Prolongation of survival following depletion of CD4+CD25+ regulatory T cells in mice with experimental brain tumors

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Object. Regulatory CD4+CD25+ T cells have been shown to play an important role in the regulation of the immune response. Whereas the presence of these cells has been associated with immune suppression, the lack of regulatory T (Treg) cells has been shown to induce autoimmunity. The purpose of this study was to define the role of Treg cells in tumors of the central nervous system (CNS).

Methods. The authors implanted syngeneic GL261 tumor cells in the brains or flanks of C57BL/6 mice. The resulting tumors were later removed at specific time points, and the presence of tumor-infiltrating lymphocytes was analyzed by performing flow cytometry for the presence of Treg cells. In a separate experiment, mice with GL261 tumors were treated with injections of anti-CD25 monoclonal antibody (mAb) to determine whether depletion of Treg cells may have an impact on the length of survival in mice with brain tumors.

Tumor-infiltrating lymphocytes isolated from mice with GL261 tumors were found to have a significant increase in the presence of Treg cells compared with control lymphocytes (p < 0.05). Moreover, Treg cells isolated in murine brain tumors expressed FoxP3, CTLA-4, and CD62L. Mice treated with anti-CD25 mAb lived significantly longer than tumor-bearing control animals (p < 0.05). An analysis of brains in surviving animals showed a depletion of CD4+CD25+ T cells.

Conclusions. The results of this study indicate that CD4+CD25+ Treg cells play an important role in suppressing the immune response to CNS tumors. These Treg cells may therefore represent a potentially novel target for immunotherapy of malignant gliomas.

KEY WORDS • brain neoplasm • immunotherapy • regulatory T cells • CD4+CD25+ T cells

Regulatory T cells represent a small subset of peripheral CD4+ cells, which is characterized by the expression of CD25 (IL-2Rα). It has recently been shown that Treg cells can be generated in vitro or in vivo from human CD4+CD25+ T cells under various stimulation conditions and that the generation of these cells correlates with the induction of FoxP3 expression. Although the precise function of these cells remains controversial, Treg cells appear to play an important role in immune regulation. Whereas the presence of Treg cells in systemic tumors has been shown to induce a state of local immune suppression, the absence of Treg cells is associated with severe autoimmunity. These cells are therefore critical to maintaining a balance between autoimmunity and tumor immunity.

Several reports have demonstrated increased frequency of Treg cells in human neoplasms, including lung, cervical, head, and neck cancers. In the majority of these studies, CD4+CD25+ T cells have been shown to be suppressive in vitro. Experimental tumor models have shown that depletion of CD4+CD25+ T cells changes the immune response to tumors in vivo and, in selected cases, results in enhanced tumor immunity and rejection of end-stage tumors in vivo. Recently, Yu and associates demonstrated that Treg cells that selectively accumulate inside a tumor act to maintain a local cytokine environment that suppresses the effector function of tumor-infiltrating CD8+ T cells. However, intratumoral depletion of Treg cells unmasked the immunogenicity of the tumors and reversed CTL tolerability, leading to rapid rejection of well-established tumors.

Although Treg cells have been studied in different cancer models, there is no published literature regarding the role of these cells in CNS tumors. Tumors of the CNS previously were considered to reside in an immunoprivileged area; however, current evidence clearly indicates that the CNS is a site of finely tuned immune responses and that immuno-therapeutic approaches can be used in the treatment of...
malignant brain tumors. Based on these considerations, an understanding of Treg cell function in CNS tumors may be important for our further understanding of the immunobiology of glial tumors and for the design of appropriate immunotherapeutic interventions.

In this study, we examined the presence of Treg cells in CNS tumors by using a murine glioma model. Moreover, we manipulated the immune response by systemically depleting CD4+ CD25+ T cells to determine what effect, if any, the absence of Treg cells would have on intracranial tumor growth and progression. In this article we report the first phenotypic and functional characterization of Treg cells in the context of malignant brain tumors.

Materials and Methods

Tumor Cell Lines and Animals
We obtained GL261 glioma cells from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 5 mM L-glutamine, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in a humidified atmosphere of 95% air/5% CO2. We acquired female C57BL/6 mice (6–12 weeks old) from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The mice were housed and maintained in pathogen-free conditions in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Chicago. At the end of the experiments, the animals were killed by cervical dislocation.

Intracranial Tumor Model
Ten mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine HCl (25 mg/ml), xylazine (2.5 mg/ml), and 14.25% ethyl alcohol (diluted 1:3 in 0.9% NaCl). For the stereotactic intracranial injection of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and Prepodyne solution. After a midline incision had been made, a 1-mm right parietal bur hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was created. The animals were then placed in a stereotactic frame and 10° GL261 tumor cells were delivered by a 26-gauge needle to a depth of 3 mm over a period of 3 minutes. The total volume of injected cells was 5 μl. The needle was removed, the site was irrigated with sterile 0.9% NaCl, and the skin was sutured with 4-0 nylon thread.

Flank Tumor Model
Ten animals were used in this experiment. After the mice had been anesthetized in the manner described in the previous section, 10° GL261 tumor cells were injected into their right flanks. The cells were delivered by a 25-gauge needle over a period of 3 minutes. The total volume of injected cells was 100 μl. The needle was removed, and the site was irrigated with sterile 0.9% NaCl.

Multicolor Flow Cytometric Analysis
Cell suspensions of tumors isolated from murine brains or flanks or cell suspensions isolated from normal spleens (five mice/group) were prepared by first crushing the tissue through a cell strainer and washing the cells in staining buffer (PBS with 2% fetal bovine serum). Erythrocytes were removed by lysis and the remaining cells were stained with the following antibodies: anti-CD4 (L3T4), anti-CD8 (53-6.7), anti-CD25 (3C7), anti-CTLA-4, and anti-CD62L (BD Biosciences, San Jose, CA). Expression of FoxP3 protein was detected using a fluorescein isothiocyanate anti–mouse FoxP3 staining set from eBioscience (San Diego, CA). The mAbs were directly coupled to fluorescein isothiocyanate, phycoerythrin, phycoerythrin–indotricarbocyanin, or allophycocyanin. One million cells were incubated with specific mAbs for 30 minutes at 4°C, washed twice with PBS, and resuspended in staining buffer for analysis. The expression of surface markers on infiltrating T-cells was visualized using an FACS-Calibur flowcytometer (BD Biosciences) and analyzed with the aid of FlowJo software (Tree Star, Inc., Ashland, OR) and CELLQuest software (BD Biosciences).

Experimental Groups
Three experimental animal groups were used for the in vivo studies (10 mice/group). The first group consisted of mice injected with GL261 alone; the second group consisted of mice injected with GL261 and treated with anti-CD25 mAb; and the third group consisted of mice without tumors, which were injected with anti-CD25 mAb alone. Mice that received the anti-CD25 mAb were first anesthetized in the manner described earlier, and injections of anti-CD25 mAb (0.1 ml diluted in PBS [50 ng/μl]) were administered to the retro-orbital venous plexus. The injections began 1 week after tumor implantation and were given three times per week for 3 weeks. All experiments were performed in triplicate.

Statistical Analysis
A statistical comparison of the level of expression of different markers used for Treg cell characterization in different experimental groups was performed using a paired Student t-test. Survival was plotted using a Kaplan–Meier survival curve, and statistical significance was determined by performing a Kruskal–Wallis nonparametric analysis of variance followed by a nonparametric analog of the Newman–Keuls multiple comparison test. A probability value less than 0.05 was considered significant.

Results

Presence of Treg Cells Within Glioma-Infiltrating Lymphocytes

Normal brains as well as brains implanted with GL261 glioma cells were compared for differences in total CD4+, CD8+, and Treg cell subsets in TILs by performing an FACS analysis. As shown in Fig. 1A, the proportion of CD4+ and CD8+ T cells was higher in the brains of animals with intracranial gliomas (CD4+ cells: mean 5.01%, range 4.7–5.5%; CD8+ cells: mean 5.07%, range 4.75–5.5%) than in the brains of normal control animals (CD4+ cells: mean 0.079%, range 0.03–0.12%; p < 0.05; CD8+ cells: mean 0.999%, range 0.08–1.45%; p < 0.05) 4 weeks after implantation of GL261 cells. Moreover, although Treg cells were present in TILs (mean 49%, range 44–57%), there was a total absence of CD4+CD25+ T cells in normal brains.

In a parallel experiment, spleens from healthy control mice were compared with spleens from mice with intracranial GL261 tumors to find differences in CD4+ and CD8+ T cell infiltration (Fig. 1B). In the spleens of host mice with GL261 tumors, the frequencies of total CD4+ T cells (mean 18%, range 15–22%) and CD8+ T cells (mean 15.6%, range 14–17.5%) were increased, compared with those of control mice (CD4+ cells: mean 14.3%, range 12–17.5%; p < 0.04; CD8+ cells: mean 8.03%, range 5.5–11%; p < 0.04). In contrast to the intracranial tumor model, however, the proportion of Treg cells was similar in both the experimental (mean 12.3%, range 11–14%) and control (mean 12.7%, range 10.8–14.3%) groups.

Elevation of Treg Cells in Intracranial Tumors Compared With Peripheral Tumors
To characterize further the presence of TILs and Treg cells in CNS tumors, we compared the results obtained in mice harboring intracranial GL261 tumors with mice harboring flank GL261 tumors. As shown in Fig. 2A, the pro-
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Fig. 1. Results of an FACS analysis of TILs in mice with intracranial GL261 tumors. Cell suspensions isolated from the brain in animals harboring intracranial tumors and in control animals were stained with anti-CD4, anti-CD8, and anti-CD25 mAbs, and then analyzed using tricolor flow cytometry. A: Dot plots depicting a significant increase in the frequency of CD4<sup>+</sup> (5.01% compared with 0.079%; p < 0.05) and CD8<sup>+</sup> (5.07% compared with 0.099%; p < 0.05) T cells as well as an expansion of CD4<sup>+</sup>CD25<sup>+</sup> cells (49% compared with 0%) in murine intracranial GL261 tumors compared with control brain tissue, after gating (contour plots show data for cells with specific characteristics) in total CD3<sup>+</sup> T cells. B: An analysis of spleens from the same animal groups also showing an increase in CD4<sup>+</sup> (18% compared with 14.3%; p < 0.04) and CD8<sup>+</sup> (15.6% compared with 8.03%; p < 0.04) T cells, but no significant difference in the level of CD4<sup>+</sup>CD25<sup>+</sup> T cells (12.3% compared with 12.7%).

Fig. 2. Results of an FACS analysis of TILs in mice with flank GL261 tumors. A: Staining of T cell infiltration with anti-CD4 and anti-CD8 mAbs showing a significant increase in CD4<sup>+</sup> (4.38% compared with 0.18%; p < 0.05) and CD8<sup>+</sup> (6.07% compared with 0.11%; p < 0.05) T cells as well as an expansion of CD4<sup>+</sup>CD25<sup>+</sup> (21.1% compared with 0%) in mice with GL261 tumors in their flanks. The CD4<sup>+</sup>CD25<sup>+</sup> cells were gated in total CD4<sup>+</sup> T cells. B: An analysis of spleen cell suspension from the same animal groups stained with the same cocktail of mAbs, showing only an increase in CD4<sup>+</sup> (9.08% compared with 6.39%; p < 0.05) T cells and no significant difference in the level of CD8<sup>+</sup> (7.81% compared with 7.38%) and CD4<sup>+</sup>CD25<sup>+</sup> T cells (7.69% compared with 7.3%).

Portion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was also higher in samples obtained from mice with flank tumors (CD4<sup>+</sup> cells: mean 4.38%, range 3.3–6%; CD8<sup>+</sup> cells: mean 6.07%, range 3.9–8.1%) than in the normal flanks of control animals.
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(CD4 cells: mean 0.18%, range 0.1–0.34%, p < 0.05; CD8 cells: mean 0.11%, range 0.09–0.26%, p < 0.05) 4 weeks after the implantation of GL261 cells. Moreover, whereas Treg cells were present in TILs (mean 21.1%, range 16.5–26%), there was a total absence of CD4^+CD25^+ Treg cells in normal (non–tumor-bearing) flanks.

In a similar experiment, the spleens of healthy mice as well as mice harboring GL261 tumors in their flanks were compared for differences in CD4^+ and CD8^+ cell infiltration (Fig. 2B). In the spleens of host mice with GL261 tumors, only the frequency of CD4^+ T cells (mean 9.08%, range 7–11.6%) was increased compared with control mice (CD4^+ T cells: mean 6.39%, range 5.15–8%, p < 0.05). As in the intracranial tumor model, the proportion of Treg cells was similar in both experimental (mean 7.69%, range 4.8–10.3%) and control (mean 7.3%, range 5.3–10%) groups.

Frequency of Treg Cells Dependent on Tumor Growth and Progression

In an attempt to determine a temporal relationship between the infiltration of Treg cells within intracranial tumors and tumor growth, we examined the presence of Treg cells at different time points of tumor progression (10, 20, and 30 days). As shown in Fig. 3A, there are increases in Treg cells in TILs from 18.2% at 10 days to 33.6% at 20 days and 49% at 30 days after tumor implantation. To exclude the possibility that our observed increase in Treg cells was due to inflammation caused by initial tumor implantation or the needle injection, this result was compared with that obtained in a group of mice that received only injections of normal saline. The data, as shown in Fig. 3B, demonstrate a total absence of Treg cells in this control group.

Phenotypic Difference Between Treg Cells in the Brain and Flank Glioma Models

The Treg cells that had been isolated from TILs were further analyzed for expression of FoxP3, CTLA-4, and CD62L (Fig. 4). The intracellular expression of FoxP3 within Treg cells was confirmed in both the brain (mean 56.4%, range 54.5–58.5%) and flank (mean 58.8%, range 55.5–64%) models (Fig. 4A). The FACS analysis also revealed Treg cells staining positively for CTLA-4 in CD4^+CD25^+ gated cells isolated from brain tumors (mean 19%, range 16–21%), as well as in those isolated from flank tumors (mean 16%, range 12–22%). The CTLA-4 was significantly elevated in tumor Treg cells compared with normal control cells (mean 8%, range 6–11%, p < 0.02) (Fig. 4B). Finally, the population of CD62L^+ cells was elevated in Treg cells isolated from brain tumors (mean 18%, range 16–22.5%) compared with Treg cells isolated from flank tumors (mean 9%, range 5–11%) and normal controls (mean 6%, range 4.6–7.7%) (Fig. 4C).

Treatment With Anti-CD25 mAb Increases Survival in Mice With Intracranial Tumors

To investigate the role of Treg cells in glioma growth, a group of animals was treated with anti-CD25 mAb. As shown in Fig. 5A, mice with intracranial GL261 tumors that were treated with anti-CD25 mAb lived longer than control mice with GL261 tumors (median survival increased from 27 to 40 days, respectively, with 40% of treated mice surviving > 70 days; p < 0.005). Examinations of animal brains showed no evidence of tumor growth in long-term survivors. An analysis of TILs showed a decrease in total
CD4<sup>+</sup> T cells from 10.5% (range 9.5–12.5%) in nontreated mice to 2.98% (range 2.1–3.5%) in treated mice (p < 0.001). Most importantly, there was a depletion of Treg cells in intracranial tumors from 46.1% (range 45–56%) in nontreated mice to 5.7% (range 4.4–7.1%) in treated mice (p < 0.001) (Fig. 5B).

Discussion

A review of the published literature on suppressive activities of Treg cells in patients with systemic malignancies<sup>15,27,44</sup> provides a rationale for analyzing this subset of T cells within glial tumors. We previously characterized the presence of Treg cells in patients with malignant gliomas.<sup>17</sup> In the present study, we examined the prevalence of CD4<sup>+</sup>CD25<sup>+</sup> T cells in a model of experimental glioma. Moreover, we explored whether the presence of CD4<sup>+</sup>CD25<sup>+</sup> T cells in glioma infiltrates modulates the immune response against intracranial tumors in vivo. This article represents the first report in which the functional role of Treg cells has been characterized in tumors of the CNS and suggests that these cells play an important role in locally suppressing the immune function against intracranial tumors.

Our results reveal that Treg cells are increased in glioma infiltrates and this increase directly correlates with tumor...
Similar results were seen in the context of the immune response in the CNS. Furthermore, it has been shown that human Treg cells are not homogeneous populations and can be split into suppressive fractions by sorting CD25+ T cells. Recently, it has been shown that human Treg cells are not homogeneous populations and can be split into suppressive fractions by sorting CD25+ T cells. It was proposed that only a subset of cells expressing high levels of CD25 and CTLA-4 is capable of inducing suppressive function, and there was a difference in the cytokine production profile depending on the level of CD25 expression within CD4+CD25+ T cells. Taken together, these results suggest that expansion of Treg cells with suppressive phenotypes in gliomas may downregulate the antitumor response in the CNS.

The forkhead/winged helix transcription factor FoxP3 is a master gene for Treg cell function and dominant tolerance. We have shown that the prevalence of FoxP3+ Treg cells is significantly elevated in tumors. This finding further supports the immunosuppressive nature of Treg cells in patients with malignant glioma. One critical question that remains unanswered is how FoxP3 causes T cells to become regulatory. Recently, Choi and colleagues demonstrated that FoxP3 can induce HO-1 expression and, subsequently, such regulatory phenotypes as suppression of nontransfected cells in a cell–cell contact-dependent manner as well as impaired proliferation and production of cytokines on stimulation in Jurkat T cells. Moreover, the authors were able to confirm that the suppressive function of the cells was relieved by the inhibition of HO-1 activity. These findings may have important implications for gliomas, in which the expression of HO-1 has been shown to be elevated and responsible for tumor progression and angiogenesis.

Our animal model of antitumor immunotherapy indicates increased survival in mice with GL261 tumors that were treated with anti-CD25 mAb. The flow cytometric analysis of TILs from mice implanted with tumor cells and treated with anti-CD25 mAb showed a significant decrease in Treg cells. This finding supports the conclusion that CD4+CD25+ T cells suppress antitumor immunity. Depletion of CD4+CD25+ T cells in vivo by anti-CD25 mAb before tumor challenge has also been shown to enhance natural tumor immunosurveillance and induce rejection of multiple immunogenic tumors in different strains of mice. Nevertheless, anti-CD25 treatment alone does not induce rejection of tumor if the tumor is poorly immunogenic. The T cells found in draining lymph nodes of CD4+CD25+ T-cell–depleted mice, which are unable to reject poorly immunogenic tumors, can still induce tumor rejection when adoptively transferred to tumor-bearing mice. These results indicate that, although the antitumor immunity enhanced by the depletion of CD4+CD25+ T cells may be insufficient to eradicate tumors completely, it augments the sensitization of immune T cells in draining lymph nodes, thus facilitating adoptive immunotherapy.

As a functional consequence of an increased proportion of Treg cells, it has been shown that these cells can suppress the immune responses of other CD4+ and CD8+ cells. It has also been shown that CD4+CD25+ Treg cells suppress the proliferation, cytokine secretion, and cytotoxic activity of Vα24+ natural killer cells. Thus, one of the explanations for impaired cell-mediated immunity in cancer-bearing hosts is the increased prevalence of Treg cells. More studies are needed to address the roles of the two Treg cells.

**Fig. 5.** Depletion of Treg cells increases survival in mice with experimental brain tumors. A: Kaplan–Meier survival graph showing a significant increase in the median length of survival in mice treated with systemic administration of anti-CD25 mAb (median survival was increased from 27 to 40 days, with 40% of mice remaining long-term survivors; p < 0.005). B: Results of an FACS analysis of TILs in the group treated with anti-CD25 mAb showing a decrease in total CD4+ T cells (from 10.5 to 2.98%; p < 0.001) as well as in CD4+CD25+ Treg cells (from 46.1 to 5.7%; p < 0.001).
These data are further supported by our own results, in which the depletion of Treg cells via an intravenous injection of anti-CD25 mAb has been shown to prolong survival in mice with experimental brain tumors, a finding that potentially could be translated to the clinical setting.

Conclusions

The identification of Treg cells within gliomas provides a new way to study the relationship between tumor development and immune suppression in the CNS. These cells are an attractive therapeutic target for immune-related diseases. Our results support this hypothesis and clearly show the following: 1) CD4+CD25+ T cells infiltrate gliomas and express FoxP3, CTLA-4, and CD62L; and 2) depletion of CD4+CD25+ Treg cells enhances survival in mice with experimental brain tumors and may allow the expansion of a critical number of tumor-reactive T cells needed to mediate tumor rejection. Effective antitumor responses in individuals with cancer depend on the presence and function of immune cells that are able to recognize and eliminate tumor cells. However, immunotherapy of brain tumors has to overcome some endogenous protective mechanisms to achieve the rejection of glioma cells, without disturbing the delicate balance of immune regulation, to avoid the induction of potential autoimmune disease in the CNS.

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