoantibodies to the gastric parietal cell $H^+K^+ATPase$ and IF

Dysregulation of T follicular helper (Tfh) cells is associated with development of autoimmunity through the promotion of autoantibody production (53). In support of the gastric autoantibody response (Fig. 1F), we observed a significant expansion of PD1⁺CXCR5⁺ Tfh cells in the SDLN at 3 wk (Fig. 2A). We also observed a concomitant increase in the absolute numbers of Tfh cells in the NDLN (Fig. 2A), likely owing to the increased total lymph node cellularity after Treg depletion (data not shown).

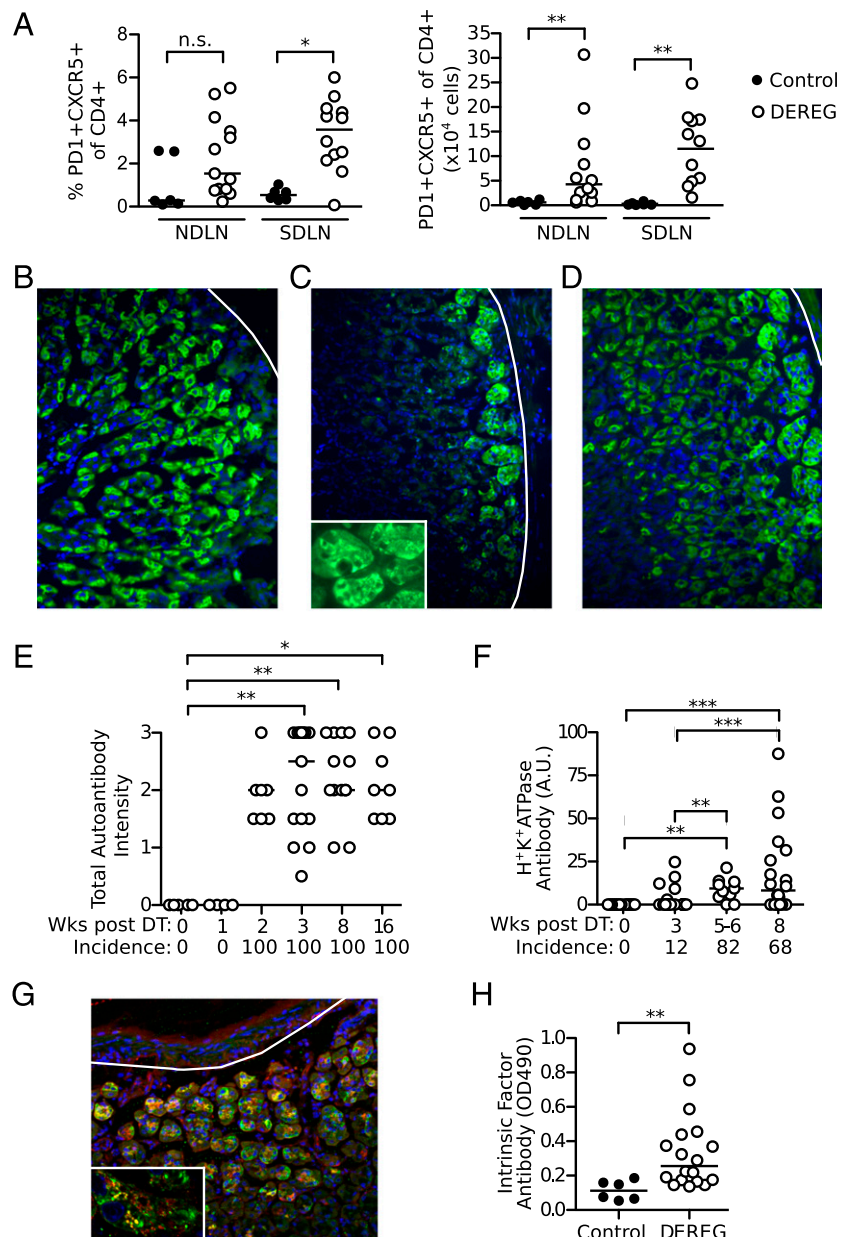
Treg-depleted DEREG mice developed autoantibodies to gastric Ags located in the basal epithelial cells as well as the parietal cells. Of the Ab-positive mice at 3 wk, 31% reacted with parietal cells only (Fig. 2B), 28% reacted with basal gland epithelial cells only (Fig. 2C), and 41% reacted with both (Fig. 2D). At 8 wk, 58% of the autoantibodies reacted with both parietal cells and basal gland epithelial cells, whereas 37% or 5% reacted with parietal cells or basal gland epithelial cells alone, respectively. Serum autoantibodies were detected by week 2 and persisted through week 16

(Fig. 2E). Autoantibodies against gastric $H^+K^+ATPase$ were detected in 82% of Treg-depleted mice by 5–6 wk (Fig. 2F).

We next investigated the specificity of the autoantibodies to the basal gland epithelial cells. Specifically, we addressed whether they targeted IF expressed primarily in murine gastric chief cells (54). Autoantibodies to IF are common in human AIG (55–57) yet were not detected in some experimental AIG models (58). To address this, we first costained normal stomach sections with sera from Treg-depleted mice with AIG and goat anti-human IF Ab. They reacted with Ags present in the same cytoplasmic locations within the basal epithelial cells (Fig. 2G), especially in vesicular structures with a punctate distribution (Fig. 2G inset). We then developed an ELISA to detect IF autoantibodies and found that ~50% of mice with AIG and serum basal epithelial cell autoantibodies targeted IF (Fig. 2H).

These findings indicate that Tfh cell responses and autoantibody production resulting from both gastric $H^+K^+ATPase$ and IF are normally controlled by Tregs in mice, and autoantibody responses to both Ags can occur spontaneously in mice with AIG following

FIGURE 2. Tfh cell and autoantibody responses in Treg-depleted DEREG mice with AIG. **(A)** Percentages (*left*) and absolute numbers (*right*) of PD1⁺CXCR5⁺CD4⁺ T cells in the NDLN and SDLN at 3 wk in control and Treg-depleted DEREG mice. **(B–D)** Immunofluorescence patterns of serum gastric autoantibodies from Treg-depleted mice: (B) parietal cells only, (C) basal gland epithelial cells only, or (D) both cell types (green, IgG; blue, DAPI; original magnification $\times 200$; white lines denote base of gastric mucosa). Inset in (C) shows patchy cytoplasmic distribution. **(E)** Kinetics of total serum gastric IgG autoantibody incidences and staining intensities after Treg depletion detected by immunofluorescence. **(F)** Kinetics of gastric $H^+K^+ATPase$ autoantibody incidence and titer after Treg depletion (ELISA). **(G)** Colocalization (yellow) of DEREG mouse serum autoantibody to basal gland epithelial cells (green) with anti-human IF Ab (red), detected by immunofluorescence on normal stomach sections (blue, DAPI; original magnification $\times 200$; white line denotes base of gastric mucosa). Punctate pattern of colocalization is shown as yellow dots in inset (original magnification $\times 800$). **(H)** ELISA detection of IF autoantibody in control and Treg-depleted mice at 3 wk. The latter mice were selected based on the presence of autoantibodies directed to basal gland epithelial cells (present in 69% of mice). Data were pooled from 2–11 independent experiments per time point. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis tests with Dunn's posttests (E and F), Mann–Whitney U test (A and H).



Treg depletion. The presence of these autoantibodies is consistent with the clinical observations in patients with AIG (20).

After DT-mediated Treg depletion, the Tregs rapidly rebound and exhibit normal tissue distribution and expression of Treg-associated functional molecules

After DT injection, the lymph node CD4⁺Foxp3⁺ Tregs rebounded within 14–21 d (Supplemental Fig. 2A). Nonetheless, AIG developed and progressed (Fig. 1) despite the return of Tregs to normal frequencies. This was not due to a major homing defect of the rebounded Tregs, because a normal frequency and absolute number of Foxp3⁺ CD4⁺ cells were detected in the SDLN, and significantly more Foxp3⁺ CD4⁺ cells were found in the stomach of DERE mice with AIG when compared to control mice at 3 wk (Supplemental Fig. 2B, 2C).

We next investigated the expression levels of CTLA-4, GITR, CD25, and IRF4 on the rebounded Tregs compared to Tregs from control mice. CTLA-4, a marker of Treg activation, is essential for their suppressive function (59–63) and for induction of Th2 cell apoptosis (64). Mice with CTLA-4-deficient Tregs develop high titers of serum IgE and autoimmune disease in several organs, including the stomach (62). GITR and CD25 are critical for Treg costimulation (65) and survival (27, 66), and Tregs with a low level of CD25 are more prone to conversion into pathogenic effector cells (67, 68). IRF4 expression has been shown to promote Treg homeostasis and effector functions, including suppression of Th2 responses (69, 70). Compared to control Tregs, the rebounded Tregs from the SDLN of DERE mice expressed equivalent or higher, rather than lower, mean fluorescent intensities of Foxp3, CTLA-4, CD25, GITR, and IRF4 (Supplemental Fig. 2D). Therefore, the rebounded Tregs appear to retain a normal phenotype.

AIG and autoantibody responses in DT-treated DERE mice are preventable by WT Treg transfer at the time of DT treatment but not at 7 d after DT treatment

To further investigate AIG development despite normal Treg rebound, we evaluated the effect of Treg reconstitution on the development of the gastric autoimmune response and AIG. When WT Treg transfer was performed on the same day as the first DT dose, the expansion of CXCR5⁺PD1⁺ Tfh cells (Fig. 3A) and activation of effector T cells (Fig. 3B) were abolished in the DERE recipients studied 3 wk later. Moreover, the stomachs were free of inflammation and loss of parietal cells, and gastric autoantibodies were no longer detected in the serum (Fig. 3C). However, when the transfer of WT Tregs was delayed to day 7 after the first DT dose, the gastric pathology and autoantibody responses were no longer prevented at 3 wk (Fig. 3C). Therefore, within just 1 wk after the initial DT treatment, the autoimmune responses in the DERE mice were less sensitive to suppression by Tregs.

The rebounded Tregs exhibit normal suppressive capacity, but the effector T cells are less susceptible to Treg-mediated suppression in vitro

We next evaluated both the functional capacity of the rebounded Tregs and the susceptibility of DERE effector T cells to Treg-mediated suppression in vitro. We isolated the CD4⁺CD25⁺ Tregs from DERE mice 3 wk after the first dose of DT and assessed their capacity to suppress the proliferation of WT CD4⁺CD25⁺ effector T cells. The rebounded Tregs from DERE mice were just as potent as WT Tregs in the in vitro suppression assay (Fig. 3D, $p = 0.0950$). Therefore, the rebounded Tregs appeared to retain a normal suppressive function. In contrast, the CD25⁺CD4⁺ effector T cells isolated from the SDLN of mice with AIG were significantly less susceptible to suppression when compared to those from AIG-free control mice (Fig. 3E, $p = 0.0280$).

Based on these in vitro and in vivo findings, we conclude that although the rebounded Treg cells retain suppressive potential, the expanded CD4⁺CD25⁺ T cells rapidly become less susceptible to Treg-mediated suppression. These findings further support the conclusion that the naturally occurring Tregs present in normal DERE mice are responsible for maintaining physiological tolerance to gastric autoantigens.

Treg-depleted DERE mice develop a Th2-dominant autoimmune response and gastric immunopathology typical of a Th2 immune response

At 1 wk, when the gastric mucosa retained normal integrity, numerous eosinophils began to infiltrate the gastric submucosa and basal mucosa (Fig. 4A). By 3 wk, when parietal cell reduction was manifested, SiglecF⁺ eosinophils further expanded and infiltrated all layers of the stomach wall (Fig. 4B). In addition, small clusters of plasma cells were detected among the eosinophils and other mononuclear cells in the submucosa and the muscle wall (data not shown). We also observed a major restructuring of the gastric mucosa, including a reduction in parietal cells, metaplasia of mucin-rich epithelial cells identified by the periodic acid–Schiff stain, and severe mucosal hyperplasia (Fig. 4C, Fig. 1A). Notably, the eosinophilic infiltration and muscular hypertrophy also affected the keratinized squamous epithelial cell lining of the adjacent gastric cardia (data not shown).

We next determined the serum gastric autoantibody isotypes as indicators of Th1 and Th2 cell responses; class-switch recombination to IgG1 and IgE is enhanced by IL-4, whereas class-switch recombination to IgG2a is enhanced by IFN- γ (71–73). The intensities of the IgG1 autoantibodies to parietal cells and basal epithelial cells were significantly higher than the intensities of the IgG2a autoantibodies (Fig. 4D). In addition, the total serum IgE levels in the DERE mice at 3 wk were 11-fold greater than those of control mice (Fig. 4E). The prevalence of the parietal cell and basal epithelial cell IgG1 versus IgG2a autoantibodies and total serum IgE levels were also significantly different ($p < 0.0001$ for all comparisons).

A significant increase in the proportion of IL-4⁺ or IL-5⁺ CD4⁺ T cells was detected in the SDLN, with a concomitant increase in the absolute numbers of IL-4⁺ and IL-5⁺ CD4⁺ T cells in both the SDLN and the NDLN (Fig. 4F, 4G). We detected only minimal IL-17 production by the lymph node CD4⁺ T cells (Supplemental Fig. 3A, 3B). Although IFN- γ production by lymph node CD4⁺ T cells was also detected (Supplemental Fig. 3A, 3B), the severity and frequency of AIG in the Treg-depleted DERE mice were not affected by treatment with an IFN- γ Ab (Supplemental Fig. 3C). As a control, the same IFN- γ Ab blocked the development of neonatal autoimmune ovarian disease (Supplemental Fig. 3D), which is IFN- γ dependent (45). Furthermore, the severity and frequency of AIG in Treg-depleted DERE mice were not affected by treatment with an IL-17 Ab (Supplemental Fig. 3E).

These findings indicate that AIG in Treg-depleted DERE mice exhibits the characteristics of a Th2 response. In the next studies, we investigated the pathogenic role of the autoimmune Th2 responses in AIG.

IL-4 and Th2 responses are critical for mediating gastric mucosal injury and eosinophilic inflammation after Treg depletion

IL-4 is a critical cytokine for Th2 responses (74). We therefore predicted that DERE mice lacking IL-4 would have diminished AIG development after Treg depletion. The total gastric IgG1 autoantibody responses and total serum IgE levels were significantly reduced in the IL-4^{-/-} DERE mice after Treg depletion

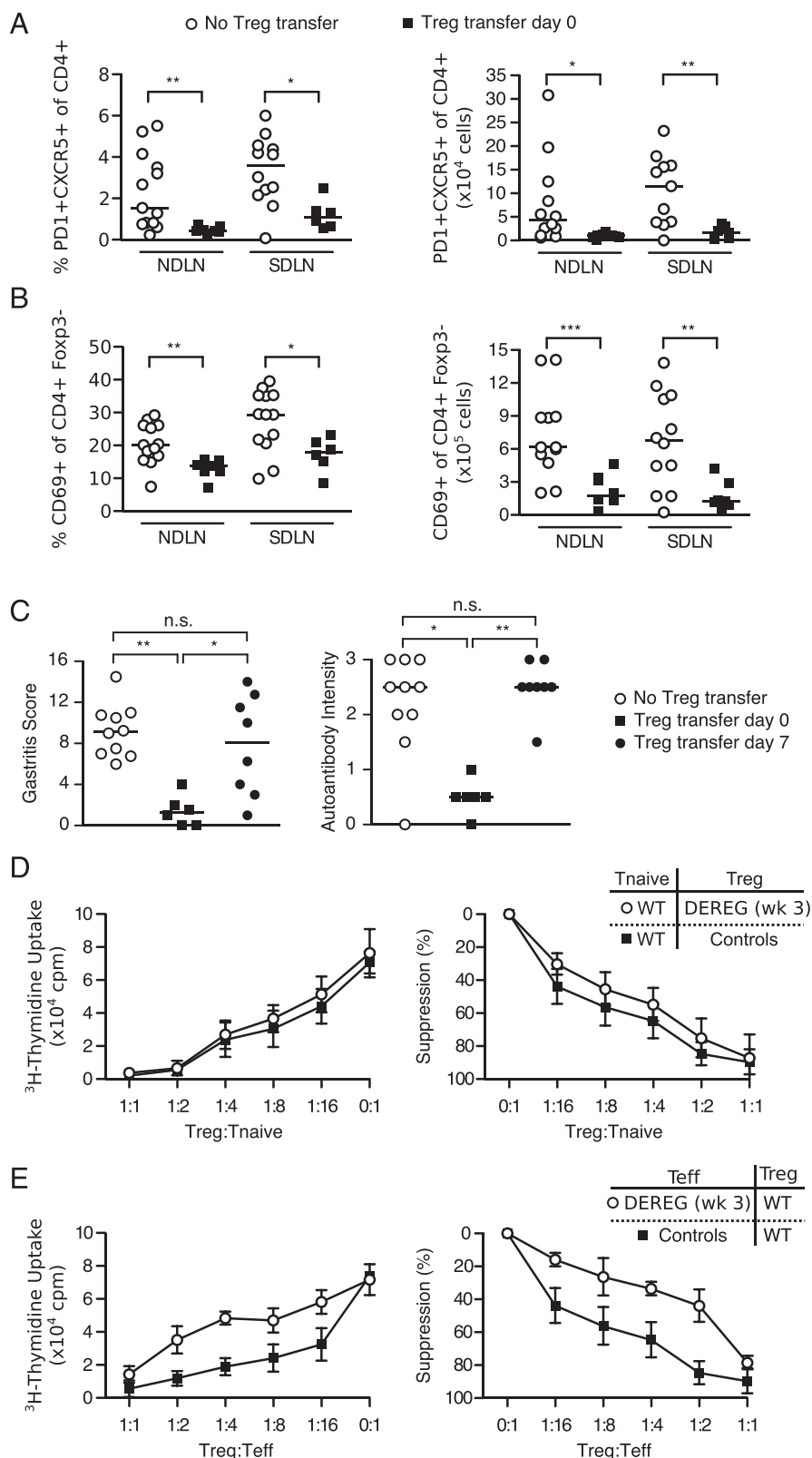


FIGURE 3. Effector T cells from DEREK mice with transient Treg depletion are less sensitive to suppression by Tregs in vitro and in vivo. Percentages (*left*) and absolute numbers (*right*) of (A) PD1⁺CXCR5⁺ of CD4⁺ Tfh cells or (B) CD69⁺ of CD4⁺ Foxp3⁻ activated T cells in the NDNLN or SDLN at 3 wk in Treg-depleted DEREK mice with (■) or without (○) WT Treg reconstitution at day 0 of DT treatment. (C) Gastritis score (*left*) and gastric IgG autoantibody intensity (*right*) at 3 wk in Treg-depleted DEREK mice with or without infusion of WT Tregs at day 0 or day 7 after the first dose of DT treatment. (D) Suppression of naive CD4⁺CD25⁻ T cell proliferation by CD4⁺CD25⁺ Tregs from WT mice or from the SDLN of DEREK mice obtained at 3 wk after DT treatment. (E) Suppression of proliferation of CD4⁺CD25⁻ T cells from WT mice or from the SDLN of DEREK mice obtained at 3 wk after DT treatment by CD4⁺CD25⁺ Tregs from WT mice. Proliferation of CD4⁺CD25⁻ T cells in (D) and (E) was assessed by uptake of [³H] thymidine (representative mean and standard deviations of cpm from five wells each in one experiment, *left*; average percent suppression of two to three experiments, *right*). $p = 0.0950$ in (D), $p = 0.0280$ in (E) (three-way ANOVA with F test). Data in (A)–(C) were pooled from three independent experiments, and each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis test with Dunn’s posttests (C) or Mann–Whitney U tests (A and B).

(Fig. 5A, 5B), whereas the incidence and intensity of total IgG2a Ab responses were unaltered (Fig. 5A).

Compared to IL-4-sufficient DEREK mice, the IL-4^{-/-} DEREK mice after Treg depletion exhibited significantly reduced overall gastritis severity (Fig. 5C) and stomach weights (Fig. 5D). This reduction in pathological characteristics was mainly accounted for by the reduction of parietal cell loss and mucin-rich cellular metaplasia (Fig. 5E). Although the extent of gastric in-

flammation was unchanged (Fig. 5E), the eosinophil-rich infiltrate of IL-4-sufficient DEREK mice was diminished and replaced by lymphomonocytic cells in IL-4^{-/-} DEREK mice (Fig. 5F, 5G). We did observe a slight increase in Ly6G⁺ neutrophils in the IL-4^{-/-} DEREK mice after Treg depletion (Fig. 5G).

These results indicate that IL-4 is a major contributor to AIG pathogenesis, including the destruction of gastric parietal cells. The reduction in mucin-rich cellular metaplasia is consistent with a role

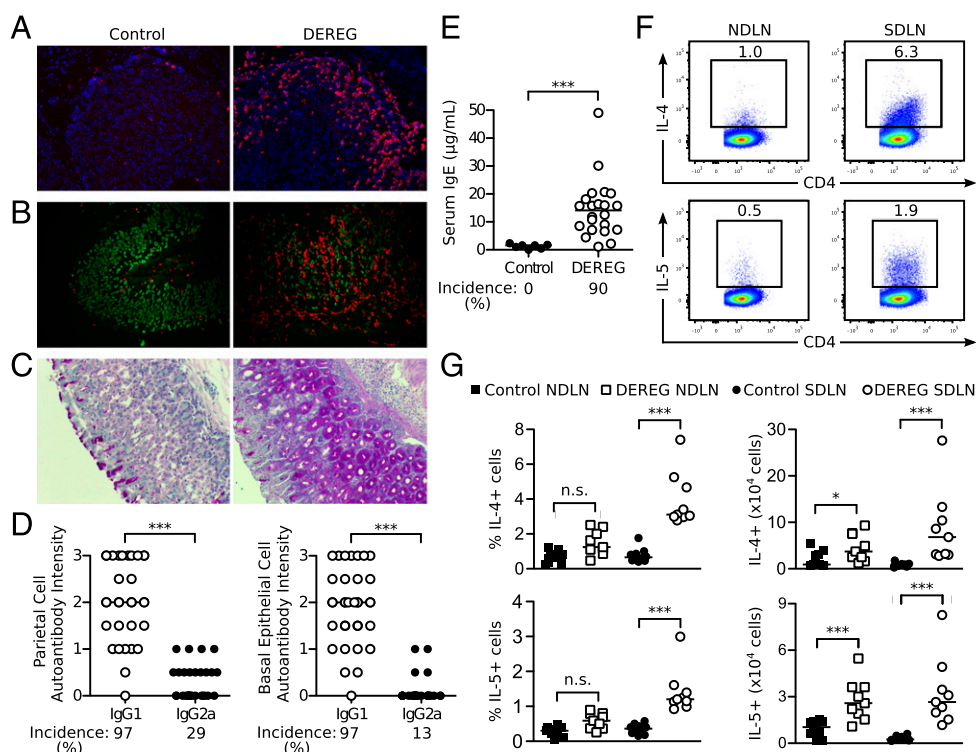


FIGURE 4. AIG in Treg-depleted DEREG mice is predominantly associated with Th2 cell responses. **(A)** Representative immunofluorescence microscopy images of eosinophils in the stomach mucosa from control (left) and Treg-depleted mice (right) at 1 wk (red, SiglecF; blue, DAPI; original magnification $\times 200$). **(B)** Colocalization of parietal cells and eosinophils in representative immunofluorescence microscopy images of stomach mucosa from control (left) and Treg-depleted DEREG mice with AIG (right) (red, SiglecF; green, mouse serum Ab to gastric parietal cells; original magnification $\times 200$). **(C)** Mucin-positive cells in stomach sections from control (left) and Treg-depleted mice (right) at 3 wk (periodic acid–Schiff stain; original magnification $\times 100$). **(D)** Intensity and prevalence of serum IgG1 and IgG2a autoantibodies to parietal cells (left) and basal epithelial cells (right) from DEREG mice detected by indirect immunofluorescence. **(E)** Total serum IgE levels and prevalence in control and Treg-depleted DEREG mice. **(F)** Representative flow cytometry dot plots of IL-4 and IL-5 producing CD4⁺ T cells in the SDLN and NDLN of Treg-depleted DEREG mice at 3 wk (cells were stimulated ex vivo by PMA and ionomycin before flow cytometry analysis). **(G)** Quantitation of (F) (left, percentage; right, absolute number). Data were pooled from three (F and G), four (E), and six (D) independent experiments. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis tests with Dunn’s posttest (G [left]), Mann–Whitney U tests (D, E, and G [right]).

for IL-4 and Th2 responses in mucinous cell expansion observed in airway remodeling in asthma models (75–77). Because eosinophilic infiltration in the stomach was IL-4 dependent, we next investigated the role of eosinophils in AIG pathogenesis.

Eosinophils expand in the lymph nodes, support IgE responses, and exert a pathogenic effect in AIG of Treg-depleted mice

Eosinophils have a pathogenic role in models of allergic asthma and helminth infection (78, 79). In addition to their expansion in the stomach (Figs. 4A, 4B, 5G), we also detected an increase in eosinophil percentages in the pooled lymph nodes within 2 wk and in absolute numbers within 1 wk after Treg depletion (Fig. 6A). Notably, the eosinophils accounted for a larger fraction of the total cellularity in the SDLN and NDLN of the Treg-depleted DEREG mice at 3 wk (Fig. 6B). To investigate the contribution of eosinophils to AIG, we studied PHIL mice that express the DT A catalytic subunit under the eosinophil peroxidase promoter and are developmentally deficient in eosinophils (80). Indeed, the loss of eosinophils led to a significant reduction in the serum IgE levels of the DEREG PHIL mice (Fig. 6C). In contrast, the incidence and intensity of the total IgG1 or IgG2a gastric autoantibody responses remained unchanged (Fig. 6D).

At 3 wk, the overall gastritis scores and disease incidence in the DEREG PHIL mice were significantly reduced, compared to the eosinophil-sufficient DEREG mice (Fig. 6E). In the absence of eosinophils, gastric inflammation and mucin-rich cellular metaplasia were significantly reduced. There was also a trend toward

reduction in parietal cell loss, although the difference did not reach statistical significance (Fig. 6F, 6G). Gastric hypertrophy was also reduced in the mice without eosinophils (Fig. 6G), accompanied by a significant reduction in the stomach weight, compared to these features in eosinophil-sufficient DEREG mice (Fig. 6H). As a control, the WT PHIL mice did not develop gastric disease or autoantibody responses after DT treatment (data not shown). These results indicate that eosinophilic inflammation is a significant contributor to multiple facets of AIG pathogenesis in Treg-depleted mice.

Intestinal microbiome alteration by oral antibiotic treatment did not affect the development of Th2-dominant AIG in Treg-depleted DEREG mice

A challenging question is why the DEREG mice with transient Treg deficiency developed a dominant pathogenic Th2 response. Environmental factors, such as the microbiome, may contribute to disease susceptibility as well as the Th2 bias in AIG of Treg-depleted DEREG mice. Alterations in commensal microbial communities have been found to influence Th2 responsiveness (81–83). We addressed whether the microbiome of the DEREG mice in our colony affected disease susceptibility and Th2 bias using an established protocol of oral antibiotic gavage to manipulate the intestinal microbiota (48). The mice responded appropriately to antibiotic treatment, as they all developed enlarged cecums and soft stool, as expected. Antibiotic-treated DEREG mice developed severe eosinophilic AIG (Supplemental Fig. 4A) with increased

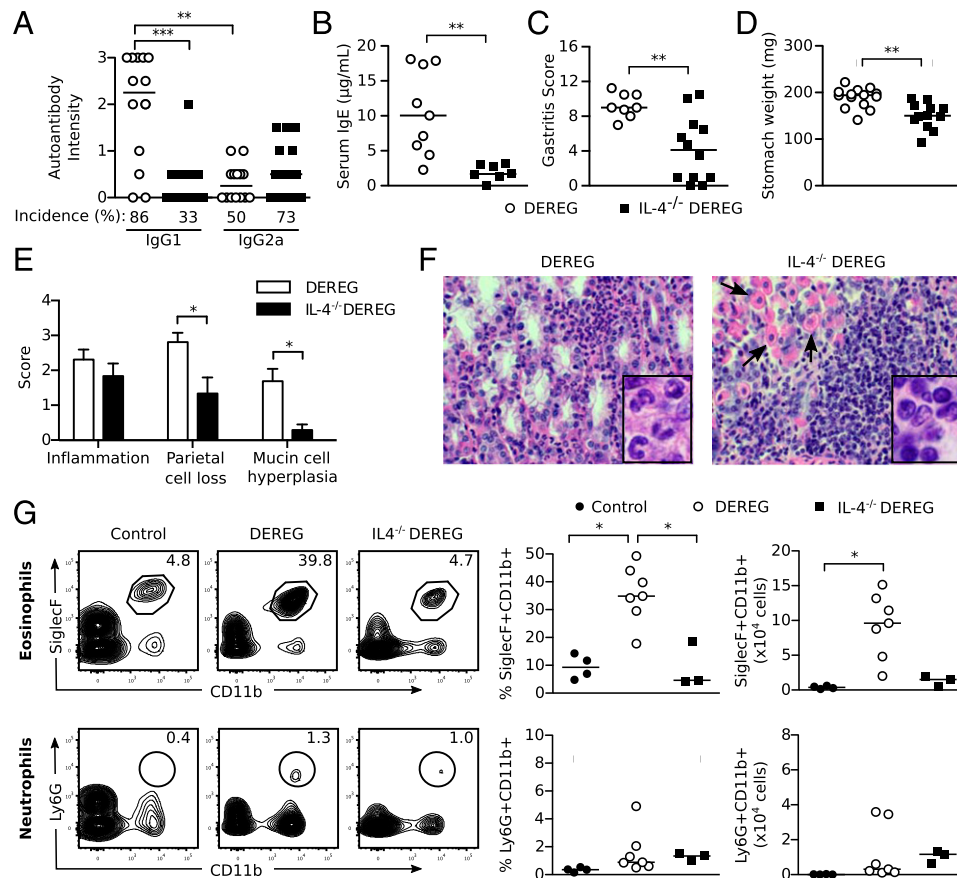


FIGURE 5. IL-4 contributes to AIG pathogenesis in Treg-depleted DERE mice. (A) Serum IgG1 and IgG2a gastric autoantibody staining intensities by immunofluorescence, (B) serum IgE levels, (C) total gastritis score, (D) stomach weights, (E) score of individual histopathological findings of AIG, and (F) stomach-infiltrating cells (H&E; original magnification $\times 400$; arrows indicate residual parietal cells) of IL-4-sufficient DERE and IL-4^{-/-} DERE mice at 3 wk. *Insets* (enlarged from $\times 1000$): Note the numerous granulocytes in stomachs of IL-4^{-/-} DERE mice (*left*) and the numerous mononuclear cells in the IL-4-sufficient DERE mice (*right*). (G) Flow cytometric analysis of SiglecF⁺CD11b⁺ eosinophils (*top*) and Ly6G⁺CD11b⁺ neutrophils (*bottom*) isolated from stomachs of control mice and Treg-depleted WT and IL-4^{-/-} DERE mice at 3 wk. Representative plots (*left*) were gated on live CD45⁺ cells (numbers indicate percentages of cells in the gates). Percent (*center*) and absolute numbers (*right*) of eosinophils and neutrophils are quantified. Data were pooled from three (G), four (B–E), and seven (A) independent experiments. Each symbol represents an individual mouse (A–E) or a pool from three mice (G). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Kruskal–Wallis tests with Dunn’s posttests (A and G), Mann–Whitney U tests (B–E).

stomach weights (Supplemental Fig. 4B) at 3 wk after DT treatment. These results were comparable to those of water-treated DERE mice after Treg depletion (Supplemental Fig. 4A, 4B). In addition, the IgG1-dominant gastric autoantibody production (Supplemental Fig. 4C) and the IL-4 and IL-5 cytokine production by SDLN CD4⁺ T cells after restimulation (Supplemental Fig. 4D) were similar in the water-treated and the antibiotic-treated DERE mice after Treg depletion. These findings indicate that the intestinal microbiome does not promote the development of Th2-dominant AIG in our DERE colony.

The DERE mice with Th2-associated AIG have expanded IRF4⁺PD-L2⁺ dendritic cells and ILT3⁺ Tregs

We further addressed why AIG in Treg-depleted mice has a Th2 bias by testing the hypothesis that the expanded dendritic cells (DCs) and the rebounded Tregs may express molecules that have recently been shown to promote Th2 responses.

A specialized subset of DCs that express PD-L2 on their cell surface and the transcription factor IRF4 have been found to regulate Th2 differentiation in vivo (84, 85). We observed a significant increase in total CD11c⁺ DCs in the pooled LN from DERE mice by 1 wk after Treg depletion (Fig. 7A). Indeed, among the expanded CD11c⁺MHCII⁺ DCs, we readily detected a higher frequency and absolute number of cells coexpressing IRF4

and PD-L2 in the pooled SDLN and NDLN of Treg-depleted mice compared to control mice (Fig. 7B, 7C).

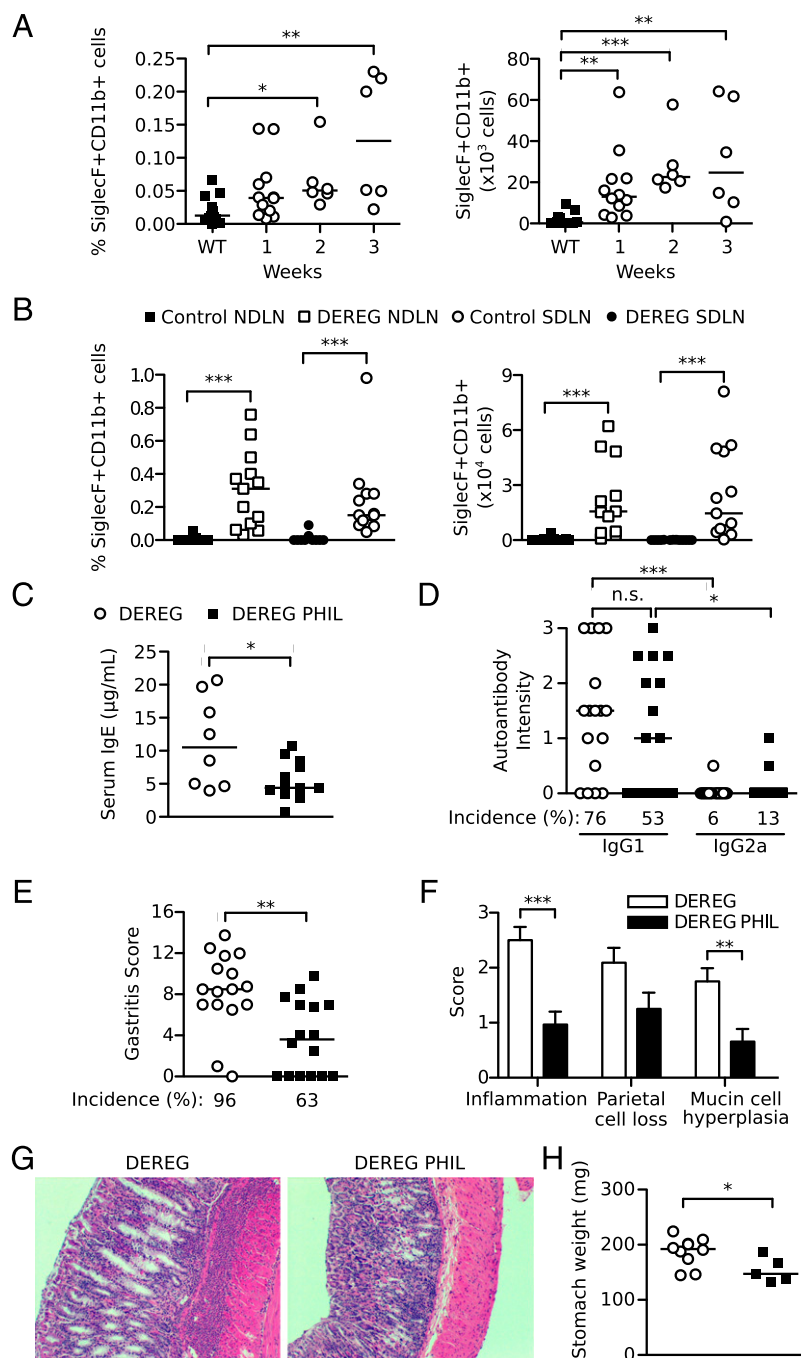
In another recent study, a newly discovered population of ILT3⁺ Tregs was found to promote the maturation of IRF4⁺PD-L2⁺ DCs and participate in controlling the development of Th2 cell responses in vitro and in vivo (86). Indeed, when we assessed the rebounded Treg population at 3 wk, a significantly elevated percentage and absolute number of ILT3⁺ Tregs were detected in the Treg-depleted mice compared to control mice (Fig. 7D, 7E). Notably, an increase in the frequency of ILT3⁺ Tregs was observed in the SDLN, but not the NDLN, of DERE mice.

Together, our findings suggest that an early expansion of IRF4⁺PD-L2⁺ DCs and a disequilibrium between ILT3⁺ and ILT3⁻ Treg subsets may contribute to the Th2-dominant immune responses of Treg-depleted DERE mice.

Discussion

We have shown that severe AIG occurs in C57BL/6 and B6AF1-DERE mice after transient Treg depletion. AIG in these mice is characterized by dominant eosinophilic inflammation, together with parietal cell loss, mucinous metaplasia of mucosal epithelial cells, production of Th2-biased gastric autoantibodies that target both H⁺K⁺ATPase and IF, and T cell activation and Th2 cytokine production in the regional lymph nodes. Our data indicate that

FIGURE 6. Eosinophils contribute to AIG pathogenesis in Treg-depleted DERE mice. **(A)** Kinetics of SiglecF⁺CD11b⁺ eosinophil expansion in pooled lymph nodes as percentage (*left*) and absolute number (*right*). **(B)** Percentage (*left*) and absolute number (*right*) of eosinophils in the NDLN and SDLN of control and Treg-depleted DERE mice at 3 wk. **(C)** Serum IgE levels, **(D)** serum IgG1 and IgG2a gastric autoantibody staining intensities, **(E)** gastritis severity and prevalence, **(F)** score of individual histopathological findings of AIG, **(G)** representative stomach histopathological characteristics (H&E, original magnification $\times 100$), and **(H)** stomach weights in DERE mice ($\circ$) and eosinophil-deficient DERE PHIL mice ($\blacksquare$) at 3 wk. Data were pooled from five (A) or seven (B–H) independent experiments. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann–Whitney U tests (B [*right*], C, E, F, and H), Kruskal–Wallis tests with Dunn's posttest (A, B [*left*], and D).



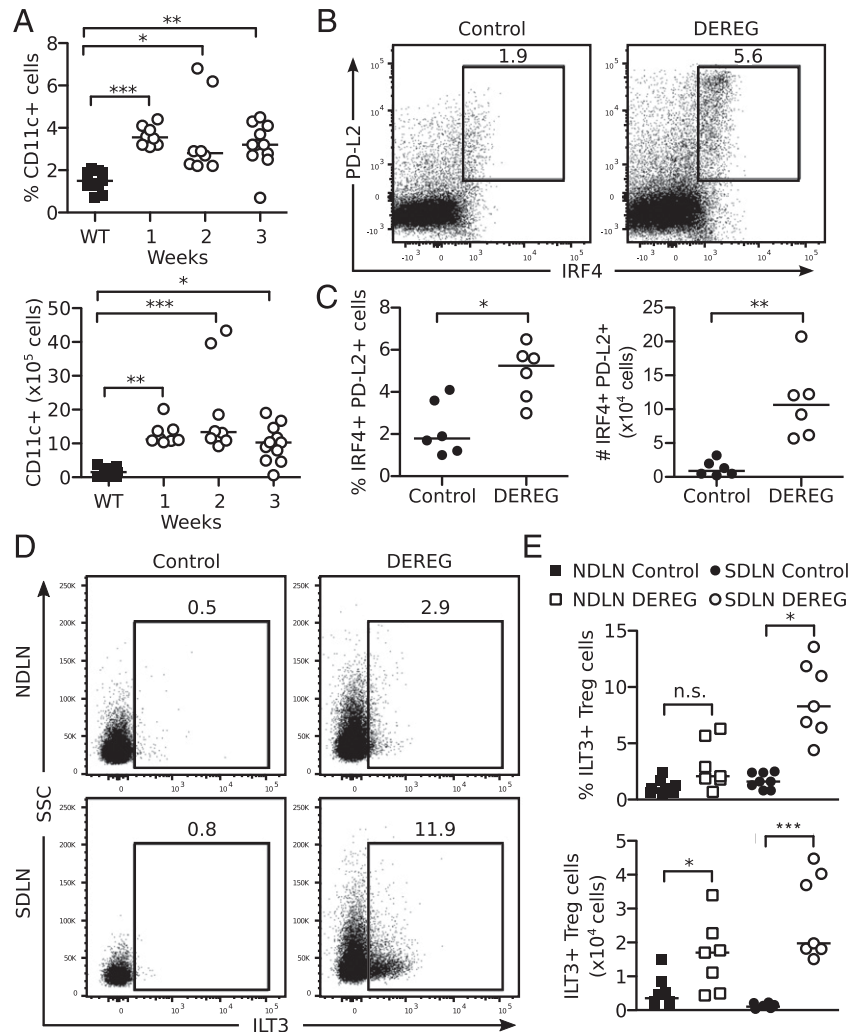
these changes are the product of the rapid loss of effector T cell susceptibility to suppression by Tregs.

Our study, along with others (40, 69), provides conclusive evidence that Tregs are critical in maintaining physiological tolerance to murine gastric autoantigens. However, our findings differ from previous AIG studies in several important aspects. First, AIG was not observed in adult C57BL/6 or BALB/c-DERE mice after a similar regimen of Treg depletion (42, 44). The different outcomes could be explained by diverse environmental factors as well as differences in the microbiomes known to influence autoimmune diseases. In this regard, our mice tested negative for pinworm and fur mite infestations known to skew toward Th2 responses (87, 88), and the AIG severity and Th2 phenotype were not altered by a standard regimen of oral antibiotic treatment. Thus, the intestinal microbiota do not appear to promote the gastric-specific autoimmune responses and Th2-biased disease in

our DERE colony. However, a role for the microbiome in suppression of AIG development in other mouse colonies is a possibility that has not been investigated. Second, our study indicates that the DERE and Foxp3^{DTR} knock-in mice (39) exhibit different responses and clinical outcomes after Treg depletion, although the nature of these differences is not fully understood. Third, similar to other AIG models induced by immunization (89) or d3tx (34), the rebounded Tregs in our model are functional. However, no immunization boost or lymphopenia is required in Treg-depleted DERE mice for the effector T cells to resist Treg-mediated suppression.

The current paradigm posits that autoimmune diseases are dependent on Th1 or Th17 responses and less reliant on Th2 responses, and this applies to studies on AIG (31, 90–92). Th2 cells are generally regarded as counterinflammatory to Th1-dominant autoimmunity, with therapeutic potential (93–96). However, path-

FIGURE 7. Increase in Th2-promoting PD-L2⁺ IRF4⁺ DC subsets as well as ILT3⁺ Treg subsets in transiently Treg-depleted DEREg mice. **(A)** Kinetics of the percentage (*top*) and absolute number (*bottom*) of live, CD11c⁺ DCs in pooled lymph nodes. **(B)** Representative flow cytometry dot plots (numbers indicate percentages of cells in the gates) and **(C)** quantitation as percentage and absolute number of IRF4⁺PD-L2⁺ DCs among live, CD4⁺, CD11c⁺, MHCII⁺ cells in pooled SDLN and NDLN of control and Treg-depleted DEREg mice at 1 wk. **(D)** Representative flow cytometry dot plots (numbers indicate percentages of cells in the gates) and **(E)** quantitation as percentage (*top*) and absolute number (*bottom*) of ILT3⁺ Tregs among live, CD4⁺, Foxp3⁺ cells in the SDLN or NDLN of control and Treg-depleted DEREg mice at 3 wk. Data were pooled from two (B), three (C), or five (A) independent experiments. Each symbol represents an individual mouse. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mann–Whitney *U* tests (C and E [bottom]), Kruskal–Wallis tests with Dunn’s post-test (A and E [top]).



ogenic Th2 responses in autoimmunity have also been suggested by previous studies. For example, studies on AIG have documented activated Th1 and Th2 autoantigen-specific T cells in the regional lymph nodes of d3tx mice. Both the H⁺K⁺ATPase-specific Th1 and Th2 cell clones can transfer severe AIG (32), and TCR transgenic mice generated from these T cell clones spontaneously develop AIG (97, 98). In addition, naive transgenic T cells were able to transfer disease to immunodeficient recipients after *in vitro* differentiation to Th1, Th2, and Th17 cells (91). Disease transfer by Th2 cells has also been documented in several other autoimmune diseases (99–102). In this study, we have provided clear evidence for the spontaneous occurrence of a pathogenic Th2 response after Treg depletion alone, without the requirement for adjuvant, lymphopenia, or use of a transgenic TCR. Our findings will likely have clinical relevance because, despite limited data on human AIG, recent studies also support a possible role for Th2 responses. Bettington and Brown (103) reported an increased eosinophil count in biopsy specimens of patients with AIG, and Bedeir et al. (104) detected numerous IgG4-positive plasma B cells in biopsy specimens of AIG patients with pernicious anemia. The latter is notable because human IgG4 production is associated with Th2 responses (105–107). Although it is possible that different individual AIG patients may exhibit different combinations of Th cell responses, our model seems appropriate for delineating a component of AIG in some patients.

Although autoantibodies to gastric H⁺K⁺ATPase are well defined (13), this study also provides documentation of autoantibody

responses to the gastric IF in experimental AIG. Autoantibodies to IF and H⁺K⁺ATPase are involved in the complications of chronic atrophic gastritis and are useful diagnostic markers of AIG (21). For example, immune-mediated depletion of parietal cells can lead to hypochlorhydria, and autoantibodies to IF, by impairing the absorption of vitamin B12, can lead to pernicious anemia (108). Patients with AIG and pernicious anemia are reported to have an increased risk of developing gastric cancer (16–18). Investigation of these important sequelae of AIG is not possible with the current models of AIG caused by Treg depletion. In this regard, the longevity of AIG in the DEREg mice and their survival after Treg depletion may provide an opportunity for investigation of these sequelae.

The evidence for a Th2 type of immune response in the pathogenesis of AIG is supported by the following findings: 1) the subclass of the autoantibody response, 2) the inflammatory cell infiltrate and epithelial cell response, and 3) the cytokine production. The pathogenicity of the Th2 response is supported by the significant reduction in stomach disease and immune responses in IL-4^{-/-} mice and eosinophil-deficient mice. Strikingly, the phenotypes of these mice are not identical. The decreased disease in Treg-depleted IL-4^{-/-} mice was mainly attributed to the reduced parietal cell loss and mucin cell hyperplasia. These changes correlated with a reduced infiltration of eosinophils in the inflamed stomach. However, the degree of inflammation remained comparable to that observed in IL-4-sufficient DEREg mice, suggesting that additional mechanisms are responsible for the residual gas-

tritis in the absence of IL-4. Although a predominant mononuclear cell infiltrate, characteristic of Th1 responses, was observed in Treg-depleted IL-4^{-/-} mice, IFN- γ Ab treatment did not prevent AIG in these mice (data not shown) or in IL-4-sufficient DERE mice. In addition, neutrophil infiltration was scarce in Treg-depleted mice, and treatment with IL-17 Ab did not inhibit AIG, arguing against a predominant role for Th17 responses. Thus, IL-4-dependent mechanisms appear to be major contributors to AIG in Treg-depleted DERE mice.

A more dramatic reduction of gastric inflammation was observed in the Treg-depleted eosinophil-deficient PHIL mice. In addition, a significant reduction in mucinous metaplasia was noted. The major loss of parietal cells in mice with eosinophils raises the possibility that eosinophils could have a cytotoxic role in AIG in DERE mice. It is known that upon activation, eosinophils can release proteases and other granule mediators and exhibit strong cytotoxic effects (109). Activated eosinophils also secrete proinflammatory cytokines and chemokines that attract other pathogenic immune cells to the site of tissue injury (109, 110).

In addition to their potential roles in the efferent phase of the autoimmune response, eosinophils may also support the induction of Ag-specific Th2 cell-dependent Ab responses in the lymph nodes. In allergy models, eosinophils infiltrate the T cell zones, where they can directly present Ags to T cells or/and release cytokines that influence Th2 differentiation and Ig class switching, directly or through DCs (111, 112). In the DERE mice, Treg depletion also led to a rapid expansion of eosinophils in the lymph nodes, and Treg-depleted eosinophil-deficient PHIL mice had a significant reduction in the levels of serum IgE. However, the precise meaning of this finding and the precise role for eosinophils in promoting AIG pathogenesis will require additional studies.

Why does Treg depletion in the DERE mice lead to a dominant Th2 response? This response could be explained by a higher Th2 cell susceptibility to apoptosis in the presence of Tregs, and, thus, a tighter Treg control of Th2 cells compared to Th1 cells (64). Notably, scurfy mice and patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome both develop hyper-IgE syndrome and eosinophilia. A dominant Th2 response was also observed in Foxp3-deficient mice (69) and in mice with Treg-specific deletion of CTLA-4 (62), ITCH (113), IRF4 (69), CK2 (86), or Nr4a (114), suggesting that these molecules expressed on Tregs are involved in the control of Th2 responses. However, the mechanisms that link the loss of Treg function to the allergic Th2-type responses are not fully understood (115–119). It is possible that factors and cytokines operating locally in the stomach also influence the expression of a Th2-dominant tissue response. For example, Buzzelli et al. (120) showed that IL-33 promoted a type 2 gastric inflammatory response in WT mice that resembles the AIG in DERE mice.

To determine why the DERE mice with transient Treg depletion develop a dominant pathogenic Th2 response, we investigated the altered expression of certain key molecules on Tregs and DCs that can influence the preferential induction of Th2 responses. Despite the development of uncontrolled Th2 responses in the Treg-depleted DERE mice, the rebounded Tregs did not show altered expression of Foxp3, GITR, CD25, CTLA-4, or IRF4. Two reports have demonstrated that the initiation of Th2 cell-driven immune responses depends on IRF4 expression in DCs, which controls the maturation of a PD-L2⁺ DC subset (84, 85). It was shown that IRF4 promotes IL-33 and IL-10 expression by DCs that induces the differentiation of Th2 cells *in vivo*. Consistent with previous reports (39), we also observed a rapid expansion of

DCs after Treg depletion in DERE mice. Furthermore, we detected a significant increase in the proportion and absolute numbers of IRF4⁺PD-L2⁺ DCs in the DERE mice as early as 1 wk after Treg depletion. This finding supports the hypothesis that the early expansion of a unique DC population may preferentially drive the development and activation of Th2 cells in AIG. In addition, we also found that a greater proportion and number of the rebounded Tregs in DT-treated DERE mice express ILT3. This is an intriguing finding because a recent study reported that ILT3 expression on Tregs is controlled by the casein kinase 2, and the ILT3⁺ Tregs could promote the expansion of IRF4⁺PD-L2⁺ DCs (86). It is tempting to speculate, as a potential mechanism, that the Th2-dominant immune responses in the Treg-depleted DERE mice may result from the cooperation of the IRF4⁺PD-L2⁺ DCs and the ILT3⁺ rebounded Treg subsets.

It is striking that a very short period of Treg deficiency was sufficient to allow for AIG development. Our analysis indicates that the suppressive function of the rebounded Tregs does not appear to be compromised. Thus, the rebounded Tregs suppress as efficiently as control Tregs *in vitro*. In contrast, we observed that the effector T cells were less sensitive than naive cells to Treg-mediated suppression within 1 wk of the first dose of DT treatment. Although this state of resistance to suppression by Tregs may be due to potential changes in the innate or adaptive immune compartments, findings from our *in vitro* studies suggest the alteration is also intrinsic to the effector T cells.

Effector T cells can resist Treg-mediated suppression through multiple ways. For example, the cytokine and growth factor composition in the environment, alterations in TCR signaling, the anatomical location, and the number and activation status of the effector T cells can all affect the mechanism and the degree of Treg-mediated suppression (121, 122). Resistance to suppression has been reported in other animal models of autoimmunity and in human autoimmunity (123–131). Although numerous signaling pathways can account for the resistance phenotype (122), one relevant to Th2 cell resistance might involve IL-4 signaling and STAT6 activation, as previously documented *in vitro* (132) and *in vivo* (133). Whether this lack of susceptibility to Treg-mediated suppression is also associated with the preferential reduction of other Treg subsets that regulate Th2 cells (86, 113, 114), the absence of gastric Ag-specific Tregs among the rebounded Tregs (34, 134), or additional possibilities (135) remains to be elucidated.

In summary, our study has shown that Th2 responses are involved in AIG, indicating that Th2 responses can participate in the pathogenesis of organ-specific autoimmune diseases in mice with transient Treg depletion. This observation is associated with the finding that the effector T cells are less sensitive to Treg-mediated suppression after a short period of Treg deficiency. These findings suggest that Treg therapy or the blockade of certain effector targets might not be as effective in the treatment of AIG and highlight the need for a better understanding of the crosstalk between the effector and regulatory mechanisms of T cell responses. For AIG, successful therapy may require dampening of the effector autoimmune response while strengthening the regulatory components.

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Disclosures

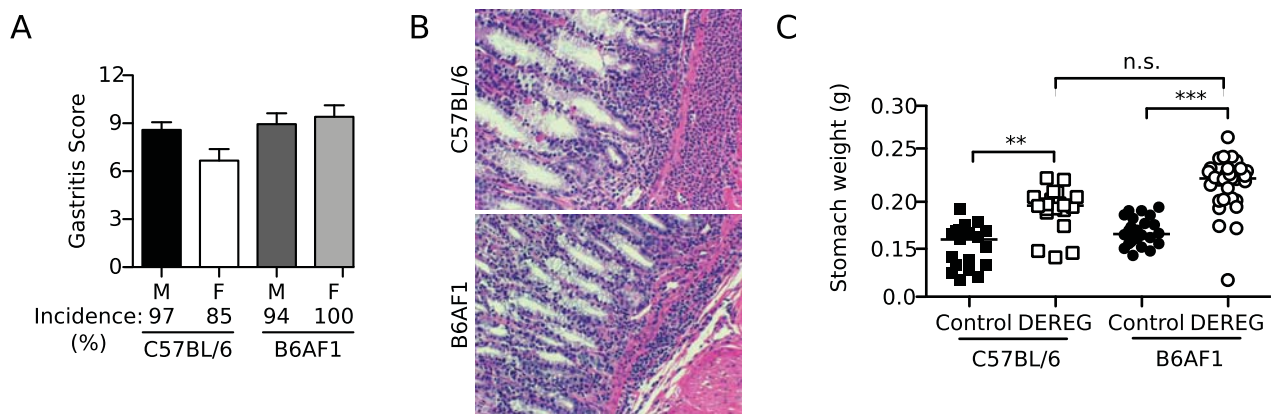
The authors have no financial conflicts of interest.

References

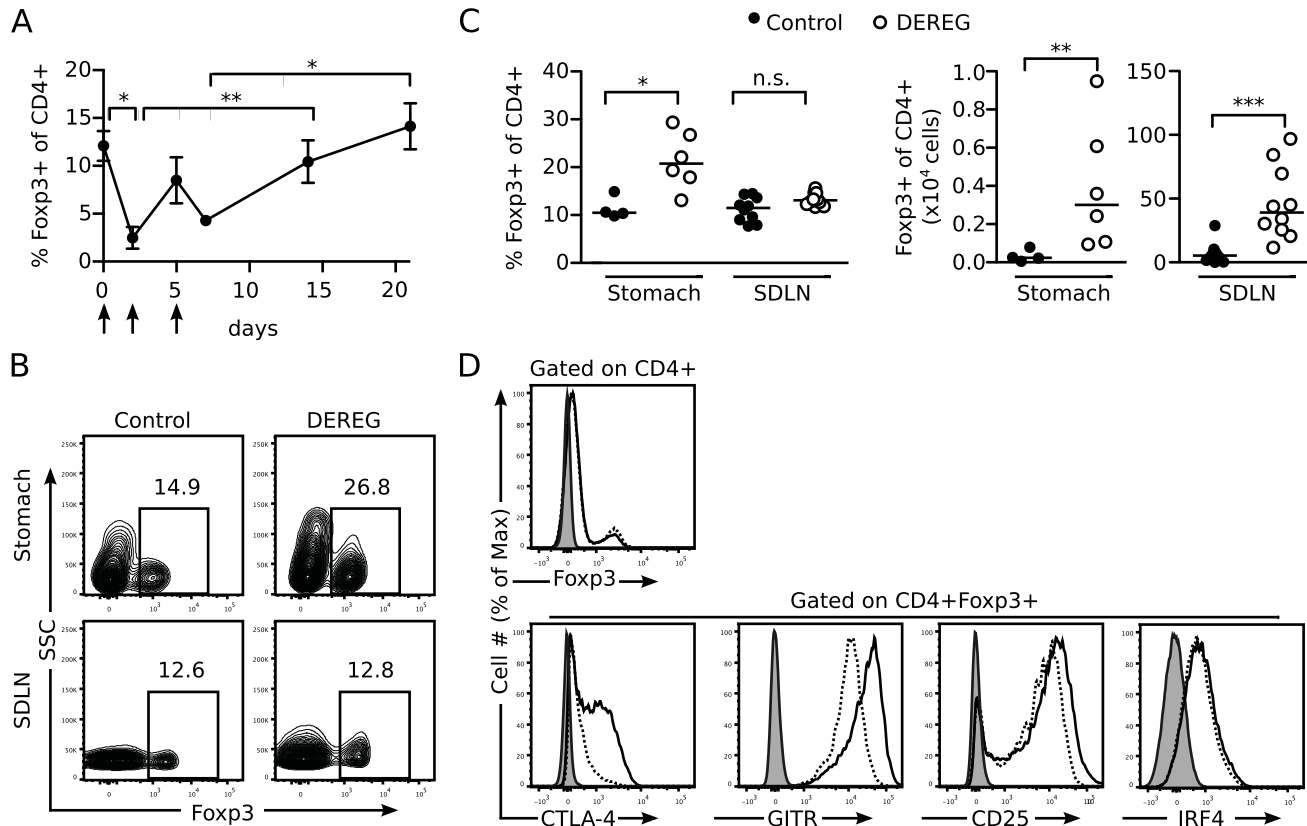
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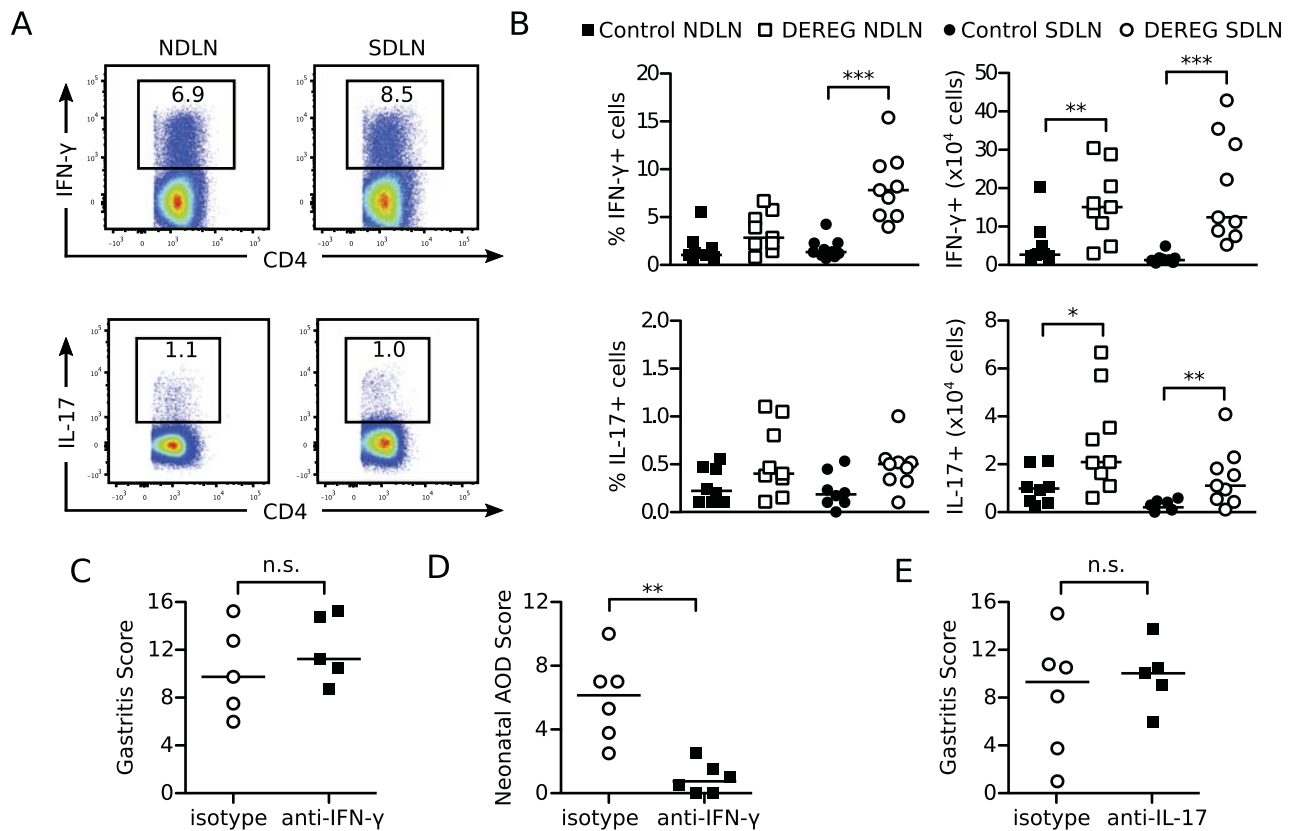
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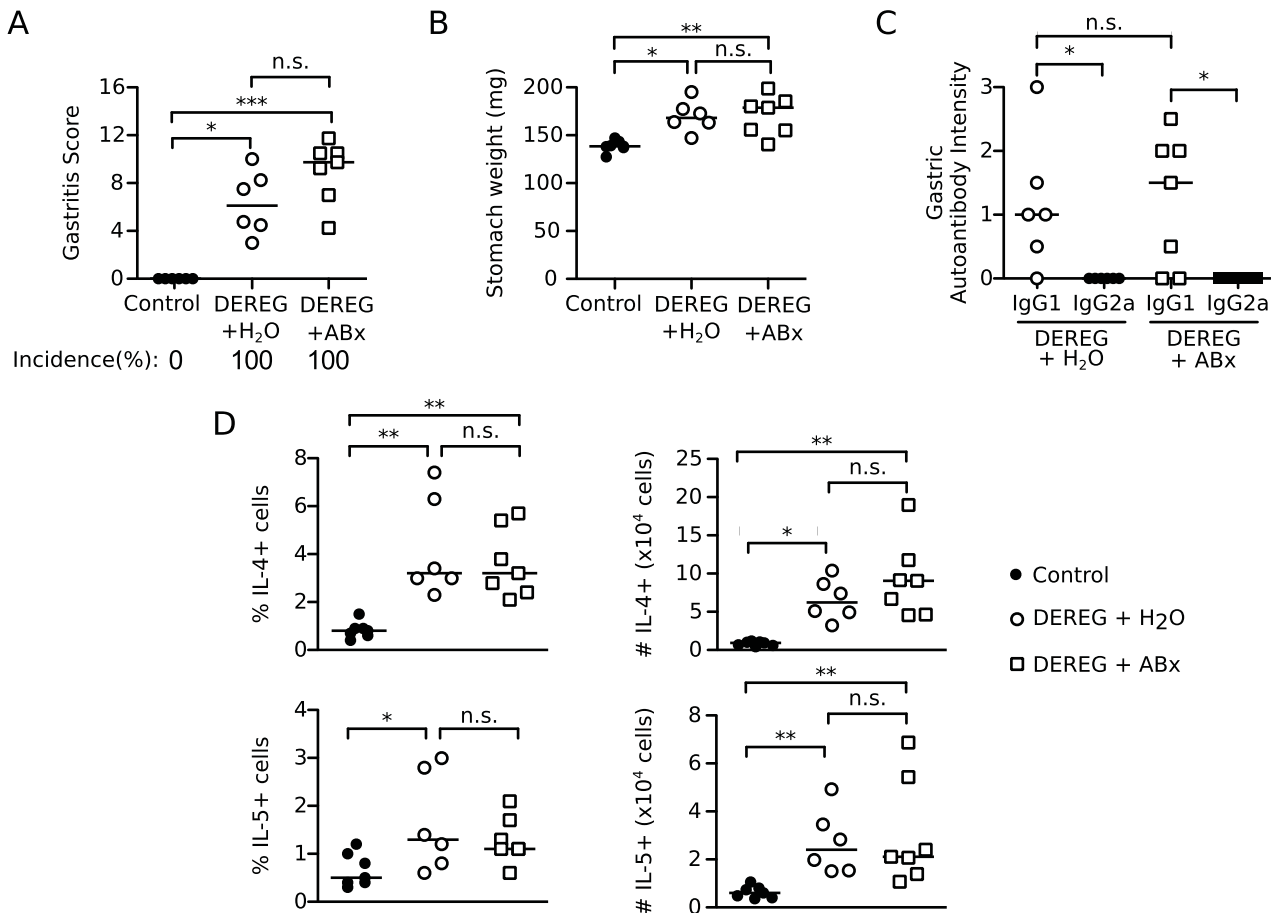
Supplemental Figure 1: AIG incidence and severity are comparable between C57BL/6 and B6AF1 mice and between male and female mice. (A) Gastritis severity and incidence at 3 weeks in male (M) and female (F) C57BL/6 and B6AF1 DEREG mice. (B) Representative stomach histopathology (H&E, x200) at 3 weeks. (C) Comparison of stomach weights between control and Treg cell-depleted C57BL/6 and B6AF1-DEREG mice. Data in (A) display the mean and SEM. Data were pooled from 3-11 independent experiments per group. Each symbol in (C) represents an individual mouse. $p > 0.05$ for all comparisons in (A), $**p < 0.01$ and $***p < 0.001$; Kruskal-Wallis test with Dunns posttest. Data in subsequent experiments were pooled from both mouse strains and genders.



Supplemental Figure 2: The stomach- and draining lymph node distribution and suppressive capacity of normal and rebounded Treg cells is comparable. (A) Kinetics of changes in the percent of Foxp3+ Treg cells among total CD4+ T cells in pooled lymph nodes after DT treatment (indicated by arrows). (B) Representative flow cytometry contour plots depicting the frequency of Foxp3+ Treg cells in the stomach and SDLN of control or Treg cell-depleted mice at 3 weeks (numbers indicate percentages of cells in the gates). (C) Quantitation of B as percentage (left) and absolute number (right). (D) Expression levels of Foxp3, CTLA-4, GITR, CD25, and IRF4 on Treg cells from the SDLN of control and Treg cell-depleted mice at 3 weeks. A representative histogram showing the MFI of Foxp3 among live CD4+ cells (top) and of CTLA-4, GITR, CD25, and IRF4 among live CD4+ Foxp3+ cells (bottom) are shown. The gray shaded peaks are isotype controls; dotted and continuous lines are control and Treg cell-depleted DEREK mice, respectively. Data were pooled from 6 (B, C) and 3 (A, D) independent experiments. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$; Kruskal-Wallis with Dunns posttest (A); Mann-Whitney t tests (C).



Supplemental Figure 3: Th1 and Th17 responses do not contribute to AIG development in Treg cell-depleted mice. (A) Representative flow cytometry dot plots of IFN- γ and IL-17 producing CD4⁺ T cells in the NDLN and SDLN of control and Treg cell-depleted DERE mice at 3 weeks. Cells were stimulated *ex vivo* with PMA and ionomycin before flow cytometry analysis (numbers indicate percentages of cells in the gates). (B) Quantitation of A. (C) Gastritis scores at 3 weeks of Treg cell-depleted DERE mice with continuous IFN- γ antibody or isotype IgG treatment. (D) Neonatal autoimmune ovarian disease (nAOD) scores at 2 weeks after disease induction in mice with continuous IFN- γ antibody or isotype IgG treatment. (E) Gastritis scores at 3 weeks of Treg cell-depleted DERE mice with continuous IL-17 antibody or isotype IgG treatment. Data were pooled from 3 (A, B, C, E) or 4 (D) independent experiments. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$; Mann-Whitney t tests (B right, C, D, E); Kruskal-Wallis test with Dunns posttests (B left).



Supplemental Figure 4: Intestinal microbiome alteration by oral antibiotic treatment did not affect the development of Th2-dominant AIG in Treg cell-depleted DERE mice. (A) Gastritis severity and prevalence, (B) stomach weights, and (C) serum IgG1 and IgG2a gastric autoantibody intensities by immunofluorescence staining at 3 weeks in DT-treated control and DERE mice treated with water or antibiotics (ABx). (D) Quantification of flow cytometry analysis of IL-4 (top) and IL-5 (bottom) producing CD4⁺ T cells in the SDLN of control and Treg cell-depleted DERE mice treated with water or ABx (cells were stimulated *ex vivo* by PMA and ionomycin before flow cytometry analysis; left: percentage; right: absolute number). Data were pooled from 2 independent experiments. Each symbol represents an individual mouse. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Kruskal-Wallis tests with Dunns posttests.