

## Incidence and Prognostic Impact of FoxP3<sup>+</sup> Regulatory T Cells in Human Gliomas

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**Abstract** **Purpose:** The incidence of regulatory T cells (Treg) in intrinsic central nervous system malignancies is unknown. Immunotherapeutic approaches that inhibit the Treg population may be limited to a subset of patients with gliomas. Our hypothesis is that only the most malignant gliomas have a prominent glioma-infiltrating Treg population that contributes to the immunosuppressive biology and that the presence of Tregs is a negative prognostic variable. **Experimental Design:** We measured the incidence of Tregs in 135 glial tumors (including all pathologic types) in a glioma microarray using immunohistochemical analysis. Results were categorized according to the total number of Tregs within the tumors. Correlation of the presence of Tregs with prognosis was evaluated using univariate and multivariate analyses. **Results:** Tregs were not present in normal brain tissue and were very rarely found in low-grade gliomas and oligodendrogliomas. We observed significant differences in the prevalence of Tregs between astrocytic and oligodendroglial tumors, between tumors of different grades, and between different pathologic types of tumors. We identified Tregs most frequently in glioblastoma multiforme (GBM) but very rarely in low-grade astrocytomas. The presence of Tregs within GBMs did not alter the median survival in patients from whom the tumors were obtained. **Conclusions:** Treg infiltration differed significantly in the tumors according to lineage, pathology, and grade. Tregs seemed to have the highest predilection for tumors of the astrocytic lineage and specifically in the high-grade gliomas, such as GBM. In both univariate and multivariate analysis, the presence of Tregs in GBMs seemed to be prognostically neutral.

Immune cell infiltrates are common in the parenchyma of human gliomas, and multiple studies have attempted to correlate the intensity of the infiltration of these cells with survival without reaching a definitive consensus regarding the prognostic effect of infiltrating immune cells within tumors (1–3). A lack of correlation between the presence of effector T cells (CD4<sup>+</sup> or CD8<sup>+</sup>) in tumors and improved survival is likely secondary to the fact that the functional activity of the infiltrating effector immune population and the presence of the CD3<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Treg) is not accounted for.

FoxP3<sup>+</sup> Tregs are inhibitors of antitumor immunity and have been shown to be present in malignant effusions and blood of patients with a variety of cancers (4–8). More specifically,

researchers have shown that an increased FoxP3<sup>+</sup> Treg to CD4<sup>+</sup> T cells ratio correlates with impairment of CD4<sup>+</sup> T-cell proliferation in peripheral blood specimens obtained from patients with glioblastoma multiforme (GBM; ref. 9). The same study also found that in murine model systems, *in vivo* depletion of Tregs led to glioma rejection. Other studies have shown that an immunosuppressive population of Tregs is present within the GBM microenvironment (10, 11). This trafficking of Tregs into the GBM microenvironment is secondary to GBM elaboration of the chemokine CCL2 and to increased expression of the CCL2 chemokine receptor CCR4 on Tregs in patients with GBM (12).

Recent studies found that the presence of FoxP3<sup>+</sup> Tregs in tumors was an unfavorable prognostic marker in patients with hepatocellular carcinoma (13, 14) and pancreatic cancer (15). However, another study found that an increased number of FoxP3<sup>+</sup> Tregs was not associated with poor survival in patients with renal cell carcinoma (16). No studies have specifically examined the prognostic role of Tregs within the infiltrating lymphocyte population in patients with glioma. A fundamental understanding of which types of glioma have immune resistance mediated by Tregs is required for developing and initiating specific immunotherapeutic approaches that may target these cells (17, 18). The purpose of our study was to determine the incidence and prognostic significance of FoxP3<sup>+</sup> Tregs in various types of gliomas, including tumors of oligodendroglial and astrocytic lineage, tumors at various grades within those lineages, and different pathologic types of tumors.

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## Materials and Methods

**Glioma tissue microarray analysis.** A tissue microarray that contained tissue specimens from 52 GBMs (WHO grade IV), 19 anaplastic astrocytomas (WHO grade III), 3 low-grade astrocytomas (WHO grade II), 21 oligodendrogliomas (WHO grade II), 16 anaplastic oligodendrogliomas (WHO grade III), 5 mixed oligoastrocytomas (WHO grade II), 13 anaplastic mixed oligoastrocytomas (WHO grade III), and 6 gliosarcomas (WHO grade IV) was assembled by the study neuropathologist (G.N.F.). The pathologic types of the tumors were confirmed by the final issued standard of care postsurgical pathology report by the neuropathology department at The University of Texas M. D. Anderson Cancer Center, and the tumors were archived embedded in paraffin blocks. As described previously (19), the microarray also contained normal brain tissue specimens (white matter, cortex, and cerebellum); these specimens were obtained from parenchyma that overlaid deep metastases in surgical specimens that were not involved with neoplasms. No autopsy tissue was used. This study was conducted according to a protocol LAB03-0228 approved by the M. D. Anderson Cancer Center Institutional Review Board.

**Immunohistochemical analysis of Tregs in paraffin-embedded glioma tissue microarray specimens.** Formalin-fixed, paraffin-embedded sections of the brain tumor specimens in the microarray were deparaffinized in xylene and rehydrated in ethanol. The endogenous peroxidase in the tissue was blocked with 0.3% hydrogen peroxide/methanol for 10 min at room temperature before antigen retrieval. Antigen retrieval was done by placing the sections in an electric kitchen pot filled with ~800 mL of 0.05% citraconic anhydride solution (pH 7.4; Immunosaver; Nissin EM Co. Ltd.) for 45 min at 98°C. They were then cooled to room temperature for 20 min and washed once in APK solution. For antigen retrieval of FoxP3 staining, the sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) for 10 min at 121°C. After blocking nonspecific binding with a protein block serum-free solution (Dako), diluted primary antibodies against CD3 (clone SK7 8-11, 1:100; Dako), CD4 (clone 4B12, 1:40; Novocastra Laboratories Ltd.), CD8 (clone 144B, 1:20; Dako), and FoxP3 (1:20; Dr. Nobuyoshi Hiraoka, National Cancer Center Research Institute, Tokyo, Japan; ref. 15) were added to the tissue arrays and incubated overnight in a humidified box at 4°C. Slides containing the specimens described above were subjected to staining with biotin-labeled secondary antibodies (biotinylated link universal solution; Dako) for 30 min at room temperature. Finally, streptavidin-horseradish peroxidase (Dako) was added to the arrays, and the slides were incubated for 30 min at room temperature. Diaminobenzidine (Dako) was used as the chromogen, and color development was stopped by gently dipping the slides in distilled water. The nuclei on the slides were counterstained with hematoxylin. Human normal spleen, lymph node, and tonsil tissue specimens were used as positive controls. The primary antibody was omitted from the immunohistochemical staining procedure and replaced with a protein block serum-free solution (Dako), which served as a negative control.

Quantitative microscopic evaluation of Tregs in the tissue was done by examining the slides using at least three different high-power fields (maximum,  $\times 40$  objective and  $\times 10$  eyepiece). Each of the specimens in the microarray, which were obtained in duplicate from different areas of the same tumor, were examined microscopically by four independent observers (M.A.G., D.S.Y., C.R.O., and G.N.F.) in a blinded fashion, and the number of cells that stained positively for CD3, CD4, CD8, and FoxP3 was recorded. The duplicate specimens from each tumor were then averaged to calculate the final number of CD3, CD4, CD8, and FoxP3 positive lymphocytes per surgical specimen. Discrepancies between the recorded numbers for any of the lymphocyte populations prompted recounting of the cells in the specimens by the conflicting observers; final arbitration was conducted by the study neuropathologist. The ratio of FoxP3<sup>+</sup> Tregs to CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells was calculated for each specimen. In the cases of negative staining for CD3, CD4, CD8, or FoxP3<sup>+</sup>, a number of 0 was recorded. Potential

mismatching of data were minimized by staining the specimens in an intact microarray with H&E and identifying the correct location of each tissue core in the microarray by visually matching the tumors based on their unique histologic elements.

**Flow cytometric analysis for the presence of Tregs in fresh oligodendroglioma specimens.** Qualitative analysis of Tregs in surgical oligodendroglioma specimens obtained directly from patients ( $n = 5$ ) was done using fluorescence-activated cell sorting as described previously (10) to verify the immunohistochemical observation that Tregs were absent from oligodendrogliomas.

**Statistical analysis.** An equal-proportion examination with respect to tumor grade, pathologic type, and glial lineage (astrocytic versus oligodendroglial) was conducted (20). Kaplan-Meier product-limit probability estimates of overall survival were calculated (21), and log-rank tests (22) were done to compare overall survival according to FoxP3 positivity (versus FoxP3 negativity), tumor grade, astrocytic and oligodendroglial lineage, and sex. In each fitted overall survival regression model, nonsignificant variables were eliminated in a step-down fashion using a  $P$  value cutoff of 0.10. Comparisons of infiltrating immune populations were done by  $t$  test assuming unequal variances with statistical significance set at 0.05.

## Results

**Study population.** The median age of the 135 patients from whom the study tumors were obtained was 44 years (range, 4-91 years). All patients received steroids at the time of surgery. The majority of the patients (98%) had a Karnofsky performance scale (KPS) score of  $\geq 70$ ; the median score was 90 at the time of diagnosis (range, 50-100). Table 1 lists the patients' ages, KPS scores, and median survival durations stratified according to the pathologic types of the tumors. The characteristics did not differ significantly from those in patients in previous studies that examined prognostic markers in glioma patients (19, 23, 24). Multifocal disease was present in 1 case of anaplastic mixed oligoastrocytoma, 3 cases of anaplastic oligodendroglioma, 3 cases of anaplastic astrocytoma, 2 cases of gliosarcoma, and 10 cases of GBM. Of the GBM cases, 9 (17%) had received prior chemotherapy and 11 (21%) had received prior radiation therapy. Table 2 details the overall composition of the glioma tissue microarray and Fig. 1 shows the immunohistochemistry staining of the infiltrating immune populations.

**The number of infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> cells in glioma vary according to tumor grade.** CD8<sup>+</sup> cells were identified in the majority of the glioma specimens despite the grade; however, the number of patients that had a CD4<sup>+</sup> population present increased with tumor grade from 39% (7 of 18) for WHO grade II to 73% (24 of 34) for WHO grade III, and 98% (44 of 45) for grade WHO grade IV ( $P < 0.001$ ; across all grades). In contrast, CD8<sup>+</sup> cells were identified in the majority of the glioma patient specimens regardless of grade (Fig. 2A). The number of both CD4<sup>+</sup> and CD8<sup>+</sup> tumor-infiltrating T cells increased with tumor grade. Specifically, in WHO grade II tumors, there was an average number of 1.4 (SD, 2.5; range, 0-10) CD4<sup>+</sup> T cells per core, which increased to 3.2 (SD, 5.0; range, 0-21) for WHO grade III and 11.6 (SD, 13.1; range, 0-70) for WHO grade IV ( $P < 0.001$ ; between grade II and IV). Similarly, in WHO grade II tumor, there was an average number of 8.6 (SD, 6.0; range, 0-22) CD8<sup>+</sup> T cells per core, which increased to 10.3 (SD, 11.5; range, 1-49) for WHO grade III and 18.0 (SD, 21.5; range, 2-103) for WHO grade IV tumors ( $P = 0.046$ ; between grade II and IV).

**Table 1.** Demographic characteristics of patients with glioma stratified according to pathology

Pathology	Age (y)			KPS score			% newly diagnosed patients	Median survival duration (mo)*
	Median	Min	Max	Median	Min	Max		
O	40	7	55	100	70	100	81	99.8
MOA	41	24	52	95	70	100	75	—
AMOA	35	22	47	95	80	100	67	89.2
AO	39.5	25	59	95	70	100	75	—
LGA	33	4	44	95	90	100	100	166.7
AA	47	24	91	90	90	100	81	27.7
GBM	54.5	17	77	90	50	100	85	13.8
GS	55.5	23	68	80	70	100	50	4.9

Abbreviations: Min, minimum; Max, maximum; O, oligodendroglioma; MOA, mixed oligoastrocytoma; AMOA, anaplastic mixed oligoastrocytoma; AO, anaplastic oligodendroglioma; LGA, low-grade astrocytoma; AA, anaplastic astrocytoma; GS, gliosarcoma; —, not analyzable due to rarity.

\*Based on Kaplan-Meier estimates.

*The incidence of Treg infiltration within gliomas varies according to lineage.* To determine whether tumors of the oligodendroglial and astrocytic lineages are predisposed to a high Treg population, we stained the specimens in the glioma tissue array with an antibody against FoxP3 and scored the number of positive cells as described above. We did not observe FoxP3<sup>+</sup> Tregs in normal brain tissue specimens ( $n = 5$ ). The patients with oligodendroglioma (WHO grade II;  $n = 21$ ), mixed oligoastrocytoma (WHO grade II;  $n = 5$ ), and anaplastic oligodendroglioma (WHO grade III;  $n = 16$ ) only rarely had faint staining of one to three FoxP3<sup>+</sup> Tregs per core. To confirm that Tregs were not present within oligodendrogliomas, we obtained surgical specimens of these tumors ( $n = 5$ ) directly from patients not included on the glioma tissue microarray and stained them for CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs. We did not observe Tregs in any of these specimens.

Although FoxP3<sup>+</sup> Tregs were barely discernable in the low-grade astrocytoma specimens (WHO grade II;  $n = 3$ ), we detected several cells of lymphocytic morphology that stained positively for FoxP3 in 10 (53%) of the 19 anaplastic astrocytoma specimens (WHO grade III). Also, 39% of the 13 anaplastic mixed oligoastrocytomas (WHO grade III) had FoxP3<sup>+</sup> cells. In addition, 48% of the 52 GBMs (WHO grade IV) had FoxP3<sup>+</sup> cells. Finally, 83% of the 6 gliosarcomas (WHO grade IV) had FoxP3<sup>+</sup> Tregs. These cumulative data indicate that Treg infiltration is more prevalent in tumors of the astrocytic lineage than in those of the oligodendroglial lineage ( $P < 0.0001$ ; Table 3).

*The incidence of Treg infiltration varies according to tumor pathology.* We observed significant differences in staining for

FoxP3 according to pathologic type. Specifically, the presence of any FoxP3 staining was most often observed in gliosarcomas (83%) followed by anaplastic astrocytomas (53%), GBMs (48%), and anaplastic mixed oligoastrocytomas (39%; Fig. 1). The pathologic types with the lowest incidence of staining for FoxP3 were the anaplastic oligodendrogliomas (6%) followed by the oligodendrogliomas (14%), mixed oligoastrocytomas (20%), and low-grade astrocytomas (33%), again indicating a general attenuation of Treg infiltration in tumors bearing oligodendroglial components (Table 3). Additionally, no statistical differences were found between the numbers of FoxP3<sup>+</sup> T cells within each tumor pathology between newly diagnosed versus recurrent tumor.

*The incidence and number of Tregs in astrocytomas vary according to tumor grade.* Our immunohistochemical analysis showed that the incidence of Tregs in tumors increased with tumor grade. The highest grade astrocytic tumors (WHO grades III and IV) had the highest numbers of FoxP3<sup>+</sup> Tregs, and these patients were more likely to have FoxP3<sup>+</sup> Tregs within their tumors compared with other glioma patients. Only 3 of the 21 oligodendrogliomas had faint staining of 1 to 3 FoxP3<sup>+</sup> Tregs per tissue core in the microarray, and the number of FoxP3<sup>+</sup> cells in patients with anaplastic oligodendroglioma was not significantly higher than that in patients with oligodendroglioma. Only one patient with anaplastic oligodendroglioma had faint staining of one or two FoxP3<sup>+</sup> Tregs per tissue core. Of the patients with low-grade astrocytoma, one rarely had very faint staining of FoxP3<sup>+</sup> Tregs. However, 10 (53%) of the 19 patients with anaplastic astrocytoma had several cells of lymphocytic morphology that stained positively for FoxP3. Of the patients with gliosarcomas, 83% had FoxP3<sup>+</sup> Tregs. Although the number of patients with GBM who had FoxP3<sup>+</sup> Tregs in tissue cores 25 (48%) was not significantly different from that in patients with anaplastic astrocytoma, the number of FoxP3<sup>+</sup> Tregs in the former patients [at least 5 per core; 7 (14%)] was markedly higher than that in the latter patients. In fact, we observed high numbers of FoxP3<sup>+</sup> Tregs (at least 5 per core) only in patients with high-grade gliomas, such as GBM ( $n = 7$ ), gliosarcoma ( $n = 2$ ), anaplastic mixed astrocytoma ( $n = 1$ ), and anaplastic astrocytoma ( $n = 1$ ). Thus, as the glioma grade increased, the number of cells that stained positively for FoxP3 increased ( $P = 0.008$ ; Table 4); this increase was even more pronounced in tumors of astrocytic lineage than in those of oligodendroglial lineage.

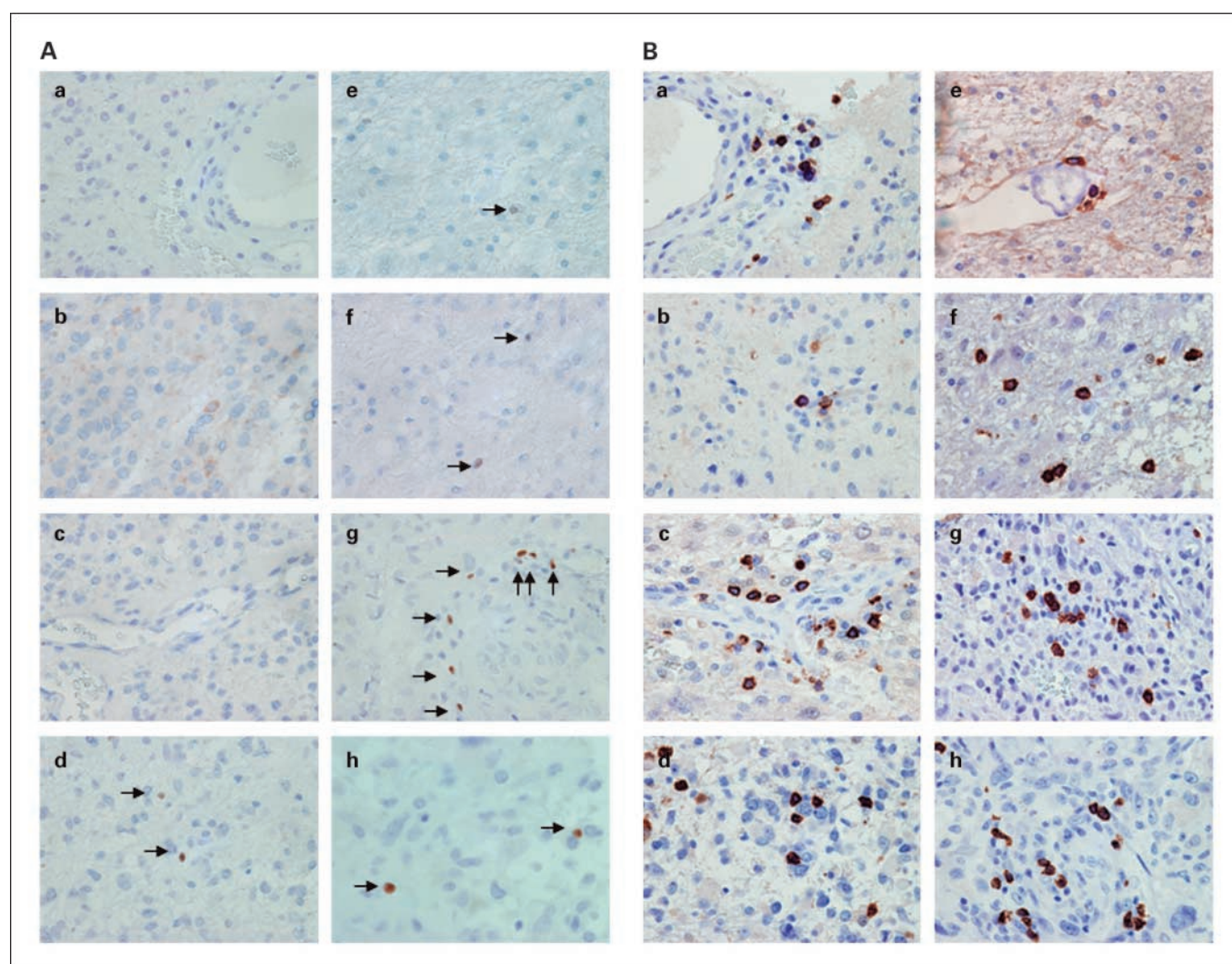
**Table 2.** Composition of the glioma tissue microarray

Lineage	n (%)	Pathology	n (%)
Oligodendroglial	55 (40.7)	O	21 (15.6)
		MOA	5 (3.7)
		AMOA	13 (9.6)
		AO	16 (11.9)
Astrocytic	80 (59.3)	GBM	52 (38.5)
		GS	6 (4.4)
		LGA	3 (2.2)
		AA	19 (14.1)

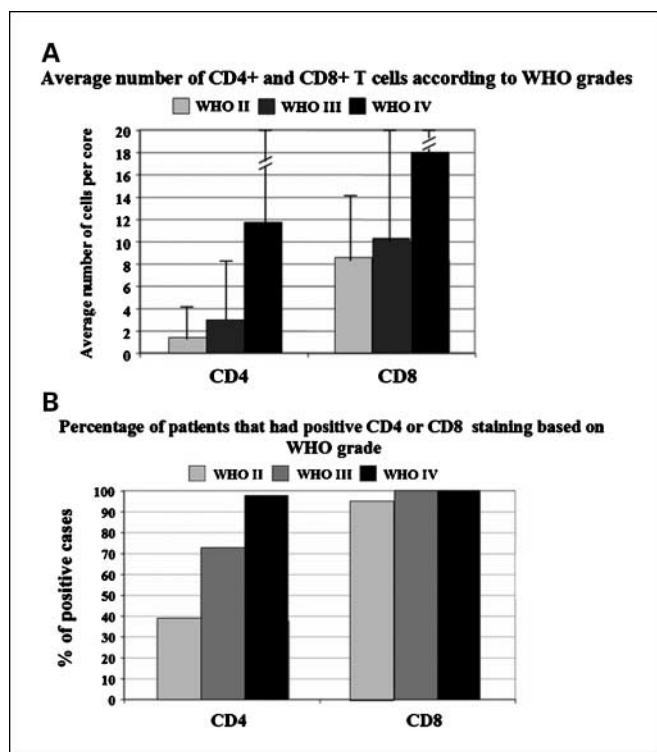
**The presence of Tregs is not a marker for survival duration.** Among all of the gliomas regardless of pathologic type, the median survival duration was 43.0 months [95% confidence interval (CI), 26.9-Not Available months] in patients whose tumors stained negatively for FoxP3. In contrast, the median survival duration was 19.2 months (95% CI, 13.8-34.0 months) in patients whose tumors contained FoxP3<sup>+</sup> Tregs ( $P < 0.001$ ). However, this finding does not account for the confounding influence of tumor grade on survival. Patients with higher grade gliomas, which portend shorter survival durations, were more likely to have Treg infiltration than patients with lower grade gliomas were. Our observed differences in survival duration according to tumor grade and pathologic type conformed to established expectations for these tumor types (Table 1). As expected, we observed that survival duration decreased as tumor grade increased.

Because the presence of FoxP3<sup>+</sup> Tregs correlated with the overall malignant behavior of astrocytic tumors, expecting this expression to function as a negative prognostic indicator would

be reasonable. According to univariate Cox proportional hazards analysis, within glioma pathologic types, the percentage of cells that stained positively for FoxP3 did not seem to correlate with survival duration. For example, the median survival duration in patients with GBM who had FoxP3<sup>+</sup> Tregs was 13.8 months (95% CI, 7.8-21.7 months), and that in patients with GBM who did not have any FoxP3<sup>+</sup> Tregs was 12.8 months (95% CI, 6.6-37.7 months), a difference that was not statistically significant ( $P = 0.56$ ; Fig. 3). Although we observed a trend of a higher probability of survival at 2 years in patients who did not have FoxP3<sup>+</sup> Tregs [0.32 (95% CI, 0.18-0.57)] than in patients who had FoxP3<sup>+</sup> Tregs [0.2 (95% CI, 0.09-0.44)], this difference was not statistically significant ( $P = 0.65$ ). Univariate analysis showed that the presence or absence of FoxP3<sup>+</sup> Tregs ( $P = 0.03$ ) and the absolute number of FoxP3<sup>+</sup> Tregs per tumor sample ( $P = 0.002$ ) were prognostic factors, similar to other established variables, such as KPS score, age, and tumor grade (Table 5). However, when we did multivariate analysis to account for confounding factors,



**Fig. 1.** Immunohistochemical staining of human glioma tissue sections demonstrating FoxP3 (A) and CD8-positive (B) lymphoid cells. FoxP3 staining is confined to the nucleus, whereas CD8 staining is noted on the cell surface. A, Tregs are more evident in astrocytic, higher grade gliomas. Arrows, FoxP3-positive cells. B, CD8 staining shows high numbers of infiltrative CD8<sup>+</sup> T cells within all glioma grades. All images were taken at  $\times 400$ . Oligodendroglioma (a), mixed oligoastrocytoma (b), anaplastic oligodendroglioma (c), anaplastic mixed oligodendroglioma (d), low-grade astrocytoma (e), anaplastic astrocytoma (f), glioblastoma (g), and gliosarcoma (h).



**Fig. 2.** The incidence of CD4+ T cells and the number of CD4+ and CD8+ T cell increases with tumor grade. **A**, CD8+ cells were identified in the majority of the glioma specimens despite the grade; however, the number of patients that had a CD4+ population present increased with tumor grade ( $P < 0.001$ ; across all grades). **B**, the number of both CD4+ and CD8+ glioma infiltrating T cells increased with tumor grade.

such as patient age and KPS score, we found that FoxP3 did not have a prognostic effect. Specifically, neither the presence nor absence of FoxP3+ Tregs ( $P = 0.45$ ; hazard ratio, 1.2) nor the absolute number of FoxP3+ cells ( $P = 0.35$ ; hazard ratio, 1.03) had a prognostic effect. We also analyzed the data set based on the ratio of FoxP3+ Tregs (immune inhibitors) to CD8+ T cells (cytotoxic effector) to determine whether the relative balance of these factors influences prognosis. Within the GBM group, we found that this ratio did not have a significant prognostic effect ( $P = 0.17$ ; hazard ratio, 1.04; 95% CI, 0.98-1.10). Upon stratification of the GBM patients based on the presence of FoxP3+ cells, no differences were identified in postoperative treatment course including radiation (96% for FoxP3+ versus 100% for no FoxP3+ staining) or chemotherapy (96% for

**Table 3.** Proportion of immunohistochemical FoxP3+ cases stratified according to pathology and WHO tumor grade

Pathology	Grade	No. of cases (%)
O	II	3/21 (14.3)
MOA	II	1/5 (20.0)
AO	III	1/16 (6.3)
AMOA	III	5/13 (38.5)
LGA	II	1/3 (33.3)
AA	III	10/19 (52.6)
GBM	IV	25/52 (48.1)
GS	IV	5/6 (83.3)

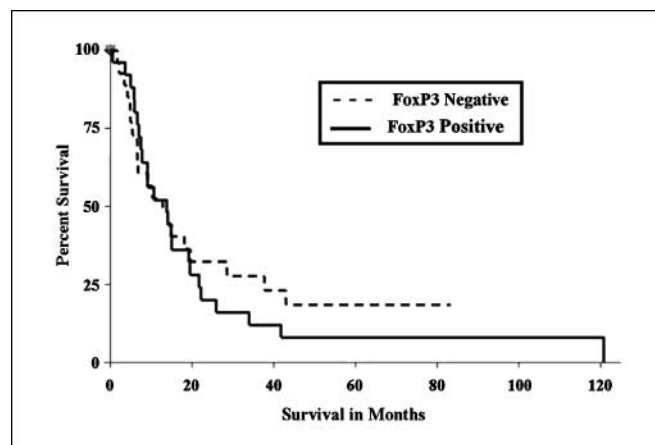
**Table 4.** Differences in the presence of FoxP3+ cells by tumor grade

Grade	Pathology	n (%)
II	LGA	29 (21.5)
	O	
	MOA	
III	AA	48 (35.6)
	AO	
	AMOA	
IV	GBM	58 (43.0)
	GS	

FoxP3+ versus 93% for no FoxP3+ staining). Furthermore, there was no statistical difference in the number of infiltrating Tregs in GBM patients that had unifocal versus multifocal disease. We cannot come to any meaningful statistical conclusions regarding the prognostic effect of FoxP3+ Tregs on survival for many of the other pathologic types of gliomas because of the relative infrequency of FoxP3+ Tregs in these tumors.

## Discussion

In this study, we showed that FoxP3+ Tregs were more commonly associated with astrocytomas than with oligodendrogliomas. Furthermore, as tumors became more malignant, the number of FoxP3+ Tregs in them increased. The number of FoxP3+ T cells with increasing tumor grade has been shown previously (25), but these studies were confined to astrocytic gliomas and were not correlated with prognosis. Similarly, among astrocytic tumors, we frequently observed FoxP3+ Tregs in glioblastomas, gliosarcomas, and anaplastic astrocytomas. However, significant Treg infiltration was not evident in many of the other types of glial tumors, such as oligodendrogliomas, which has not been previously described. Because we used a brain tumor microarray, we cannot completely exclude the possibility that Tregs were present in these other types of glial tumors in low frequency. However, in an attempt to negate this as a possibility, we used duplicate tissue cores from



**Fig. 3.** Kaplan-Meier survival estimates as stratified by the presence or absence of FoxP3+ immunohistochemical staining within GBM patients. Median survival in GBM patients that had positive FoxP3+ staining was 13.8 mo (95% CI, 7.8-21.7) and was 12.8 mo (95% CI, 6.6-37.7) in GBM patients that did not have FoxP3 staining, which is not statistically significant ( $P = 0.56$ ).

**Table 5.** Univariate Cox proportional hazards model estimates, hazard ratio, and significance of the study variables

Variable	Estimate	HR	P
KPS score	-0.02	0.98	0.03
Age at diagnosis	0.04	1.04	<0.0001
Sex	0.09	1.1	0.67
Tumor grade	1.06	2.9	<0.0001
Tumor lineage	1.33	3.79	<0.0001
FoxP3 positivity (vs none)	0.49	1.64	0.03
Actual FoxP3 <sup>+</sup> cell number	0.1	1.1	0.002

Abbreviation: HR, hazard ratio.

different areas of the tumors in the microarray and analyzed large volumes of tumor from surgical specimens of tumors obtained directly from patients; we were unable to identify Tregs in oligodendrogliomas as defined by the presence of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs according to flow cytometry analysis.

Most of the promising clinical trials of immunotherapy for gliomas have assessed patients with anaplastic astrocytoma or GBM (26–32). However, this therapeutic approach has great potential for low-grade gliomas, given that the long survival durations in patients with these tumors afford sufficient time to generate immune responses and, as evidenced in the present study, that these patients may have less overall immunosuppression compared with high-grade astrocytomas patients. A variety of approaches to negating the negative immune modulatory properties of Tregs in patients with glioma in clinical trials are being considered, including treatment with CTLA-4 blockade (33), an anti-CD25 antibody (17), cyclophosphamide (18, 34), and temozolomide (12). The use of these agents would seem to be justified in clinical trials of immunotherapy for high-grade gliomas to inhibit Tregs but not necessarily for other types of glioma, such as oligodendroglioma. Furthermore, the variability of the infiltrating Treg population within tumors and the systemic circulation (9) suggests that not all patients will uniformly benefit from these approaches and that the greatest clinical responses to these agents may be seen in patients with significant tumor Treg infiltration and/or an enhanced Treg fraction in a diminished CD4 compartment in the systemic circulation. Although low-grade gliomas do not seem to exploit Treg infiltration as a mechanism of immunosuppression, this does not mean that other mechanisms of immunosuppression are not involved, such as immunosuppressive cytokines, antigen loss, T-cell apoptosis, and induction of anergy by tumor antigen-presenting cells (35). The limitation of this study is that we are only determining the influence of Tregs within the tumor microenvironment and have not addressed the presence of Tregs within the systemic circulation (9), which may inhibit the activation of immune response in the periphery (i.e., outside the central nervous system). The identification of glioma patients who may be optimal candidates for immune therapies directed at inhibiting Tregs should consider taking into account both variables for enrollment.

T-cell infiltration is common in the parenchyma of human gliomas (2). Several studies have attempted to correlate the intensity of this infiltration with survival (1, 2). Although these

studies found prolonged survival durations that correlated with the presence of lymphocytic infiltration, this has not been a consistent finding in other studies (36). This is likely because the absolute number of T cells in a glioma does not necessarily correlate with functional activity (10) or account for the subpopulation of Tregs, which also have a CD3<sup>+</sup>CD4<sup>+</sup> surface phenotype. This study was an attempt to account for the confounding factor of immune inhibitor Tregs in this type of analysis. We did not observe prognostic significance of the presence of Tregs in gliomas in the group of patients for which sufficient statistical analysis could be done: those with GBM. This was likely secondary to use of multiple redundant immunosuppressive mechanisms in these patients.

Investigators have reported the presence FoxP3<sup>+</sup> Tregs in a variety of cancers, including hepatocellular carcinoma, colorectal cancer, ovarian cancer, and others. Studies have shown that the presence of FoxP3<sup>+</sup> Tregs in ovarian cancer was not only a predictor of poor prognosis but also an independent predictor of overall survival and progression-free survival (6, 37). However, in other cancers such as anal squamous cell carcinoma, the presence of Tregs has not been shown to be a prognostic influence (38). In head and neck squamous cell carcinoma, Treg infiltration has proven to correlate positively with locoregional control of the cancer (39). A hypothesized variable factor that may be more predictive of prognosis than solely the presence of Tregs is the balance between cytotoxic T cells and Tregs (i.e., the effector to suppressor ratio). Authors have reported that this ratio is more valuable than the presence of a single tumor-infiltrating lymphocyte subset for cancer prognostic purposes in ovarian cancer (37), lymphoma (40), and hepatocellular carcinoma after tumor resection (14). However, in colorectal cancer, analysis of this ratio did not predict poor survival (41). In the present study, we examined both variables as potential prognostic factors for GBM. Using both criteria, we did not find that FoxP3 was an independent negative prognostic factor for survival. The low incidence of Tregs in the oligodendrogliomas and low-grade gliomas did not allow for definitive conclusions regarding the prognostic effect of the presence of FoxP3 Tregs in these gliomas.

Although some cancers may mediate immunosuppression predominantly by using Tregs, researchers have shown that high-grade gliomas have multiple mechanisms of mediating immunosuppression (35); thus, the lack of a prognostic effect of one mechanism such as the presence or absence of FoxP3<sup>+</sup> Tregs in this setting is not entirely surprising. Furthermore, the ratio of FoxP3<sup>+</sup> Tregs to CD8<sup>+</sup> T-cell effectors may not be valid as a prognosticator in gliomas because the effector cells in gliomas are not activated (10) and likely are not functional (42, 43).

In conclusion, Tregs frequently infiltrate high-grade malignant gliomas of astrocytic lineage and are viable targets for immunotherapy. However, their presence does not confer a negative prognosis, likely confounded by multiple other redundant immunosuppressive pathways.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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