The Role of Tregs in Human Glioma Patients and Their Inhibition With a Novel STAT-3 Inhibitor

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Several recent clinical trials for high-grade gliomas have demonstrated promising results. Nonetheless, despite improvement in survival, these patients ultimately die of tumor progression. Malignant glioma patients are profoundly immunosuppressed, and 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.

Tregs Incidence and Frequency in Gliomas

Immune cell infiltrates are present in human gliomas, and previous studies have attempted to correlate the number of tumor-infiltrating immune cells with a positive prognosis; however, the findings of these studies have not been definitive.[5,46,50,51] This lack of correlation between the presence of effector T cells ($CD4^+$ or $CD8^+$) in gliomas and improved survival is likely secondary to these analyses not accounting for the immunosuppressive cell populations such as Tregs and the lack thereof of functional activity in the infiltrating effector immune population.[27] This current study was an attempt to account for the confounding factor of the immunoinhibitory Tregs in this type of analysis. $FoxP3^+$ Tregs ($CD3^+CD4^+CD25^+FoxP3^+$) are inhibitors of antitumor immunity and have been shown to be present in the blood and malignant effusions of patients with cancer.[7,39,42,44,52] In glioblastoma multiforme (GBM) patients, there is an increased number of Tregs in peripheral blood relative to the $CD4^+$ T cells, and this directly correlates with the impairment of $CD4^+$ T-cell proliferation.[10] Tregs are also present in the GBM microenvironment,[3,27] likely secondary to gliomas elaborating CCL-2, a Treg chemokine CCL2.[29]

In many systemic, non–central nervous system (CNS) cancers, the presence of Tregs in the tumor is an unfavorable prognostic marker[14,24,32]; however, this is not universally seen in all malignancies.[17] The prognostic role of Tregs present in gliomas has not been previously evaluated, and therefore we determined the incidence and prognostic significance of $FoxP3^+$ Tregs in various pathologies and grades. A glioma tissue microarray was assembled from archived paraffin-embedded tumors containing 52 GBMs (World Health Organization [WHO] grade IV), six gliosarcomas (WHO grade IV), 19 anaplastic astrocytomas (WHO grade III), three low-grade astrocytomas (WHO grade II), 21 oligodendrogliomas (WHO grade II), 16 anaplastic oligodendrogliomas (WHO grade III), five mixed oligoastrocytomas (WHO grade II), and 13 anaplastic mixed oligoastrocytomas (WHO grade III). This previously described array[22] also contained normal brain tissue (white matter, cortex, and cerebellum) and was stained for CD3, CD4, CD8, and FoxP3 (Dr. Nobuyoshi Hiraoka) as previously described.[23,24] The number of infiltrating immune cells was determined in duplicate from different areas of the same tumor by four independent observers in a blinded fashion. The duplicate specimens from each tumor were then averaged to calculate the final number of $CD3^+$, $CD4^+$, $CD8^+$, and $FoxP3^+$ lymphocytes per surgical specimen. An equal-proportion examination with respect to tumor grade, pathological type, and glial lineage (astrocytic versus oligodendroglial) was conducted.[49] Kaplan-Meier product-limit probability estimates of overall survival (OS) were calculated,[30] and log-rank tests[40] were performed to compare OS according to FoxP3 positivity (versus FoxP3 negativity), tumor grade, astrocytic, and oligodendroglial lineage, and sex. In each fitted OS 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 performed using t tests, assuming unequal variances with statistical significance set at 0.05.

In the 135 study patients, parameters such as age and Karnofsky Performance Scale were similar to those of patients in previous studies that examined prognostic markers in glioma patients.[4,21,22] Of the GBM patients, nine (17%) had received previous chemotherapy and 11 (21%) had received previous radiation therapy. *Table 18.1* details the overall

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composition of the glioma tissue microarray and *Figure 18.1* demonstrates the immunohistochemistry staining of the infiltrating immune populations. A $CD8^+$ cell population was identified in the majority of the glioma specimens regardless the grade (*Fig. 18.2A*); however, the number of patients who had a $CD4^+$ population present increased with tumor grade from 39% (7/18) for WHO grade II to 73% (24/34) for WHO grade III and 98% (44/45) for grade WHO grade IV ($P < 0.001$; across all grades). The absolute number of both $CD4^+$ and $CD8^+$ tumor-infiltrating T cells increased with tumor grade (*Fig. 18.2B*). Specifically, in WHO grade II tumors, there was an average number of 1.4 (standard deviation [SD] = 2.5; range, 0–10) $CD4^+$ 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 grades II and IV). Similarly, in WHO grade II tumor, there was an average number of 8.6 (SD = 6.0; range, 0–22 $CD8^+$) 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).

In the case of $FoxP3^+$ staining, no Tregs were present 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 1 to 3 $FoxP3^+$ Tregs per core. Additionally, $FoxP3^+$ Tregs were barely discernable in the low-grade astrocytoma specimens (WHO grade II; n = 3). In contrast, there was positive staining of FoxP3 in 10 (53%) of the 19 anaplastic astrocytoma specimens (WHO grade III), 39% of the 13 anaplastic mixed oligoastrocytomas (WHO grade III), 48% of the 52 GBMs (WHO grade IV), and 83% of the six gliosarcomas (WHO grade IV). These cumulative data indicate that Treg infiltration is more prevalent in tumors of the astrocytic lineage compared with the oligodendroglial lineage ($P < 0.0001$) (*Table 18.2*). Furthermore, the highest grade astrocytic tumors (WHO grades III and IV) had the highest numbers of $FoxP3^+$ Tregs.

Although the number of patients with GBM who had $FoxP3^+$ Tregs in their tissue cores was not significantly

TABLE 18.1. Composition of the Glioma Tissue Microarray[a]

Lineage	No. (%)	Pathology	No. (%)
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)

[a]O, oligodendroglioma; MOA, mixed oligoastrocytoma; AMOA, anaplastic mixed oligoastrocytoma; AO, anaplastic oligodendroglioma; GBM, glioblastoma multiforme; GS, gliosarcoma; LGA, low-grade astrocytoma; AA, anaplastic astrocytoma.

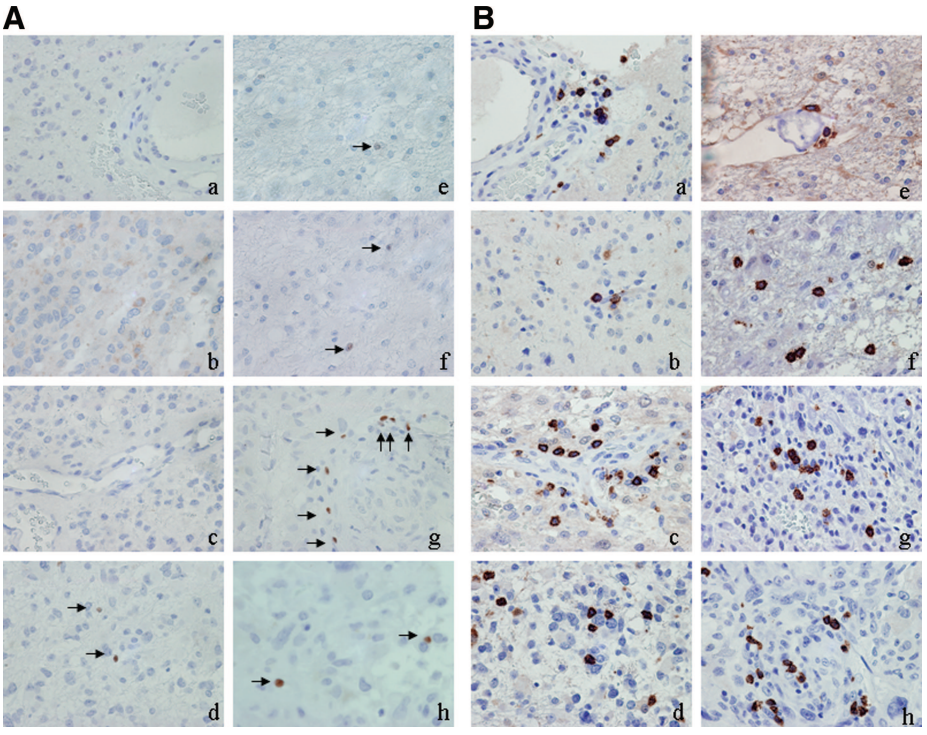

FIGURE 18.1. Immunohistochemical staining of human glioma tissue sections demonstrating FoxP3 (**A**) and $CD8^+$ (**B**) lymphoid cells. FoxP3 staining is confined to the nucleus, whereas CD8 staining is noted on the cell surface. **A,** Tregs are more evident in astrocytic higher grade gliomas. Arrows demonstrate $FoxP3^+$ cells. **B,** CD8 staining demonstrates high numbers of infiltrative $CD8^+$ T cells in all glioma grades. All images were taken at ×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*).

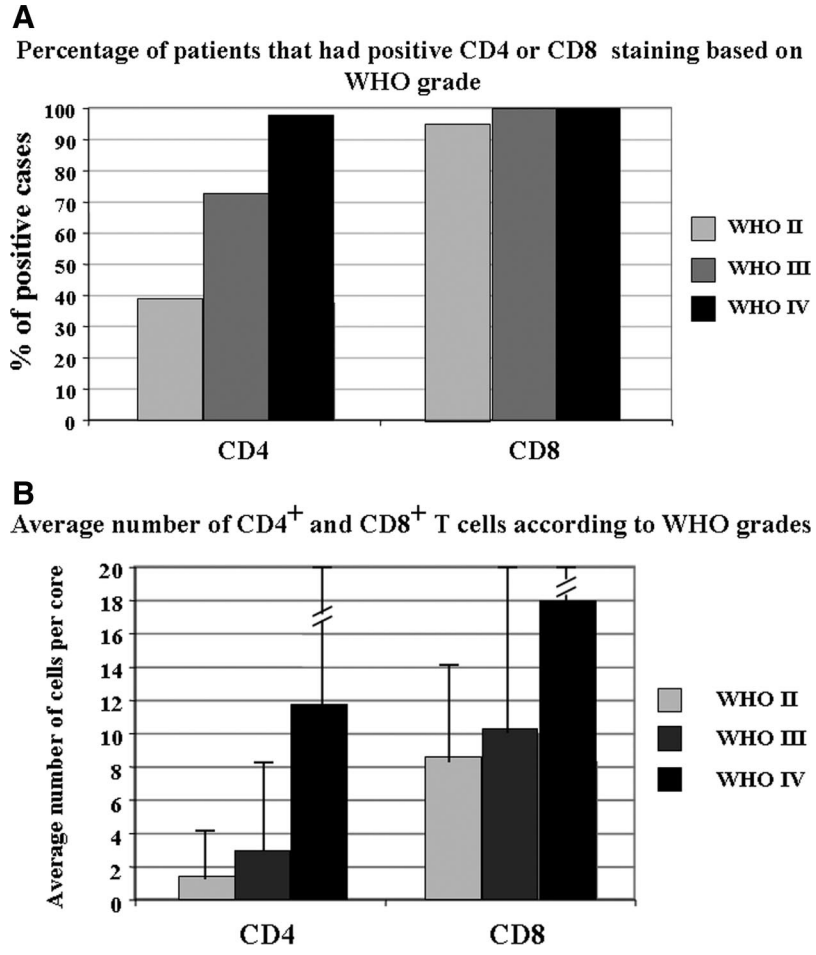

FIGURE 18.2. The incidence of $CD4^+$ T cells and the number of $CD4^+$ and $CD8^+$ T-cell increases with World Health Organization (WHO) tumor grade. **A,** $CD8^+$ cells were identified in the majority of the glioma specimens despite the grade; however, the number of patients who had a $CD4^+$ population 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.

TABLE 18.2. Proportion of Immunohistochemical $FoxP3^+$ Cases Stratified According to Pathology and World Health Organization Tumor Grade[a]

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)

[a]O, oligodendroglioma; MOA, mixed oligoastrocytoma; AO, anaplastic oligodendroglioma; AMOA, anaplastic mixed oligoastrocytoma; LGA, low-grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; GS, gliosarcoma.

different from that in patients with anaplastic astrocytoma, the number of $FoxP3^+$ Tregs present in the tumor of GBM patients (at least 5 per core; 7 [14%]) was markedly higher than that in the anaplastic astrocytoma patients. Thus, as the glioma grade increased, the number of cells that stained positively for FoxP3 increased ($P = 0.008$) (*Table 18.3*); this increase was even more pronounced in tumors of astrocytic lineage than in those of oligodendroglial lineage. These data are consistent with the findings of a previous study demonstrating that number of $FoxP3^+$ T cells increased in astrocytic glioma grade.[9]

Influence of Tregs on Survival in Glioma Patients

Because the presence of $FoxP3^+$ Tregs correlated with the overall malignant behavior of astrocytic tumors, we next determined whether the presence of $FoxP3^+$ Tregs was a prognosticator of survival in glioma pathologies. The median survival duration in patients with GBM who had intratumoral $FoxP3^+$ Tregs was 13.8 months (95% confidence interval [CI], 7.8–21.7), and in patients with GBM who did not have any intratumoral $FoxP3^+$ Tregs, it was 12.8 months (95% CI, 6.6–37.7) ($P = 0.56$) (*Fig. 18.3*). Although there was a trend of a higher probability of survival at 2 years in patients who did not have $FoxP3^+$ Tregs (0.32; 95% CI, 0.18–0.57)

TABLE 18.3. Differences in the Presence of FoxP3$^+$ Cells by Tumor Grade

Grade	Pathology	No. (%)
II	LGA	29 (21.5)
	O	
	MOA	
III	AA	48 (35.6)
	AO	
	AMOA	
IV	GBM	58 (43.0)
	GS	

Abbreviations: LGA, low-grade astrocytoma; O, oligodendroglioma; MOA, mixed oligoastrocytoma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AMOA, anaplastic mixed oligoastrocytoma; GBM, glioblastoma multiforme; GS, gliosarcoma.

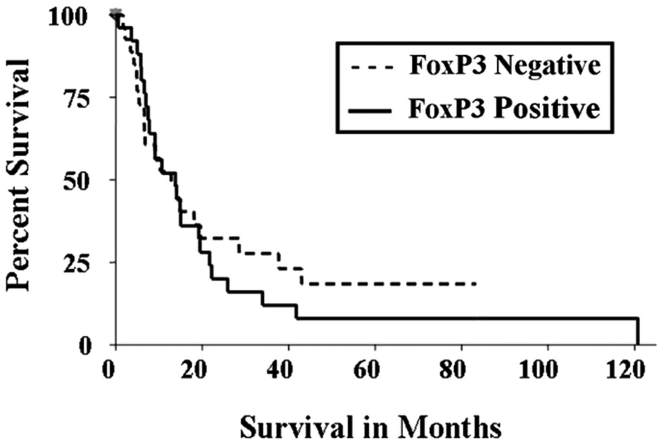

FIGURE 18.3. Kaplan-Meier survival estimates as stratified by the presence or absence of FoxP3$^+$ immunohistochemical staining in glioblastoma multiforme (GBM) patients. Median survival in GBM patients who had FoxP3$^+$ staining was 13.8 (95% confidence interval [CI], 7.8–21.7) months and was 12.8 (95% CI, 6.6–37.7) months in GBM patients who did not have FoxP3 staining, which is not statistically significant ($P = 0.56$).

compared with patients who had FoxP3$^+$ Tregs (0.2; 95% CI, 0.09–0.44), this difference was not statistically significant ($P = 0.65$). Univariate analysis demonstrated that the presence or absence of FoxP3$^+$ Tregs ($P = 0.03$) and the absolute number of FoxP3$^+$ Tregs per tumor sample ($P = 0.002$) were prognostic factors, similar to other established parameters, such as Karnofsky Performance Scale score, age, and tumor grade (*Table 18.4*). However, a multivariate analysis to account for confounding factors, such as patient age and Karnofsky Performance Scale score, demonstrated that FoxP3$^+$ did not have a prognostic impact. Specifically, neither the presence nor absence of FoxP3$^+$ Tregs ($P = 0.45$; hazard ratio, 1.2) nor the absolute number of FoxP3$^+$ cells

TABLE 18.4. Univariate Cox Proportional Hazards Model Estimates, Hazard Ratio, and Significance of the Study Variables[a]

Variable	Estimate	HR	*P* Value
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 (versus none)	0.49	1.64	0.03
Actual FoxP3$^+$ cell number	0.1	1.1	0.002

[a]HR, hazard ratio; KPS, Karnofsky Performance Scale.

($P = 0.35$; hazard ratio, 1.03) had a prognostic impact. On 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) and chemotherapy (96% for FoxP3$^+$ versus 93% for no FoxP3$^+$ staining). We cannot come to a meaningful statistical conclusion regarding the prognostic impact of FoxP3$^+$ Tregs on survival for many of the other pathological types of gliomas because of the relative infrequency of FoxP3$^+$ Tregs in these tumors.

A hypothesized parameter that may be more predictive of prognosis than solely the presence of Tregs is the balance between cytotoxic and regulatory T cells (i.e., the effector-to-suppressor ratio). Other investigators[2,14,47] reported that this ratio is more valuable for prognostic purposes than the presence of a single tumor-infiltrating lymphocyte subset. 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). This lack of prognostic influence is likely secondary to multiple redundant immunosuppressive mechanisms in the glioma patients. Although some cancers may mediate immunosuppression predominantly by using Tregs, researchers have shown that high-grade gliomas have multiple mechanisms for mediating immunosuppression[8]; thus, the lack of a prognostic impact of a single mechanism of immune suppression such as the presence or absence of FoxP3$^+$ Tregs in this setting is not entirely surprising. Furthermore, the ratio of FoxP3$^+$ Tregs to CD8$^+$ T-cell effectors may not be valid as a prognosticator in gliomas because the effector cells in gliomas are not activated[27] and likely are not functional.[41,43] A further 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 in the systemic circulation[10] as a prognostic influence.

Inhibition of Tregs With STAT3 Blockade

Although the presence of Tregs was not an independent prognostic factor for survival in glioma patients, their influence on restraining antiglioma immune responses has been clearly established.[10,11,12] In a study of a syngeneic murine model of glioma, in vivo depletions of Tregs resulted in complete tumor rejection and markedly enhanced survival.[10] A variety of approaches to negating the negative immunomodulatory properties of Tregs in patients with glioma are being considered for clinical trials, including treatment with CTLA-4 blockade that confers resistance to Treg-mediated suppression,[11] an anti-CD25 antibody that binds to the surface of Tregs and disrupts their functional activity,[12] cyclophosphamide that selectively depletes the number of Tregs,[15,16] and temozolomide that inhibits Treg trafficking to the glioma.[29] Some of these aforementioned agents have already been used in clinical trials of patients with systemic cancers with notable progression of disease occurring in CNS.[45] The variability of the infiltrating Treg population within tumors and the systemic circulation[10] 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.

The Janus kinases/signal transducers and activators of transcription 3 (JAK/STAT3) pathway is a key signaling pathway that drives the fundamental components of tumorigenesis[37,53,54] and transduces extracellular signals such as the epidermal growth factor receptor (EGFR) and interleukin-6, which is expressed in the CNS. The STAT3 protein is overexpressed in most cancers, including gliomas,[1] and fosters tumorigenesis by preventing apoptosis and enhancing proliferation, angiogenesis, and invasiveness[25,53] and is a key regulator of immunosuppression in cancer patients.[36,54] Previous studies in mice have shown that the ablation of STAT3 in the hematopoietic system was accompanied by a reduction in the number of tumor-infiltrating Tregs.[36] STAT3 has also been shown to be required for both transforming growth factor β and interleukin-10 production by $CD4^+$ T cells,[31] factors necessary for the generation of tumor-associated Tregs. Interleukin-2 has been shown to regulate FoxP3 expression in human $CD4^+CD25^+$ Tregs by STAT3 binding of the first intron of the *FoxP3* gene.[55] Thus, blockade of the STAT3 pathway should theoretically potently inhibit Tregs and reverse immunosuppression while exerting a direct antitumor effect, the latter of which is a property that the other previously mentioned Treg inhibitory agents either lack or is minimal.

WP1066, a novel low molecular weight agent, has been shown to effectively block the JAK2/STAT3 pathway[6,13,18,28,34,38] and can inhibit glioma (U-87) growth in vivo.[28] WP1066 can penetrate the CNS in mice and, in physiologically relevant doses in vitro, reverse tolerance in immune cells isolated from GBM patients.[26] WP1066 activates the immune system by inducing expression of costimulatory molecules, stimulating the production of the immunostimulatory cytokines, and inducing T-cell effector responses, even in cancer patients who are refractory to CD3 stimulation.[26]

To evaluate whether WP1066 could improve survival in mice, C57BL/6J mice with intracerebral B16EGFRvIII were treated by oral gavage with WP1066 starting on day 3 after tumor cell implantation. Kaplan-Meier product-limit survival probability estimates of OS were calculated[30] and log-rank tests[40] were performed to compare OS between treatment groups and the control arm. A P value of ≤ 0.05 was considered statistically significant. The median survival time for the control group was 17.5 days (95% CI, 16 to not available). For the mice treated by oral gavage with WP1066 at 40 mg/kg (n = 10), 80% survived long term (>78 days) ($P < 0.0001$ compared with the control group), and there was at least a 324% increase in median survival time when the experiment was terminated to perform tumor rechallenge experiments (*Fig. 18.4A*).

To determine whether mice with intracerebral tumors treated with WP1066 were able to generate long-lasting protection against tumor regrowth, mice that survived for 78 days after the initial tumor cell implantation were reinoculated with B16EGFRvIII cells, but in the contralateral hemisphere. On this rechallenge, in the animal group that had received WP1066 by oral gavage, the median survival time was 18 days (95% CI, 17 to not available), which was significantly different from 11 days, the median survival time of naïve animals challenged at the same time (95% CI, 10 to not available; $P < 0.001$); however, only 10% of the mice were long-term survivors (*Fig. 18.4A*). The longer median survival time compared with naïve control animals demonstrates a partial protective immune effect, yet, ultimately, these mice were not long-term survivors, indicating that maintenance of the immune effects of WP1066 will require sustained dosing if a tumor recurs.

To further clarify that the immune system is the primary mediator of the in vivo efficacy in the CNS, we implanted the B16EGFRvIII tumors in the CNS of nude animals and then treated with WP1066. In contrast, to our previous findings in which WP1066 resulted in marked long-term survival, in nude mice that had established intracerebral B16EGFRvIII, there was no enhancement of long-term survival with WP1066, and all animals died of progressive tumor in the CNS (*Fig. 18.4B*). Furthermore, in the syngeneic murine model when we performed in vivo depletions of the CD4 and CD8 T cell population (*Fig. 18.4C*), therapeutic

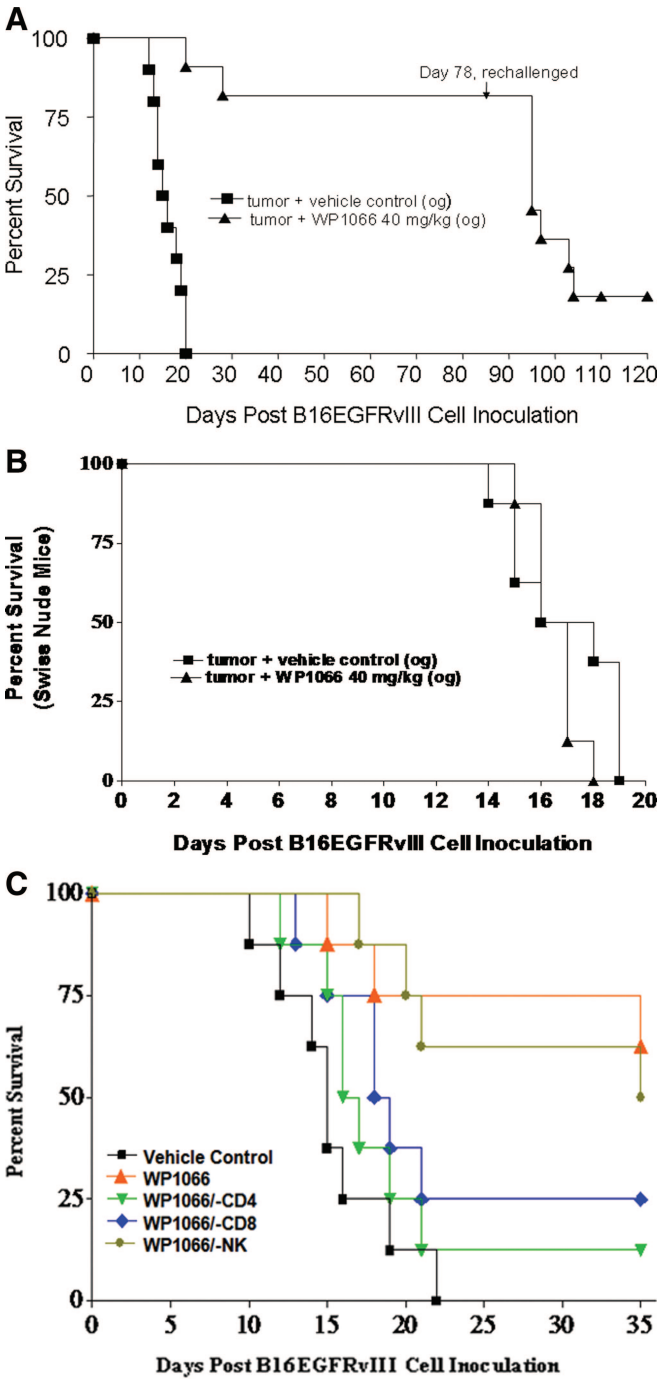

FIGURE 18.4. Survival data from C57BL/6J mice treated with WP1066 (WP) after intracerebral B16EGFRvIII cells were established in the brain. **A,** C57BL/6J mice with established intracerebral B16EGFRvIII cells treated with WP1066 via oral gavage (og) (n = 10) showed at least a 324% increase in their median survival time, and 80% achieved long-term survival compared with the vehicle-treated controls (n = 10). For the group treated with 40 mg/kg WP1066 by oral gavage, the *P* values were 0.04, 0.18, 0.007, 0.002, 0.001, and 0.001 at days 25, 30, 35, 40, 45, and 50, respectively, compared with the vehicle control group. In animals that survived longer than 78 days, subsequent rechallenge by injection of tumor cells into the contralateral hemisphere indicated that minimal immunological memory was induced. **B,** The therapeutic effect of WP1066 was lost when WP1066 was used to treat established B16EGFRvIII tumors in immunoincompetent nude mice. **C,** Monoclonal antibodies GK1.5 (anti-$CD4^+$), 2.43 (anti-$CD8^+$), and polyclonal anti-asialo GM1 (anti–natural killer) were injected once intravenously 3 days before tumor challenge and intraperitoneally every 5 days thereafter with pretitrated amounts of the antibody. The efficacy of WP1066 in suppressing intracerebral tumor was abrogated when $CD4^+$ or $CD8^+$ T cells were depleted. The experiment was terminated on day 35. NK, natural killer.

efficacy were lost. However, in vivo depletion of natural killer cells demonstrated no loss in therapeutic efficacy, suggesting that the tumor cytolytic mechanism may to be a major histocompatibility complex–restricted $CD8^+$ T cell–mediated cytolysis. Cumulatively, these data indicate that the immune system contributes to the clearance of CNS tumors with anti-STAT3 agents.

The underlying mechanism of WP1066-mediated CNS immune clearance of gliomas was further assessed by evaluating whether WP1066 could enhance humoral responses. C57BL/6J mice were vaccinated with phosphate-buffered saline (PBS), WP1066, PEP-3-KLH/CDX-110 (a 14-mer peptide spanning the epidermal growth factor variant III (EGFRvIII) that is currently in phase II testing for GBM patients; www.celldextherapeutics.com), PEP-3-KLH + WP1066, and PEP-3-KLH + complete Freund's adjuvant (positive control) and evaluated for humoral responses after the first and third vaccinations as described.[48] None of the animals treated with PBS (n = 3), PEP-3-KLH (n = 3), or PEP-3-KLH + complete Freund's adjuvant (n = 3) had detectable humoral responses to EGFRvIII after the first vaccination. EGFRvIII humoral responses were not detected in the mice that were vaccinated 3 times with PBS (n = 5), PEP-3-KLH (n = 5), or WP1066 alone (n = 4). As expected, mice that were vaccinated three times with PEP-3-KLH + complete Freund's adjuvant all (n = 5) produced significant quantities of immunoglobulin G antibody, ranging from 1,156 to 10,308 ng/mL. In contrast, mice treated with the combination of PEP-3-KLH + WP1066 (n = 5) showed no detectable EGFRvIII antibody responses (*Fig. 18.5A*). Furthermore, in animals (n = 8) that survived intracerebral tumor treatment with WP1066 (*Fig. 18.4A*); none demonstrated the induction of EGFRvIII-specific responses, indicating that WP1066 does not appear to exert antiglioma activity by EGFRvIII antibody responses.

However, WP1066 significantly enhanced cytotoxic responses directed against PEP-3. Splenocytes from naïve C57BL/6J mice and C57BL/6J mice vaccinated with PEP-3-KLH, WP1066, PEP-3-KLH + WP1066, and PEP-3-KLH + complete Freund's adjuvant were stimulated in vitro with

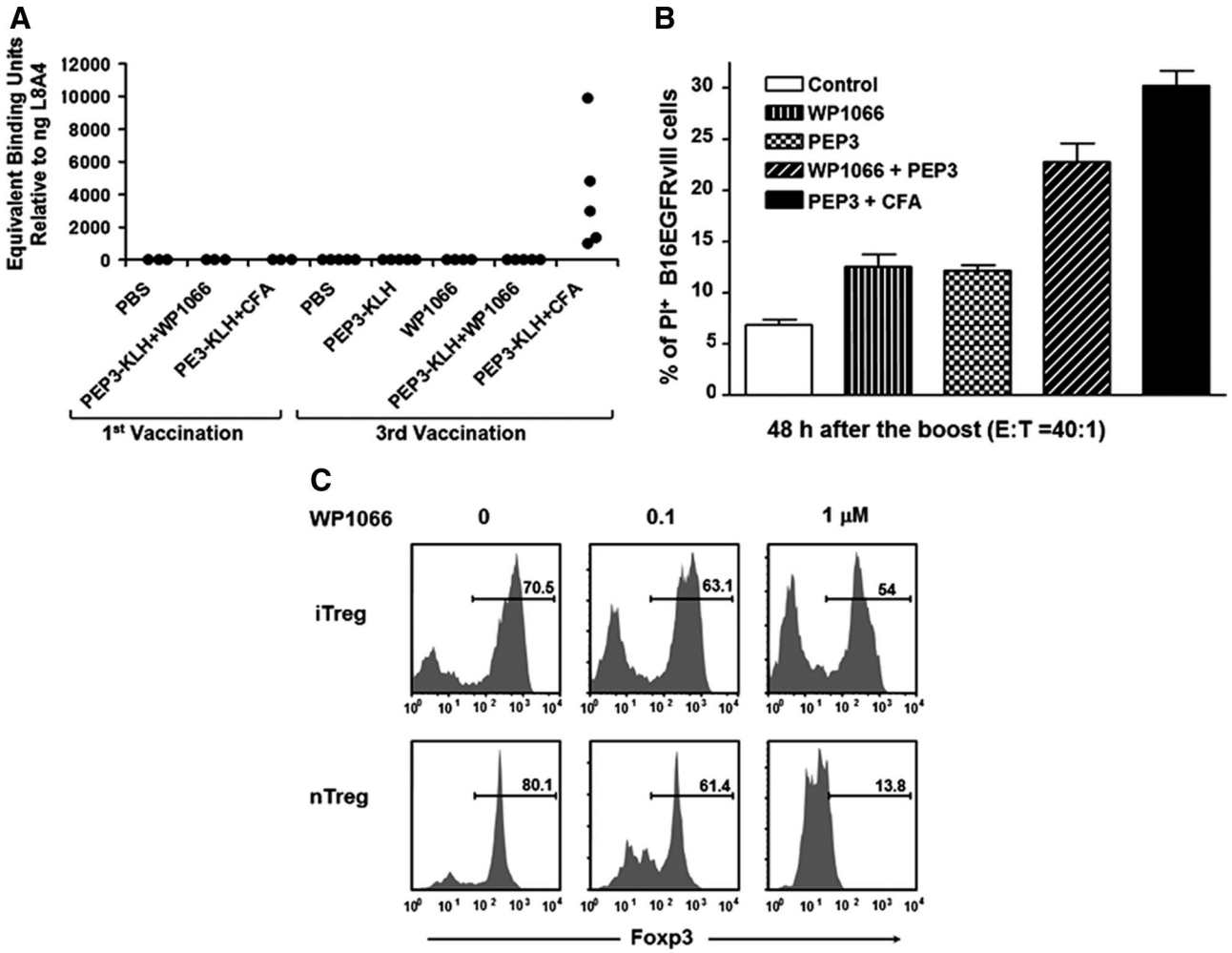

FIGURE 18.5. **A,** Humoral responses were not induced in mice vaccinated with PEP-3-KLH and WP1066 but were induced, as anticipated, with PEP-3-KLH and complete Freund's adjuvant (CFA). Phosphate-buffered saline (PBS), **B,** Cytotoxicity of the B16EGFRvIII cells in vitro produced by splenocytes from mice vaccinated with PEP-3-KLH or with PEP-3-KLH + WP1066. The splenocyte effector cells from mice that were vaccinated with PEP-3-KLH induced minimal lysis. However, splenocyte effector cells from mice that were vaccinated with PEP-3-KLH + WP1066 potently enhanced EGFRvIII-specific lysis ($P < 0.05$). Error bars show 1 standard deviation from mean values. **C,** WP1066 inhibits FoxP3 induction in T cells in peripheral blood and down-regulates FoxP3 in natural Tregs. $CD4^+CD25\text{-}CD62Lhi$ naïve T cells from C57BL/6J mice were stimulated by plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (2 μg/mL) in the presence of transforming growth factor β_1 (1 ng/mL) and interleukin-2 (200 U/mL) with 0, 0.1, and 1.0 μM WP1066 for inducible Tregs (iTreg) differentiation; $CD4^+CD25^+$ T cells (natural Tregs, nTreg) were stimulated by plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (2 μg/mL) in the presence of interleukin-2 (200 U/mL) with 0, 0.1, and 1.0 μM WP1066. Ninety-six hours after stimulation, the cells were analyzed for intracellular FoxP3 expression by flow cytometry. The percentage numbers for the indicated population are shown.

B16EGFRvIII cells, and cytotoxicity was assessed against carboxyfluorescein succinimidyl ester–labeled B16EGFRvIII target cells. The naïve mice showed minimal lysis of the B16EGFRvIII target cells. The PEP-3-KLH- or WP1066-treated mice had increased cytotoxic clearance of the B16EGFRvIII target cells compared with naïve mice (*Fig. 18.5B*). In mice treated with both PEP-3-KLH- and WP1066, there was further significant enhanced cytotoxic clearance of the B16EGFRvIII target cells compared with mice that were treated with either WP1066 or PEP-3-KLH alone ($P < 0.05$; *Fig. 18.5B*) indicating that WP1066 enhances direct tumor clearance.

Finally, to investigate the effects of WP1066 on the peripheral induction of $FoxP3^+$ Tregs, we used an in vitro Treg induction system in which FoxP3 expression was induced in naïve $CD4^+$ ($CD4^+CD25\text{-}CD62L^{hi}$) T cells isolated from the spleens of C57BL/6J mice.[33,35] $FoxP3^+$ Treg generation was directly measured by intracellular staining for

FoxP3 protein expression. In this system, naïve $CD4^+$ T cells underwent robust $FoxP3^+$ Treg differentiation when they were activated by polyclonal stimulation in the presence of exogenous transforming growth factor-β. In contrast, WP1066 inhibited $FoxP3^+$ Treg induction, compared with the control, from 70% to 54% with WP1066 (*Fig. 19.5C*). Moreover, WP1066 reduced $Foxp3^+$ natural Tregs (nTregs) to 13.8% under polyclonal stimulation (Fig. 5*C*). We also recently showed that WP1066 enhances $CD3^+$ (which contain Tregs) T cell–mediated tumor cytotoxicity but fails to do so when the Tregs were excluded, indicating that WP1066 does not primarily activate T-cell responses but secondarily via inhibition of Tregs.[33] In conclusion, WP1066 appears to inhibit the induction of Tregs, which contributes to the antitumor responses observed with its use in vivo.

Implications for Therapeutics

Patients with advanced malignancies are known to be profoundly immunosuppressed and even if a systemic immune response can be generated, it is likely negated in the tumor microenvironment by a wide variety of factors including Tregs.[9,10,27] STAT3 blockade appears to be a highly promising therapeutic approach given the ability of WP1066 to inhibit Tregs, reverse immunosuppression, and favorably modulate the tumor microenvironment. WP1066 could potentially be used in combination with other immunotherapeutic approaches such as dendritic cells,[19] peptide vaccination,[20] and cytokine immunotherapy or adoptive immunotherapy. In addition to immunomodulatory properties, WP1066 also acts directly on the process of tumorigenesis by inhibiting the phosphorylation of STAT3 and the subsequent downstream molecules, such as survivin and c-Myc. Given its oral bioavailability, inherent immunomodulatory properties, direct tumor cytotoxicity activity, ability to enter the CNS, and efficacy against established CNS tumors, WP1066 is a compelling agent for further development and translation to applications for patients with gliomas.

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Disclosure

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