Systemic Anti-CD25 Monoclonal Antibody Administration Safely Enhances Immunity in Murine Glioma without Eliminating Regulatory T Cells

Peter E. Fecci, Alison E. Sweeney, Peter M. Grossi, Smita K. Nair, Christopher A. Learn, Duane A. Mitchell, Xiuyu Cui, Thomas J. Cummings, Darell D. Bigner, Eli Gilboa, and John H. Sampson

Abstract

Purpose: Elevated proportions of regulatory T cells (Treg) are present in patients with a variety of cancers, including malignant glioma, yet recapitulative murine models are wanting. We therefore examined Treg in mice bearing malignant glioma and evaluated anti-CD25 as an immunotherapeutic adjunct.

Experimental Design: CD4+CD25+Foxp3+GITR+ Treg were quantified in the peripheral blood, spleens, cervical lymph nodes, and bone marrow of mice bearing malignant glioma. The capacities for systemic anti-CD25 therapy to deplete Treg, enhance lymphocyte function, and generate antiglioma CTL responses were assessed. Lastly, survival and experimental allergic encephalitis risks were evaluated when anti-CD25 was combined with a dendritic cell – based immunization targeting shared tumor and central nervous system antigens.

Results: Similar to patients with malignant glioma, glioma-bearing mice show a CD4 lymphopenia. Additionally, CD4+CD25+Foxp3+GITR+ Treg represent an increased fraction of the remaining peripheral blood CD4+ T cells, despite themselves being reduced in number. Similar trends are observed in cervical lymph node and spleen, but not in bone marrow. Systemic anti-CD25 administration hinders detection of CD25+ cells but fails to completely eliminate Treg, reducing their number only moderately, yet eliminating their suppressive function. This elimination of Treg function permits enhanced lymphocyte proliferative and IFN-γ responses and up to 80% specific lysis of glioma cell targets in vitro. When combined with dendritic cell immunization, anti-CD25 elicits tumor rejection in 100% of challenged mice without precipitating experimental allergic encephalitis.

Conclusions: Systemic anti-CD25 administration does not entirely eliminate Treg but does prevent Treg function. This leads to safe enhancement of tumor immunity in a murine glioma model that recapitulates the tumor-induced changes to the CD4 and Treg compartments seen in patients with malignant glioma.

It is currently well established that the peripheral CD4+ T-cell repertoire is equipped with its own means for the enforcement of tolerance (1–9). Focused within the CD25+GITR'Foxp3+ compartment (10, 11), this regulatory T-cell constituent (Treg) also seems to play a significant role in hindering immunity to tumor antigens (12, 13). Accordingly, Treg come to represent an increased fraction of CD4+ T cells in the peripheral blood or tumor of patients with a variety of malignancies (13–16). Recent work also indicates that such increased “Treg fractions” exist amidst severe CD4 lymphopenia in patients harboring malignant glioma (17) and assume responsibility for cellular immune defects described for these patients over the last three decades (reviewed in ref. 18). Therefore, provided an appropriate, recapitulative murine model of malignant glioma, targeting Treg activity could gain justification as a means to counter immunosuppression and enhance antitumor immune responses in these patients. This strategy currently remains untested in brain tumor models.

We report here that our murine model of glioma indeed recapitulates malignant glioma–induced changes to the human peripheral blood CD4 and Treg compartments. Specifically, tumor-bearing mice exhibit CD4 lymphopenia whereas CD4+CD25+Foxp3+GITR+ Treg come to represent an increased fraction of the peripheral blood CD4+ T cells that remain despite themselves being reduced in number. Extending the study to other sites, similar phenomena are observed in the spleens and cervical lymph nodes whereas the reverse scenario emerges in bone marrow.
We employed this model to investigate the in vivo effects of Treg removal on antigen immunity responses. The current doctrine is that effective depletion of Tregs may be achieved simply by systemic administration of anti-CD25 monoclonal antibody (mAb; refs. 19–21). Likewise, in non–central nervous system (CNS) tumor models, the administration of anti-CD25 mAb has been employed in attempts to remove Tregs and has effectively elicited prolonged survival to s.c. tumor challenge (22–24). These studies, however, predated our ability to examine Foxp3 expression in Tregs with antibody staining.

Following in vivo administration of anti-CD25 mAb, we discovered that CD4+Foxp3+GITR+ cells failed to entirely disappear despite the present thinking. Instead, they persisted at significant levels in all sites tested. When isolated based on CD4 and glucocorticoid-induced tumor necrosis factor–like receptor (GITR) expression, however, these cells showed none of the typical suppressive capacities of CD4+CD25+GITR+ Tregs in vitro. Accordingly, systemic anti-CD25 mAb proved capable of enhancing T-cell proliferation, IFN-γ production, and glioma-specific CTL responses in treated mice. When combined with a dendritic cell–based immunization strategy, anti-CD25 mAb elicited glioma rejection in 100% of challenged mice without attendant induction of experimental allergic encephalitis. Systemic anti-CD25 mAb administration therefore seems to counter the suppressive effects of Tregs, without comprehensively eliminating the cells in vivo. This activity proves permissive for potent antitumor immunity in a murine glioma model that aptly recapitulates tumor-induced changes to the CD4 and Treg compartments.

**Materials and Methods**

**Tumor cell line and experimental animals.** The SMA-560 cell line was derived from a spontaneous intracerebral malignant glioma arising in inbred VM/Dk mice. VM/Dk mice were obtained from the McLaughlin Research Institute (Great Falls, MT) and an inbred colony was established at Duke University. Mice are maintained in a pathogen-free environment.

**Antibodies.** Antibodies to CD3 (145-2C11), CD4 (L3T4), CD16/32 (2.4G2), CD25 (PC61 and 7D4), and rat immunoglobulin G1 (IgG1) and appropriate isotype controls were obtained from BD PharMingen (San Diego, CA). Anti-α-GITR (108619) was obtained from R&D Systems (Minneapolis, MN) and a-Foxp3 (FJK-16s) was obtained from eBioscience (San Diego, CA). Anti-CD25 (PC61) ascites for in vivo administration was obtained from Accurate Chemical (Westbury, NY).

**Flow cytometry.** Spleens and cervical lymph node were harvested, minced, and pushed through cell screens to create single-cell suspensions. Whole blood was obtained by retro-orbital bleed. Bone marrow was harvested from tibias. RBC lysis was done on spleens, bone marrow, and whole blood as needed with 1× ammonium chloride lysing solution (BD PharMingen). Cells were incubated first with antibodies against surface markers for 30 minutes at 4°C in the dark. Cells were fixed and permeabilized using 1× Fix/Perm solution (eBioscience) according to the instructions of the manufacturer. Following incubation period, cells were washed in 1× Permeabilization Buffer (eBioscience), Fc-blocked with α-CD16/32, and stained with a-Foxp3 for 30 minutes at 4°C in the dark. Cells were washed, fixed, and analyzed on a FACSFlow cytometer (BD Biosciences).

**Isolation of T cells and Treg.** Spleen and cervical lymph node cells were harvested as above. Miltenyi mouse CD4+ Isolation Kit (Miltenyi Biotec, Auburn, CA) was used to isolate CD4+ cells, without engaging the CD4 molecule, according to the instructions of the manufacturer. Briefly, a biotinylated antibody cocktail specific for non-CD4+ cells (CD8a, CD11b, CD45R, DX5, and Ter119) was added at 10 μL/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. Unless otherwise indicated, nonlabeled fraction (CD4+) was counted, labeled with allopurinol, and CD4+ and phycoerythrin-α-GITR, and sorted into CD25+GITR+ and CD25− populations on a FACSAvantage SE flow cytometer (BD Biosciences). Purity of each population was always >98% to 99%.

**T-cell cultures.** For proliferation and suppression assays, 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 CD4+CD25− and CD4+CD25+ GITR+ cells (unless otherwise indicated) were purified as above from VM/Dk mice. Cells were cultured alone or mixed in varying proportions in triplicate wells in 200 μL T-cell medium consisting of RPMI 1640 + 10% FCS, supplemented with HEPS buffer, sodium pyruvate, penicillin/streptomycin, L-glutamine, β-mercaptoethanol, and nonessential amino acids (all from Life Technologies, Inc., Grand Island, NY).

For T-cell proliferative responses following in vivo Treg depletion, spleens were harvested 5 days after depletion as above. Nonadherent cells were plated in triplicate wells in T-cell medium alone or mixed 1:1 with 5-μm latex beads (Interfacial Dynamics, Tualatin, OR) coated with α-CD3e (145-2C11) and α-CD28 (clone 37.51, BD Pharmingen).

**Measurement of proliferation.** In all cases, after 72 hours of culture, 1 μCi [3H]thymidine (Amersham, Piscataway, NJ) was added to each well. Cells were cultured for an additional 16 hours and then harvested on a FilterMate cell harvester (Perkin-Elmer, Boston, MA). [3H] counts were done using a Wallac 1450 Microbeta Trilux Liquid Scintillation/Luminescence Counter (Perkin-Elmer). Data were taken as means of triplicate wells.

**IFN-γ ELISA.** Experimental supernatants were assayed for IFN-γ levels using eBioscience IFN-γ ELISA sets (eBioscience) according to the instructions of the manufacturer. Briefly, ELISA plates were coated with capture antibody and incubated overnight at 4°C. The following day, wells were washed and blocked before addition of standards and samples to the appropriate wells. Plates were later incubated with detection antibody and detected with avidin-horseradish peroxidase, followed by addition of substrate and stop solutions. Plates were read at 450 nm.

**Generation of murine bone marrow–derived dendritic cells.** Tibias, femurs, and sternums were removed using sterile technique and marrow was harvested. RBCs were lysed with ammonium chloride. Cells were suspended in complete dendritic cell medium and plated in six-well plates. Dendritic cell medium consists of T-cell medium + 18 ng/mL murine granulocyte-macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ) + 18 ng/mL murine interleukin 4 (Peprotech). Medium was replaced on day 3. On day 7, nonadherent cells were collected and replated. On day 8, nonadherent cells were harvested and electroporated with total tumor RNA isolated from the SMA-560 glioma cell line using RNeasy Midi Kit (Qiagen, Valencia, CA) at 300 V × 400 μs using an ECM 830 electroporator (BTX, San Diego, CA). On day 9, nonadherent cells were harvested for use in vaccines.

**CTL assay.** Target cells (5 × 10^6–10^8) were labeled with europium for 20 minutes at 4°C. Europium-labeled targets (10^6) and serial dilutions of effector cells at varying effector/target ratios were incubated in T-cell medium for 4 hours. Supernatant was harvested and europium release was measured by time-resolved fluorescence. Specific cytotoxic activity was determined using the following formula: % specific release = [(experimental release – spontaneous release) / (total release – spontaneous release)] × 100. Spontaneous release of the target cells was ≤25% of total release by detergent in all assays. SE of triplicate cultures was <5%.

**Intracranial tumor challenge.** Tumor cells were harvested, mixed with an equal volume of 10% methylcellulose in PBS, and loaded into...
250-μL Hamilton syringes (Hamilton, Reno, NV). Mice (VM/Dk or athymic BALB/c) were anesthetized with a mixture of xylazine/ketamine and placed into a stereotactic frame (Kopf Instruments, Tujunga, CA). The injection needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull. Cells (1.0 × 10^7) in a volume of 5 μL were delivered into the right cerebral hemisphere. After injection, the skull hole was closed with bone wax and the wound was closed with surgical staples (Stoelting Co., Wood Dale, IL).

**Experimental allergic encephalitis induction and evaluation.** VM/Dk mice were immunized i.d. in the left groin with 100 μL of an emulsion composed of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) peptide (200 μg/mouse) in PBS and an equal volume of complete Freund’s adjuvant (Difco, Detroit, MI). Mice were checked daily for signs of experimental allergic encephalitis. The clinical severity of experimental allergic encephalitis 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; and 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.

**Statistical analysis.** Unless otherwise stated, comparisons of T-cell numbers, Treg fractions, and proliferation levels among groups were made using unpaired t tests. To compare lysis curves generated in CTI assays, a generalized linear model for normal data that accounted for correlation of measurement replication within groups was used. 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**

**Validation of Treg phenotype and function in normal and tumor-bearing mice.** Tregs were defined phenotypically as CD4+ T cells coexpressing the surface markers CD25 and GTR, as well as the intracellular T reg-specific transcription factor Foxp3. Using flow cytometry, we validated that Foxp3 was found specifically within those CD4+ T cells expressing both CD25 and GTR. A representative analysis is depicted in Fig. 1A. These analyses were done in peripheral blood, cervical lymph node, spleen, and bone marrow. Nearly all CD4+CD25+GITR T cells present at each site were Foxp3+ (spleen, 87%; lymph node, 77%; whole blood, 90%; bone marrow, 86%). This was true in naive and tumor-bearing mice alike, indicating that the vast majority of CD4+CD25+GITR T cells present at each site in mice bearing glioma were Tregs and not activated T cells (Fig. 1B). This finding also permitted us to use a CD4+CD25+GITR surface phenotype for Tregs when sorting cells for entry into subsequent functional validation experiments (sorting based on Foxp3, which is intracellular, is not possible if viable cells are required).

To obtain functional validation then, CD4+CD25+GITR T cells present in both naive and tumor-bearing mice were sorted and tested in standard in vitro assays. We confirmed that CD4+CD25+GITR T cells in both naive and tumor-bearing mice were anergic to T-cell receptor stimulation and exhibited a dose-dependent capacity to suppress the proliferation of surrounding CD4+CD25 T cells.

To this end, CD4+ T cells were enriched through negative selection (leaving the CD4 molecule untouched) and were sorted into CD25+GITR and CD25- populations. The abilities of each population to proliferate in response to anti-CD3 + anti-CD28 mAbs were tested, and whereas CD4+CD25- cells in all instances proliferated well, CD4+CD25+GITR+ cells from tumor-bearing mice failed to respond, in a manner identical to those isolated from naive mice (Fig. 1C). Furthermore, CD4+CD25+GITR+ cells from each group exhibited comparable dose-dependent capacities to suppress the proliferative responses of a fixed number of CD4+CD25+ cells (Fig. 1D).

The use of a fixed number of responders in these assays ruled out the prospect of a dilutional effect due to addition of nonresponsive Tregs and likewise indicated true suppressive activity.

**Alterations to the Treg and CD4 compartments in mice bearing glioma.** Patients with malignant glioma show a markedly increased Treg fraction in their peripheral blood, which serves to hinder their cellular immune responses (17). We therefore evaluated the Treg fraction in mice harboring intracranial SMA-560 glioma. Treg fraction was defined a priori as the percentage of CD4+ T cells coexpressing CD25, GTR, and Foxp3. In addition to peripheral blood, however, Treg fraction was also analyzed in the cervical lymph nodes, spleens, and bone marrow of naive VM/Dk mice, as well as of VM/Dk mice implanted intracranially 21 days earlier with 10,000 SMA-560 glioma cells. Median survival following this manner of implantation is typically 24 to 27 days (data not shown).

The Treg fraction assessed in the peripheral blood of naive mice was 2.61 ± 0.17% of CD4+ T cells (mean ± SE; n = 10). In VM/Dk mice bearing malignant glioma, however, this fraction was raised by 65% to 4.31% ± 0.34% (n = 10, P = 0.004; Fig. 2A). Elevations in Treg fraction were also found in the cervical lymph nodes of tumor-bearing mice (Fig. 2A). As tumors were implanted within the right cerebral hemisphere, however, Treg fractions in the ipsilateral (right) and contralateral (left) cervical lymph nodes were measured individually. In general, Treg represented a larger proportion of CD4+ T cells in cervical lymph nodes than in peripheral blood (mean Treg fraction in cervical lymph nodes of naive mice was 9.51 ± 0.26%; tumor-bearing, 7.61 ± 0.17% in peripheral blood, P < 0.0001). As expected, no significant differences were observed between the left and right cervical lymph nodes of naive mice (P = 0.548). Whereas the Treg fraction was elevated bilaterally in the cervical lymph nodes of tumor-bearing mice relative to naive mice, however (Fig. 2A), in tumor-bearing mice this elevation was significantly higher within ipsilateral nodes, where Treg fraction reached as high as 37% (mean, 21.94 ± 2.67%; P = 0.011). A representative staining from the ipsilateral cervical lymph nodes is depicted in Fig. 2B. In the spleen, a small but nonsignificant increase in Treg fraction was also observed among mice bearing tumor (naive, 9.83 ± 1.02%; tumor-bearing, 10.83 ± 1.30%; P = 0.567; Fig. 2A).

Interestingly, whereas increases in Treg fraction were observed in the tissues above, a decrease in Treg fraction was observed within the bone marrow of mice bearing glioma. Notably, in naive mice, Treg represented a disproportionately large percentage of CD4+ T cells present (mean, 40.70 ± 1.07%) as compared with the other sites evaluated. In tumor-bearing mice, however, this value dropped precipitously, such that Treg came to represent only 16.92 ± 3.42% of CD4+ T cells (P = 0.0021; Fig. 2A). A representative analysis of bone marrow is depicted in Fig. 2C.

We also sought to determine the relative presence of Tregs at the tumor site in mice with glioma. To this end, SMA-560 tumors were isolated from VM/Dk mice 21 days following
tumor implantation. Samples were fixed, paraffin embedded, and subsequently examined by immunohistochemistry for Foxp3. Surrounding normal brain and SMA-560 tumors likewise isolated from athymic BALB/c mice served as controls. Scattered Foxp3+ cells were found exclusively within the tumors of immunocompetent mice, confirming a T reg infiltration of murine gliomas (Fig. 2D).

In patients with malignant glioma, increases in the peripheral blood T reg fraction exist amidst a dramatic CD4 lymphopenia. Consequently, the absolute number of peripheral blood T reg in these patients is actually reduced (17). We therefore assessed the absolute CD4 and T reg counts in the peripheral blood, cervical lymph node, spleen, and bone marrow of glioma-bearing mice (day 21) versus naive mice. Complete blood cell, total cervical lymph node cell, total splenocyte, and total tibial bone marrow cell counts were determined and cells were subjected to flow cytometric analysis. Live-cell and lymphocyte gates were created on forward and side scatter plots and the percentages of cells that represented viable CD3+, CD3+CD4+, and CD4+CD25Foxp3+GITR+ lymphocytes were subsequently determined. These percentages were multiplied by total cell counts to determine absolute counts of the respective cells and these counts were compared among groups.

As may be observed in Fig. 3A and Table 1, the presence of SMA-560 within the intracranial compartment elicited dramatic reductions in the number of CD4+ T cells in both the ipsilateral and contralateral cervical lymph nodes (P = 0.001 and P = 0.002, respectively). This was especially pronounced in the ipsilateral cervical lymph nodes (P = 0.014 for comparison with contralateral cervical lymph nodes). Similar decreases were also present in the spleen and peripheral blood.

Absolute counts of T reg were subsequently quantified in the same locations. Despite representing an increased fraction of CD4+ T cells in both the peripheral blood and cervical lymph nodes of tumor-bearing mice (Fig. 2A), T reg in these mice were reduced in absolute number at each site (Fig. 3B). The decreases in the cervical lymph nodes were significant (ipsilateral,
In the spleens of glioma-bearing mice, where Tregs had not exhibited a significantly increased representation among CD4+ T cells (Fig. 2A), absolute reductions were also present and were indeed significant (naïve, 2,113,465 ± 90,120; tumor-bearing, 433,099 ± 105,100; P < 0.0001; Fig. 3B). At none of the above sites then were Tregs observed to have actually expanded in number in the tumor-bearing state.

Interestingly, the opposite scenario was observed in the bone marrow. Although no significant differences in total bone marrow cell counts were observed among naïve and tumor-bearing mice (P = 0.3118; data not shown), CD4+ T cells came to be present at both increased percentages [P = 0.0024; observed in Fig. 2C (left)] and increased numbers (P = 0.002; Fig. 3C) in the marrow of mice harboring glioma. These increases were dramatic, approaching and exceeding 400%, respectively. Accordingly, despite exhibiting diminished Treg fractions in the bone marrow, glioma-bearing mice showed a nearly 70% increase in the absolute number of Tregs present in this compartment (P = 0.011; Fig. 3C). A more striking and disproportionate increase, however, was thus observed in the absolute number of CD4+CD25+ cells, which were expanded >6-fold in the marrow of tumor-bearing mice (P = 0.002; Fig. 3C). Therefore, whereas CD4+ T cells and Tregs were found to be reduced in absolute number in the peripheral blood, cervical lymph nodes, and spleen of tumor-bearing mice, these cells were conversely found at greater numbers in the bone marrow of the same animals.

Administration of anti-CD25 in vivo disrupts Treg function without eliciting a complete Treg depletion. Given the ability of Tregs to influence immune responses in patients with malignant glioma, we wished to evaluate the effects of in vivo anti-CD25 administration on immune responses in our recapitulative murine model. It has been widely supposed, including by us, that administration of anti-CD25 antibody in vivo results in the long-lived depletion of CD25+ Tregs thus permitting enhanced T-cell responses (19). The availability of mAbs to Foxp3, however, now permits us to more closely follow the fate of Tregs.

![Graph A](image1.png)

**Fig. 2.** Measurement of Treg fraction. A, Treg fraction (percent CD4+ lymphocytes that are CD25+Foxp3+GITR+) in peripheral blood, cervical lymph nodes, spleen, and bone marrow of naïve mice (n = 10) and mice with intracranial glioma (tumor-bearing, n = 10). Significant elevations are found in the blood (P = 0.0004), ipsilateral cervical lymph nodes (P = 0.001), and contralateral cervical lymph nodes (P = 0.008) of tumor-bearing mice. Furthermore, within tumor-bearing mice, ipsilateral cervical lymph nodes exhibit a higher Treg fraction than contralateral cervical lymph nodes (P = 0.011). In contrast, a decrease in Treg fraction was observed within the bone marrow of tumor-bearing mice (P = 0.0021). Columns, mean; bars, SE. Comparisons between groups were made using unpaired t tests. B, representative analysis of CD25, Foxp3, and GITR levels in ipsilateral (right) cervical lymph nodes isolated from naïve (N) and tumor-bearing (TB) mice. Left, gated on CD4+ lymphocytes and depict CD4+CD25+Foxp3+ cells. Middle, levels of Foxp3 in CD4+CD25+ (○) and CD4+CD25− (□) cells. Right, gated on the Foxp3+ cells, as shown, and render GITR × Foxp3 staining. C, alternative analysis depicting representative scenarios in bone marrow of naïve and tumor-bearing mice. Left, cells pulled through a lymphocyte forward and side scatter gate and further CD4 gating. Increased percentages of CD4+ cells are found in tumor-bearing mice. Middle, CD4+ cells and the percentage of CD25+ cells (decreased in TB mice), which are also subsequently gated; right, levels of Foxp3 and GITR on these CD25+ cells. D, Tregs are found at the tumor site, as tumor-infiltrating lymphocytes (not found in athymic mice harboring tumor) stain positively for Foxp3 by immunohistochemistry.
in mice following administration of anti-CD25. Accordingly, we examined the time course of CD4, CD25, Foxp3, and GITR levels in mice given a single i.p. injection of anti-CD25 (PC61) mAb. We found, in agreement with very recent work by Kohm et al. (25), that anti-CD25 may not work entirely by eliminating Tregs, as originally thought.

Following administration of 0.5 mg anti-CD25 ascites (PC61), VM/Dk mice were sacrificed at various time points, and the levels of CD4+CD25+ and CD4+Foxp3+GITR+ cells assessed independently by flow cytometry. Measurements were taken in the peripheral blood, cervical lymph node, spleen, and bone marrow. As may be observed in Fig. 4A, CD25 (PC61) became rapidly undetectable on the surface of cells at all sites evaluated (Fig. 4A). A moderate decrease in their levels was observed over time, and this change generally reached a plateau before a gradual recovery initiated. These results suggested to us

Table 1. Mean CD4 counts in naïve and tumor-bearing mice

<table>
<thead>
<tr>
<th></th>
<th>Naïve</th>
<th>Tumor-bearing</th>
<th>P for comparison</th>
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<tbody>
<tr>
<td>Ipsilateral cervical lymph nodes</td>
<td>3,007,265</td>
<td>387,275</td>
<td>0.001</td>
</tr>
<tr>
<td>Contralateral cervical lymph nodes</td>
<td>3,232,272</td>
<td>768,396</td>
<td>0.002</td>
</tr>
<tr>
<td>Spleen</td>
<td>17,950,868</td>
<td>2,328,728</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Peripheral blood (cells/μL)</td>
<td>2,245</td>
<td>1,209</td>
<td>0.0045</td>
</tr>
<tr>
<td>Bone marrow (per tibia)</td>
<td>26,662</td>
<td>117,990</td>
<td>0.002</td>
</tr>
</tbody>
</table>

NOTE: Differences at all sites measured are significant as indicated by the included P values. Additionally, differences between ipsilateral and contralateral cervical lymph nodes within tumor-bearing mice are significant (P = 0.014), with ipsilateral cervical lymph nodes showing greater reduction in counts.
that Tregs in fact persisted at some level at each site; it seemed dramatically less likely that a population of CD4+CD25-Foxp3-GITR+ cells had instead arisen de novo.

To examine this further, however, we analyzed the levels of CD4, Foxp3, GITR, and two epitopes of CD25 (PC61 and 7D4) present in the lymphocytes of mice in the minutes and hours immediately following in vivo PC61 administration. Furthermore, as the administered PC61 antibody is a rat IgG1 isotype, we assessed the levels of rat IgG1 present on the surface of cells over the same time periods (Fig. 4B). We found that although levels of CD25 detected with the same PC61 clone rapidly declined (<45 minutes), CD25 levels detected with a different anti-CD25 clone, 7D4, remained at normal levels for nearly 24 hours. These levels then began a gradual decline that mimicked exactly the time course of Foxp3 levels. Additionally, whereas rat IgG1 was not detectable on the surface of cells before in vivo PC61 administration, by 45 minutes after injection, its levels matched those of Foxp3 and 7D4, a pattern that continued out to 40 days. Both 7D4 and rat IgG1 were found specifically on the surface of Foxp3+ and GITR+ cells (correlative surface presence of rat IgG1 and GITR shown in Fig. 4C). Together, these data strongly suggest that the

![Fig. 4. Systemic anti-CD25 fails to eliminate CD4+Foxp3+GITR+ cells but interferes with their suppressive function. A, relative percentages of CD4+ T cells in which CD25 (PC61) or Foxp3 and GITR are detectable in the days following in vivo anti-CD25 (PC61) administration. Although CD25 becomes rapidly undetectable, CD4+Foxp3+GITR+ cells experience only a slow and moderate decline. B, time curves of CD25 (PC61), CD25 (7D4), Foxp3, and rat IgG1 detection on CD4+ T cells in the immediate minutes and hours following in vivo administration of anti-CD25 (PC61). PC61 is a rat IgG1 isotype. C, representative analysis showing that following in vivo PC61 administration, rat IgG1 is found specifically on the surface of GITR+ cells. Staining was done 10 days following antibody administration. Cervical lymph node is depicted. Gating is on CD4+ lymphocytes. D, anti-CD25 blocks suppressive function. Mice were given 0.5 mg PC61 (anti-CD25) or isotype control antibody (control). After 5 days, spleens and cervical lymph nodes were removed and CD4+ T cells isolated. Although CD25 was not seen on cells from anti-CD25-treated mice, GITR remained detectable at nearly the same levels and was used to sort CD4+GITR+ cells from these mice. The ability of these CD4+GITR+ cells to suppress the proliferation of CD4+CD25- T cells from control mice was compared with that of Treg, isolated from control mice on the basis of either CD25 or CD25 and GITR expression. Whereas Treg isolated from control mice suppressed T-cell proliferation in a dose-dependent fashion, CD4+GITR+ cells from anti-CD25 treated mice did not.
CD4+Foxp3+GITR+ cells that remained following PC61 administration represented the initial Treg population. Furthermore, this population seemed to retain a level of surface CD25 that remained bound with the administered antibody, making CD25 detectable to 7D4, but not to PC61, anti-CD25 clones.

Given these findings, we examined whether the CD4+GITR+ cells present in mice that received anti-CD25 retained characteristic Treg function in vitro. To this end, CD4+ T cells were enriched by negative selection as above from VM/Dk mice 5 days following administration of either isotype control or anti-CD25 antibody. In the case of mice given anti-CD25, CD4+ cells were sorted based on GITR expression, as this was the best remaining nonbound surface marker available for sorting. In control mice, CD4+ cells were sorted based instead on expression of either CD25 (PC61) or both CD25 and GITR. The abilities of CD4+CD25+ and CD4+CD25+GITR+ cells from control animals and CD4+GITR+ cells from anti-CD25−treated animals to suppress the proliferation of CD4+CD25+ responder T cells from control animals were then compared. (The testing of CD4+CD25+GITR+ cells from control animals ensured that sorting based on GITR had no effect on suppressive function). Whereas CD4+CD25+ and CD4+CD25+GITR+ cells from control animals suppressed T-cell proliferation to an equivalent extent, CD4+GITR+ cells from anti-CD25−treated animals failed to suppress the identical T-cell population (Fig. 4D).

Although it seems then that anti-CD25 administration in vivo does elicit a limited decline in Treg, this depletion is clearly not comprehensive. Anti-CD25, however, does show the ability to functionally inactivate those Tregs that persist at significant levels in vivo, thereby interfering with their ability to suppress T-cell proliferation.

T-cell function and antitumor immunity following anti-CD25 administration. As anti-CD25 administration did seem to affect Treg function, we examined what effects systemic anti-CD25 administration might have on the general proliferative and IFN-γ-elaborating capacities of T cells isolated from VM/Dk mice. To this end, we removed the spleens of VM/Dk mice 7 days following either anti-CD25 or isotype control antibody administration. Lymphocyte-enriched populations were isolated and cultured with α-CD3- and α-CD28-coated latex beads as stimulators. The proliferative response of cells was measured by [3H]thymidine uptake whereas IFN-γ elaboration was measured by ELISA. In vivo anti-CD25 significantly enhanced the ability of the lymphocyte compartment to proliferate (Fig. 5A) and secrete IFN-γ (Fig. 5B) in response to polyclonal T-cell receptor stimulation.

Experiments were subsequently initiated to determine the ability of anti-CD25 to enhance glioma-specific CTL responses. T cells were initially primed in vivo with a dendritic cell vaccine targeting malignant glioma. Mice were given antiCD25 or an isotype control antibody 4 days before receiving a single s.c. vaccination with syngeneic dendritic cells electroporated with total tumor RNA isolated from the SMA-560 glioma cell line (DC-SMA). Vaccinations were delivered bilaterally at the base of each ear, in proximity to the cervical lymph nodes.

Ten days pursuant to vaccination, spleens from all mice were harvested and restimulated in vitro with DC-SMA. Splenocytes were then entered into a CTL assay using SMA-560 cells (H2Kb) and two control tumor cell lines, B16F10.9 (H2Kb) and EL4 (H2Kb), as targets. DC-SMA vaccination alone produced a significant specific lysis of SMA-560 targets. This CTL response, however, was dramatically enhanced in those mice that received anti-CD25 before vaccination, in which specific lysis of glioma targets approached 80% (Fig. 5C).

Survival and experimental allergic encephalitis studies. The strength of the effects of anti-CD25 on in vitro T-cell responses and CTL-mediated tumor cell lysis led us to test whether the same antibody would be a successful adjunct to a dendritic cell–based immunization strategy targeting glioma in vivo. We therefore examined the effects of anti-CD25 alone, dendritic cell vaccination alone, or a combination of both therapies on the ability of mice to reject intracranial glioma challenge.

To this end, all dendritic cells were electroporated with total tumor RNA isolated from the SMA-560 glioma cell line, and all mice were challenged with 10,000 SMA-560 cells placed intracranially under stereotactic guidance. Mice receiving anti-CD25 were given a single i.p. injection of PC61 antibody 4 days before vaccination whereas controls received an equivalent dose of isotype control antibody. Vaccines were delivered 7 days before tumor challenge and were injected s.c. at the base of each ear. Anti-CD25 as a lone modality extended median survival and proved capable of rejecting tumor in 50% of treated mice (P = 0.0198, in comparison with PBS group). Vaccine alone also produced significant survival benefits (P = 0.0153; median survival, 31 days versus 17 days in PBS group), but combining this strategy with anti-CD25 therapy significantly enhanced its efficacy (P = 0.0486 for comparison with vaccine only group), evoking complete tumor rejection in all treated mice and eliciting 100% long-term survival (P = 0.0018, in comparison of combination group with PBS group; Fig. 6A).

To convince ourselves of an immune-based mechanism for anti-CD25, we initiated a set of experiments to rule out a direct effect of anti-CD25 on the SMA-560 tumor. To begin, the SMA-560 cell line was tested for surface expression of CD25 by flow cytometry. No CD25 was detected (data not shown). Additionally, SMA-560 tumor cells were cultured in the presence of various doses of anti-CD25 antibody. No effects on either proliferation (Fig. 6B) or cell viability (P = 0.9187; Fig. 6C) were identified. Lastly, we examined the ability of anti-CD25 to elicit tumor rejection in immunocompromised mice. Anti-CD25 or isotype control antibody was administered in vivo in the same manner as above to athymic BALB/c mice, 4 days before intracranial challenge with 10,000 SMA-560 tumor cells. No effect on survival was elicited (P = 0.1875; Fig. 6D). Together, these data indicate the absence of a direct effect of anti-CD25 antibody on SMA-560 and suggest instead an immune-dependent mechanism of action.

Successful attempts to remove barriers to immunity against tumors situated within the CNS not infrequently carry risks for instigating autoimmune reactions similar to experimental allergic encephalomyelitis (26). Evidence supports an experimental allergic encephalitis-protective role for Tregs (27, 28), substantiating concerns associated with employing Treg depletion as an adjunct to brain-tumor directed immunotherapy. As anti-CD25 then seemed to enhance immune responses to glioma in VM/Dk mice, we evaluated the risk that such strengthened antitumor immunity might pose concomitantly for the precipitation of experimental allergic encephalitis.

VM/Dk mice are a strain susceptible to experimental allergic encephalitis, such that autoimmunity can be evoked by a single
i.d. vaccination with MOG35-55 peptide in complete Freund's adjuvant (Fig. 6E and F). We therefore evaluated all mice in the above VM/Dk survival experiments for signs of experimental allergic encephalitis. No mice showed clinical signs of experimental allergic encephalitis and the absence of inflammation was confirmed histologically with staining of brain and spinal cord sections with H&E and luxol fast blue. Thus, anti-CD25 provided a powerful immune-based adjunct to dendritic cell vaccination and, despite being combined with a platform targeting shared tumor and CNS antigens, elicited no observable experimental allergic encephalitis in a susceptible mouse model.

Discussion

Cellular immune defects are frequently associated with malignancy and are particularly severe in patients with malignant glioma (18). Increased Treg fractions among CD4+ T cells have presently been shown in patients harboring a variety of malignancies and are believed to play a role in hindering antitumor immunity (13–16). In patients with malignant glioma, such increased fractions in peripheral blood have been shown to correlate with the manifestation of the cellular immune defects typical for these patients, including impaired T-cell proliferative responses and counterproductive shifts toward TH2 cytokine production (17). Given the immune impairments elicited by alterations to the Treg fraction, an appropriate murine cancer model for exploring Treg-directed interventions would be one in which similar alterations are aptly recapitulated. Thus, our studies here validate our murine model of malignant glioma as a suitable model for investigating means of manipulating the Treg pool and exploring Treg effects on antitumor immune function.

Specifically, we have found here that VM/Dk mice harboring syngeneic SMA-560 gliomas exhibit dramatic reductions in both CD4 and Treg counts in the peripheral blood, spleens, and in the tumor ipsilateral and contralateral cervical lymph nodes, where these changes were most dramatic. This mirrors the situation observed in the peripheral blood of patients with malignant glioma, as does the relative persistence of Tregs as an increased fraction of the remaining CD4+ T-cell compartment despite their reduced numbers. It also suggests the cervical lymph nodes as a potentially important site for immune analysis in patients with malignant glioma.

It is noteworthy, however, that this scenario of increased Treg fractions and diminished CD4 and Treg counts is not extrapolated into the bone marrow, which reveals instead an apparent true expansion of Treg numbers in the peripheral blood, spleens, and in the tumor ipsilateral and contralateral cervical lymph nodes, where these changes were most dramatic. This mirrors the situation observed in the peripheral blood of patients with malignant glioma, as does the relative persistence of Tregs as an increased fraction of the remaining CD4+ T-cell compartment despite their reduced numbers. It also suggests the cervical lymph nodes as a potentially important site for immune analysis in patients with malignant glioma.
more so, a selective trafficking to the marrow from other sites. Others have reported selective trafficking of memory CD8+ T cells to the marrow in incidences of cancer (29, 30) but the observations here are unique and invite future study. We have suggested in the past that the CD4 compartment seems to “disappear” in patients with malignant glioma (17), but our findings here now provoke more appropriate discussions of tumor-induced alterations to the distributions of the CD4 and T_{reg} compartments.

One of our most salient findings is that anti-CD25 antibody, when given systemically, fails to eliminate CD4+Foxp3+GITR+ cells from the peripheral blood, cervical lymph node, spleen, or bone marrow. A similar finding was very recently reported by Kohm et al. (25), who concluded after an eloquent study that T_{reg} remained in circulation following anti-CD25 mAb injection but that CD25 became undetectable on their surface, likely as a result of either receptor internalization or shedding. Based on the ability of anti-CD25 to exacerbate pathology in their model of acute experimental allergic encephalitis, the authors concluded that anti-CD25 must also interfere with T_{reg} function.

Our findings confirm and extend those of Kohm et al. albeit with some subtle differences to highlight. First, whereas our predecessors saw no decline in adoptively transferred T_{reg} numbers following anti-CD25 mAb administration, we analyzed physiologic T_{reg} and did detect a slow and moderate decrease in the number of CD4+Foxp3+GITR+ cells in the time following anti-CD25 injection. This was seen at a variety of sites tested. Although it remains possible that an unprecedented population of CD4+Foxp3+GITR+ cells arose de novo instead of depleted CD25+ cells, a number of our results argue strongly against this.

**Fig. 6.** Systemic anti-CD25 enhances antitumor immunity *in vivo* without eliciting experimental allergic encephalitis. A, combination of anti-CD25 and dendritic cell vaccine produces 100% survival following intracranial tumor challenge. VM/Dk mice (n = 5 per group) were given 0.5 mg PC61 (anti-CD25) or isotype control antibody i.p. on day −10. On day −7, mice were vaccinated with 2.5 × 10^{6} dendritic cells electroporated with total tumor RNA from the SMA-560 glioma cell line or injected with an equivalent volume of PBS. Injections were delivered s.c. at the base of each ear. On day 0, all mice were challenged i.c. with 10,000 syngeneic SMA-560 cells. Kaplan-Meier survival data are presented as the percentage of mice surviving in each group. Differences in survival were determined by log-rank test. Anti-CD25 (P = 0.0198) and dendritic cell vaccine (P = 0.0153) alone each produced significant survival benefits, but the combination of the two elicited 100% long-term survival (P = 0.0018). B and C, anti-CD25 has no direct antitumor effect *in vitro*. SMA-560 cells were cultured in the presence of various doses of anti-CD25 for 3 days; after which, proliferation was assessed by [3H]thymidine uptake (B) and viability was assessed by Annexin-V and propidium iodide staining (C). No significant effects were encountered. D, anti-CD25 delivered systemically to athymic BALB/c mice fails to protect against tumor challenge. Athymic mice were challenged intracranially as above with 10,000 SMA-560 tumor cells 4 days following i.p. administration of either anti-CD25 or isotype control antibody. No survival benefit was conferred (P = 0.1875). E and F, VM/Dk mice are susceptible to experimental allergic encephalitis, which develops following a single i.d. injection with MOG35-55. E, mean clinical experimental allergic encephalitis scores following MOG35-55 injection. F, representative experimental allergic encephalitis—positive histology from an affected spinal cord. Histologic staining is with H&E and luxol fast blue. Arrows, focused areas of lymphocytic infiltrate.
First, we observed a precipitous drop in CD25+ (PG61) cells and no change in the levels of CD4+Foxp3+GITR+ cells in the immediate minutes and hours following anti-CD25 injection. Furthermore, our persistent ability to detect both the administered antibody (a rat IgG1 isotype) and an alternative epitope of CD25 (7D4) specifically on the surface of CD4+Foxp3+GITR+ cells insinuates that these were indeed the same cells and that CD25 was neither entirely shed nor internalized, with at least some amount remaining on the cell surface bound by antibody. The continued presence of the anti-CD25 rat IgG1 on these cells may have effectively blocked CD25 detection and may also have been the impetus for an inefficient antibody-dependent cell-mediated cytotoxicity–based depletion. This would likewise explain the slow but somewhat steady decline in CD4+Foxp3+GITR+ cell number following anti-CD25 mAb injection.

We also provide a more direct demonstration that anti-CD25 administered in vivo may explicitly interfere with Treg function. Within 5 days of mAb injection, we established that when the remaining CD4+GITR+ cells were isolated, they no longer possessed the suppressive function shown by GITR+ cells in naive mice. Together with those by Kohm et al., these findings dramatically alter our understanding of how anti-CD25 may work to enhance immune responses. New studies into similar reagents available clinically, their mechanisms of action and their implications for employment (such as the potential for interference with activated CD25+ T cells), are warranted.

Despite the paradigm shift, however, our results in this model show the safety and utility of an anti-CD25 strategy for the improvement of glioma-directed immunotherapy. Given resilient notions of CNS immune privilege (31–33), the ability to generate a systemic antitumor immune response that is capable of extending itself into the CNS remains as a coveted goal of brain tumor immunotherapy. We find that systemic anti-CD25 mAb enhances T-cell proliferation and IFN-γ elaboration, strengthens antigen-specific anti-glioma CTL responses, and, most importantly, dramatically improves survival rates in a murine model of intracranial glioma without eliciting experimental allergic encephalitis. An immune-based mechanism for anti-CD25 is suggested, as the antibody fails to elicit direct antitumor effects or to eliminate tumor in immunocompromised athymic mice. Furthermore, the moderate survival benefit provided by anti-CD25 alone in our model (i.e., without pursuant dendritic cell vaccination) suggests that a spontaneous systemic physiologic immune response capable of targeting tumors in the CNS exists, and that peripheral Tregs are important in attenuating this response.

Acknowledgments
We thank John F. Whitesides, Patrice McDermott, and Danielle King (Duke Human Vaccine Institute Flow Cytometry Core Facility) for their technical assistance.

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Peter E. Fecci, Alison E. Sweeney, Peter M. Grossi, et al.


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