

## Systemic CTLA-4 Blockade Ameliorates Glioma-Induced Changes to the CD4<sup>+</sup> T Cell Compartment without Affecting Regulatory T-Cell Function

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**Abstract Purpose:** Patients with malignant glioma suffer global compromise of their cellular immunity, characterized by dramatic reductions in CD4<sup>+</sup> T cell numbers and function. We have previously shown that increased regulatory T cell (T<sub>reg</sub>) fractions in these patients explain T-cell functional deficits. Our murine glioma model recapitulates these findings. Here, we investigate the effects of systemic CTLA-4 blockade in this model.

**Experimental Design:** A monoclonal antibody (9H10) to CTLA-4 was employed against well-established glioma. Survival and risks for experimental allergic encephalomyelitis were assessed, as were CD4<sup>+</sup> T cell numbers and function in the peripheral blood, spleen, and cervical lymph nodes. The specific capacities for anti-CTLA-4 to modify the functions of regulatory versus CD4<sup>+</sup>CD25<sup>-</sup> responder T cells were evaluated.

**Results:** CTLA-4 blockade confers long-term survival in 80% of treated mice, without eliciting experimental allergic encephalomyelitis. Changes to the CD4 compartment were reversed, as anti-CTLA-4 reestablishes normal CD4 counts and abrogates increases in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>GITR<sup>+</sup> regulatory T cell fraction observed in tumor-bearing mice. CD4<sup>+</sup> T-cell proliferative capacity is restored and the cervical lymph node antitumor response is enhanced. Treatment benefits are bestowed exclusively on the CD4<sup>+</sup>CD25<sup>-</sup> T cell population and not T<sub>regs</sub>, as CD4<sup>+</sup>CD25<sup>-</sup> T cells from treated mice show improved proliferative responses and resistance to T<sub>reg</sub>-mediated suppression, whereas T<sub>regs</sub> from the same mice remain anergic and exhibit no restriction of their suppressive capacity.

**Conclusions:** CTLA-4 blockade is a rational means of reversing glioma-induced changes to the CD4 compartment and enhancing antitumor immunity. These benefits were attained through the conferment of resistance to T<sub>reg</sub>-mediated suppression, and not through direct effects on T<sub>regs</sub>.

Despite being restricted to the intracranial compartment, malignant gliomas (MG) elicit defects in host systemic cellular immune responses that are notably severe (1). These defects are characterized by dramatic reductions in both CD4<sup>+</sup> T cell numbers and function. Accordingly, patients with MG suffer

severe CD4 lymphopenia (2, 3), with substantial T cell anergy characterizing the remaining CD4 compartment (4, 5). We have recently shown that a disproportionate presence of regulatory T cells (T<sub>regs</sub>) amid CD4<sup>+</sup> T cells provides an explanation for diminished T-cell function in these patients (2). Furthermore, our murine model of intracranial MG recapitulates these findings. Specifically, tumor-bearing mice show dramatically reduced CD4 counts in the peripheral blood, spleen, and cervical lymph nodes (CLN), as well as increased T<sub>reg</sub> fractions and reduced T cell responsiveness (6).

Here, we investigate the effects of systemic CTLA-4 blockade in this model. CTLA-4, a homologue of CD28, is expressed on the surface of activated T lymphocytes (7, 8) and has been shown to competitively bind both B7-1 and B7-2 with affinities 50 to 2,000 times greater than those of CD28 (9, 10). Interaction between CTLA-4 on activated T cells and B7-1 and B7-2 on the surface of antigen-presenting cells promotes the inhibition of costimulatory T cell signaling pathways, thereby decreasing T cell responsiveness (11). Conversely, blockade of CTLA-4 results in enhanced T cell responses and has shown therapeutic efficacy when directed against a number of peripherally located tumors (12–18). As an alternative mechanism, CTLA-4 is also constitutively expressed on T<sub>regs</sub> (19) and may play a role in their function. Therefore, CTLA-4 blockade may

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**Note:** P.E. Fecci and H. Ochiai contributed equally to this work.

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lead to enhanced T cell responses by either directly or indirectly reducing  $T_{reg}$ -suppressive capacities (20).

In this study, we employ CTLA-4 blockade against gliomas that are well-established. Despite this, long-term survival is elicited in up to 80% of treated mice, without the concurrent emergence of experimental allergic encephalomyelitis (EAE). Significantly, both CD4 lymphopenia and T-cell functional defects are reversed. More specifically, CTLA-4 blockade reestablishes normal CD4 counts in mice bearing gliomas and abrogates the increases in  $T_{reg}$  fraction elicited by tumor, without decreasing absolute  $T_{reg}$  numbers. Furthermore, CTLA-4 blockade restores CD4<sup>+</sup> T-cell proliferative capacity and enhances the *in vitro* antitumor response of CLN cells. The benefits of treatment seem to be bestowed exclusively on the CD4<sup>+</sup>CD25<sup>-</sup> T cell population and not  $T_{regs}$ , as CD4<sup>+</sup>CD25<sup>-</sup> T cells taken from treated mice (whether tumor-bearing or tumor-naïve) show both improved proliferative responses and  $T_{reg}$  resistance, whereas  $T_{regs}$  from the same mice remain anergic *in vitro* and exhibit no restriction of their suppressive capacity.

## Materials and Methods

**CTLA-4 antibody and administration.** The anti-CTLA-4-secreting hybridoma (clone 9H10) was provided by Dr. J. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY). To effect systemic CTLA-4 blockade, mice were loaded i.p. with a single 100 µg dose of sterile 9H10 in 500 µL of PBS (Gibco, Grand Island, NY). Two maintenance doses were delivered at 3-day intervals and consisted of 50 µg of 9H10 in 250 µL of PBS. All animal studies were approved by the Institutional Animal Care and Use Committee.

**SMA-560 glioma and intracranial injection.** This has been reported previously (21–23). Briefly, the spontaneous murine astrocytoma cell line 560 (SMA-560) was derived from a spontaneous intracerebral malignant glioma arising in inbred, immunocompetent VM/Dk (H2K<sup>b</sup>/Ia<sup>b</sup>) mice (22, 24). For injection purposes, SMA-560 cells were grown *in vitro* in antibiotic-free zinc option medium (Life Technologies, Inc., Bethesda, MD) containing 10% fetal bovine serum (Gibco). Cells were harvested in logarithmic growth phase. Tumor cells in PBS were then mixed 1:1 with 3% methylcellulose and loaded into a 250 µL syringe (Hamilton, Reno, NV). The needle was positioned 2 mm to the right of the bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments, Tujunga, CA). Cells ( $5 \times 10^3$ ) were delivered in a total volume of 5 µL.

**EAE evaluation.** Mice were checked daily for signs of EAE. The clinical severity of EAE was graded into six categories: grade 0, no sign; grade 1, tail paralysis; grade 2, mild hind limb weakness; grade 3, moderate to severe hind limb paresis and/or mild forelimb weakness; grade 4, complete hind limb paralysis and/or moderate to severe forelimb weakness; grade 5, quadriplegia or moribund; grade 6, death. Brains and spinal cords were sectioned and stained with H&E and luxol fast blue. Slides were evaluated for evidence of lymphocytic infiltrates and demyelination.

**Immunohistochemistry.** Immunohistochemistry was done by the Duke Immunopathology core facility to evaluate for the presence of tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes. Briefly, tumor slides were deparaffinized by immersion in xylene. Slides were rehydrated in a graded ethanol series and trypsinized. Samples were microwaved in citrate buffer and endogenous peroxidase activity quenched with 3% H<sub>2</sub>O<sub>2</sub> in methanol. Slides were washed, blocked, and incubated with primary antibodies to CD4 (Santa Cruz Biotechnology, Santa Cruz, CA) and CD8 (DAKO, Carpinteria, CA). Samples were then incubated in sequence with biotinylated secondary antibodies, avidin-peroxidase complex, and peroxidase substrate solution prior to being stained with hematoxylin and examined microscopically.

**Flow cytometry.** Propidium iodide solution and antibodies to CD3e (145-2C11), CD4 (L3T4), CD16/32 (2.4G2) CD25 (PC61), Annexin V, and CTLA-4 (UC10-4F10-11), as well as appropriate isotype controls were obtained from BD PharMingen (San Diego, CA). Anti-(α)-GITR (108619) was obtained from R&D Systems (Minneapolis, MN), and α-Foxp3 (FJK-16s) was obtained from eBioscience (San Diego, CA). Cells were incubated first with antibodies against surface markers for 30 min at 4°C in the dark. For intracellular staining, cells were then fixed and permeabilized using 1× Fix/Perm solution (eBioscience) according to the instructions of the manufacturer. Following the incubation period, cells were washed in 1× permeabilization buffer (eBioscience), Fc-blocked with α-CD16/32, and stained with α-Foxp3 ± CTLA-4 for 30 min at 4°C in the dark. Cells were then washed, resuspended in 1× PBS + 2% FCS + 1% buffered neutral formalin and analyzed on a FACS Vantage SE or LSRII flow cytometer (BD Biosciences, San Diego, CA). Data analysis was conducted with BD FloJo software.

**Isolation of T cells and  $T_{regs}$ .** Spleens and CLN were harvested, minced, and pushed through 70-µm cell screens to create single cell suspensions. RBC lysis was done as needed with ammonium chloride. Cells were spun, counted, and resuspended in PBS + 2 mmol/L of EDTA + 0.5% bovine serum albumin at  $10^7$  cells/40 µL. Miltenyi mouse CD4<sup>+</sup> isolation kit (Miltenyi Biotec, Auburn, CA) was used to isolate CD4<sup>+</sup> cells, without engaging the CD4 molecule, according to the instructions of the manufacturer. Briefly, a biotinylated antibody cocktail specific for non-CD4<sup>+</sup> cells (CD8a, CD11b, CD45R, DX5, and Ter119) was added at  $10 \mu\text{L}/10^7$  cells. Samples were incubated and then mixed with antibiotin microbeads. Samples were reincubated, washed, and run over AUTOMACS (Miltenyi) set to program DEplete. The nonlabeled fraction (CD4<sup>+</sup>) was counted, labeled with antigen-presenting cells α-CD25 and PE-α-GITR, and sorted into CD25<sup>+</sup>GITR<sup>+</sup> and CD25<sup>-</sup> populations on a FACS Vantage SE flow cytometer (BD Biosciences). The purity of each population was always >98% to 99%.

**T cell cultures.** Cells were cultured in triplicate in 96-well plates and stimulated with either soluble or plate-bound α-CD3e antibody (145-2C11; BD PharMingen) at 2 µg/mL or with 5,000, 10,000, or 50,000 irradiated SMA-560 tumor cells as stimulator as indicated. For proliferation and suppression assays, CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> cells were purified as above from naïve VM/Dk mice. Cells from each group were cultured alone or mixed in varying proportions in triplicate wells in 200 µL of T cell media (RPMI + 5% fetal bovine serum + HEPES buffer + sodium pyruvate + nonessential amino acids + β-mercaptoethanol + penicillin/streptomycin + L-glutamate).

**Measurements of proliferation.** In all cases in which proliferation was assessed, after 72 h of culture, 1 µCi of <sup>3</sup>[H]thymidine (Amersham, Piscataway, NJ) was added to each well. Cells were cultured for an additional 16 h and then harvested on a FilterMate cell harvester (Perkin-Elmer, Boston, MA). <sup>3</sup>[H] counts were done using a Wallac 1450 Microbeta Trilux liquid scintillation/luminescence counter (Perkin-Elmer). Data were taken as means of triplicate wells.

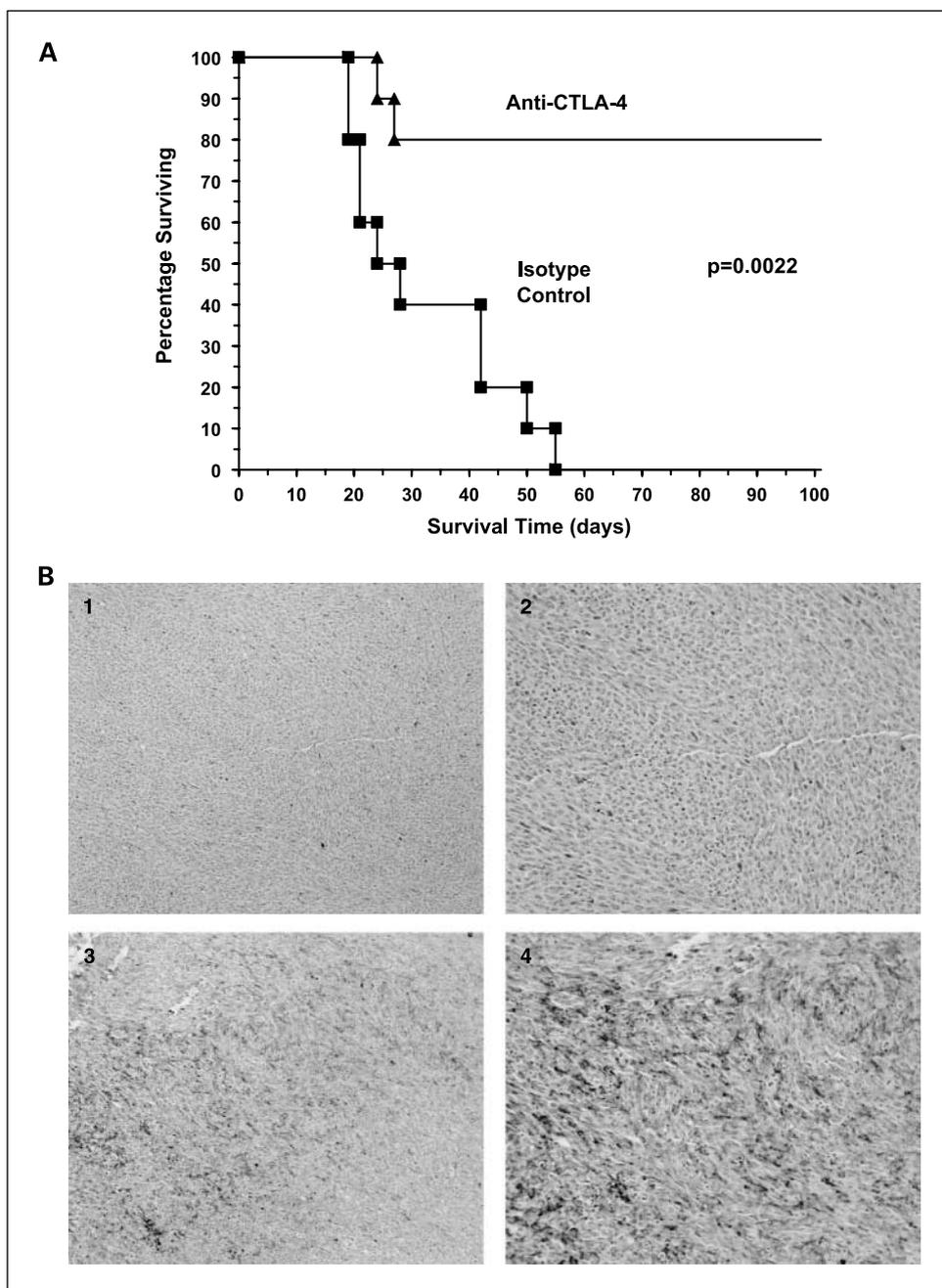
**Statistics.** Comparisons of T cell numbers,  $T_{reg}$  fractions, and proliferation levels among groups were made using unpaired *t* tests. Survival estimates and median survivals were determined using the method of Kaplan and Meier. Survival curves for each group were compared using the log-rank test.

## Results

**Survival and EAE studies.** SMA-560 is an astrocytoma line that originally arose spontaneously in the inbred, immunocompetent VM/Dk mouse strain (H2K<sup>b</sup>/Ia<sup>b</sup>). For these experiments, a lethal dose ( $5.0 \times 10^3$  cells) of SMA-560 tumor cells was introduced stereotactically into the right cerebral hemisphere of VM/Dk mice. Tumor-bearing mice were subsequently treated i.p. with injections of anti-CTLA-4 (9H10) on days 7, 10, and 13 following implantation, when the tumor was

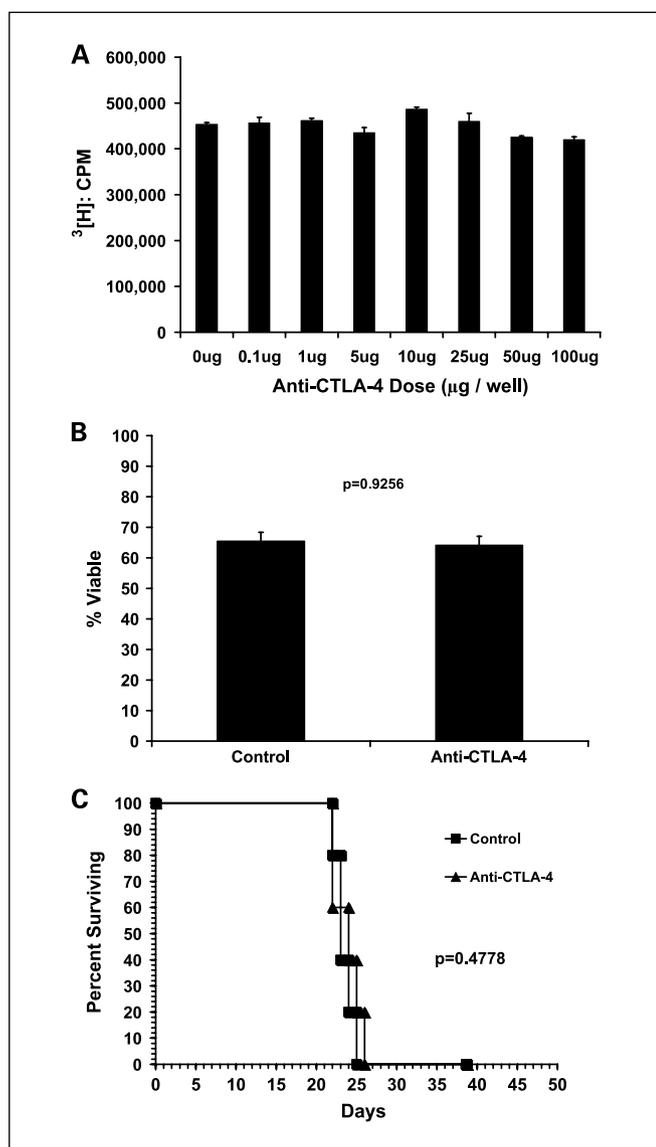
well-established. A 100 µg loading dose was administered at the day 7 time point, followed on days 10 and 13 by 50 µg maintenance doses. Control tumor-bearing mice received equivalent doses and schedules of hamster IgG<sub>2</sub> (isotype control antibody). Animals receiving isotype control therapy exhibited a median survival of 26 days, with all animals eventually succumbing to tumor. Treatment with CTLA-4 blockade, however, elicited long-term survival in 80% of treated animals ( $P = 0.0022$  by log-rank test; Fig. 1A). In those animals receiving anti-CTLA-4 therapy that eventually presented with tumor, a markedly greater infiltration of CD8<sup>+</sup> T cells into the tumor site was nonetheless noted ( $P = 0.008$  by Fisher exact test; Fig. 1B).

CTLA-4 blockade possesses the documented ability to exacerbate EAE in susceptible murine models (25–28). VM/Dk mice reproducibly develop EAE when injected intradermally with myelin oligodendrocyte glycoproteins 35 to 55 (MOG<sub>35-55</sub>) peptide, and therefore represent an EAE-susceptible strain (data not shown). In order to evaluate the risk of EAE imposed by systemic CTLA-4 blockade, mice in the above experiments were clinically evaluated each day for presenting signs of EAE. All mice in these experiments received a clinical grade of 0 throughout, corresponding with the absence of clinical signs of EAE. Additionally, the brains and spinal cords of all mice were sectioned and stained with H&E, as well as luxol fast blue, which histologically confirmed the absence of lymphocytic infiltrates



**Fig. 1.** CTLA-4 blockade prolongs survival and elicits increased tumor infiltration of CD8<sup>+</sup> cells. **A**, Kaplan-Meier survival curves showing long-term survival following systemic CTLA-4 blockade in mice harboring well-established glioma. SMA-560 cells ( $5.0 \times 10^3$ ) were implanted intracranially on day 0, and mice were treated with i.p. injections of either anti-CTLA-4 monoclonal antibody ( $n = 10$ ) or control hamster IgG (isotype control,  $n = 10$ ). Both anti-CTLA-4 and isotype control were administered in 100 µg loading doses on day 7, followed by 50 µg maintenance doses on days 10 and 13. Treatment with systemic anti-CTLA-4 increased median survival and resulted in 80% long-term (>100 days) survivors ( $P = 0.0022$  by log-rank test). **B**, increased CD8<sup>+</sup> T cell infiltrate in tumors of mice receiving CTLA-4 blockade. Mice were implanted intracranially with SMA-560 and treated as above. On day 21, those mice possessing tumor were sacrificed and intracranial tumors were fixed in formalin, paraffin-embedded, and analyzed by immunohistochemistry for the presence of CD4<sup>+</sup> and CD8<sup>+</sup> T cell infiltrates. 1 and 2, CD8 staining of intracranial astrocytoma from a control-treated animal and showing only background levels of immunoreactivity and occasional scattered lymphocytes (magnifications,  $\times 100$  and  $\times 200$ , respectively). 3 and 4, the same staining from an animal treated with anti-CTLA-4 showing prominent and diffuse infiltration of tumor with CD8<sup>+</sup> lymphocytes. None of the three control tumors revealed significant CD8<sup>+</sup> T cell infiltrates ( $>10$  cells per high-power field), whereas seven of seven anti-CTLA-4-treated animals revealed significant CD8<sup>+</sup> T cell infiltrates ( $P = 0.008$ ; Fisher exact test). No evidence of CD8<sup>+</sup> T cell accumulations were found in areas of normal brain. Staining of tumors for CD4<sup>+</sup> lymphocytes revealed occasional and scant lymphocytic infiltrates in both control and anti-CTLA-4-treated animals with no significant difference between the two treatment groups (data not shown).

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**Fig. 2.** Anti-CTLA-4 possesses no direct antitumor effects. *A* and *B*, SMA-560 cells were cultured in the presence of various doses of anti-CTLA-4 for 3 d, after which proliferation was assessed by <sup>3</sup>[H]thymidine uptake (*A*) and viability was assessed by Annexin V and propidium iodide staining (*B*). No significant effects were encountered. *C*, anti-CTLA-4 delivered systemically to athymic BALB/c mice fails to protect against tumor challenge. Athymic mice were injected intracranially with 5,000 SMA-560 tumor cells. Anti-CTLA-4 or isotype control antibody was administered i.p. as above on days 7, 10, and 13. No survival benefit was conferred by treatment with anti-CTLA-4 (*P* = 0.4778).

and demyelination, respectively (data not shown). The absence of both clinical and histological evidence of EAE suggests strongly that EAE did not accompany CTLA-4 blockade over the course of these experiments.

**Anti-CTLA-4 causes no direct cytotoxicity to SMA-560 cells.** Prior to concluding an immune-based mechanism for CTLA-4 blockade in the above experiments, we investigated the possibility that 9H10 antibody acted directly on the tumor. To this end, we first examined both surface and intracellular expression of CTLA-4 protein in SMA-560 glioma cells in order to determine the likelihood of a specific interaction between 9H10 and either intact or membrane-compromised tumor cells.

When analyzed by flow cytometry, SMA-560 cells showed no expression of CTLA-4 protein (data not shown), reducing the probability of a directed interaction between CTLA-4 antibody and the SMA-560 cells themselves.

Furthering our investigation of direct antitumor effects, we cultured SMA-560 cells *in vitro* alone or in the presence of varying concentrations of either isotype control or 9H10 antibody to determine any direct effects on growth or viability. Neither isotype control nor 9H10 significantly affected tumor cell proliferation, as measured by <sup>3</sup>[H]thymidine uptake (Fig. 2A), or viability, as measured by trypan blue exclusion and combined Annexin V and propidium iodide staining (Fig. 2B).

In the apparent absence of a direct antitumor effect, we examined the ability of 9H10 *in vivo* to prolong survival when administered to tumor-bearing immunocompromised mice. For these purposes, SMA-560 cells were injected at a lethal dose ( $5 \times 10^3$ ) into the right cerebral hemisphere of nude thy<sup>-/-</sup> mice, which subsequently were divided into a control group and a group receiving treatment with 9H10 as above. 9H10 failed to prolong survival in the athymic tumor-bearing mice (Fig. 2C). Together, these data belie a direct antitumor effect for 9H10 and suggest an *in vivo* mechanism that is instead immune-dependent.

**Restoration of CD4<sup>+</sup> T cell numbers.** Dramatic reductions in CD4 counts are frequently observed in patients with MG and are similarly present in our murine SMA-560 tumor model. These reductions in cell number represent an easily identified contributor to diminished immune responses in patients. We therefore began our investigations of the immune effects accompanying systemic CTLA-4 blockade by examining CD4 counts in tumor-bearing mice receiving 9H10 or isotype control treatment. CD4 counts were measured in age-matched naïve mice as well for comparison. Assessments began with peripheral blood, which was obtained by retro-orbital bleed on day 21 following tumor implantation. CLN and spleen were also examined at the same time point. As tumors were implanted within the right cerebral hemisphere, CD4 counts in the ipsilateral (i.e., on the side of tumor = right) and contralateral (i.e., opposite side of tumor = left) CLN were measured individually. For CLN and spleen, total cell counts were determined, whereas for peripheral blood, complete blood counts with differentiation were done. In all cases, cells were stained for CD3, CD4, and CD8 and analyzed by flow cytometry, with initial scatter gating on lymphocytes. The obtained percentages of cells staining positively for each marker were combined with total cell counts for each site to determine absolute counts of the respective cells, and these counts were compared among groups.

As observed in Fig. 3A, the presence of SMA-560 within the intracranial compartment elicited a dramatic reduction in the number of CD4<sup>+</sup> T cells present bilaterally in the CLN of control-treated tumor-bearing mice, when compared with CLN in the naïve group (ipsilateral CLN, *P* = 0.001; contralateral CLN; *P* = 0.002). This reduction was consistently more pronounced within the ipsilateral CLN (*P* = 0.01 for comparison of ipsilateral and contralateral CLN within control-treated tumor-bearing mice; Fig. 3A). Significant reductions in CD4 counts were also observed in the peripheral blood (*P* = 0.005; Fig. 3B) and spleens (*P* < 0.0001; Fig. 3C) of glioma-bearing mice.

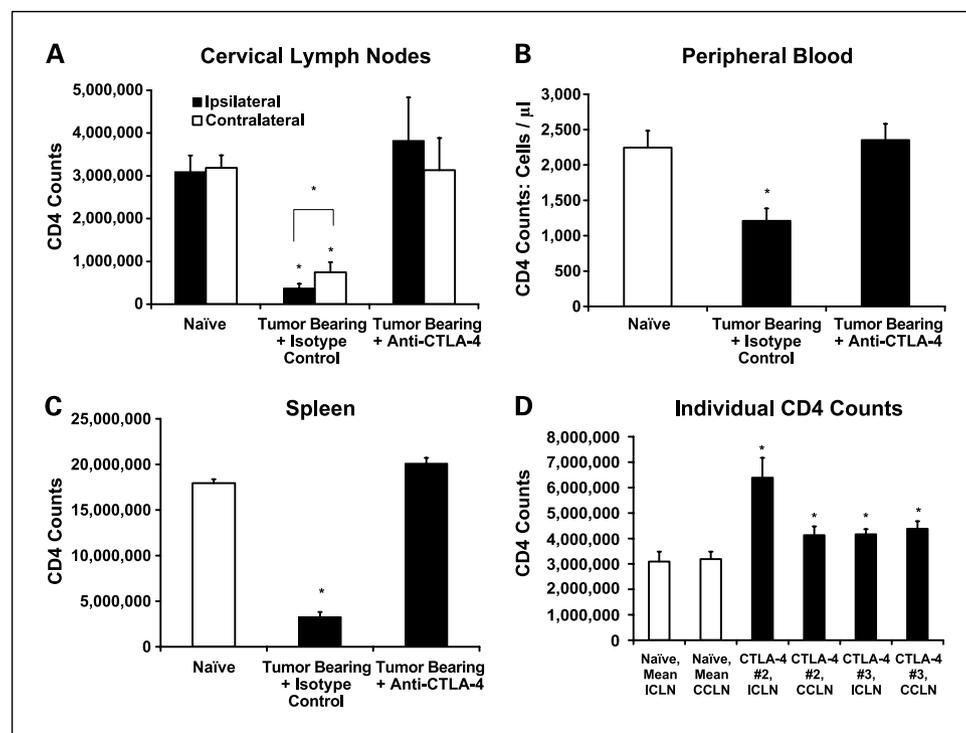
Interestingly, systemic therapy with 9H10 showed a uniform ability to restore CD4 numbers in all locations tested (Fig. 3A-C). Specifically, following 9H10 therapy, no significant differences in mean CD4 counts remained at any location between treated and naïve mice (all *P* values for comparison between 9H10-treated and naïve groups  $\geq 0.05$ ). Significant increases in total cell and CD4 counts, however, were observed in the CLN and spleens of ~20% to 25% of individual 9H10-treated mice when compared with those in the naïve group, suggesting that these changes were not merely the product of trend reversal secondary to any tumor destruction elicited (elevated CD4 counts in the CLN of two 9H10-treated mice; Fig. 3D).

**Effects of CTLA-4 blockade on  $T_{reg}$  fraction.** As patients with MG often exhibit an increased  $T_{reg}$  representation among  $CD4^+$  T cells but normal or reduced  $T_{reg}$  counts (a paradox borne out by a pervasive  $CD4$  lymphopenia), we next examined the implications of CTLA-4 blockade for  $T_{reg}$  fractions in the context of restored  $CD4$  counts.  $T_{reg}$  fraction was defined as the percentage of  $CD4^+$  T cells that were  $CD25^+Foxp3^+GITR^+$ , with the expression of each marker assessed by flow cytometry (Fig. 4A). Within control-treated tumor-bearing mice, significant elevations in the  $T_{reg}$  fraction (above the naïve fraction) were found in the bilateral CLN (ipsilateral CLN, *P* = 0.0001; contralateral CLN, *P* = 0.0008; Fig. 4B) and peripheral blood (*P* = 0.02; Fig. 4C). A notable, but nonsignificant increase was observed in the spleens (*P* = 0.37; Fig. 4D). The increased  $T_{reg}$  fractions did not correspond with an absolute expansion of this compartment, but rather with an increased representation of these cells among diminished  $CD4$  numbers (absolute  $T_{reg}$  counts in CLN; Fig. 4E). In tumor-bearing mice receiving systemic 9H10 therapy, however, these increases in  $T_{reg}$  fraction were abrogated, such that treated mice displayed  $T_{reg}$  fractions not significantly different from those of naïve mice (all *P* values

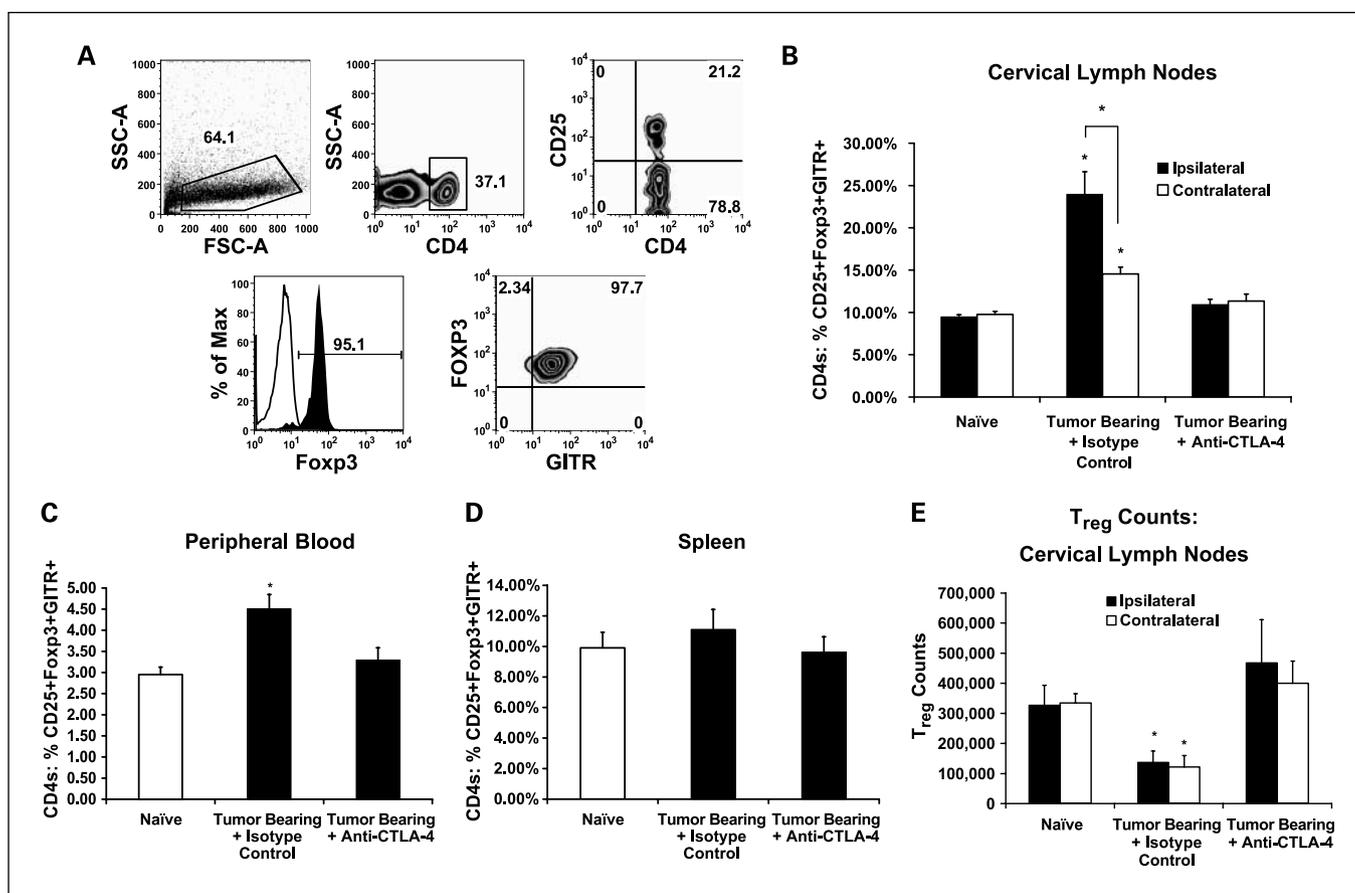
for comparison between 9H10-treated and naïve mice  $\geq 0.05$ ; Fig. 3B-D). Neither tumor nor 9H10 therapy affected the percentages of  $CD4^+CD25^+$  expressing either Foxp3 or GITR (data not shown), such that although  $T_{reg}$  proportions among  $CD4^+$  T cells varied with both tumor and treatment, their proportions among  $CD4^+CD25^+$  T cells did not.

CTLA-4 blockade also did not cause an absolute decrease in the number of  $T_{regs}$ , as shown in the depiction of CLN counts in Fig. 4E, but rather, elicited an increase that precipitated normalization of the ratio of  $T_{regs}$  to  $CD4^+CD25^-$  T cells. The absolute number of  $T_{regs}$ , although decreased in animals bearing tumor (*P* < 0.0001 for both ipsilateral and contralateral CLN), were not significantly different between 9H10-treated and naïve mice as a group (ipsilateral CLN, *P* = 0.2168; contralateral CLN, *P* = 0.1961). We did, however, note that  $T_{reg}$  numbers increased significantly within the CLN (especially the ipsilateral CLN) of ~40% to 50% of individual 9H10-treated mice, as compared with naïve mice. This result accounts for the visible trend toward higher  $T_{reg}$  numbers in the CLN of 9H10-treated mice observed in Fig. 4E. It also once again suggests immune mechanisms for CTLA-4 blockade operating independent of a simple tumor removal mechanism.

**Systemic CTLA-4 blockade improves polyclonal and antiglioma lymphocyte responses.** Substantial proliferative defects persist among lymphocytes from patients with MG, even when cell numbers were equated *in vitro* (4, 5). We therefore examined the effects of CTLA-4 blockade on  $CD4^+$  T-cell proliferative capacity.  $CD4^+$  T cells were harvested (through a negative selection technique that left the  $CD4$  molecule uncontacted by antibody) from the spleens and CLN of naïve mice, as well as from day 21 tumor-bearing mice that had received treatment with either 9H10 or isotype control antibody as earlier. Proliferative responses of equal numbers of cells from each group to polyclonal stimulation with anti- $CD3$  were tested.



**Fig. 3.** CD4 counts in the CLN (A), peripheral blood (B), and spleens (C) of naïve mice, glioma-bearing mice receiving isotype control treatment, and glioma-bearing mice receiving anti-CTLA-4 therapy. Anti-CTLA-4 reverses tumor-induced changes to  $CD4$  compartment size. CD4 counts were assessed on day 21 after tumor implantation, whereas therapy with either isotype control or anti-CTLA-4 was administered on days 7, 10, and 13. Tumor elicited a dramatic reduction in the number of  $CD4^+$  T cells present in the CLN (A; ipsilateral CLN, *P* = 0.001; contralateral CLN, *P* = 0.002), peripheral blood (B; *P* = 0.005), and spleens (C; *P* < 0.0001) of mice, as compared with naïve animals. Following anti-CTLA-4 therapy, no significant differences in mean CD4 counts remained at any location between treated and naïve mice (all *P* values for comparison between anti-CTLA-4 treated and naïve groups  $\geq 0.05$ ). D, significant increases in total cell and CD4 counts were observed in individual mice treated with anti-CTLA-4 when compared with mean counts in the naïve group, suggesting a mechanism independent of tumor destruction. Elevated CD4 counts in the bilateral CLN of two anti-CTLA-4 treated mice are depicted. ICLN, ipsilateral cervical lymph nodes; CCLN, contralateral cervical lymph nodes.



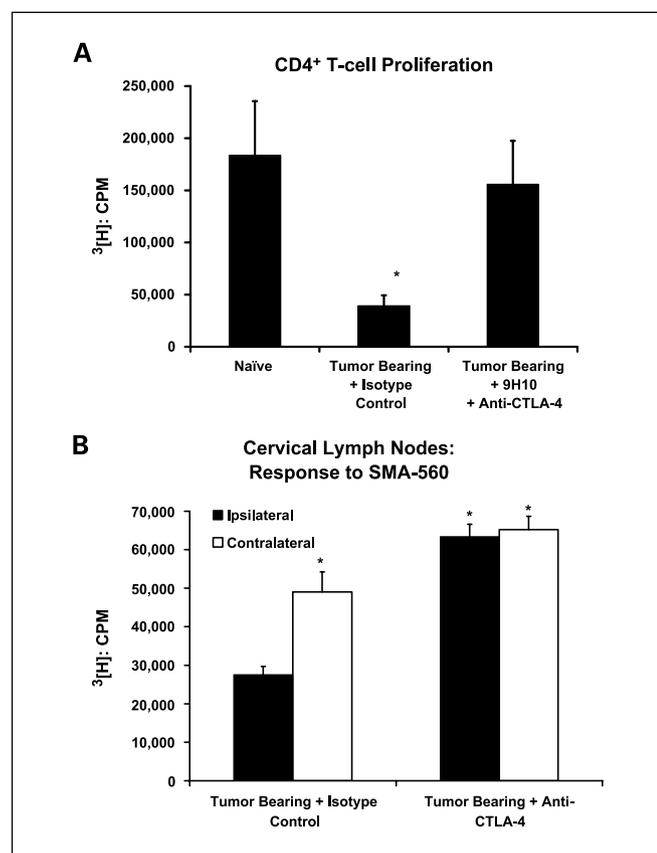
**Fig. 4.** Assessment of  $T_{reg}$  fractions and absolute counts. *A*, representative flow cytometric analysis of  $T_{reg}$  fraction in the ipsilateral CLN of a tumor-bearing (isotype control-treated) mouse. Initial forward versus side scatter gating on lymphocytes, and subsequent gating on  $CD4^+$  cells. Top right,  $CD25 \times CD4$  staining on these  $CD4^+$  lymphocytes. Bottom, gating on the  $CD25^+$  cells:  $Foxp3$  (left) and  $Foxp3 \times GTR$  (right) levels for this population. The  $T_{reg}$  fraction was defined as the percentage of  $CD4^+$  cells that were  $CD25^+ Foxp3^+ GTR^+$ . In this case,  $T_{reg}$  fraction would be 97.7% of 21.2%, or 20.71%. *B* to *D*,  $T_{reg}$  fraction in the CLN (*B*), peripheral blood (*C*), and spleens (*D*) of naive mice or of tumor-bearing mice treated with either isotype control or anti-CTLA-4 antibody (variables as earlier).  $T_{reg}$  fraction was significantly elevated in the tumor-ipsilateral CLN ( $P = 0.0001$ ), tumor-contralateral CLN ( $P = 0.0008$ ), and peripheral blood ( $P = 0.02$ ) of control-treated glioma-bearing mice, as compared with the naive group. A nonsignificant increase was observed in the spleens ( $P = 0.37$ ). In all cases, these increases were abrogated by systemic treatment with anti-CTLA-4, such that no significant differences in mean  $T_{reg}$  fraction remained at any location between treated and naive mice (all  $P$  values for comparison between anti-CTLA-4-treated and naive groups  $\geq 0.05$ ). *E*, increases in  $T_{reg}$  fraction (seen in *B-D*) do not represent increases in the absolute number of these cells, which are shown here to be reduced bilaterally in the CLN of control-treated tumor-bearing mice. Anti-CTLA-4 therapy increases  $T_{reg}$  counts beyond numbers in naive mice, although the differences between means for the two groups are not significant (ipsilateral CLN,  $P = 0.2168$ ; contralateral CLN,  $P = 0.1961$ ).

These assays thus provided a measure of relative T cell responsiveness on a per cell basis.

As seen in Fig. 5A, mice harboring glioma, in a manner akin to patients with MG, showed a significant proliferative defect among their  $CD4^+$  T cells, even when numbers were controlled ( $P < 0.0001$ ). Systemic therapy with 9H10, however, reversed this defect and reestablished a normal  $CD4$  proliferative capacity among mice, such that no difference between 9H10-treated and naive mice remained ( $P$  value for comparison of naive and 9H10-treated mice = 0.597; for comparison of isotype control-treated and 9H10-treated tumor bearing mice;  $P < 0.001$ ).

In mice bearing glioma, the CLNs represent an important site for glioma immune reactivity. As shown above, they are also one of the most visible sites of tumor-induced changes to the  $CD4$  compartment (Fig. 3A) and  $T_{reg}$  fraction (Fig. 4B). This is especially true of the ipsilateral nodes. Therefore, following our observation that CTLA-4 blockade can restore polyclonal  $CD4$  responses, we examined its ability to enhance antitumor responses in the CLN bilaterally. On day 21 following tumor implantation, right and left CLN were harvested from tumor-

bearing animals receiving either isotype control treatment or 9H10 treatment. Equal numbers of whole lymph node cells from each group and from each orientation regarding tumor (i.e., ipsilateral and contralateral) were cultured with irradiated SMA-560 glioma cells as stimulators. As depicted in Fig. 5B, cells from the ipsilateral CLN of glioma-bearing animals showed a comparatively diminished antitumor lymphocyte proliferative response relative to the contralateral nodes, when no treatment was given ( $P = 0.019$ ). Systemic treatment with 9H10, however, enhanced the antitumor proliferative responses within the contralateral CLN to levels beyond those found in the analogous nodes in isotype control-treated mice ( $P = 0.04$ ). A more dramatic effect of treatment, however, was observed in the ipsilateral CLN, as cells from the ipsilateral lymph nodes now responded at levels that were well above those in isotype control-treated animals ( $P = 0.0001$ ) and that were equivalent to those of the contralateral CLN in 9H10-treated mice ( $P = 0.705$ ). Therefore, CLN cells from animals treated with 9H10 showed an enhanced faculty for antitumor proliferation that appeared bilaterally within the CLN.



**Fig. 5.** Lymphocyte-proliferative responses before and after CTLA-4 blockade. *A*, polyclonal responses of CD4<sup>+</sup> T cells to stimulation with anti-CD3. Cells were cultured for 72 h, following which proliferation was assessed by <sup>3</sup>[H]thymidine uptake. Mice bearing glioma show a severe CD4 proliferative defect ( $P < 0.0001$ ) that is alleviated by systemic treatment with anti-CTLA-4 ( $P = 0.597$ , anti-CTLA-4 – treated versus naïve mice). *B*, responses of ipsilateral and contralateral CLN cells from the same groups when cultured with irradiated SMA-560 glioma cells as stimulators. Again, cells were cultured for 72 h, following which proliferation was assessed by <sup>3</sup>[H]thymidine uptake. Ipsilateral CLN of tumor-bearing mice were limited in their response, as compared with the contralateral CLN ( $P = 0.019$ ). Systemic anti-CTLA-4 enhanced the antitumor response in both the contralateral ( $P = 0.04$ ) and ipsilateral ( $P = 0.0001$ ) CLN, and erased the difference originally observed between right and left hemispheres ( $P = 0.705$ ).

**Systemic CTLA-4 blockade confers enhanced proliferation and T<sub>reg</sub> resistance on CD4<sup>+</sup>CD25<sup>-</sup> T cells but does not alter T<sub>reg</sub> suppressive function.** The relative contributions of CTLA-4 to T<sub>reg</sub> versus CD4<sup>+</sup>CD25<sup>-</sup> T-cell function have been controversial (29). We thus attempted to isolate the effects of *in vivo* 9H10 administration on these two functionally opposed populations. Initially, we conducted our experiments in non-tumor-bearing mice in order to definitively eliminate any contributions that 9H10-mediated tumor destruction and antigen presentation might indirectly have on observed T cell behavior. To this end, naïve mice received three injections (100, 50, and 50 μg) of either 9H10 or isotype control antibody spaced 3 days apart. All cells were harvested 8 days following the last injection (akin to day 21 in earlier experiments). CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> T<sub>regs</sub> and their CD4<sup>+</sup>CD25<sup>-</sup> counterparts were purified from each group for entry into functional studies, as the use of Foxp3 as a variable for sorting cells was precluded. Typically, however, in our experience, 87% to 97% of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup> T cells also expressed Foxp3, and these values were not affected by the presence of tumor (data not shown).

We next combined varying numbers of T<sub>regs</sub> ( $5 \times 10^3 - 2.5 \times 10^4$ ) from either isotype control antibody-treated (referred to hereafter for simplicity as “normal”) or 9H10-treated mice with fixed numbers ( $5 \times 10^4$ ) of CD4<sup>+</sup>CD25<sup>-</sup> T cell responders from normal mice. This permitted us to isolate and observe the propensity for CTLA-4 blockade to damage the ability of T<sub>regs</sub> to suppress their CD4<sup>+</sup>CD25<sup>-</sup> counterparts. At the same time, however, we combined varying doses of T<sub>regs</sub> from either group (normal or 9H10-treated) with fixed numbers of CD4<sup>+</sup>CD25<sup>-</sup> T cells taken from 9H10-treated mice instead. The combination of T<sub>regs</sub> from 9H10-treated mice with CD4<sup>+</sup>CD25<sup>-</sup> T cells from 9H10-treated mice allowed us to determine T-cell proliferative status in these animals; mixing T<sub>regs</sub> from normal mice with CD4<sup>+</sup>CD25<sup>-</sup> T cells from 9H10-treated mice allowed us to gauge more specifically the ability of 9H10 treatment to confer T<sub>reg</sub> resistance on CD4<sup>+</sup>CD25<sup>-</sup> T cells. Lastly, culture of CD4<sup>+</sup>CD25<sup>-</sup> T cells or T<sub>regs</sub> from either group alone permitted us to determine any direct effects of CTLA-4 blockade on CD4<sup>+</sup>CD25<sup>-</sup> T-cell proliferative capacity or on the “anergic” state of T<sub>regs</sub> themselves.

As seen in Fig. 6A, T<sub>regs</sub> taken from normal and 9H10-treated mice showed equivalent suppressive capacities when mixed with normal CD4<sup>+</sup>CD25<sup>-</sup> T cells. Conversely, CD4<sup>+</sup>CD25<sup>-</sup> T cells taken from 9H10-treated mice failed to be suppressed by T<sub>regs</sub> from either normal or 9H10-treated mice (Fig. 6B). Only at extraordinarily high T<sub>reg</sub> doses did suppression begin to become apparent. Furthermore, the levels of proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells isolated from 9H10-treated mice (Fig. 6B) were substantially higher than those of CD4<sup>+</sup>CD25<sup>-</sup> T cells from normal mice (Fig. 6A) at all T<sub>reg</sub> doses tested. These differences were dramatic enough that Fig. 6A and B were plotted on separate graphs.

Differences in the levels of CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation were more clearly shown when  $7.5 \times 10^4$  CD4<sup>+</sup>CD25<sup>-</sup> T cells from normal and 9H10-treated mice were cultured alone, in the absence of T<sub>regs</sub>. CD4<sup>+</sup>CD25<sup>-</sup> T cells from 9H10-treated mice showed a markedly enhanced proliferative capacity ( $P = 0.0018$ ; Fig. 6C). T<sub>regs</sub> taken from both groups were also cultured alone, and as observed in Fig. 6C, T<sub>reg</sub> anergy was not broken by treatment with 9H10. Together, these results indicate that CTLA-4 blockade acts to enhance CD4<sup>+</sup>CD25<sup>-</sup> T cell-proliferative capacity, whereas simultaneously making these cells resistant to T<sub>reg</sub>-mediated suppression. No direct effects on T<sub>regs</sub> were apparent, however, with regard to either their suppressive function or their anergic state.

Whereas the above experiments were conducted in tumor-naïve mice, we repeated these experiments in tumor-bearing mice to confirm that these mechanisms remained operative in the context of gliomas. To this end, T<sub>regs</sub> and CD4<sup>+</sup>CD25<sup>-</sup> T cells were harvested from tumor-bearing mice treated with isotype control antibody (referred to hereinafter as “tumor-bearing”) and from 9H10-treated tumor-bearing mice on day 21 after tumor implantation. The same results were obtained in these experiments. For the purpose of highlighting a difference in the setup of this portion of the experiment, however, relevant results are depicted in Fig. 6D. More specifically, in order to appropriately test the effects of 9H10 treatment on T<sub>reg</sub> function, the abilities of T<sub>regs</sub> from tumor-bearing and 9H10-treated mice to suppress CD4<sup>+</sup>CD25<sup>-</sup> responders from naïve mice (i.e., a third party) were tested. The use of CD4<sup>+</sup>CD25<sup>-</sup> T cells from naïve animals as responders in these assays allowed

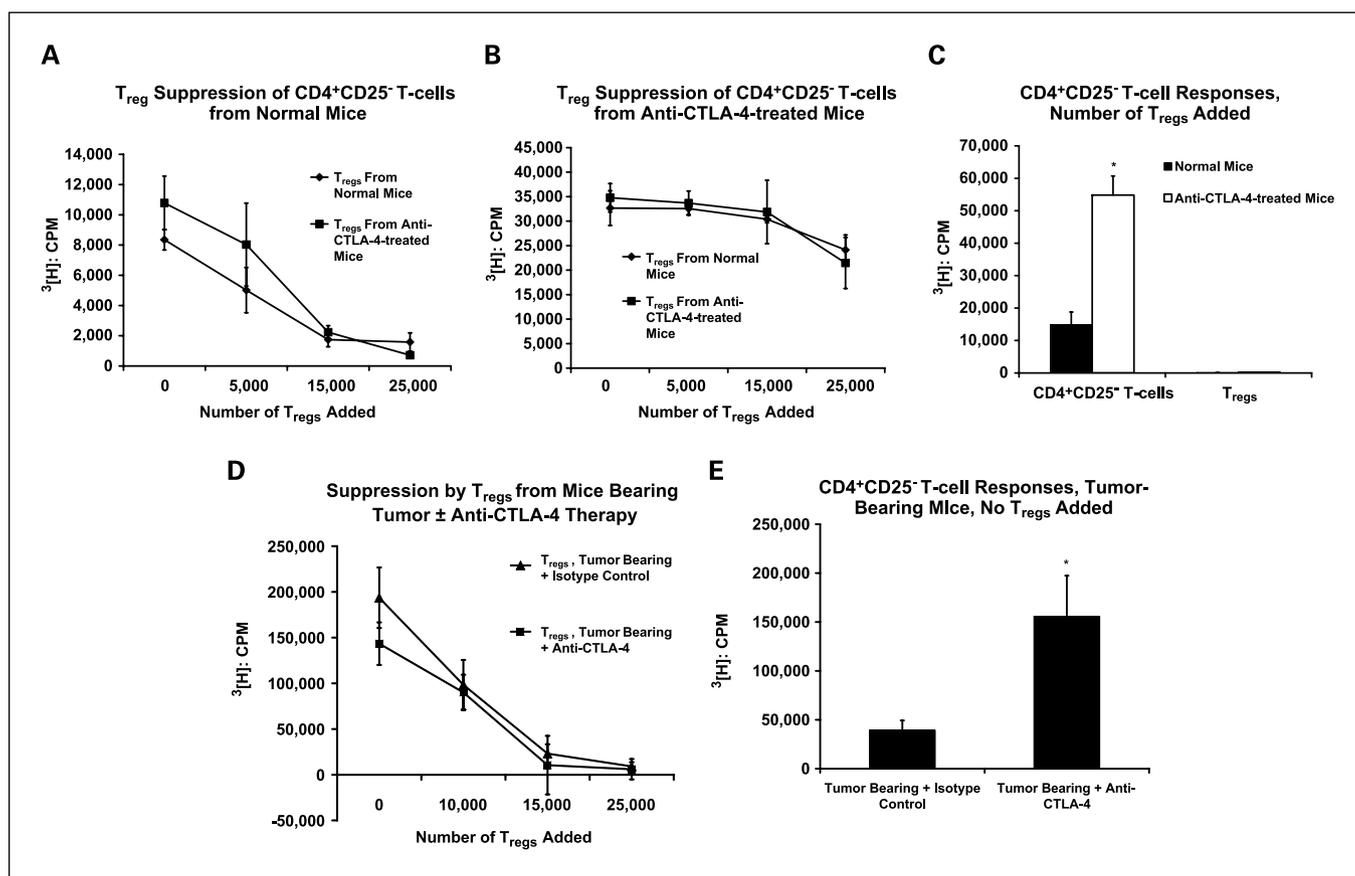
us to isolate  $T_{reg}$ s as the variable. Once again, treatment with 9H10 produced no effect on the suppressive function of  $T_{reg}$ s. Meanwhile, however,  $CD4^+CD25^-$  T cells from 9H10-treated mice showed a markedly enhanced proliferative response ( $P = 0.0091$ ; Fig. 6E). These results suggest that the activity of CTLA-4 blockade in mice bearing gliomas remains focused on the  $CD4^+CD25^-$  T cell population.

## Discussion

CTLA-4 blockade has shown efficacy as an antitumor strategy in both animal models (15, 30–32) and clinical studies (12, 16). Prior to this study, it had not been tested in primary brain tumor models, but its ability to exacerbate EAE (25–28) and to mediate the regression of brain metastasis in a patient with melanoma (16) suggested a capacity to elicit responses that could penetrate the immunologically “privileged” central nervous system. In this study, systemic CTLA-4 blockade proved permissive for established tumor rejection within the

intracranial compartment, without attendant EAE. This rejection was effected without additional therapy, despite the fact that the SMA-560 murine astrocytoma employed in this study was only weakly immunogenic (22). The ability of CTLA-4 blockade administered alone to evoke the rejection of a weakly immunogenic tumor in the central nervous system implies either a central nervous system-specific component to the antitumor mechanism, or perhaps, that tumor rejection in the central nervous system poses an issue more of immune threshold than of attenuation.

To our knowledge, we are the first to report that CTLA-4 blockade can reverse both the quantitative (CD4 counts) and qualitative (T-cell function) components of a resident tumor-based immunosuppression. One group has shown in an i.p. E.G7 tumor model that adoptively transferred  $CD8^+$  T cells (OT-I) may undergo “split energy” that is reversed on CTLA-4 blockade (30), whereas another group reported the somewhat contrasting result that tumor-tolerized  $CD4^+$  T cells could not have their tolerance broken (31). Our findings here are thus



**Fig. 6.** Systemic CTLA-4 blockade enhances  $CD4^+CD25^-$  T cell proliferation and confers resistance to  $T_{reg}$ -mediated suppression, but does not alter  $T_{reg}$  function. *A* to *E*, mice were administered standard dosing of either isotype control or anti-CTLA-4 antibody. Eight days after the final dose,  $T_{reg}$ s and  $CD4^+CD25^-$  T cells were isolated from spleens and CLN. For initial experiments (*A-C*), tumor-naïve mice were used, in order to eliminate any contributions that anti-CTLA-4-mediated tumor destruction might indirectly have on T cell behavior. Mice administered isotype control antibody were indicated as “normal” (*A-C*). *A* and *B*, varying numbers of  $T_{reg}$ s isolated from both normal and anti-CTLA-4-treated mice were mixed with fixed numbers of  $CD4^+CD25^-$  T cell responders ( $5 \times 10^4$ ) taken exclusively from normal (*A*) or anti-CTLA-4-treated (*B*) mice. Cells were stimulated with anti-CD3. *A*, normal  $CD4^+CD25^-$  T cells were suppressed at similar levels by  $T_{reg}$ s from both normal and anti-CTLA-4-treated mice. *B*,  $CD4^+CD25^-$  T cells from anti-CTLA-4-treated mice, however, were resistant to  $T_{reg}$ s from both normal and anti-CTLA-4-treated mice. At all  $T_{reg}$  doses,  $CD4^+CD25^-$  T cells from anti-CTLA-4-treated mice (*B*) proliferated at higher levels than those from normal mice (*A*). This is highlighted further in (*C*), in which  $CD4^+CD25^-$  T cells from anti-CTLA-4-treated mice show enhanced proliferation compared with those from normal mice when cultured in the absence of  $T_{reg}$ s (*C, left*). Anti-CTLA-4, however, failed to reverse  $T_{reg}$  anergy (*C, right*), as  $T_{reg}$ s from both normal and anti-CTLA-4-treated mice failed to proliferate to anti-CD3. *D* and *E*, experiments (from *A-C*) were repeated in glioma-bearing mice. *D*, the ability of  $T_{reg}$ s isolated on day 21 from control-treated versus anti-CTLA-4-treated tumor-bearing mice to suppress  $CD4^+CD25^-$  T cells from naïve mice was tested. Once again, anti-CTLA-4 did not alter  $T_{reg}$ -suppressive capacity. *E*,  $CD4^+CD25^-$  T cells from anti-CTLA-4-treated tumor-bearing mice showed enhanced proliferation compared with those from control-treated mice with tumor.

unique as they address the effects of CTLA-4 blockade on tumor-induced changes in the resident CD4 compartment and show the reversal of tumor-elicited defects in both CD4<sup>+</sup> T cell number and function.

There has been significant debate over the role of CTLA-4 in T<sub>reg</sub> versus CD4<sup>+</sup>CD25<sup>-</sup> T-cell function (29). Although some studies have shown that T<sub>regs</sub> fail to suppress in the presence of anti-CTLA-4 and have thereby asserted an essential role for CTLA-4 in T<sub>reg</sub> function (20), these have not offered conclusive evidence that CD4<sup>+</sup>CD25<sup>-</sup> T cells were not instead simply made resistant to T<sub>reg</sub> activity. Indeed, a number of studies now seem to indicate that T<sub>regs</sub> and anti-CTLA-4 act independently, and that the effects of CTLA-4 blockade are not focused on T<sub>regs</sub> (17, 19, 33). This issue, however, has not been resolved.

Here, we employ CTLA-4 blockade *in vivo* and subsequently isolate the relevant T cell populations to address these questions, rather than simply adding antibody into *in vitro* mixing experiments. We isolate the effects of CTLA-4 blockade on both T<sub>regs</sub> and non-T<sub>reg</sub> CD4<sup>+</sup>CD25<sup>-</sup> T cells and show fairly decisively that *in vivo* CTLA-4 blockade does not remove the suppressive capacity of T<sub>regs</sub>. Rather, it confers an enhanced proliferative capacity to response-capable CD4<sup>+</sup>CD25<sup>-</sup> T cells, whereas also rendering these cells resistant to T<sub>reg</sub>-mediated suppression. Thus, CD4<sup>+</sup>CD25<sup>-</sup> T cells from anti-CTLA-4-treated animals show striking levels of proliferation whether in the presence of T<sub>regs</sub> or not. The absence of a direct effect of anti-CTLA-4 on T<sub>regs</sub> also strengthens the notion that CTLA-4 blockade and T<sub>reg</sub> removal may show synergy as a combined modality (17).

Importantly, the effects of anti-CTLA-4 treatment were first measured in tumor-naïve animals and the results were then simply recapitulated in tumor-bearing mice. Our findings

therefore suggest that the immune mechanisms of CTLA-4 blockade are independent of the tumor and not merely mediated by, or indicative of, tumor rejection. This argument was strengthened further by the absence of a direct effect of anti-CTLA-4 on tumor, the failure of anti-CTLA-4 to elicit tumor rejection in the absence of a functional immune system, and our observation that CTLA-4 blockade often increased CD4 and T<sub>reg</sub> counts beyond physiologic levels in mice bearing tumor. All of these suggest an active immunologic response to CTLA-4 blockade that precedes, if not elicits, tumor rejection. Given the resistance that CD4<sup>+</sup>CD25<sup>-</sup> T cells from treated animals showed toward the suppressive activities of T<sub>regs</sub>, a mechanism involving the direct action of CTLA-4 blockade on CD4<sup>+</sup>CD25<sup>-</sup> T cells is advanced.

In conclusion, CTLA-4 blockade shows efficacy against established intracranial tumors, despite a systemic route of administration, and accomplishes this without concomitant EAE induction. It also shows the abilities to alleviate changes sustained by the CD4 compartment in the context of tumor and to restore a normal ratio of T<sub>regs</sub> to non-T<sub>regs</sub> within this population. A component of its activity seems to be through extending T<sub>reg</sub> resistance and enhanced proliferative responses to CD4<sup>+</sup>CD25<sup>-</sup> T cells, without eliciting changes in T<sub>reg</sub>-suppressive function. These findings further our understanding of the mechanisms of CTLA-4 blockade and provide a rational approach to investigate toward the end of enhancing antitumor immunity in patients with MG.

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