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$CD4^+$ $CD25^+$ Regulatory T Cells Modulate the T-Cell and Antibody Responses in *Helicobacter*-Infected BALB/c Mice

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Gastric *Helicobacter* spp. induce chronic gastritis that may lead to ulceration and dysplasia. The host elicits a T helper 1 (Th1) response that is fundamental to the pathogenesis of these bacteria. We analyzed immune responses in *Helicobacter*-infected, normal mice depleted of $CD4^+$ $CD25^+$ T cells to investigate the in vivo role of regulatory T cells (Tregs) in the modulation of *Helicobacter* immunopathology. BALB/c and transgenic mice were depleted of $CD4^+$ $CD25^+$ T cells by administration of an anti-CD25 antibody either at the time of infection with *Helicobacter* or during chronic infection and gastritis. Depletion of $CD25^+$ Tregs prior to and during infection of mice with *Helicobacter* spp. did not affect either bacterial colonization or severity of gastritis. Depletion of $CD25^+$ Tregs was associated with increased *Helicobacter*-specific antibody levels and an altered isotype distribution. Paragastric lymph node cells from $CD25^+$ Treg-depleted and control infected mice showed similar proliferation to *Helicobacter* antigens, but only cells from anti-CD25-treated animals secreted Th2 cytokines. $CD25^+$ Tregs do not control the level of gastritis induced by gastric *Helicobacter* spp. in normal, thymus-intact BALB/c mice. However, $CD25^+$ Tregs influence the cytokine and antibody responses induced by infection. Autoimmune gastritis is not induced in *Helicobacter*-infected mice depleted of $CD25^+$ Tregs but is induced in $CD25^+$ Treg-depleted mice, which have a higher frequency of autoreactive T cells.

Helicobacter pylori is a chronic pathogen of the human gastric mucosa (40), infecting approximately half the world's population (20). Only 10 to 15% of infected individuals develop disease, which may range from acute gastric inflammation (38, 39) to duodenal and gastric ulcers, gastric adenocarcinoma, and mucosal-associated lymphoid tissue (MALT) lymphoma (10, 24, 51).

H. pylori-infected individuals develop cellular and humoral immune responses (44) that are ineffective in clearing the infection. Infected individuals develop a predominantly inflammatory T helper 1 (Th1) response in the gastric mucosa, the extent of which is linked to the severity of gastritis in humans (15, 34, 64). The dominance of Th1 gastric T cells in *H. pylori* infection may explain the failure of infected individuals to induce immunity to *H. pylori*, and the production of gamma interferon (IFN-γ) is thought to contribute to the pathology (8, 45). The infiltration of IFN-γ-producing cells in the infected mucosa is accompanied by increased numbers of cells producing transforming growth factor β (TGF-β), suggesting that *H. pylori*-induced inflammatory responses may be partially regulated by $CD25^+$ T regulatory cells ($CD25^+$ Tregs) (34).

$CD25^+$ Tregs constitute 5 to 10% of all peripheral $CD4^+$ T cells in normal naïve mice and healthy humans (59) and possess potent regulatory activity both in vitro (73) and in vivo (5, 56, 67). $CD25^+$ Tregs are thought to mediate their immunosuppressive activity through the release of soluble factors, including TGF-β and interleukin-10 (IL-10) (76, 78) and/or directly by a cell contact-dependent mechanism (71, 73). $CD25^+$ Tregs not only play a key role in the maintenance of self-tolerance and protection from autoimmune diseases, such as autoimmune gastritis, but also influence the nature of the immune responses to a range of infectious organisms (6, 9, 58).

Gastric autoantibodies can be detected in approximately 30% of *H. pylori*-infected individuals (21). The frequency of *H. pylori* infection in human subjects with early gastric autoimmunity, as indicated by the presence of parietal cell-specific antibodies, suggests that infection with *H. pylori* may affect the induction or maintenance of stomach-specific autoimmunity (54), possibly as a result of molecular mimicry resulting from epitopes that are common to the gastric mucosa and *H. pylori*, such as H^+/K^+-ATPase gastric antigen (4, 12, 14, 47, 48).

We examined the role of $CD4^+$ $CD25^+$ T cells in regulation of *Helicobacter*-driven immune responses and autoimmune gastritis in a BALB/c mouse model, using a monoclonal anti-CD25 antibody to deplete $CD25^+$ Tregs during or after *Helicobacter* infection of BALB/c mice. These studies were designed to address the role of $CD25^+$ Tregs in the maintenance of *Helicobacter*-induced gastritis and the development of autoantibody responses against gastric antigens.

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MATERIALS AND METHODS

Animals. Six- to 9-week-old female BALB/c and 1E4-TCR transgenic (tg) mice were bred and housed under specific-pathogen-free conditions at the Department of Microbiology and Immunology Animal Facility, The University of Melbourne, Parkville, Australia. All work with animals was performed with approval of The University of Melbourne Animal Ethics and Experimentation Committee.

Antibodies and administration to mice. 1H9 (mouse immunoglobulin G1 [IgG1] anti-H^+/K^+ ATPase α-subunit) and 2B6 (mouse anti-H^+/K^+ ATPase β-subunit) (46) hybridoma supernatants were used as controls for enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. RA3-6B2 (rat anti-mouse B220) (7) and H129.19 (rat anti-mouse CD4) (53) hybridoma supernatants were used for immunohistochemistry. PC61 (rat IgG2a anti-CD25) (35) hybridoma supernatant was purified as described elsewhere (23). The sources of other antibodies were as follows: rat anti-mouse CD8 (53-6.7), rat anti-mouse CD4-PE (GK1.5), and rat anti-mouse CD25–fluorescein isothiocyanate (FITC) (7D4) were from Pharmingen, San Diego, CA; rat anti-mouse Ig–FITC, sheep anti-mouse Ig–biotin, and streptavidin-horseradish peroxidase (HRP) were from Silenus Laboratories, Australia; goat anti-rat IgG–HRP and goat anti-rabbit IgG–HRP were from Chemicon International, California; rabbit anti-mouse Ig–HRP was from DAKO, Denmark; rabbit anti-mouse IgG1 and rabbit anti-mouse IgG2a were from ICN Biomedicals, Ohio; and rat IgG (technical grade) was from Sigma, Germany.

Purified PC61 or rat IgG (0.3 mg), diluted in phosphate-buffered saline (PBS), was injected into mice intraperitoneally (i.p.) using a 1-ml syringe and a 26-gauge needle.

***Helicobacter felis* and *H. pylori* growth conditions.** *H. felis* CS1 (52) and *H. pylori* SS1 (33) were obtained from A. H. Mitchell at The University of New South Wales, Sydney, Australia, and were cultured as described by Sakagami et al. (57) and Lee et al. (33), respectively.

Preparation of *H. felis* and *H. pylori* antigens. Bacteria were harvested from broth culture or agar plates in PBS and sonicated while on ice. The bacterial sonicate was stored at −70°C, and the protein concentration was determined by a Bradford protein assay (Bio-Rad Laboratories).

Infection of mice with *H. felis* and *H. pylori*. *H. felis* CS1 was scraped from plates into brain heart infusion (BHI) broth, washed, and resuspended in BHI broth to approximately 10^8 bacteria per 200 μl. *H. pylori* SS1 was grown in BHI broth, washed, and resuspended in PBS to approximately 10^9 bacteria per 200 μl. Prior to infecting mice, bacteria were analyzed in wet mounts for motility and morphology, as well as by urease test (25) and by Gram stain. Mice were infected on days 1, 3, and 5 by oral gavage with 200 μl of bacteria under light anesthesia. Viable counts of the *H. pylori* SS1 inoculum were determined immediately after infection of mice by culturing the bacteria on *Campylobacter* selective agar plates under microaerophilic conditions.

Assessment of *H. felis* and *H. pylori* colonization. Stomachs were removed from euthanized mice and opened along the greater curvature. Contents were scraped, and the stomach was washed twice in PBS and sectioned in small strips along its length to include the greater curvature. The stomach strips were either fixed in 10% (vol/vol) formalin in 0.1 M Na-phosphate buffer (10% NBF), pH 7.2, washed with PBS, and frozen for immunohistochemistry or fixed in 10% NBF, processed, and embedded in paraffin, or used to enumerate the bacterial load.

H. felis colonization of the gastric mucosa was analyzed by histology. Paraffin-embedded tissues were cut (4 μm) and silver stained using the Warthin-Starry method (42) to visualize the bacteria. The number of bacteria within the crypts of the antrum and body regions of the stomach was enumerated in sections, and colonization was graded using a scoring method previously described (69).

H. pylori colonization was quantified by determining the number of CFU per gram of stomach tissue. Stomach strips were weighed, homogenized in 5 ml PBS, and serially diluted in PBS. The Miles and Misra dilution technique was used to enumerate CFU within each dilution (43). Aliquots were plated on Glaxo selective supplement agar plates (33).

Histological examination and grading of gastritis. Hematoxylin and eosin-stained, formalin-fixed paraffin-embedded sections were used to grade the inflammatory response, based on a previously described method (68). The stomach mucosa was divided into upper, mid-, and lower body and antrum. Mild inflammation was defined as an influx of inflammatory cells in the basal zone of the mucosa, moderate describes inflammatory cells extending up to the mid-zone, and in severe inflammation the infiltrate is spread through the full thickness of the mucosa. Lymphoid follicles were defined as collections of lymphocytes forming a central cortex and an outer marginal zone. Focal inflammation was defined as small aggregates of inflammatory cells often around a small blood vessel; diffuse inflammation describes cells forming a band in the lamina propria. The following six-point scale was used to define mononuclear cell infiltration: 1, mild multifocal; 2, mild widespread or moderate multifocal; 3, mild widespread and moderate or severe multifocal; 4, moderate widespread; 5, moderate widespread and severe multifocal; 6, severe widespread. The first 2 mm of the upper body at the junction with the esophageal mucosa were disregarded, since there was a consistent follicle in this zone in all infected mice. All sections were graded blindly.

Flow cytometry. Heparinized blood (50 to 100 μl) was collected, and cells were stained and analyzed using a FACSCalibur (Becton Dickinson) as previously described (31).

In vitro T-cell proliferation assays. In vitro T-cell proliferation assays were performed using paragastric lymph node cells (1×10^5) in the presence or absence of 5 μg/ml *H. pylori* sonicate as previously described (31).

Detection of cytokines in T-cell culture supernatants. Cytokines (IL-2, IL-4, IL-5, IFN-γ, and tumor necrosis factor alpha [TNF-α]) produced during in vitro T-cell proliferation assays were measured in the supernatant using the BD cytometric bead array mouse Th1/Th2 cytokine CBA kit (BD Biosciences, San Diego, Calif.) following the manufacturer's instructions. Samples were analyzed using a BD FACSCalibur flow cytometer and BD CellQuest software. Data were formatted and further analyzed using BD CBA software.

***H. pylori*- and *H. felis*-specific ELISAs.** Serum anti-*Helicobacter* antibody levels were determined by standard ELISA. Microtiter plates (NUNC, Denmark) were coated overnight at 4°C with 50 μg/ml *H. felis* sonicate or 10 μg/ml *H. pylori* sonicate in PBS. Plates were washed with 0.05% (vol/vol) Tween 20–PBS and blocked with 5% (wt/vol) skim milk–PBS for 1 h at 37°C. Bound antibody was detected using sheep anti-mouse Ig conjugated to HRP and visualized using Immunopure *o*-phenylenediamine (Pierce) with H_2O_2 as a substrate. Antigen-specific IgG1 and IgG2a antibody titers were determined using rabbit anti-mouse IgG1 and IgG2a antibodies and HRP-conjugated anti-rabbit Ig. Titers are expressed as the reciprocal of the dilution of serum that gave an optical density at 492 nm five times the value of the background. Sera with known specific *Helicobacter* antibody titers were included in all ELISA studies.

Statistical analysis of data. The nonparametric two-tailed Mann-Whitney U-test was used for statistical analysis of the results. Differences were considered statistically significant when P was <0.05. Antibody and cytokine levels below the detection limit of the assay were assigned the minimum level of detection of the assay, to enable statistical analysis.

RESULTS

MAb PC61 depletes $CD4^+$ $CD25^+$ lymphocytes from the peripheral blood of BALB/c mice. To investigate the contribution of $CD4^+$ $CD25^+$ regulatory T cells in the regulation of immune responses to *Helicobacter* antigens in infected mice, we depleted $CD25^+$ T cells from *H. pylori* SS1-infected animals using the anti-CD25 monoclonal antibody (MAb) PC61 (30, 50). Treatment of mice with a single dose of 0.3 mg of PC61 resulted in a reduction of blood, splenic, and lymph node $CD4^+$ $CD25^+$ T-cell populations by 75% by 2 days after treatment (not shown), consistent with published data (50). In addition, we have previously demonstrated that multiple treatments with the anti-CD25 MAb PC61 result in depletion of $CD4^+$ $CD25^+$ T cells in the peripheral blood to 5% of the starting population (30). Based on these data, in this study 0.3 mg MAb PC61 was injected i.p. six times over a period of 3 weeks into BALB/c mice, resulting in removal of greater than 96% of $CD4^+$ $CD25^+$ T cells from the peripheral blood 3 days after the final antibody injection (3.68% ± 2.65% [mean ± standard deviation]) (Fig. 1 shows a representative example). As a control, mice received purified rat IgG. Flow cytometric analysis of blood cells was used throughout this study, using the anti-CD25 MAb 7D4, which recognizes a different epitope than PC61, to ensure that the numbers of $CD4^+$ $CD25^+$ T cells were reduced to <4% following injection of PC61.

Depletion of $CD25^+$ Tregs does not alter gastric pathology and bacterial colonization in *Helicobacter*-infected mice. The

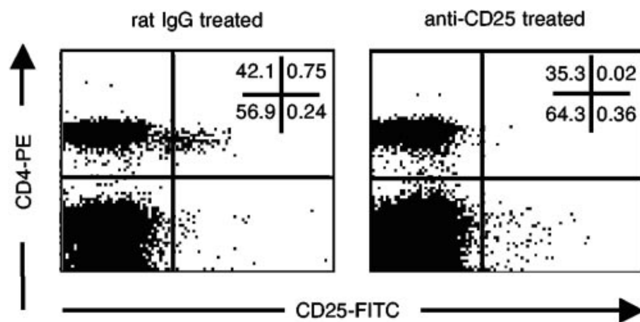


FIG. 1. In vivo treatment with anti-CD25 MAb efficiently eliminates CD25$^+$ Treg cells. Thymus-intact adult BALB/c mice were injected (i.p.) six times with 0.3 mg anti-CD25 (PC61) or rat IgG over a period of 3 weeks. Three days after the final injection, peripheral blood lymphocytes were stained with anti-CD25 MAb (7D4)–FITC and anti-CD4 (GK1.5)–phycoerythrin and analyzed by flow cytometry. Shown is a representative profile of 1 out of 10 mice per treatment group. The percentage of lymphocytes in each quadrant is indicated.

role of CD25$^+$ Tregs in the modulation of the *Helicobacter*-induced inflammatory response was investigated in mice infected with either *H. pylori* or *H. felis*. BALB/c mice were infected with *H. pylori* and treated with anti-CD25 or rat IgG as shown in Fig. 2A. Gastric pathology (Fig. 2B) of anti-CD25- and rat IgG-treated animals was examined by histology 4 and 14 weeks after infection with *H. pylori* (Fig. 2C). *H. pylori* infection resulted in a mononuclear cell inflammation of the antrum and body regions of the stomach at 4 weeks, which progressed in severity 14 weeks after infection (Fig. 2C and D). The chronic (14 weeks), predominantly lymphocytic cellular inflammation within the gastric mucosa of anti-CD25-treated mice did not differ significantly from control mice throughout the course of the experiment ($P = 0.2$, $P = 0.07$, $P = 0.8$, and $P = 1$ in the upper, mid-, and lower body and antrum at 4 weeks; $P = 0.2$, $P = 1$, $P = 0.3$, and $P = 1$ at 14 weeks after infection, respectively). Acute inflammation in all infected mice was minimal, and no atrophy was evident in any animals (data not shown). The level of *H. pylori* colonization of anti-CD25- and rat IgG-treated mice was similar at 4 ($1.5 \times 10^7 \pm 1.5 \times 10^7$ and $2.1 \times 10^7 \pm 1.2 \times 10^7$ CFU/gram of stomach, respectively; $P = 0.42$) and 14 weeks after infection ($2.3 \times 10^6 \pm 2.1 \times 10^6$ and $3.4 \times 10^6 \pm 2.7 \times 10^6$ CFU/gram of stomach, respectively; $P = 0.69$).

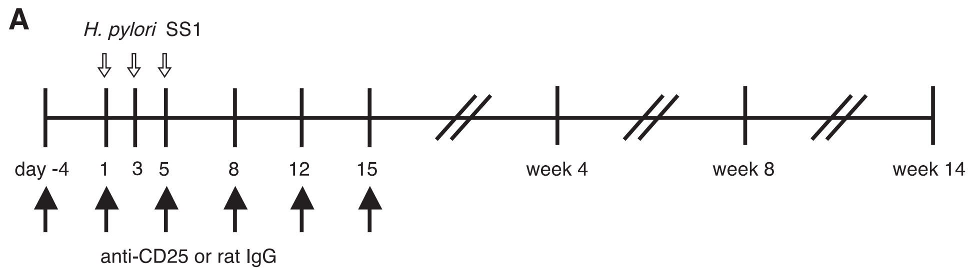
We next examined whether elimination of CD25$^+$ Treg cells affected gastric pathology induced by a *Helicobacter* species (*H. felis* CS1) that evokes a stronger inflammatory response in mice than *H. pylori*. Groups of 10 BALB/c mice were infected with *H. felis* and treated with either anti-CD25 or rat IgG as shown in Fig. 3A. The level of gastritis was determined by histological examination of stomach sections at 12 and 26 weeks after infection. Inflammation was observed in the antrum and body regions of the stomachs of all *H. felis*-infected mice at both time points. No differences in the pattern or severity of the gastric infiltrate were observed in anti-CD25-treated mice compared with rat IgG-treated mice in any region of the stomach (Fig. 3B) ($P = 0.7$, $P = 0.55$, $P = 0.7$, and $P = 0.55$ in the upper, mid-, and lower body and antrum at 12 weeks; $P = 0.5$, $P = 0.3$, $P = 0.8$, and $P = 1$ at 26 weeks after infection, respectively). Immunohistochemical analysis was also used to phenotype the cellular infiltrate in the gastric mucosa. Formalin-fixed, frozen stomach sections were stained with MAbs specific for either CD4, CD8, B cells, or macrophages. There was no difference in the phenotype, number, or distribution of mononuclear cells that infiltrated into the stomachs of mice treated with anti-CD25 antibody or rat IgG (data not shown).

H. felis colonization was determined in silver-stained histological sections of gastric tissue. *H. felis* was detected in the stomachs of all infected mice throughout the duration of the experiment (26 weeks). Using the scale adapted from that of Sutton et al. (69), anti-CD25-treated and control mice had similar levels of *H. felis* stomach colonization at 12 weeks (1.4 ± 0.55 and 2.5 ± 0.57, respectively, in the antrum; $P = 0.06$) and 26 weeks (2.4 ± 1.14 and 2.2 ± 1.3, respectively, in the antrum; $P = 0.8$) after infection.

Depletion of CD25$^+$ Tregs during infection with *Helicobacter* spp. increases the specific antibody response and alters the isotype distribution of *Helicobacter*-specific antibodies. Sera obtained from anti-CD25- and rat IgG-treated, *H. pylori*-infected mice were analyzed by ELISA (Fig. 4A). *H. pylori*-specific antibody levels were significantly increased ($P = 0.0001$, $P = 0.008$, and $P = 0.015$ at 4, 8, and 14 weeks, respectively) in anti-CD25-treated mice compared with rat IgG-treated mice. Further examination of the antibody response revealed that, in mice treated with anti-CD25, IgG1 dominated the *H. pylori*-specific antibody response ($P = 0.0001$), whereas rat IgG-treated mice had undetectable levels of *H. pylori*-specific IgG1 and IgG2a (Fig. 4B) at 4 weeks after infection. After 14 weeks, IgG1 remained the predominant isotype of *H. pylori*-specific antibodies produced by anti-CD25-treated mice ($P = 0.15$), although the IgG2a titer was increased compared with that seen in the sera of these animals 4 weeks after infection (Fig. 4C). In rat IgG-treated mice, approximately equal levels of IgG1 and IgG2a *H. pylori*-specific antibodies were detected 14 weeks after infection, but specific antibody levels remained below the detection limit in some animals (Fig. 4C).

Serum obtained from anti-CD25-treated *H. felis*-infected mice at 6, 9, and 12 weeks after infection also contained significantly increased anti-*Helicobacter* antibody levels compared to rat IgG-treated animals ($P = 0.005$, $P = 0.0001$, and $P = 0.008$ at 6, 9, and 12 weeks, respectively) (Fig. 4D). At 12 and 26 weeks after infection, levels of *H. felis*-specific IgG1 antibodies were higher ($P = 0.05$ and $P = 0.008$ at 12 and 26 weeks, respectively) than IgG2a antibodies in anti-CD25-treated animals, whereas in the rat IgG-treated mice IgG1 and IgG2a levels were comparable ($P = 1$ and $P = 0.5$ at 12 and 26 weeks) (Fig. 4E and F).

Cytokine profile of *H. pylori*-specific T cells is different in CD25$^+$ Treg cell-depleted mice. To further study the role of CD25$^+$ Treg cells in regulation of *H. pylori*-specific immune responses, T-cell responses were compared in anti-CD25- and rat IgG-treated *H. pylori*-infected mice. Paragastric lymph node cells were cultured with *H. pylori* antigens 14 weeks after *H. pylori* infection. Splenocytes from naïve mice were used as a negative control, since the yield of lymphocytes from the paragastric lymph nodes of naïve mice was very low. Lymphocytes obtained from the paragastric lymph nodes of anti-CD25-treated mice showed an increase in proliferation compared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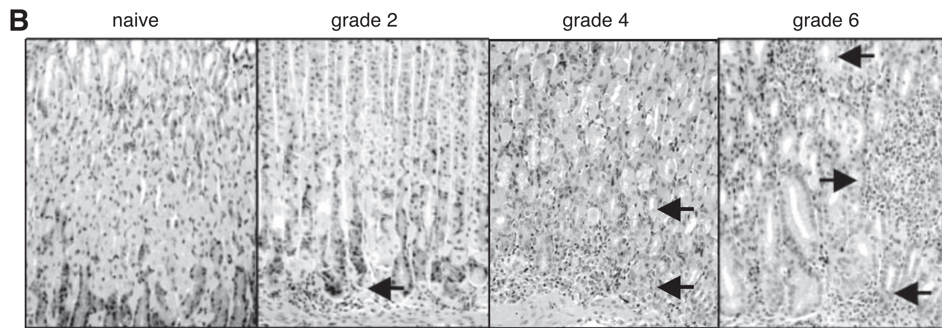
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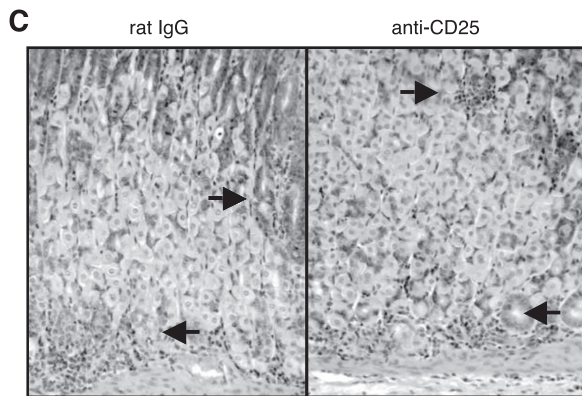
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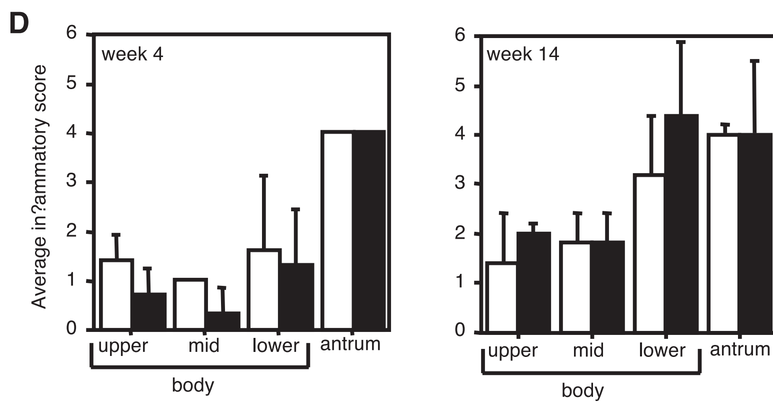
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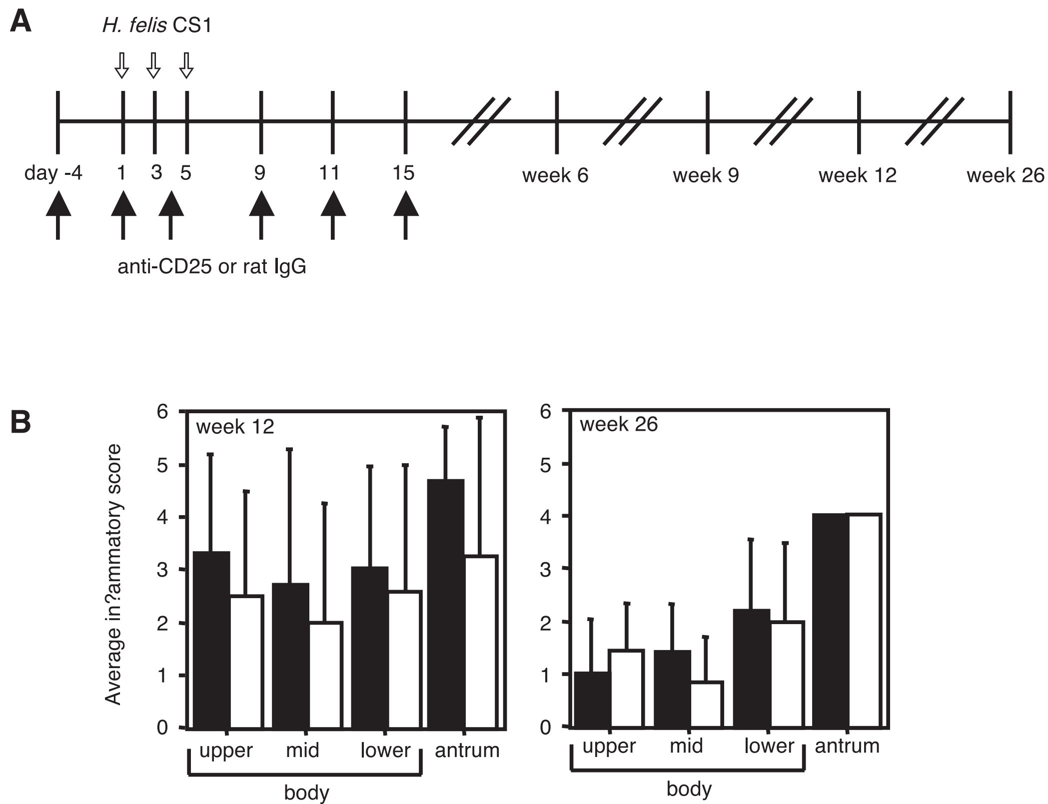

FIG. 3. *H. felis*-induced gastritis is not affected by in vivo elimination of $CD25^+$ Treg cells. (A) Groups of 10 BALB/c mice were infected with *H. felis* CS1 on days 1, 3, and 5 (white arrows) and were injected (i.p.) six times (black arrows) with either 0.3 mg of anti-CD25 MAb (white bars) or rat IgG (black bars). (B) Formalin-fixed paraffin-embedded stomach sections (4 μm) were stained with hematoxylin and eosin and examined by light microscopy. Shown are the average inflammatory score ± standard deviation ($n = 5$ rat IgG- or anti-CD25-treated animals observed 12 or 26 weeks after infection).

with control *H. pylori*-infected mice when incubated with *H. pylori* antigens (Fig. 5A) that was not statistically significant when data were analyzed as the stimulation index (average stimulation indexes, 12.6 and 8.8 for rat IgG-treated and anti-CD25-treated groups, respectively; $P = 0.15$).

Supernatants from paragastric lymph node cell cultures were used to measure cytokine production following antigen-specific stimulation in vitro. Lymph node cells from *H. pylori*-infected rat IgG-treated mice restimulated in vitro with *H. pylori* antigen produced IFN-γ, TNF-α, and IL-2 (Fig. 5B to F), whereas IL-4 and IL-5 were not detected. In contrast, T cells from anti-CD25-treated mice produced IFN-γ, TNF-α, and IL-2 as well as IL-4 and IL-5 when stimulated with *H. pylori* antigens ($P = 0.008$ and $P = 0.036$ for IL-4 and IL-5 cytokine production, respectively) (Fig. 5B to F). There were no significant differences in the levels of IFN-γ, TNF-α, IL-2, and IL-5 produced by T cells from anti-CD25-treated mice compared with rat IgG-treated mice restimulated in vitro with *H. pylori* antigen ($P = 0.42$, $P = 0.84$, $P = 0.06$, and $P = 0.036$ for IFN-γ, TNF-α, IL-2, and IL-5 production, respectively); however, the levels of *H. pylori*-specific IL-4 secretion were significantly increased ($P = 0.008$). Therefore, $CD25^+$ Treg depletion during chronic infection with *H. pylori* results in enhanced production of Th2 cytokines.

We next examined the role of $CD25^+$ Tregs in established inflammatory responses in the gastric mucosa of *H. pylori*-infected mice. Mice were infected with *H. pylori* and 1 month later treated with anti-CD25 or rat IgG for 3 weeks as shown in Fig. 6A. Chronic inflammation was observed at 12 and 20 weeks after *H. pylori* infection in the gastric mucosa of all mice,

FIG. 2. Elimination of $CD25^+$ Treg cells does not affect *H. pylori*-induced gastritis in the stomach. (A) BALB/c mice were infected with *H. pylori* SS1 on days 1, 3, and 5 (white arrows) and injected (i.p.) six times (black arrows) with either 0.3 mg of anti-CD25 MAb or rat IgG. Groups of five anti-CD25 MAb-treated or rat IgG-treated mice were killed at 4 and 14 weeks after infection. Formalin-fixed paraffin-embedded stomach sections (4 μm) were stained with hematoxylin and eosin and examined by light microscopy. (B) Representative sections of stomach taken 14 weeks after infection with *H. pylori*, illustrating mononuclear cell infiltrates scored as naïve, and grades 2, 4, and 6. The arrows indicate the levels of infiltrate into the gastric tissue. (C) Representative sections of stomach from rat IgG-treated (one of five) or anti-CD25-treated (one of five) animals taken 14 weeks after infection with *H. pylori* and assigned a grade of 4. (D) Four or 14 weeks after infection with *H. pylori*, stomach sections of rat IgG-treated (black bars) or anti-CD25-treated (white bars) animals were examined for mononuclear cell infiltrates. Shown are the average inflammatory scores ± standard deviation ($n = 5$ for rat IgG- or anti-CD25-treated animals at 4 and 14 weeks).

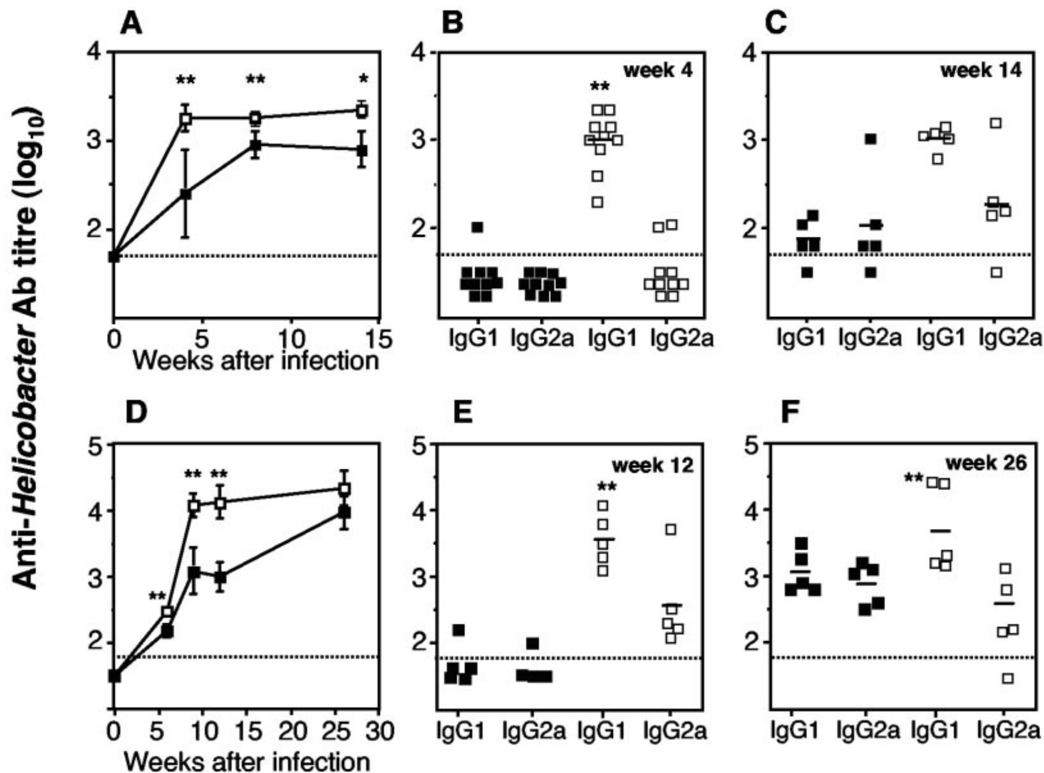

FIG. 4. The antigen-specific antibody response in *Helicobacter*-infected mice is increased and dominated by IgG1 following treatment with anti-CD25. Groups of 10 BALB/c mice were infected with either *H. pylori* SS1 (A to C) or *H. felis* CS1 (D to F) and treated with either rat IgG (black symbols) or anti-CD25 MAb (white symbols) according to Fig. 2A or 3A, respectively. Serum was collected at several time points after infection, and *Helicobacter*-specific antibody levels were determined by ELISA (A and D). Isotype-specific antibodies were used to determine the levels of *Helicobacter*-specific IgG1 and IgG2a at weeks 4 and 14 after infection with *H. pylori* (B and C) or weeks 12 and 26 after infection with *H. felis* (E and F). Shown are either the mean ± standard deviation of groups of mice (A and D) (n = 10 per group at weeks 4 and 12 after infection with *H. pylori* and *H. felis*, respectively, and n = 5 per group at weeks 14 and 26 after infection with *H. pylori* and *H. felis*, respectively), or the responses of individual animals and the average (horizontal bar) per group (B, C, E, and F). The dotted line indicates the detection limit of the assays. *, $P < 0.05$; **, $P < 0.01$.

in both the body and antrum regions, irrespective of the antibody treatment. No difference was observed in the pathology in anti-CD25-treated and rat IgG-treated mice at either time point, and bacterial colonization was equivalent in anti-CD25- and rat IgG-treated mice (data not shown).

Compared with rat IgG-treated mice, *H. pylori*-specific antibody levels were elevated ($P = 0.0007$) in anti-CD25-treated mice 1 week after the cessation of antibody treatment (i.e., 8 weeks after infection), but this difference was not evident at later time points examined ($P = 1$) (Fig. 6B). Furthermore, IgG1 dominated the *H. pylori*-specific antibody response in anti-CD25-treated mice at 8 weeks after infection ($P = 0.0003$) (Fig. 6C), whereas *H. pylori*-specific antibody responses were undetectable at 8 weeks after infection in 9 of 10 rat IgG-treated mice (Fig. 6C). At 20 weeks after infection, *H. pylori*-specific IgG2a antibodies were detectable in four of five rat IgG-treated mice, and one of five mice produced detectable levels of *H. pylori*-specific IgG1, whereas anti-CD25-treated mice produced *H. pylori*-specific IgG1 (four of five mice) and IgG2a antibodies (two of five mice) (Fig. 6D). There was no longer a dominance of IgG1 *H. pylori*-specific antibody response in anti-CD25-treated mice at 20 weeks after infection ($P = 0.54$).

Antigen-induced proliferation of paragastric lymph node cells was comparable in both groups of mice at 20 weeks after infection ($P = 0.15$) (Fig. 6E). In response to antigen, paragastric lymph node cells from anti-CD25- and rat IgG-treated mice produced similar levels of TNF-α, IL-2, and IFN-γ (data not shown), while IL-4 and IL-5 were produced by anti-CD25-treated mice; however, only the levels of IL-5 production were significant ($P = 0.15$ and $P = 0.008$ for IL-4 and IL-5 production, respectively) (Fig. 6F and G).

$CD25^+$ Treg depletion coupled with localized *Helicobacter* infection does not result in autoimmunity in normal mice; however, autoimmune gastritis develops if the frequency of gastric-specific T cells is increased. A link between *H. pylori* infection and gastric autoimmunity has been suggested in a number of human studies (4, 49, 54). To gain a better insight into the relationship of *Helicobacter* infection, the role of $CD25^+$ Tregs and the development of autoimmunity, we analyzed the development of autoimmune gastritis in wild-type BALB/c mice infected with either *H. pylori* or *H. felis* and treated with either rat IgG or anti-CD25 (Fig. 2A and 3A). No anti-H^+/K^+ ATPase autoantibodies, which are a marker of autoimmune gastritis, were detected by ELISA in any animals up to 14 weeks (*H. pylori*) or 26 weeks (*H. felis*) after infection

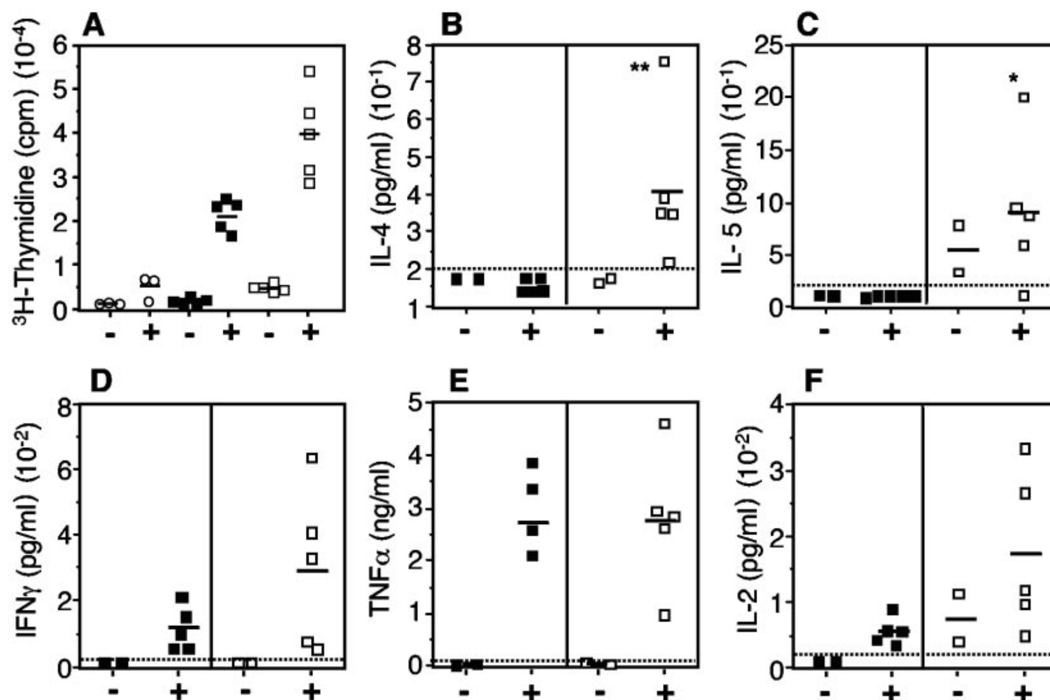

FIG. 5. Anti-CD25 treatment during infection with *H. pylori* skews the T-lymphocyte response towards a Th2 response. BALB/c mice were infected with *H. pylori* and treated with either rat IgG (black squares) or anti-CD25 MAb (white squares) according to Fig. 2A (n = 10 per group). Paragastric lymph node cells were isolated 14 weeks after infection and cultured in vitro in the presence (+) or absence (-) of *Helicobacter* antigen. Splenocytes from untreated BALB/c mice (white circles) were also included. Antigen-specific proliferation was measured by incorporation of [^{3}H]thymidine (A), and secretion of IL-4 (B), IL-5 (C), IFN-γ (D), TNF-α (E), and IL-2 (F) into culture supernatant was determined using the BD cytometric bead array mouse Th1/Th2 cytokine kit. Represented are the responses of individual mice as well as the mean of each group (horizontal bars). The dotted lines indicate the detection limits of the assays. *, $P < 0.05$; **, $P < 0.01$.

(data not shown). These data demonstrate that stomach-localized *Helicobacter* infection, coupled with removal of CD25$^+$ Tregs, does not result in autoimmunity in BALB/c mice with a normal repertoire of conventional CD4$^+$ T cells.

To determine if an increase in the precursor frequency of H$^+$/K$^+$ ATPase-specific T cells influenced the outcome of autoimmunity in *Helicobacter*-infected mice, we exploited T-cell receptor (TCR) tg mice that express a low-affinity, pathogenic TCR specific for the H$^+$/K$^+$ ATPase β-subunit (1E4-TCR tg mice) (2). 1E4-TCR tg mice spontaneously develop autoimmune gastritis only at a low incidence (<10%) (29). 1E4-TCR tg mice were infected with *H. pylori* and either depleted of CD25$^+$ Tregs with anti-CD25 or treated with rat IgG as outlined in Fig. 2A. At 12 weeks after infection, 1E4-TCR tg mice were killed and autoimmune gastritis was assessed by the presence of anti-H$^+$/K$^+$-ATPase autoantibodies in the serum and by the severity of gastritis graded from 1 to 3, as previously described (29). An autoimmune gastritis grading of 1 is readily distinguished from the low level of gastritis caused by the bacterial infection. None of the five infected, rat IgG-treated 1E4-TCR tg mice was autoantibody positive or developed gastric infiltrate typical of autoimmune gastritis. In contrast, four out of six (66%) *H. pylori*-infected, anti-CD25-treated 1E4-TCR tg mice showed a mononuclear cell infiltrate that was characteristic of autoimmune gastritis. Furthermore, two of the four gastritis-positive 1E4-TCR tg mice were also positive for anti-parietal cell autoantibodies by immunofluorescence and positive for anti-gastric H$^+$/K$^+$ ATPase by ELISA (not shown).

DISCUSSION

Murine *Helicobacter* infection mimics many aspects of human *H. pylori* disease, including the development of a chronic inflammation characterized by T cells expressing Th1 cytokines (16, 22, 32). The murine model therefore provides an excellent opportunity to analyze the genesis and regulation of *Helicobacter*-induced chronic gastritis and allows manipulation of various putative pathological factors, including cells and their effectors, such as cytokines (45, 61) and antibodies (1, 70). CD25$^+$ Tregs have been implicated as important in defining the nature of immune responses in a number of infections, including *Helicobacter* infections (19, 36, 37). However, the impact of CD25$^+$ Tregs on immune responses to *Helicobacter* infection has been analyzed in vitro or in mouse models associated with a T-cell deficiency, while the contribution of CD25$^+$ Tregs in defining the immune response to *Helicobacter* infection in normal individuals is not known. In this study, we investigated the influence of CD25$^+$ Tregs on the immunopathology of *Helicobacter*-mediated disease in BALB/c mice, which are genetically prone to develop both *Helicobacter*-induced gastritis (13) and autoimmune disease (27, 59, 74). Overall, our studies show that CD25$^+$ Tregs are important in maintaining a predominant Th1 response to the bacterium.

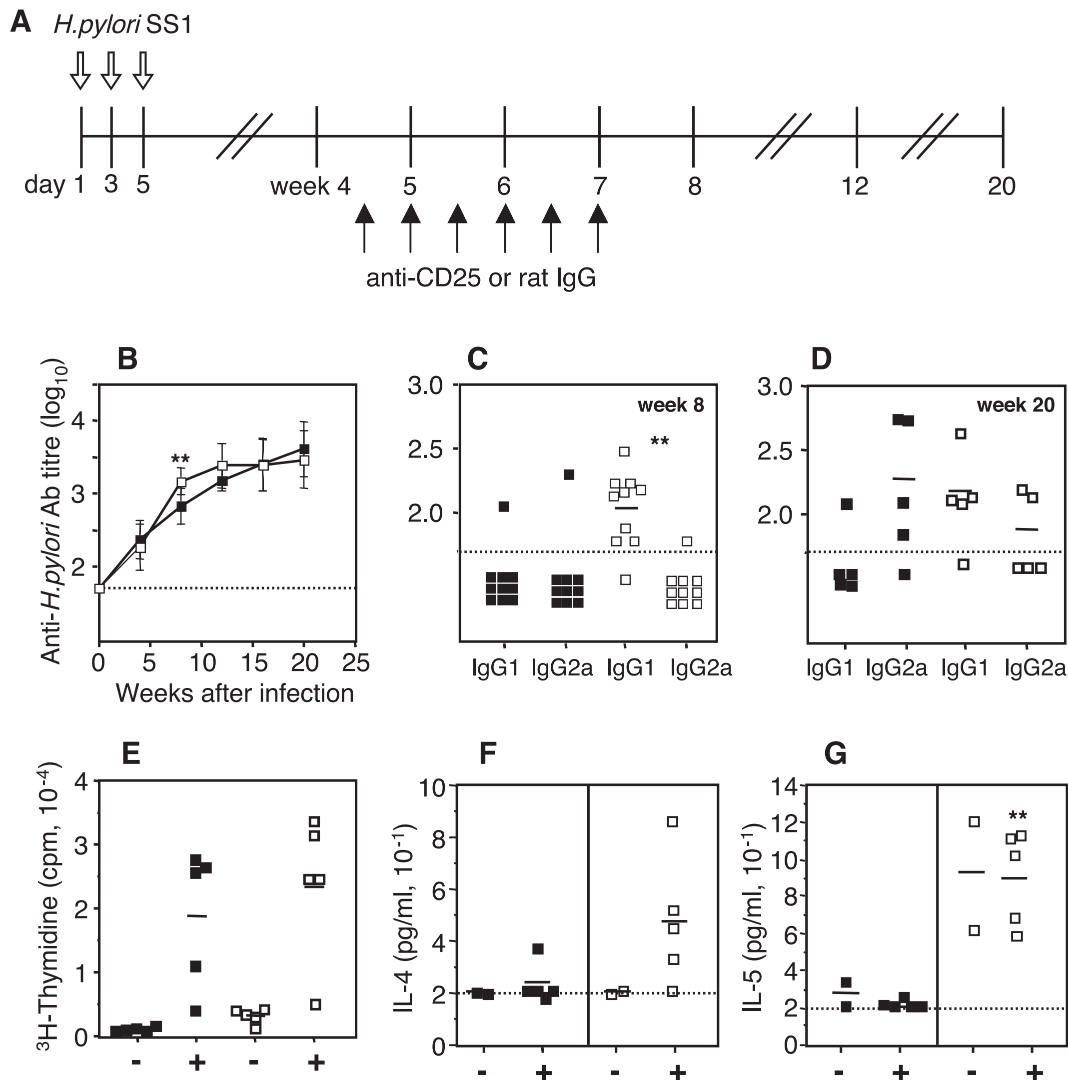

FIG. 6. Effect of anti-CD25 treatment on the immunopathology of an established *H. pylori* infection. (A) BALB/c mice were infected with *H. pylori* SS1 on days 1, 3, and 5 (white arrows) and were injected (i.p.) six times (black arrows) with either 0.3 mg of anti-CD25 MAb or rat IgG (n = 10 per group). (B) Serum was collected at several time points after infection, and *H. pylori*-specific antibody levels were determined by ELISA. (C and D) Isotype-specific antibodies were used to determine the levels of *Helicobacter*-specific IgG1 and IgG2a at weeks 8 and 20 after infection with *H. pylori*. (E to G) Paragastric lymph node cells were isolated 20 weeks after infection and cultured in vitro in the presence (+) or absence (-) of *Helicobacter* antigen. Antigen-specific proliferation was measured by incorporation of [^{3}H]thymidine (E), and secretion of IL-4 (F) and IL-5 (G) into the culture supernatant was determined using the BD cytometric bead array mouse Th1/Th2 cytokine kit. Represented are the means ± standard deviations of groups of five mice (B) or the responses of individual mice as well as the means of each group (horizontal bars) (C to G). The dotted lines indicate the detection limits of the assays. **, $P < 0.01$.

The absence of CD25$^+$ Tregs resulted in an increased production of Th2 cytokines by lymphocytes in response to *H. pylori* antigens and an increase in *Helicobacter*-specific antibodies in the circulation. This study has highlighted a key role for CD25$^+$ Tregs in defining the balance of Th1/Th2 responses following *Helicobacter* infection and suggests that these cells are likely to play an important role in the long-term outcome of chronic *Helicobacter* infection.

In vivo depletion of CD25$^+$ Tregs in BALB/c mice with anti-CD25 antibody PC61 resulted in a 96% reduction in peripheral CD4$^+$ CD25$^+$ T cells. The depletion of CD25$^+$ Tregs is transient, and the population returns to normal levels within a few weeks (30). Removal of CD25$^+$ Tregs during the establishment of infection with *Helicobacter* spp. did not alter the level of colonization with either *H. pylori* or the more inflammatory *H. felis*. In addition, depletion of CD25$^+$ Tregs did not reduce the level of *Helicobacter*-induced gastritis, nor was there any indication of gastric atrophy and gastric metaplasia over the 14-week infection period. The depletion of CD25$^+$ Tregs in mice 1 month after *H. pylori* infection was established equally and did not result in major changes to the induced gastric pathology or bacterial colonization levels. Our findings demonstrate that transient depletion of CD25$^+$ Tregs does not perturb *Helicobacter*-induced gastritis in a mouse model with a normal immune repertoire, irrespective of the time of depletion. These results are consistent with those of Eaton et al.

(17), who analyzed the role of $CD4^+$ T-cell subsets in the pathogenesis of *H. pylori* by adoptive transfer of splenocyte subsets to infected C57BL/6 SCID mice. Bacterial colonization levels and gastritis were equivalent in recipients of $CD4^+$ splenocytes and $CD4^+$ $CD45RB^{high}$ splenocytes, a subset of $CD4^+$ splenocytes devoid of $CD25^+$ Tregs.

Mice depleted of $CD25^+$ Tregs at the time of initial infection showed a significant increase in *Helicobacter*-specific antibodies by 4 weeks after infection, a difference that persisted throughout the 14-week experimental period. An increase in *Helicobacter*-specific antibodies was also observed when $CD25^+$ Tregs were depleted 4 weeks after the infection was established. The striking feature of the elevated antibody response in the absence of $CD25^+$ Tregs was a dominance of the IgG1 subtype. In addition, lymphocytes from $CD25^+$ Treg-depleted, infected mice produced IL-4 and IL-5, whereas these cytokines were absent in cultures from rat IgG-treated, infected animals. Production of IL-4 and IL-5 and IgG1 antibodies are indicative of a Th2 response, a response not typical of *H. pylori* infection in either humans or rodents (8, 63, 65). Depletion of $CD25^+$ Tregs 1 month after an *H. pylori* infection also resulted in the generation of an increased Th2 response. Therefore, $CD25^+$ Tregs are critical, not only in establishing an exclusive Th1 response during the initial infection period, but also in maintaining Th1 immune responses once the *H. pylori*-induced gastritis is established. Furthermore, these studies clearly demonstrated that depletion of $CD25^+$ Tregs in *H. pylori*-infected animals induced a Th2 immune response that persisted over many weeks, despite only a transient depletion of the $CD25^+$ Tregs.

An enhanced Th2 response to *H. pylori* antigens has also been reported after treatment of C57BL/6 mice with anti-CTLA4 antibodies during the first 7 days of infection with *H. pylori* (77). CTLA4 blockade resulted in an increased *H. pylori*-specific IgG1/IgG2a ratio in the serum and a higher IL-4/IFN-γ ratio by splenocytes in response to *H. pylori* antigens. CTLA4 is a costimulatory molecule that has an important inhibitory role in T-cell proliferation and regulation of Th responses (75). Significantly, CTLA4 is expressed constitutively by $CD25^+$ Tregs and is considered to be required for their suppressor function (56, 60, 72). In addition to a shift to a Th2 response, infected C57BL/6 mice treated with anti-CTLA4 also showed a reduction in the severity of gastritis, whereas in our study the depletion of $CD25^+$ Tregs in BALB/c mice had no apparent effect on development of gastric inflammation. This difference may reflect the diverse functions of the CTLA4 molecule on different subsets of T cells, the reduction in IFN-γ production by T cells as a consequence of CTLA4 blockade, or the strains of mice used in the two studies. Nonetheless, it is possible that more prolonged ablation of $CD25^+$ Tregs in the BALB/c model would also influence the level of gastritis.

H. pylori memory T-cell responses from the peripheral blood of infected patients were shown to be suppressed by *H. pylori*-specific $CD25^+$ Tregs in vitro (36), a finding which could explain why *H. pylori* infection does not give rise to protective immunity. Regulation of immune responses by $CD25^+$ Tregs has been reported in murine *Helicobacter hepaticus*-induced inflammatory bowel disease and colitis (19). The role of Tregs in maintaining gastric inflammation has also been investigated by reconstituting athymic C57BL/6 *nu/nu* mice with lymphocyte populations either containing or depleted of $CD25^+$ Tregs, followed by infection with *H. pylori* bacteria (55). In this system, $CD25^+$ Tregs were shown to reduce the immunopathology associated with *H. pylori* infection but to allow a higher load of bacterial colonization. The authors proposed that $CD25^+$ Tregs reduced the activation of IFN-γ-producing $CD4^+$ T cells, therefore reducing the severity of gastritis. However, potential complications of this model are, firstly, that donor T cells will undergo homeostatic expansion in the lymphopenic environment of the athymic mouse (66); secondly, $CD25^+$ Tregs are known to regulate the efficiency of this homeostatic proliferation of polyclonal T cells (3); thirdly, the T-cell repertoire has been shown to be highly perturbed following expansion of T-cell populations transferred into lymphopenic hosts (28). The presence of antigen during homeostatic expansion is likely to promote an even more exaggerated expansion of individual clones. Thus, the observed immune responses to an infection in an environment where there is homeostatic expansion of T cells may not be representative of those in a normal individual. A further complication of using athymic mice is the likelihood of partial defects in B-cell development (26). Therefore, our study involving the depletion of $CD25^+$ Tregs in the context of a normal immune repertoire is likely to represent a more robust model for examination of regulation of *H. pylori*-specific immune responses.

How might $CD25^+$ Tregs influence specific antibody production during *Helicobacter* infections? One mechanism may be indirect via the effect on antigen-specific Th1/Th2 responses. However, it is also possible that $CD25^+$ Tregs influence the activation and survival of antigen-specific B cells directly. Regulatory T cells have been shown to inhibit activation-induced blast activity and proliferation of B cells in vitro (11). Our finding of an increased *H. pylori*-specific antibody level in the serum of *Helicobacter*-infected mice following the depletion of $CD25^+$ Tregs, even though antigen-specific proliferative T-cell responses were similar, suggests that $CD25^+$ Tregs may play a direct role in the regulation of B-cell responses in vivo.

B-cell lymphomas are induced by *Helicobacter* infection either in humans (41, 62) or in mouse models after a long-term (22-month) infection with *Helicobacter* (18). It is possible that $CD25^+$ Tregs may control or inhibit the development B-cell lymphomas. The effect of $CD25^+$ Tregs on the development of B-cell lymphomas would require a lengthy infection period of up to 2 years. Strategies need to be developed for removal of $CD25^+$ Tregs in adult mice for prolonged periods so that the consequence of the absence of $CD25^+$ Tregs throughout the entire infection period can be monitored. It should be noted that the removal of $CD25^+$ Tregs early in life influences the establishment of a normal repertoire (30, 31); therefore, prolonged depletion of $CD25^+$ Tregs should commence after the peripheral immune system is established. An approach involving the regulated disruption of the Foxp3 gene may prove useful.

We found that depletion of Tregs did not result in gastric autoimmune disease unless the frequency of gastric-specific T cells was elevated. While the examined cohorts of mice were small, the high incidence of autoimmune gastritis in the anti-CD25-treated 1E4-TCR tg mice suggests that *Helicobacter* infection coupled with depletion of $CD25^+$ Tregs may result in

enhanced autoimmunity in individuals with an increased frequency of gastric-specific T cells.

This study demonstrates a fundamental link between $CD25^+$ regulatory T cells and the maintenance of a Th1 response. Moreover, removal of Tregs led to the appearance of a functional Th2 immune response in animals where a Th1 response was already established. This finding may have important implications for long-term *Helicobacter* disease in that the B cells which ultimately form MALT lymphomas will initially be regulated by Th cells. Our studies show that B-cell activity is increased in the absence of Tregs, and it may be important to establish whether the MALT lymphomas, which appear following chronic *Helicobacter* gastritis, represent a failure by Tregs to control the emergence of a Th2 immune response.

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