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Anti-CD25 Antibody-Mediated Depletion of Effector T Cell Populations Enhances Susceptibility of Mice to Acute but Not Chronic Toxoplasma gondii Infection

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Natural regulatory T cells (Tregs) constitutively express the IL-2R α-chain (CD25) on their surface. Consequently, administration of anti-CD25 Abs is a commonly used technique to deplete Treg populations in vivo. However, activated effector T cells may also transiently express CD25, and are thus also potential targets for anti-CD25 Abs. In this study using Toxoplasma gondii as a model proinflammatory infection, we have examined the capacity of anti-CD25 Abs to target effector T cell populations during an inflammatory episode, to determine to what extent that this action may modulate the outcome of disease. Anti-CD25 Ab-treated C57BL/6 mice displayed significantly reduced CD4⁺ T cell IFN-γ production during acute T. gondii infection and exhibited reduced weight loss and liver pathology during early acute infection; aspects of infection previously associated with effector CD4⁺ T cell responses. In agreement, anti-CD25 Ab administration impaired parasite control and caused mice to succumb to infection during late acute/early chronic stages of infection with elevated tissue parasite burdens. In contrast, anti-CD25 Ab treatment of mice with established chronic infections did not markedly affect brain parasite burdens, suggesting that protective T cell populations do not express CD25 during chronic stages of T. gondii infection. In summary, we have demonstrated that anti-CD25 Abs may directly abrogate effector T cell responses during an inflammatory episode, highlighting important limitations of the use of anti-CD25 Ab administration to examine Treg function during inflammatory settings.


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tural regulatory T cells (Tregs)³ constitutively express high levels of the IL-2R α-chain (CD25) and until recently this marker was the most commonly used to identify these cells. Natural Tregs, which comprise 5–10% of CD4⁺ T cells in naïve mice, have potent regulatory capacity and have been shown to have important roles in a number of autoimmune and infectious diseases (reviewed in Refs. 1, 2). Importantly, however, CD25 is not a specific marker of natural Tregs and it may be expressed by a number of other cell populations, including activated T and B cells, monocytes, and some dendritic cells (reviewed in Ref. 3). Moreover, the identification of the transcription factor Foxp3 as a lineage specific marker for natural Tregs has enabled more accurate differentiation of regulatory and effector CD4⁺ T cell populations, and has shown that populations of CD25⁻ Foxp3⁺ Tregs also exist (4).

Nevertheless, administration of anti-CD25 Abs remains a commonly used method to deplete natural Tregs in vivo. Some studies have obtained results consistent with the depletion of regulatory cell populations, as evidenced by augmented protective immune responses and enhanced pathogen control (for example see Refs. 5–8), whereas other studies have observed no difference in immunity following anti-CD25 administration, possibly due to the disease model used or the strain and immune status of mice in the study (for example see Refs. 9–13). In addition, anti-CD25 Abs are also routinely used in vitro for the positive selection of natural Tregs from naïve and infected mice for the subsequent use in adoptive transfer systems or in vitro suppression assays. In general, these experiments have almost entirely focused on the effects of anti-CD25 Abs on CD4⁺ natural Tregs, even though many cells other than natural Tregs, including effector T cells, could potentially be targeted by anti-CD25 treatment (3). Thus, anti-CD25 treatment in vivo may have effects beyond simply depleting CD25⁺ expressing natural Tregs or affecting their regulatory capability, potentially leading to inaccurate conclusions on the role of natural Tregs in any particular disease setting.

In this study, we use oral Toxoplasma gondii infection as a model of inflammatory disease to directly investigate the effect of anti-CD25 Ab treatment on the effector arm of the immune response. The T. gondii infection model has several useful features for this purpose. During acute infection, which lasts 1–2 wk, strong proinflammatory innate and adaptive responses develop as rapidly proliferating single cell tachyzoites develop from dormant encysted parasites and disseminate from the intestine to liver, lung, brain, and other sites. Cooperation among neutrophils, NK cells, and macrophages is required for the production of IL-12 and IFN-γ, which then play critical protective roles during both acute and chronic infections (14–19). However, in susceptible mouse strains such as C57BL/6 (B6), type I cytokines cause immunopathology in small intestine and liver that is not evident in resistant mouse strains (e.g., BALB/c) (20). Notably, CD4⁺ cells, although

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Abbreviations used in this paper: Treg, regulatory T cell; yFP, yellow fluorescent protein.

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not required for control of parasites in the first week or so of acute infection, are nonetheless important mediators of the immunopathology seen in C57BL/6 mice (20).

Following the development of sufficient adaptive T cell-mediated immune responses, the acute stage of infection is controlled, and tachyzoites are cleared in the intestine, liver, and lungs and parasites revert to a semidormant state within cysts in the brain. In this chronic infection phase, acquired immune mechanisms dependent on CD4+ and CD8+ T cells and B cells, along with IFN-γ and TNF-α are required to control tachyzoite dissemination and to prevent the development of toxoplasmic encephalitis (21–24). Thus, activated effector CD4+ T cells mediate damaging immunopathology early during acute infection, yet contribute to parasite control during late acute/chronic stages of infection and prevent the development of encephalitis during chronic infection.

Our results support the conclusion that anti-CD25 Ab administration directly targets CD25 expressing effector CD4+ T cells during acute T. gondii infection. We show that anti-CD25 Ab administration, while depleting ~40% of Foxp3+ naïve Tregs, also significantly reduces effector CD4+ T cell numbers and diminishes IFN-γ production during acute T. gondii infection, ameliorating early immune-mediated pathology but significantly impairing disease control and elevating mortality during late acute/early chronic stage T. gondii infection. Thus, our results strongly imply that anti-CD25 Abs should be used with caution in vivo during highly proinflammatory disease models, and that the potential effects on all cells that can express CD25 should be considered when they are used to examine the function and importance of natural Tregs.

Materials and Methods

Mice
C57BL/6/J, Thy1.1 C57BL/6 (B6), and bicysticine IFN-γ reporter (Yeti) male mice (25) were used between 6 and 12 wk of age. Mice were bred in the Trudeau Institute Animal Breeding Facility or obtained from The Jackson Laboratory or Taconic Farms. All experiments were reviewed and approved by the Trudeau Institute Institutional Animal Care and Use Committee. The Trudeau Institute is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Parasites and infections
ME49 cysts were obtained from brains of chronically infected B6 mice. Infections were initiated by peroral administration of 10 cysts in 0.1 ml of dilute brain suspension as previously described (26). Sham-infected mice received similarly diluted brain suspension containing no cysts. Weight loss was monitored as a sign of morbidity and is expressed as the percentage of baseline starting weight. Mice were monitored daily for survival.

Ab treatments
Mice were given i.p. injections of 1 mg of anti-CD25 (clone PC61) or a control rat IgG (clone HRPN) obtained from BioXcell (previously Bio-Express). Abs were administered 5–7 days before infection or during infection on days stated in the experiment. PC61 administration promoted long-lasting depletion/neutralization of CD25 expression (longer than 2 wk) in naive mice, consistent with previously published reports (27). Enzyme-linked immunospots (ELISPOT) of clones PC61 and HRPN were below 1 EU/mg.

Flow cytometry
Single cell suspensions were prepared from spleens, liver, blood, and mesenteric lymph nodes. Brains were gently mashed using a Teflon pestle, cell populations separated on a Percoll gradient, and lymphocytes recovered for analysis. Cells were counted using a hemacytometer. The surface Ag phenotype was determined by flow cytometry using the following fluorochrome-conjugated Abs: anti-CD4 (clone GK1.5), anti-CD25 (clone PC61 or clone 7D4), or anti-CD44 (clone IM7). Cells were preincubated with Fc receptor blocking (clone 2.4G2) before staining. All Abs were obtained from BD Biosciences. Foxp3 staining was performed using anti-Foxp3 (FJK-16s) Ab according to the manufacturer’s instructions (eBioscience). It is important to note that fluorochrome-labeled 7D4 was used for flow cytometric detection of CD25 expression in all experiments in which PC61 was administered in vivo to deplete CD25-expressing cells; PC61 does not inhibit 7D4 detection of cell surface CD25 (27).

Isolation of CD4+ CD25− and CD4+ CD25high T cells
CD4+ T cells were purified from the spleens and brains of C57BL/6 mice using anti-CD4 MidiMac beads according to the manufacturer’s instructions (Miltenyi Biotec). Following separation, CD4+ T cells were stained with anti-CD4 (GK1.5) and anti-CD25 (PC61) and sorted into CD4+ CD25− and CD4+ CD25high populations using a FACSVantage cell sorter. The sorted cell populations were routinely >99% pure.

In vitro restimulation experiments
CD4+ CD25high and CD4+ CD25− cells, obtained as described, were cultured individually or at a ratio of 1:1. Cells were either stimulated with naive APCs and anti-CD3 Ab (1 µg/ml) or with T. gondii-pulsed APC (without anti-CD3 Ab costimulation). APCs were prepared by gamma irradiation (3000 rad) of splenocyte cultures from naive mice as indicated. Irradiated tachyzoites and APC were cultured for 12 h at 5% CO2 and 37°C before being washed and added at required cell concentration to wells including purified CD4+ cells. The cells were then incubated at 5% CO2 and 37°C for 60 h. Supernatant wells were then harvested to assay cytokine production and proliferation was determined by pulsing cells with 0.2 µCi 3H-thymidine (Amersham Biosciences) for an extra 12 h. [3H]-Thymidine incorporation and proliferation was measured using a beta counter.

ELISA and real-time PCR
Serum IFN-γ levels were measured by ELISA as previously described (18). Tachyzoites were quantified by a real-time PCR-based method (28) based on the expression of the tachyzoite-limited Sag1 gene (29). RNA was isolated from tissues, cDNA prepared, amplified, and quantitated using the following primer to probe sets: T. gondii Sag1 (forward) TTTCGGAAGACGTGAGAGCAG (reverse) GGATCCGATGCCATAGCG, probe TTGCGGCGCACACTGTAGT; IFN-γ (forward) CAGTGAGACG, (reverse) GGATCCGATGCCATAGCG, probe TTGCGGCGCACACTGTAGT; IL-10 (forward) AGAGAC GGCAGAGATGG (reverse) ACCGCTCCACTGCTTCTTCC, probe TGGAGCGCTGATCATGTTCTTCCC. Foxp3 (forward) CCCAGGA AAGACAAGACACCTT, (reverse) CTCTCACAACGGCCACTTGTG, probe ATCTACACTCCCTGGCAGAATGACTGAGC, and GAPDH (forward) CTGTCCCGTGATACAAATGTG, (reverse) AATTCCTCACTT GCACTGCA, probe GGAGTGTGGGCTAATTGCCC. Values were normalized to GAPDH expression. IFN-γ message levels in brain are shown as fold change (log10) relative to control mAb-treated infected mice. Tachyzoite numbers were calculated relative to a standard curve generated by inoculating known numbers of tachyzoites into uninfecte d liver samples.

Histological exam
On day 8 postinfection, livers and ileums from anti-CD25 Ab-treated mice and control Ab-treated mice were removed and processed for H&E staining. Liver and ileal lesions were semiquantitatively scored based on the following scoring systems (28, 30) as follows: liver damage 1, degeneration and necrosis of individual hepatocytes occasionally seen; 2, clusters or small aggregates of hepatocyte degeneration and necrosis; 3, medium to large aggregates of hepatocyte necrosis; 4, large areas of hepatocyte necrosis with loss of normal liver anatomic architecture; intensity of the inflammatory response: 1, small inflammatory infiltrates with a few inflammatory cells; 2, medium-sized inflammatory infiltrates with a small to moderate number of inflammatory cells; 3, large inflammatory infiltrates with a moderate to large number of inflammatory cells; 4, extensive infiltration with a large number of inflammatory cells. The ileal lesions were scored for the degeneration and necrosis of villous epithelial cells, transmural inflammation, and lamina propria and crypt inflammation and an integer score between 0 (unremarkable) and 4 (severe) was assigned.

Statistics
Unless otherwise noted, group means were compared by Student’s t test. Intergroup survivals were compared by log-rank test. All statistical analyses were performed using GraphPad Prism software.


Results

Anti-CD25 Ab increases susceptibility of mice to acute T. gondii infection

The administration of anti-CD25 Ab (clone PC61) significantly increased the susceptibility of mice to acute oral T. gondii infection (Fig. 1). The majority of C57BL/6 mice treated with PC61 succumbed to infection by day 20 of infection, and this correlated with a biphasic modulation of weight loss (Fig. 1A). PC61 pretreated mice exhibited reduced weight loss during early acute T. gondii infection, with significant differences compared with control Ab treated mice on days 6–8 of infection (p < 0.01). However, in contrast to control Ab-treated mice that survived acute infection and recovered weight, PC61-treated mice were unable to resolve the acute infection and exhibited significantly elevated weight loss during mid to late acute infection (p < 0.05) (Fig. 1A). Surviving PC61 pretreated mice also harbored significantly increased brain cyst numbers compared with control mice on day 30 postinfection (p < 0.033) (Fig. 1C). Anti-CD25 Abs modulate development of immune-mediated pathology and significantly impair parasite control during acute T. gondii infection

As anti-CD25 Ab administration elevated mortality, modulated weight loss, and increased brain cyst numbers, we next examined whether anti-CD25 Ab administration impeded tachyzoite control or limited acute stage immunopathology. In agreement with this hypothesis, we observed significantly reduced liver pathology in anti-CD25 Ab-treated mice compared with control Ab-treated mice on day 8 of infection, with ameliorated inflammatory cell infiltration (predominantly mononuclear cells and neutrophils) throughout the parenchymal and portal areas and significantly reduced levels of hepatic coagulation necrosis (Fig. 1D and Table I). In addition, moderately severe perivascularitis, vasculitis, and fibrinuous thrombosis was observed in medium to large blood vessels of control Ab-treated mice, which was relatively absent in anti-CD25 Ab-treated mice (Fig. 1D). Nevertheless, parasite control in the liver was relatively unaffected by anti-CD25 Ab treatment, as comparable tachyzoite numbers were observed in control Ab- and PC61-treated mice on day 8 postinfection (Fig. 1E), indicating that parasite control in the liver during early acute T. gondii infection

FIGURE 1. Anti-CD25 Ab administration significantly modulates the outcome of acute T. gondii infection. Groups of five B6 males were given 1 mg of control mAb (clone HRPN) or anti-CD25 (clone PC61) 5 or 7 days before oral infection with 10 ME49 cysts. A, Changes in body weight were monitored and are presented as a percentage change relative to starting weight. Data show mean ± SD. *p < 0.05 for significant difference. B, Compiled survival data from three experiments comparing anti-CD25-treated with control T. gondii-infected B6 male mice. Survivals differed significantly (p < 0.01) by log-rank test. C, Brain cysts of three anti-CD25-treated B6 male mice, and five controls 28 days after mice were sham-infected or infected with 10 ME49 cysts. Horizontal bar denotes median values. D, On day 8 postinfection, livers and ileums were removed and stained with H&E for histological examination of tissue pathology with arrows marking areas of inflammation or necrosis (also marked by an asterisk). E and F, On day 8 postinfection, livers (E) and ileums (F) were removed for real-time PCR (Taqman) quantification of tissue parasite burdens. Results shown are mean ± SD of the group (n = 5) and are representative of two independent experiments. **, p < 0.01.
is CD25-independent. In contrast, significantly elevated pathology was observed in the ileum of anti-CD25 Ab-treated mice compared with control Ab-treated mice on day 8 postinfection; extensive infiltration of a moderate number of mononuclear cells was observed in the lamina propria of anti-CD25 Ab-treated mice, which was absent in control Ab-treated mice (Fig. 1D and Table I). Elevated pathology was directly correlated with increased parasite burdens in the ileum of anti-CD25 Ab-treated mice on day 8 postinfection (Fig. 1F), suggesting that the failure of parasite control, rather than partial depletion or inactivation of natural Tregs following anti-CD25 Ab administration, was responsible for the increased pathology evident in the ileum. Combined, these data highlight the pathological role of CD25-expressing effector cells in mediating hepatic pathology during early acute T. gondii infection and demonstrate the importance of CD25-expressing effector cells for control of T. gondii parasites in the ileum during the acute stage of infection.

CD25 is transiently up-regulated on CD4 T cells during acute T. gondii infection

To examine the kinetics of CD25 expression during T. gondii infection, to establish when it is up-regulated and on which cells, we determined the expression level of CD25 on CD4 T cells expressing high levels of CD25. A similar transient up-regulation of CD25 on splenic CD8 T cells was also observed between days 4 and 12 of infection, to establish when it is up-regulated and on which cells. Thus, to investigate the ability of CD4 CD25− T cells to up-regulate CD25 during acute T. gondii infection could potentially have been due to either the proliferation of CD4 CD25high Tregs (present in naive mice) or due to the up-regulation of CD25 on activated precursor CD25− naive CD4 T cells. To study the ability of CD4 CD25− T cells to up-regulate CD25 during acute T. gondii infection we purified and adoptively transferred Thy1.1+ CD4 CD25− cells into congenic Thy1.2+ mice before oral T. gondii infection. Importantl, significant up-regulation of CD25 expression was observed on the CD4 CD25− donor T cell population (Fig. 2B). Moreover, the kinetics (and magnitude) of CD25 up-regulation on the donor CD4+ T cells mirrored the up-regulation of CD25 in the total CD4+ T cell population in wild-type mice (Fig. 2A), indicating that CD4+ CD25+ cells are the primary precursor cells of the CD4+ CD25high cells generated during acute T. gondii infection.

Although there were significantly more CD4+ CD25high spleen cells in infected mice than in sham-infected mice on day 8 postinfection, there was no significant difference in the number of total CD4+ Foxp3+ cells, or the number of CD4+ Foxp3+ cells expressing CD25 (Fig. 2C), suggesting that the majority of CD4+ CD25high T cells were not Tregs, as shown from day 8 of infection.
versely, CD4⁺CD25ᵢₖₑₑₚ cells derived from acutely infected mice failed to suppress CD4⁺CD25⁻ cell responses, produced significant quantities of IFN-γ and proliferated rapidly following mitogenic and T. gondii antigenic challenge (Fig. 3, C–F). Importantly, CD4⁺CD25ᵢₖₑₑₚ T cells derived from acutely infected mice produced more IFN-γ and proliferated more rapidly than infection-derived CD4⁺CD25⁻ cells following specific T. gondii stimulation (Fig. 3, E and F), suggesting that during acute T. gondii infection, the majority of parasite-specific effector CD4⁺ T cells are found within the CD4⁺CD25ᵢₖₑₑₚ T cell population.

Reduced numbers of effector T cells and suppressed IFN-γ production in anti-CD25 Ab-treated T. gondii-infected mice

Numerous studies have shown that anti-CD25 Ab (clone PC61) treatment leaves mice essentially devoid of cells expressing CD25 for an extended period (27, 32), an observation we confirm in this study during T. gondii infection (Fig. 4); CD25 expressing splenic CD4⁺ T cells were almost completely undetectable following anti-CD25 Ab administration (detection day 9 postinfection with Ab injected i.p. 4 days before infection) (Fig. 4A). These results were largely reproduced when examining mesenteric lymph node cells (data not shown). Importantly, and consistent with previous reports (27, 32), PC61 administration failed to induce complete depletion of Foxp3⁺ Tregs, instead promoting a maximal 40% reduction in Foxp3-expressing cell numbers (Fig. 4, B and C). As we have demonstrated that during acute stage T. gondii infection the overwhelming majority of CD4CD25ᵢₖₑₑₚ cells display effector rather than regulatory characteristics, these results directly indicate that anti-CD25 Abs primarily deplete effector T cells during T. gondii infection. In agreement, anti-CD25 Abs principally targeted activated T cell populations, as shown by the significant decrease in the number of CD4⁺ T cells expressing high levels of the activation marker CD44 following PC61 administration (28.9 ± 11.6 × 10⁶ in control mAb-treated vs 13.3 ± 4.5 × 10⁶ in anti-CD25-treated) (Fig. 4A).

Consequently, to examine whether the depressed effector T cell responses observed during T. gondii infection following anti-CD25 Ab administration correlated with a functional defect in proinflammatory immune responses, we next determined the levels of the prototypic type I cytokine IFN-γ in T. gondii-infected mice treated with anti-CD25 Abs. Our results show that PC61 administration reduced systemic plasma IFN-γ levels (detected on day 8 postinfection) compared with control Ab-treated infected mice (Fig. 4D). Although the reduction in IFN-γ production failed to reach statistical significance in any individual experiments, a reproducible reduction in IFN-γ production was observed in four separate experiments, with the percentage of reductions relative to controls of 11, 20, 27, and 52%. Furthermore, the reduced serum IFN-γ levels were paralleled by significantly reduced levels of IFN-γ mRNA in the liver of anti-CD25 Ab-treated mice on day 8 postinfection (Fig. 4E).

CD4⁺CD25ᵢₖₑₑₚ T cells are primarily effector T cells during acute T. gondii infection

It is well established that CD4⁺CD25ᵢₖₑₑₚ T cells derived from naive mice express high levels of Foxp3 and exhibit potent regulatory capabilities. In contrast, extremely strong systemic proinflammatory immune responses develop during T. gondii infection and the majority of CD4⁺CD25ᵢₖₑₑₚ cells do not express Foxp3 (Fig. 2), indicating that these cells may exert effector rather than regulatory functions. To address this likelihood, we purified CD4⁺ CD25⁻ and CD4⁺CD25ᵢₖₑₑₚ cells from uninfected mice and mice acutely infected with T. gondii and assessed the ability of these cells to respond to mitogenic (anti-CD3) and T. gondii Ag-specific stimulation. As expected, CD4⁺CD25ᵢₖₑₑₚ cells derived from uninfected mice closely matched the functional characteristics of natural Tregs; they failed to produce IFN-γ or proliferate following mitogenic stimulation and suppressed proliferation and IFN-γ production by CD4⁺CD25⁻ cells (p < 0.05) (Fig. 3, A and B). Consequently, to examine whether the depressed effector T cell responses observed during T. gondii infection following anti-CD25 Ab administration correlated with a functional defect in proinflammatory immune responses, we next determined the levels of the prototypic type I cytokine IFN-γ in T. gondii-infected mice treated with anti-CD25 Abs. Our results show that PC61 administration reduced systemic plasma IFN-γ levels (detected on day 8 postinfection) compared with control Ab-treated infected mice (Fig. 4D). Although the reduction in IFN-γ production failed to reach statistical significance in any individual experiments, a reproducible reduction in IFN-γ production was observed in four separate experiments, with the percentage of reductions relative to controls of 11, 20, 27, and 52%. Furthermore, the reduced serum IFN-γ levels were paralleled by significantly reduced levels of IFN-γ mRNA in the liver of anti-CD25 Ab-treated mice on day 8 postinfection (Fig. 4E).
Anti-CD25 Ab administration suppresses IFN-γ production by CD4+ T cells

To examine the T cell populations affected by anti-CD25 Ab administration leading to impaired IFN-γ expression, we used IFN-γ-bispecific reporter mice (YETI) (25), enabling the detection and quantification of cell-specific IFN-γ production directly ex vivo, negating the requirement for subsequent restimulation in vitro. Importantly, yellow fluorescent protein (yFP) expression in YETI mice not only marks cells actively producing IFN-γ, but also marks cells that have expressed IFN-γ, providing an accurate representation of both current and historical cellular function (25). As expected, the majority of CD4+ T cells in naive mice were found to be yFP-negative (25), indicating that few CD4+ T cells were polarized type 1 effector/memory cells (Fig. 5A). The CD4+ yFP-positive cells found in liver, spleen, blood, and mesenteric lymph nodes in naive mice displayed heterogeneity in CD25 expression, suggesting the existence of a number of different populations of IFN-γ-producing CD4+ T cells at various stages of cellular activation. All yFP-positive cells irrespective of CD25 expression or tissue/organ location were, however, uniformly CD44hi (results not shown).

In agreement with Fig. 2, up-regulation of CD25 on CD4+ T cells was observed on day 8 postinfection in all tissues examined and this response correlated with significant up-regulation of yFP expression; importantly in all the tissues examined the majority of yFP-expressing CD4+ T cells coexpressed CD25 (Fig. 5A). Thus, virtually all CD4+CD25hi cells derived from liver and blood were yFP-positive, marking CD4CD25hi cells as predominantly effector rather than Tregs in these tissues during acute infection. In contrast, a number of CD4+CD25hi cells derived from spleen and mesenteric lymph nodes of T. gondii-infected mice did not coexpress yFP, suggesting that these cells are either Tregs or activated T cells that have failed to produce IFN-γ (Fig. 5A). Irrespective of this finding, our results in Fig. 3 clearly show that splenic CD4+CD25hi cells predominantly exert effector rather than regulatory functionality during acute stage T. gondii infection.

In support of our hypothesis that anti-CD25 Ab administration directly suppresses proinflammatory protective immune responses during acute T. gondii infection, we observed significantly reduced frequency of yFP-positive CD4+ T cells in the liver, mesenteric lymph node, blood, and spleen of T. gondii-infected mice treated with anti-CD25 Abs as compared with control Ab-treated infected mice (Fig. 5B). Similarly, significantly reduced frequency of yFP-positive CD8+ T cells were observed in the liver of T. gondii-infected mice treated with anti-CD25 Abs as compared with control Ab-treated infected mice (Fig. 5B). Similarly, significantly reduced frequency of yFP-positive CD8+ T cells were observed in the liver of T. gondii-infected mice treated with anti-CD25 Abs as compared with control Ab-treated infected mice (results not shown). In contrast anti-CD25 Ab administration did not significantly reduce yFP expression by CD8+ T cells in the spleen, mesenteric lymph node, or blood, suggesting that effector CD8+ T cells are less affected by anti-CD25 Ab than CD4+ T cells (results not shown).

Heterogeneous expression of yFP by CD4+ T cells occurs during T. gondii infection, as shown in infected, isotype control group (Fig. 5) and as published in Mayer et al. (34). Importantly, the expression level of yFP is directly correlated to the capacity to produce IFN-γ following in vitro restimulation, with cells expressing highest levels of yFP capable of producing most IFN-γ protein (34). Interestingly, the highest level of yFP expression was routinely observed in CD4+ T cells expressing highest levels of CD25 (Fig. 5A), marking these potent IFN-γ-producing cells as principal targets of anti-CD25 Abs. Thus, anti-CD25 Ab administration
significantly reduced the yFP<sup>bright</sup> CD4<sup>+</sup> T cell subsets in the liver and blood. A reduction in the frequency of yFP<sup>bright</sup> CD4<sup>+</sup> T cells was also observed in the mesenteric lymph node and spleen, but the reduction failed to reach statistical significance (results not shown). Combined, these results demonstrate that anti-CD25 Abs can directly target effector IFN-γ-producing CD4<sup>+</sup> T cells during acute <i>T. gondii</i> infection leading to systemic reductions in IFN-γ production and impaired protective T cell immunity.

**Anti-CD25 Ab administration does not impair resistance to chronic stage <i>T. gondii</i> infection**

It is well established that anti-parasitic CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells are required for the control of parasite reactivation in the brain and prevention of <i>Toxoplasma</i> encephalitis during chronic <i>T. gondii</i> infection (22). However, the activation status of these CD4<sup>+</sup> and CD8<sup>+</sup> T cells is relatively unknown. Consequently it is unclear whether protective CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen or brain express CD25 during chronic infection, and whether these cells may also be affected by anti-CD25 Ab administration. Additionally, it has previously been shown that natural Tregs that co-express Foxp3 and CD25 can suppress protective immunity during a number of infections, including <i>Leishmania major</i> infection, enabling parasite persistence and the establishment of chronic infections (35, 36). Thus, CD25-expressing CD4<sup>+</sup> T cells may also play an important regulatory role during chronic <i>T. gondii</i> infection, lowering proinflammatory responses, moderating immune-mediated pathology but impairing parasite control. As a result, anti-CD25 Ab administration during chronic <i>T. gondii</i> infection could potentially augment anti-parasitic immune responses and enhance parasite control. In support of this hypothesis, Foxp3 mRNA levels in the brain were increased during chronic infection compared with uninfected control levels (results not shown). Moreover, we have shown that CD25 up-regulation on effector T cells is transient during <i>T. gondii</i> infection and occurs primarily during the acute phase.

**FIGURE 5.** Anti CD25 Ab administration suppresses IFN-γ production by CD4<sup>+</sup> T cells. Groups of three bicistronic IFN-γ-enhanced yFP (IFN-γ-yFP) reporter male mice (Yeti) were given 1 mg of anti-CD25 (clone PC61) or control mAb (clone HRPN) i.p. 5 days before oral infection with brain homogenate containing 10 ME49 cysts or sham uninfected brain homogenate. A, Representative dot plots showing yFP expression relative to CD25 expression (using 7D4 to detect) on CD4<sup>+</sup> T cells from spleen, mesenteric lymph, liver, and blood on day 8 postinfection. B, Relative frequency of CD4<sup>+</sup> T cells expressing yFP in anti-CD25 Ab-treated and control Ab-treated <i>T. gondii</i>-infected and sham-infected mice on day 8 postinfection. Results shown are mean ± SD of the group (<i>n</i> = 3) and are representative of two independent experiments. *, <i>p</i> < 0.05.
of infection, suggesting that CD4+CD25high cells are functionally disparate during acute and chronic stages of *T. gondii* infection (Fig. 2). In agreement with a regulatory rather than effector role of CD25-expressing cells during chronic infection, CD4+CD25high cells isolated from both brain and spleen during chronic infection failed to produce significant quantities of IFN-γ following *T. gondii* antigenic restimulation (as compared with CD4+CD25− cells) and splenic CD4+CD25high cells moderately suppressed IFN-γ production by CD4+CD25− cells (Fig. 6, A and B).

To directly determine the relative importance of CD25-expressing natural Tregs during chronic *T. gondii* infection, we administered anti-CD25 Abs to chronically infected mice (day 56 postinfection) and determined whether Ab administration significantly modulated disease control. Anti-CD25 Ab administration effectively neutralized CD25 expression in the spleen (results not shown) and brain (Fig. 6C) 5 and 10 days postadministration (days 5 and 10 postadministration were examined in separate experiments with results from day 5 postadministration shown). However, Foxp3 levels were not significantly reduced in anti-CD25 Ab-treated mice (Fig. 6C). Importantly, it has previously been shown that Foxp3+ cells that survive anti-CD25 Ab treatment are functionally inactive (32), indicating that anti-CD25 Ab administration should still ablate Treg functionality during chronic *T. gondii* infection even if all Foxp3+ T cells are not eliminated. Nonetheless, Ab administration did not significantly alter the level of weight loss (relative to preantibody administration baseline levels) or mortality over a 30-day period posttreatment (Fig. 6, D and E) and this correlated with unaltered parasite control (measuring invasive tachyzoite forms of parasite) and IFN-γ production in the brain of anti-CD25 Ab-treated mice compared with control treated groups (Fig. 6F). Combined these results demonstrate that CD25-expressing natural Tregs do not suppress anti-parasitic immune responses during chronic *T. gondii* infection and are not required to limit excessive inflammation. In support of this conclusion, the frequency of CD4+Foxp3+ cells in the brain (of total CD4+ T cells) was not elevated in chronically infected mice compared with naive mice. Thus, the increase in brain Foxp3 mRNA expression during chronic infection is due to the increase in total CD4+ T cells that migrate to and reside in brains of chronically infected mice, rather than specific localization and migration of Tregs to the site of infection, as is observed during *L. major* infection (35).

### Discussion

In this study, we demonstrate that during acute *T. gondii* infection transient up-regulation of CD25 expression occurs on CD4+ T cells during the expansion phase of the T cell response. Importantly, the vast majority of CD4+CD25high cells generated during acute *T. gondii* infection do not coexpress Foxp3, clearly defining the cells as effector cells rather than Tregs. Anti-CD25 Abs directly target these CD25-expressing effector CD4+ T cell populations leading to impaired proinflammatory immune responses, exemplified by significantly reduced T cell-derived IFN-γ production. Thus anti-CD25 Ab administration initially reduced the severity of T cell-dependent immune-mediated pathology during early acute *T. gondii* infection, but led to the failure of parasite control and ultimately death in a large number of anti-CD25 Ab-treated mice. In contrast, anti-CD25 Ab administration did not affect parasite control during chronic *T. gondii* infection, suggesting that CD25-expressing T cells do not significantly regulate anti-parasitic immunity during the later chronic stage of *T. gondii* infection.

The results observed in this study are entirely consistent with a direct effect of anti-CD25 Ab administration on proinflammatory effector T cell populations. Thus, although (as expected) anti-CD25 Ab administration reduced the number of Foxp3+ natural Tregs, it also reduced circulating IFN-γ levels (in all four experiments examined) and resulted in significantly reduced IFN-γ mRNA levels in the liver. Using IFN-γ-YFP reporter (YETI) mice, we subsequently showed that anti-CD25 Ab administration leads to reduced IFN-γ production by CD4+ T cells in the liver, spleen, blood, and mesenteric lymph node. Consequently our results differ from those obtained by Lund et al. (37), where specific natural Treg depletion reduced localized proinflammatory immune responses at the mucosal site of HSV infection, but enhanced proinflammatory immune responses in tissue draining lymph nodes. Therefore, whereas in their model, the specific depletion of natural Tregs (performed in Foxp3 diphtheria toxin receptor transgenic mice) inhibited effector T cell migration to nonlymphoid tissue sites of infection, we show that anti-CD25 Ab administration promoted
systemic suppression of effector T cell responses. Combined, these data demonstrate the lack of specificity of anti-CD25 Abs to target natural Tregs compared with the more recently generated Foxp3-dt system (38).

It has previously been demonstrated that effector CD4+ T cells are responsible for the onset of immune-mediated pathology and severe weight loss in B6 mice following oral T. gondii infection. (20, 31, 39, 40). Consistent with this demonstration, anti-CD25 Ab administration modulated body weight loss during acute T. gondii infection and significantly reduced the severity of liver pathology on day 8 of infection, indicating a direct effect on effector T cell populations. Nevertheless, it has also previously been reported that Th1 CD4+ T cells that coproduce IFN-γ are the primary source of host-protective IL-10 during T. gondii infection (33). Consequently, given the effect of anti-CD25 Ab administration on CD4+ T cell-derived IFN-γ production, it was foreseeable that Ab administration could also have impaired IL-10 production, and that this secondary Ab effect may have contributed to the enhanced susceptibility of anti-CD25 Ab-treated mice during acute T. gondii infection. Our results show that IL-10 mRNA levels were not significantly reduced in the liver following anti-CD25 Ab administration, suggesting that CD25 expression is not critical for the production of host-protective IL-10 during acute T. gondii infection. These results appear to further underline the effects of anti-CD25 Ab administration on effector proinflammatory responses, rather than regulatory based responses during acute T. gondii infection. As we did not specifically examine the cellular source or protein levels of IL-10 additional experiments are, however, required to determine the extent that anti-CD25 Ab treatment alters IL-10 production by Th1 CD4+ T cells during infection. Interestingly, although we highlight the importance of CD25 expression on effector T cells during the acute stage of primary T. gondii infection, administration of anti-CD25 Abs did not affect vaccine-induced protection during secondary challenge infection. Thus, anti-CD25 Ab administration to mice previously infected with the noncyt forming attenuated TS-4 strain of parasites did not inhibit protective immune responses and anti-CD25 Ab- and control-treated vaccinated mice displayed equivalent ability to prevent brain cyst development (results not shown). These results indicate that CD25-expressing effector T cell populations are not critically required for vaccine-induced protection to T. gondii infection.

Despite the pathological role of CD4+ T cells during early acute T. gondii infection, it is well established that effector CD4+ T cells and CD8+ T cells are required for protection and parasite control during late acute and chronic stages of infection (41–43). Consequently, as anti-CD25 Ab treatment reduced the number of activated (CD44(high)) CD4+ spleen cells by more than 50% on day 9 of infection, it is not surprising that anti-CD25 Ab treated mice displayed elevated parasite levels in the ileum on days 8 and 15 postinfection, exhibited more brain cysts on day 30 post infection and succumbed more rapidly to late/acute and chronic stage infection. Nonetheless, we have also demonstrated that anti-CD25 Ab administration during established chronic stage of T. gondii infection did not affect brain-localized parasite control or anti-T. gondii immunity, suggesting that anti-CD25 Abs do not negatively affect protective T cell responses at this stage. In agreement, transient expression of CD25 on effector T cells was observed only during acute T. gondii infection and CD25 expression on splenic CD4+ and CD8+ T cells during chronic stages of infection was comparable to that observed in naive mice. Moreover, brain infiltrating CD4+ T cell populations did not express higher levels of CD25 than splenic CD4+ T cells during chronic T. gondii infection, indicating that CD25 expressing lymphocytes do not preferentially migrate to and reside at the local site of parasite persistence during chronic infection (data not shown). In addition, CD4+CD25(high) cells isolated from the spleen and brain of chronically infected mice displayed regulatory rather than effector phenotypes following in vitro Ag-specific stimulation. Thus, CD25-expressing CD4+ T cells are primarily regulatory rather than effector cells during chronic T. gondii infection and effector CD4+ and CD8+ T cells do not require CD25 expression to mediate parasite control during the later stages of infection. Importantly, these results also demonstrate that natural Tregs do not control anti-parasitic immune responses during chronic T. gondii infection, as administration of anti-CD25 Abs did not augment parasite control. Consequently, it is unlikely that natural Tregs are the source of IL-10 that is required for the limitation of immune-mediated pathology during chronic T. gondii infection (44).

Most studies that use anti-CD25 Ab administration to investigate Tregs assume that the Ab affects only that subset and, for the most part, these studies have disregarded effects on cells other than Tregs that can up-regulate CD25 in response to infection. This decision is surprising because Ag-stimulated effector T cells (and others) up-regulate CD25 and IL-2 drives T cell proliferation in response to Ag stimulation. Notably, anti-CD25 Abs have been used clinically to dampen autoimmunity and reduce rejection of transplants (45), uses that run counter to the notion that anti-CD25 augments immunity. The reason for the pronounced effect of anti-CD25 Ab on effector T cell populations during acute T. gondii infection, but not in other models is unclear, but is likely correlated with the extreme polarized systemic proinflammatory immune response that develops during T. gondii infection. As CD25 is up-regulated on a significant percentage of the CD4+ and CD8+ T cell population during acute T. gondii infection, it is foreseeable that any effect of anti-CD25 Ab treatment on effector T cell populations is more pronounced during acute T. gondii infection, and might not be as apparent in other models.

In conclusion, we have shown that during acute T. gondii infection, anti-CD25 Abs directly target effector CD4+ T cell populations, significantly modulating the proinflammatory immune response to T. gondii infection. Although initially this limits immune-mediated pathology, it also leads to the failure to control parasite dissemination and encystment and finally death. Thus, although highlighting the role of CD25 expressing T cells for the control of acute T. gondii infection, we importantly also demonstrate the significant limitations of using anti-CD25 Abs in vivo during inflammatory diseases to investigate the importance of Tregs. On the basis of our findings, we propose that studies that use anti-CD25 Abs during inflammatory settings should take note of the effects on the entire T cell population, not solely those expressing markers associated with Tregs.

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Disclosures

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References

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