Increased Regulatory T-Cell Fraction Amidst a Diminished CD4 Compartment Explains Cellular Immune Defects in Patients with Malignant Glioma

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Abstract

Immunosuppression is frequently associated with malignancy and is particularly severe in patients with malignant glioma. Anergy and counterproductive shifts toward T_H2 cytokine production are long-recognized T-cell defects in these patients whose etiology has remained elusive for >30 years. We show here that absolute counts of both $CD4^+$ T cells and $CD4^+CD25^+FOXP3^+CD45RO^+$ T cells (T_{regs}) are greatly diminished in patients with malignant glioma, but T_{regs} frequently represent an increased fraction of the remaining CD4 compartment. This increased T_{reg} fraction, despite reduced counts, correlates with and is sufficient to elicit the characteristic manifestations of impaired patient T-cell responsiveness *in vitro*. Furthermore, T_{reg} removal eradicates T-cell proliferative defects and reverses T_H2 cytokine shifts, allowing T cells from patients with malignant glioma to function *in vitro* at levels equivalent to those of normal, healthy controls. Such restored immune function may give license to physiologic antiglioma activity, as *in vivo*, T_{reg} depletion proves permissive for spontaneous tumor rejection in a murine model of established intracranial glioma. These findings dramatically alter our understanding of depressed cellular immune function in patients with malignant glioma and advance a role for T_{regs} in facilitating tumor immune evasion in the central nervous system. (Cancer Res 2006; 66(6): 3294-302)

Introduction

Patients with malignant glioma exhibit a comprehensive depression in cellular immune function that has been documented for more than three decades (reviewed in ref. 1). Although malignant gliomas remain within the intracranial compartment and rarely metastasize, immunosuppression in patients is both systemic and profound. Deficiencies in T-cell function are especially well-documented and patient lymphocytes respond poorly to T-cell mitogens, anti-CD3 monoclonal antibody, and T-dependent B-cell mitogens (2, 3). Although present, neither lymphopenia nor immunosuppressive factors present in the sera of patients entirely explain these deficits, as purified T cells retain a significant degree of nonresponsiveness even when cultured in normal sera (4, 5). A substantial measure of dysfunction is therefore intrinsic to the T-cell compartment. More specifically, this dysfunction seems manifested within the $CD4^+$ T-cell subset (6), whose relative anergy is characterized most broadly by weakened proliferative responses and insufficient synthesis of the T_H1 cytokine interleukin 2 (IL-2; refs. 7, 8). Production of other T_H1-type cytokines also suffers, whereas T_H2-type cytokines are typically elaborated at elevated levels (9, 10). The etiology of these T-cell proliferative defects and T_H2 cytokine shifts remains poorly understood.

We noted that the cellular immune defects delineated for patients with malignant glioma show remarkable overlap with the typical consequences of regulatory T-cell (T_{reg}) activity. T_{regs} are a physiologic subset of $CD4^+$ T cells expressing high levels of CD25 (IL-2Rα; ref. 11), and they potently inhibit T-cell activation and proliferation. Some of this is achieved through down-regulation of IL-2 production in their target cells (12), a predilection that recapitulates a central component of the T-cell anergy observed in patients with malignant glioma (7, 8). T_{regs} also inhibit IFN-γ production (13, 14) and prompt target cells to secrete predominantly T_H2 cytokines (15), which can propagate the regulatory phenotype (16, 17). T_{regs} may elicit tolerance to tumor antigens and supraphysiologic T_{reg} fractions have been found in the peripheral blood and tumors of patients with a variety of cancers (18–21).

We examine the contribution made by T_{regs} to depressed cellular immunity in patients with glioblastoma multiforme (GBM), the most common and malignant form of malignant glioma. We find that T_{regs} are actually reduced in absolute number in these patients who exhibit a substantial reduction in the number of circulating $CD4^+$ T cells. T_{regs}, however, come frequently to represent an increased fraction of the remaining CD4 compartment. Patients with increased T_{reg} fractions are those that show diminished lymphocyte responsiveness *in vitro*, and increasing T_{reg} fractions among normal lymphocytes to such elevated levels proves sufficient to elicit the same impairments. Most importantly, removal of T_{regs} *in vitro* eradicates the differences in responsiveness observed among $CD4^+$ T cells isolated from patients and healthy volunteers and reverses the predilection toward T_H2 cytokine production.

Together, these results indicate that patient $CD4^+$ T cells are capable of normal immune function, but are instead hindered by T_{regs}, increased fractions of which seem necessary and sufficient to produce much of the "intrinsic" T-cell dysfunction in patients with GBM. Restored normal immune function may consist of physiologic antiglioma activity, as the depletion of T_{regs} *in vivo* leads frequently to spontaneous tumor rejection in a murine model of intracranial glioma. These data dramatically alter our approach toward understanding and addressing depressed cellular immune function in patients with GBM.

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Materials and Methods

Human samples. All samples were obtained at Duke University Medical Center from individuals who had given written, informed consent. Histopathologic diagnoses were established by the Department of Pathology. Those eligible underwent 4-hour leukapheresis at the Duke Apheresis Unit to obtain peripheral blood mononuclear cells (PBMC). All studies were done with approval of the institutional review board.

Fluorescence-activated cell sorting analysis of whole blood. Whole blood was incubated at room temperature for 15 minutes with combinations of titrated antibodies to CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD45RO (UCHL1), and CD25 (M-A251; BD Biosciences, San Jose, CA). Following incubation, Optilyse B (Immunotech, Marseilles, France) was added to each sample. Cells were reincubated at room temperature for 15 minutes and diluted with distilled water. Samples were analyzed within 48 hours on a FACSVantage SE flow cytometer (BD Biosciences). Data analysis was done using BD FloJo software.

Fluorescence-activated cell sorting analysis of tumor. Tumor samples were subjected to overnight digestion at room temperature in 0.1% collagenase (Sigma, St. Louis, MO). Following digestion, RBCs were lysed with 8.4 mg/mL NH_4Cl (Sigma). Cells were washed, counted, and labeled with biotinylated anti-(α)-CD45 (HI30; BD Biosciences), followed by incubation with antibiotin microbeads (Miltenyi Biotec, Auburn, CA) and positive selection using AUTOMACS (Miltenyi Biotec). Enriched $CD45^+$ cells (leukocytes) were subsequently stained with the same antibodies as those for whole blood. Cells were analyzed as above.

T_{reg} retrieval from leukapheresis. Leukapheresis samples were diluted, underlayered with Ficoll (Histopaque 1077, Sigma), and spun for 25 minutes. Interphases were collected, washed extensively, and subjected to a 2-hour adherence step to remove monocytes. Nonadherent PBMCs were frozen until needed. Upon use, cells were rested overnight at 37°C, 5% CO_2. A $CD4^+$ T-Cell Isolation kit II (Miltenyi Biotec) was used to isolate untouched $CD4^+$ cells according to the instructions of the manufacturer. Briefly, a biotinylated antibody cocktail specific for non-$CD4^+$ cells (CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, and glycophorin A) was added, followed 10 minutes later by antibiotin microbeads. Samples were washed and run over AUTOMACS set to program DEPLETE. Nonlabeled fractions ($CD4^+$) were labeled with phycoerythrin-α-CD25 (M-A251) and FITC-α-CD45RO (UCHL1) and sorted into $CD25^+CD45RO^+$ (T_{reg}) and $CD25^-$ populations on a FACSVantage SE flow cytometer. Purity of obtained populations was always >98%.

FOXP3 reverse transcription-PCR. Purified T_{regs} and $CD4^+CD25^-$ T cells were retrieved from leukapheresis samples as above. cDNA was synthesized from appropriate amounts of each cell type (stored at −137°C) by oligo(dT) with uMACS One-Step cDNA kit (Miltenyi Biotec) according to the protocols of the manufacturer. Human FOXP3 (hFOXP3) mRNA expression levels were measured by real-time PCR detected with SYBR Green probe (Bio-Rad, Hercules, CA) on a Bio-Rad iCycler in 25 μL PCR reaction at 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, 72°C for 30 seconds. Each sample was run in triplicate and normalized with human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH). All primers spanned intron/exon boundaries to minimize genomic DNA amplification. hFOXP3 PCR products (100 bp) were amplified from hFOXP3-3 (5′-GAAACAGCACATTCCCAGAGTTC-3′) and hFOXP3-4 (5′-ATGGCCCAGCG-GATGAG-3′), and 114 bp hGAPDH were produced from hGAPDH-1 (5′-CCACATCGCTCAGACACCAT-3′) and hGAPDH-2 (5′-GGCAACAATATC-CACTTTACCAGAGT-3′). The PCR products were confirmed with melting curve during real-time PCR and loaded onto a 2% agarose gel to verify size.

Intracellular staining for FOXP3. PBMCs were labeled with antibodies to relevant surface markers (CD3, CD4, CD25, and CD45RO). Following incubation, cells were washed and incubated for 1 hour with Fix/Perm buffer (eBioscience, San Diego, CA). Cells were subsequently washed and labeled with anti-FOXP3 (PCH101, eBioscience) for 30 minutes in the dark at 4°C in the presence of permeabilization buffer (eBioscience). Samples were washed and analyzed on an LSRII flow cytometer (BD Biosciences).

T_{reg} proliferation and suppression assays. To verify anergy (proliferation assay) in the T_{reg} population, 1×10^5 T_{regs} or $CD4^+CD25^-$ cells were plated alone in triplicate wells using either immobilized or soluble α-CD3 (OKT3) as stimulator at 2 μg/mL. To verify suppressive capacity among T_{regs} (suppression assay), 5×10^4 $CD4^+CD25^-$ responders were plated alone or with varying doses of T_{regs} to a maximum ratio of 1:1 T_{regs}/responders. Complete T-cell medium (CTM) consisted of RPMI + 10% FCS, supplemented with HEPES buffer, sodium pyruvate, penicillin/streptomycin, L-glutamine, β-mercaptoethanol, and nonessential amino acids. After 72 hours of culture, levels of proliferation were assessed by [^{3}H]thymidine uptake.

$CD4^+$ T-cell cultures. Untouched $CD4^+$ T cells were cultured in triplicate in CTM (or in CTM with autologous serum substituted for FCS) at 5×10^4 to 2×10^5 per well in 96-well plates using either 0.4, 2, or 10 μg/mL soluble or plate-bound α-CD3 (OKT3); 0.1, 1, 5, 10, or 20 μg/well soluble phytohemagglutinin (PHA); or 0.1, 1, 5, 10, 20 μg/well soluble concanavalin A (ConA) as stimulator.

$CD25^+$ cell depletion from $CD4^+$ T cells. $CD25^+$ depletion was done using CD25 Microbeads (Miltenyi Biotec) according to the instructions of the manufacturer. Briefly, $CD4^+$ T cells were incubated with 20 μL beads per 10^7 cells for 15 minutes, washed, and depleted over AUTOMACS using the program DEPLETES. Negative fractions ($CD4^+CD25^-$) were collected for parallel culture with $CD4^+$ T cells.

[^{3}H] counting. In all experiments assessing proliferation, after 72 hours of culture, 1 μCi [^{3}H]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). [^{3}H] counts were done using a Wallac 1450 Microbeta Trilux Liquid Scintillation/Luminescence Counter (Perkin-Elmer). Data were taken as means of triplicate wells.

Cytokine analysis. $CD4^+$ T cells were cultured in 96-well plates with 0, 5, or 10 μg PHA as stimulator. At time points following 16 and 72 hours of culture, supernatants were harvested and processed in duplicate with a custom Bio-Rad Bio-Plex 7-plex [IL-2, IL-4, IL-6, IL-10, IL-12 (p70), IFN-γ, tumor necrosis factor-α (TNF-α)] Cytokine Reagent kit (Bio-Rad) according to the instructions of the manufacturer. Briefly, supernatants were incubated with anticytokine-conjugated beads, followed by incubation with biotinylated detection antibody. Reaction mixture was detected with streptavidin-phycoerythrin and analyzed on a Luminex 100 machine (Luminex Corporation, Austin, TX). Unknown cytokine concentrations were calculated by BioPlex Manager software using standard curves derived from a recombinant cytokine standard.

Intracranial tumor implantation. SMA-560 tumor cells were harvested in logarithmic growth phase. Tumor cells in PBS were then mixed 1:1 with 3% methylcellulose and loaded into a 250 μL syringe (Hamilton, Reno, NV). The needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments, Tujunga, CA). Cells (1×10^4) were delivered in a total volume of 5 μL.

Statistical analysis. For comparisons of means among groups (i.e., T_{reg} levels in patients versus healthy volunteers or proliferation levels in patients with and without increased T_{reg} fraction), unpaired t tests were used. A paired t test was used to compare cytokine levels in patients before and after CD25 depletion. For correlation between T_{reg} levels and proliferation levels, a Pearson coefficient was calculated using the null hypothesis that the coefficient was equal to zero. To compare proliferative response curves among groups, a generalized linear model for normal data that accounted for correlation of measurement replication within subjects was used. For *in vivo* experiments, the survival experiences among defined experimental groups were compared with a log-rank test. Within each experimental group, the survival experience was described using the Kaplan-Meier estimator.

Results

T_{reg} fraction is increased in patients bearing GBM. We determined the T_{reg} fraction in the peripheral blood of patients with GBM. Based on recent studies of FOXP3 expression in T-cell subsets (22), "T_{regs}" were defined as lymphocytes that possessed the

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cell surface phenotype $CD4^+CD25^+CD45RO^+$. Defining a surface phenotype permitted subsequent sorting for verification of function. Cells included under this definition showed appropriate levels of FOXP3 expression by reverse transcription-PCR (RT-PCR) and by intracellular staining for flow cytometry (78.10 ± 7.26% of $CD4^+CD25^+CD45RO^+$ cells were $FOXP3^+$; Fig. 1*A* and *B*). Conversely, our results also confirmed that FOXP3 resides specifically (95.58 ± 2.24% of $FOXP3^+$ cells) within the $CD25^+CD45RO^+$ subset of $CD4^+$ T cells (Fig. 1*B*). Sorted $CD4^+CD25^+CD45RO^+$ T cells from patients showed requisite *in vitro* anergy (Fig. 1*C*) and suppressive function (Fig. 1*D*) when entered into standard assays. T_{reg} "fraction" was thus defined as the percentage of $CD4^+$ T cells that coexpressed CD25 and CD45RO by flow cytometry. To reduce the contribution of potentially confounding variables introduced by surgery, T_{reg} fraction was assessed in patients at preresection time points.

T_{reg} fraction was determined in the peripheral blood of 20 patients with GBM (10 newly diagnosed, 10 recurrent, median age 50.5 years, range 31-67 years), and the mean value was compared initially to the mean fraction in blood obtained from 10 healthy, age-matched volunteers (median age 42.5 years, range 31-62 years). The proportion of T_{regs} in the peripheral blood of patients with GBM (15.94 ± 2.20%) was 2.63 times that found in the blood of normal volunteers (6.07 ± 0.39%, $P = 0.004$; Fig. 2*A*). This increase also corresponded to a significant shift in mean fluorescence intensity in the CD25 channel ($P = 0.02$, data not shown). Increased T_{reg} fraction was not present in all patients as can be observed in the data distribution plot. A representative analysis is depicted in Fig. 2*B*. Of note, and as may be seen in the analysis, the majority of $CD4^+CD25^+$ T cells in both patients in healthy volunteers were $CD45RO^+$. Overall, among the healthy volunteers enrolled, the mean percentage of $CD4^+CD25^+$ T cells that were $CD45RO^+$ was 88.53% with a range from 69.84% to 98.80%. In patients with GBM, the mean did not differ significantly at 83.33% ($P = 0.6673$).

A protocol was also devised for detecting and quantifying T_{regs} among tumor-infiltrating lymphocytes (TIL) in GBMs. No significant difference between T_{reg} fraction in blood and tumor was detected in any patient or across patients (data not shown). Using an unpaired *t* test, the *P* value for comparison of mean blood and tumor fractions across patients was 0.88. Despite a small sample size ($n = 4$), this test had >95% power to detect differences as small

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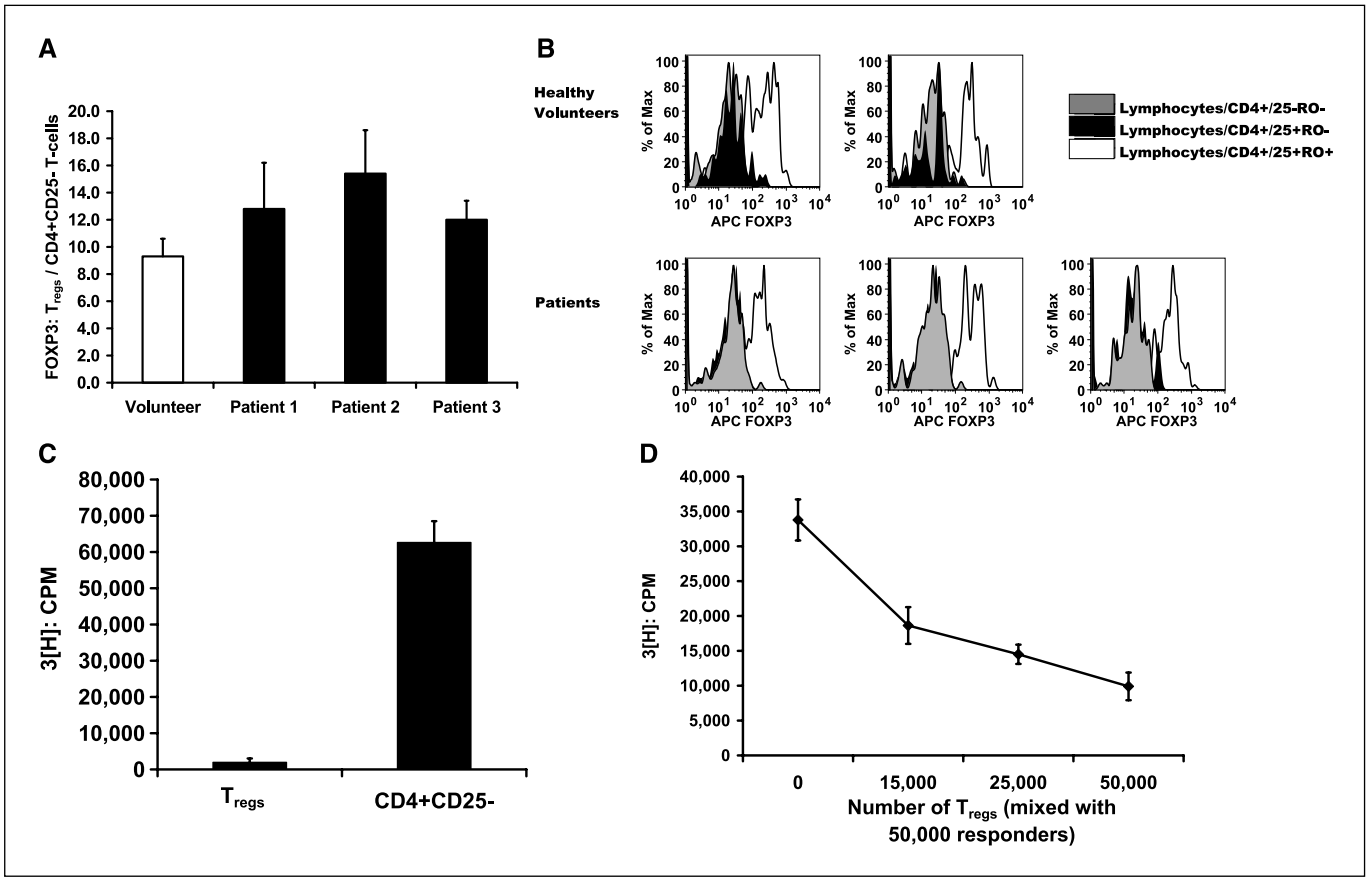

Figure 1. Verification of FOXP3 expression and T_{reg} function. *A,* FOXP3 semiquantitative RT-PCR. $CD4^+CD25^+CD45RO^+$ T_{regs} and $CD4^+CD25^-$ lymphocytes were purified from leukaphereses obtained from eight patients with GBM and three healthy volunteers. RT-PCR for FOXP3 was done normalizing to GAPDH. Representative ratios of FOXP3 expression in T_{regs} to $CD4^+CD25^-$ cells is depicted for one experiment analyzing levels in three patients and one healthy volunteer (done in triplicate). In all experiments, ratios in patients were greater than or equal to those in volunteers. *B,* confirmation that FOXP3 expression localizes to $CD4^+CD25^+CD45RO^+$ T cells by intracellular flow cytometry. Representative panels from two healthy volunteers ($n = 10$) and three patients with GBM ($n = 20$). All panels depict $CD4^+$ lymphocytes. FOXP3 expression is compared in $CD25^-CD45RO^-$ (*gray*), $CD25^+CD45RO^-$ (*black*), and $CD25^+CD45RO^+$ (*white*) subsets. The $CD25^+CD45RO^+$ subset accounts for 95.58 ± 2.24% of cells demonstrating FOXP3 staining. [Note that $CD25^-CD45RO^-$ and $CD25^+CD45RO^-$ populations are given different priorities (i.e., shown in front or back) in top and bottom panels to more clearly show their degree of overlap.] *C,* representative proliferation assay demonstrating relative T_{reg} anergy. T_{regs} (1×10^5) or $CD4^+CD25^-$ (1×10^5) cells were stimulated with anti-CD3 (OKT3). Proliferation after 72 hours was measured by [^{3}H]thymidine uptake. *Columns,* mean counts over triplicate wells; *bars,* SD. *D,* representative suppression assay (done in triplicate for each of eight patients) demonstrating ability of T_{regs} in patients with GBM to inhibit T-cell proliferation. $CD4^+CD25^-$ responder cells (5×10^4) and the indicated number of T_{regs} were plated in combination. Proliferation was measured as above. *Points,* mean counts over triplicate wells; *bars,* SD.

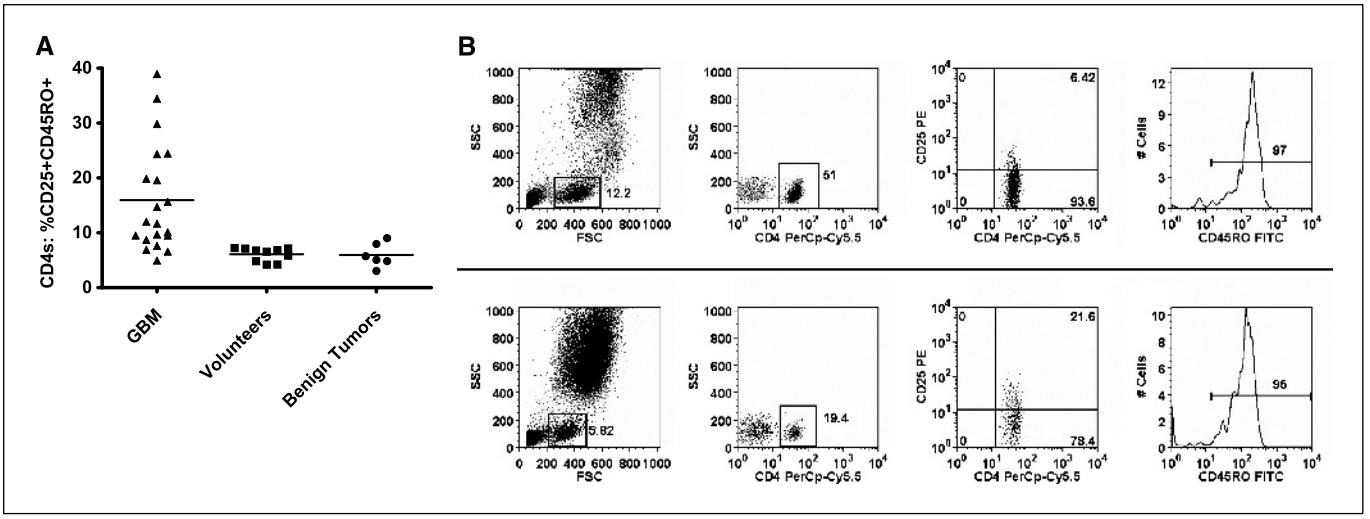

Figure 2. Flow cytometric detection of T_{regs} in peripheral blood. *A,* comparison of T_{reg} fraction in peripheral blood from patients with GBM (n = 20) with fraction in healthy volunteers (n = 10) and patients with benign intracranial tumors (n = 6; meningioma, n = 5; acoustic neuroma, n = 1). Data are expressed as the percentage of $CD4^+$ cells that are $CD25^+CD45RO^+$. *Horizontal hash bars,* mean levels for each group (volunteers = 6.07 ± 0.39%, GBM = 15.94 ± 2.20%, benign tumors = 5.94 ± 0.89%). Mean fraction in patients with GBM is significantly elevated over that in both volunteers (P = 0.004) and patients with benign intracranial tumors treated with equivalent doses of perioperative steroids (P = 0.023). *B,* representative schematic depicting flow cytometric analysis of a sample from a healthy volunteer (*top*) and a patient with GBM (*bottom*). *Leftmost panels,* detection and gating of lymphocytes on a forward versus side scatter plot and relative lymphopenia in patient with GBM. *Second panels,* detection and gating of $CD4^+$ lymphocytes. CD4 lymphopenia in the patient with GBM. *Third panels,* CD4 × CD25 staining on gated $CD4^+$ lymphocytes. *Fourth panels,* CD45RO staining on gated $CD4^+CD25^+$ lymphocytes. In this patient with GBM, 20.52% of $CD4^+$ cells (95% of 21.6%) were $CD25^+CD45RO^+$ T_{regs}. In the healthy volunteer, 6.23% were $CD25^+CD45RO^+$ T_{regs}.

as 5%. This suggests that T_{regs}, although present in GBMs, are not concentrated at the tumor site relative to the systemic circulation.

To eliminate the possibility that the T_{reg} fraction seen in blood was attributable to standard preoperative steroid treatments (23), patients receiving identical doses of pericraniotomy steroids but undergoing surgery for benign, nonglial intracranial tumors were similarly assayed preoperatively for T_{reg} fraction (n = 6, median age 50.5 years, range 40-82 years). The mean fraction observed above in patients with GBM was significantly elevated over the mean value determined for patients with benign tumors (5.94 ± 0.89%, P = 0.023). Despite steroid therapy, the values in patients with benign tumors did not vary significantly from those in healthy controls (P = 0.88; Fig. 2*A*). T_{reg} fraction in these patients also did not differ significantly from other patients with benign tumors not requiring steroid therapy (data not shown).

Reduction in absolute numbers of T_{regs}. To determine whether increased T_{reg} fraction in patients represents a true expansion of this compartment, complete blood counting with manual differentiation and calculation of $CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts was done with blood collected from healthy controls and from patients with GBM at preoperative time points. A severe $CD4^+$ T-cell lymphopenia was uncovered in the patients with GBM, who possessed a mean $CD4^+$ T-cell count of 168.2 ±50.0 cells/μL (n = 8) compared with a count of 668 ± 52.8 cells/μL determined for healthy volunteers (n = 6; P < 0.0001; reference range, 400-1,400 cells/μL; Fig. 3). Despite the elevated T_{reg} fractions among $CD4^+$ T cells in this subset of patients with GBM (mean 16.74%), such elevated fractions corresponded to an absolute T_{reg} count of only 19.2 ± 2.7 cells/μL. This number was less than half that found among healthy volunteers (40.8 ± 4.8 cells/μL, n = 6, P = 0.0011), although T_{regs} represented only 6.2% of $CD4^+$ T cells in this control population.

Increased T_{reg} fraction correlates with $CD4^+$ T-cell proliferative defects. As we noted that cellular immune defects delineated for patients with malignant glioma show remarkable overlap with the typical consequences of T_{reg} activity, we examined whether the uncovered elevations in T_{reg} fraction might be linked to the manifestation of T-cell proliferative defects in patients with GBM. Leukapheresis were obtained from eight patients with GBM and four healthy controls. T_{reg} fraction for each patient was determined, with significant elevations in T_{reg} fraction defined *a priori* as values exceeding 9.1% (mean normal value + 2 SD). Of the eight patients with GBM, five possessed significant elevations in their peripheral blood T_{reg} fractions (Fig. 4*A, top, white columns*).

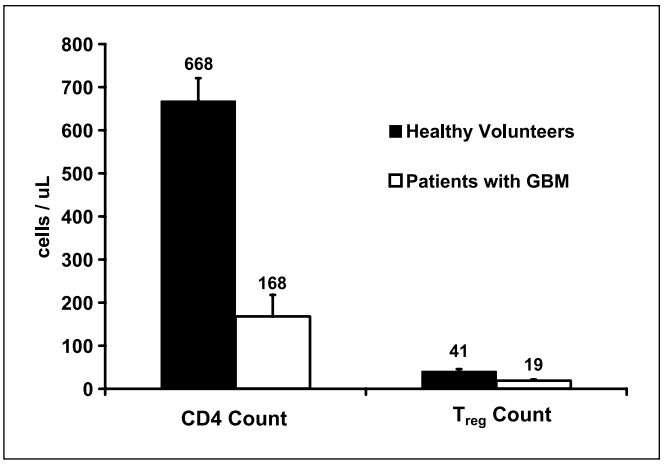

Figure 3. Absolute counts of $CD4^+$ T cells and T_{regs} in the peripheral blood of preresection patients with GBM and healthy controls. Absolute CD4 and T_{reg} counts were determined by flow cytometry in clinical laboratories for eight patients with GBM and six healthy volunteers. *Columns,* mean; *bars,* SE. Patients showed a marked CD4 lymphopenia (reference range for absolute CD4 counts = 400-1,400 cells/μL) such that T_{regs}, which represented an increased proportion of $CD4^+$ T cells on average, were still reduced in absolute number compared with healthy volunteers. Differences among volunteers and patients were significant with respect to both CD4 (P < 0.0001) and T_{reg} counts (P = 0.0011).

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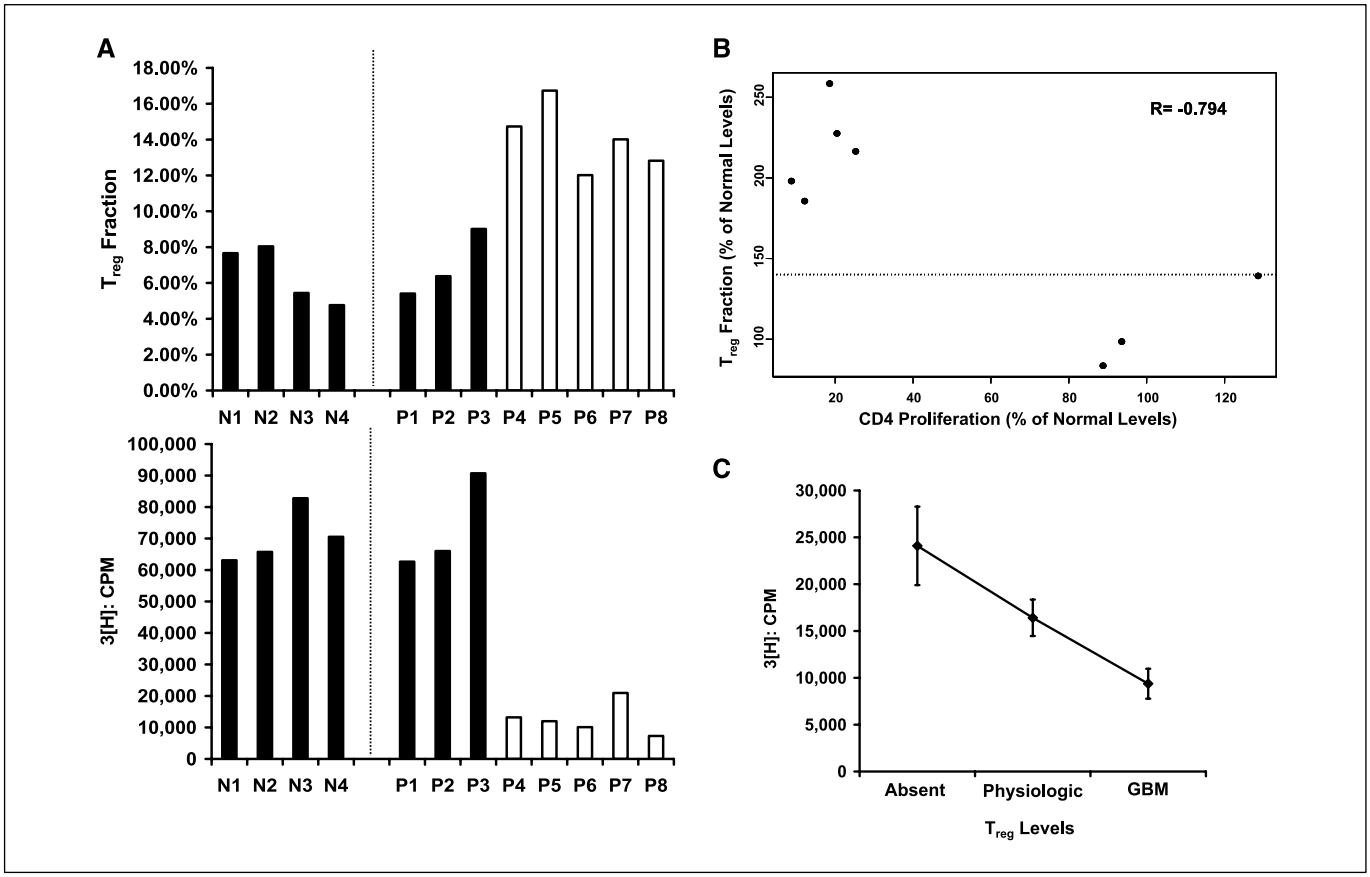

Figure 4. T_{reg} fraction correlates with proliferative defects among $CD4^+$ T cells in patients with GBM. *A,* T_{reg} fraction (*top*) and CD4 proliferation levels (*bottom*) in eight patients with GBM and four healthy controls who underwent leukapheresis to examine $CD4^+$ T-cell proliferative responses. Five patients (*white columns*) showed significantly elevated T_{reg} fractions (>2 SD above normal mean; range, 12.02-16.73%, $P = 0.0003$), and these patients had CD4 proliferation levels significantly below those of controls ($P < 0.0001$). *B,* scatter plot demonstrating negative correlation between T_{reg} fraction in patients and $CD4^+$ T-cell proliferation (each expressed as percentages of normal). The Pearson correlation coefficient for the plot is -0.794 ($P = 0.019$). *C,* increased T_{reg} fraction in patients is sufficient to elicit proliferative defects among normal $CD4^+$ lymphocytes. T_{regs} were isolated from leukaphereses taken from four healthy controls and mixed with constant numbers of autologous $CD4^+CD25^-$ cells to create T_{reg} percentages equivalent to those representing an absence of T_{regs} (0%), physiologic fraction of T_{regs} (8%), or the mean T_{reg} fraction in patients with GBM (16%). Cells were stimulated with anti-CD3. Increases in T_{reg} fraction to those levels found in patients with GBM were sufficient to produce significant deficits in the ability of T cells to proliferate. Representative result of three experiments with each of four donors.

The proliferative capacities of T-cell subsets from patients with GBM were assessed and compared with controls. To control for the existence of lymphopenia in patients and to therefore highlight only "intrinsic" proliferative defects within T-cell populations, equal numbers of patient and control cells were evaluated in a PHA-based proliferation assay.

Consistent with prior research, we found that patient T-cell proliferative defects were not present in all patients but, when present, were concentrated within the $CD4^+$ subset. The bottom panel of Fig. 4*A* plots the individual CD4 proliferation values for the same controls and patients whose T_{reg} fractions are depicted in the top panel. Interestingly, those patients possessing elevated T_{reg} fraction (mean, 14.06%; range, 12.02-16.73%; $P = 0.0003$ for comparison to normals; Fig. 4*A, white columns*) showed significant $CD4^+$ T-cell proliferative dysfunction (mean, 12,706 cpm; range, 7,297-20,949; $P < 0.0001$), whereas those patients without T_{reg} elevation (mean, 6.89%; range, 5.41-8.88%; $P = 0.76$; Fig. 4*A, black columns*) possessed $CD4^+$ T cells that proliferated at normal levels (mean, 73,120 cpm; range, 62,638-90,712; $P = 0.79$).

These results were suggestive of a correlation between elevations in T_{reg} fraction and diminished CD4 proliferation in patients with GBM. To investigate this relationship in patients more directly, we plotted the respective T_{reg} fraction and CD4 proliferation values for each patient against each other as a percentage of normal. Figure 4*B* reveals a strong negative correlation between T_{reg} fraction and $CD4^+$ T-cell proliferative capacity (Pearson coefficient = -0.794, $P = 0.019$). The predetermined threshold for T_{reg} elevation is included on the plot and proved to be a remarkably accurate separator with regard to proliferative capacities.

In light of this correlation, we sought to determine whether creating a shift in the T_{reg} fraction from normal levels to those levels observed in patients with GBM could, alone, lead to the appearance of proliferative defects among otherwise normal lymphocytes. To accomplish this, we added varying numbers of T_{regs} to a fixed number of $CD4^+CD25^-$ lymphocytes obtained from normal donors to produce *in vitro* T_{reg} fractions that corresponded to physiologic T_{reg} levels and levels in patients with GBM. The addition of T_{regs} to a fixed number of $CD4^+$ lymphocytes ensured that any decreases in proliferation observed would be due to true suppression and not to dilution of responses with simply nonresponsive T_{regs}. Increasing the T_{reg} fraction among normal lymphocytes to those levels found in patients with GBM was sufficient to elicit substantial declines in $CD4^+$ T-cell proliferative responses (Fig. 4*C*). Therefore, increases in T_{reg} fraction not only

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correlate with the appearance of CD4 proliferative defects in patients with GBM, but also prove sufficient to elicit the same defects among normal lymphocytes.

$CD4^+$ T cells from patients function normally in the absence of T_{regs}. T cells were harvested from the five patients above who exhibited substantial proliferative defects, as well as from three healthy controls. To eliminate the contributions of factors present in patient sera, the proliferative responses of lymphocytes from all individuals were tested at varying concentrations of PHA, anti-CD3 monoclonal antibody (OKT3), or ConA in medium containing FCS. The stimulators and doses chosen were those historically revealing the immunosuppressed phenotype (4, 7), and we confirmed substantial proliferative defects among $CD4^+$ T cells from patients with GBM (cumulative responses to PHA depicted in Fig. 5*A*). Significant differences were consistently observed at 5 and 10 μg PHA, but never at doses of ≤1 μg. Cumulative response curves of patients and controls were significantly different, $P < 0.001$. The persistence of dysfunction in the presence of FCS verified the existence of defects "intrinsic" to the $CD4^+$ T-cell compartment.

We next depleted $CD25^+$ cells from patient and control $CD4^+$ T cells and cultured equal numbers with the same stimulation profile. Depletion successfully erased the differences in proliferative responses originally seen among the two groups ($P = 0.689$; Fig. 5*B*). These proliferative defects were similarly reversed when $CD25^+$-depleted T-cells from patients with GBM were cultured in medium containing autologous sera. As made evident by the cumulative data depicted in Fig. 5*A* and *B*, depletion commonly produced a moderate increase in the proliferative capacity of normal $CD4^+$ T cells while instigating a dramatic increase in the proliferative capacity of $CD4^+$ T cells from patients with GBM. These observations cohere with an elevated T_{reg} fraction, and therefore elevated T_{reg} contribution, among $CD4^+$ T cells in this patient population.

To eliminate the prospect that the proliferative defects observed among patients owed their etiology simply to dilution of responsive $CD4^+CD25^-$ T cells with anergic T_{regs}, and not to suppressive effects of T_{regs} per se, assays were conducted in which T_{regs} from these same patients and controls were "added back" to fixed numbers of their own $CD4^+CD25^-$ responders. The addition of T_{regs} produced dose-dependent decreases in proliferative responses, demonstrating genuine suppression (a dilutional effect would have produced no difference in proliferation among fixed responder numbers). These assays are akin to those represented in Fig. 1*D*.

To determine the contribution of T_{regs} to the cytokine dysregulation also typical among these patients, we examined the elaboration of IL-2, IL-4, IL-6, IL-10, IL-12 (p70 subunit), TNF-α, and IFN-γ by $CD4^+$ T cells from the patients above (as well as from the same healthy controls), when stimulated with or without prior removal of T_{regs}. PHA was used for stimulation, and, initially, all cells were cultured in medium containing FCS. Levels of cytokine

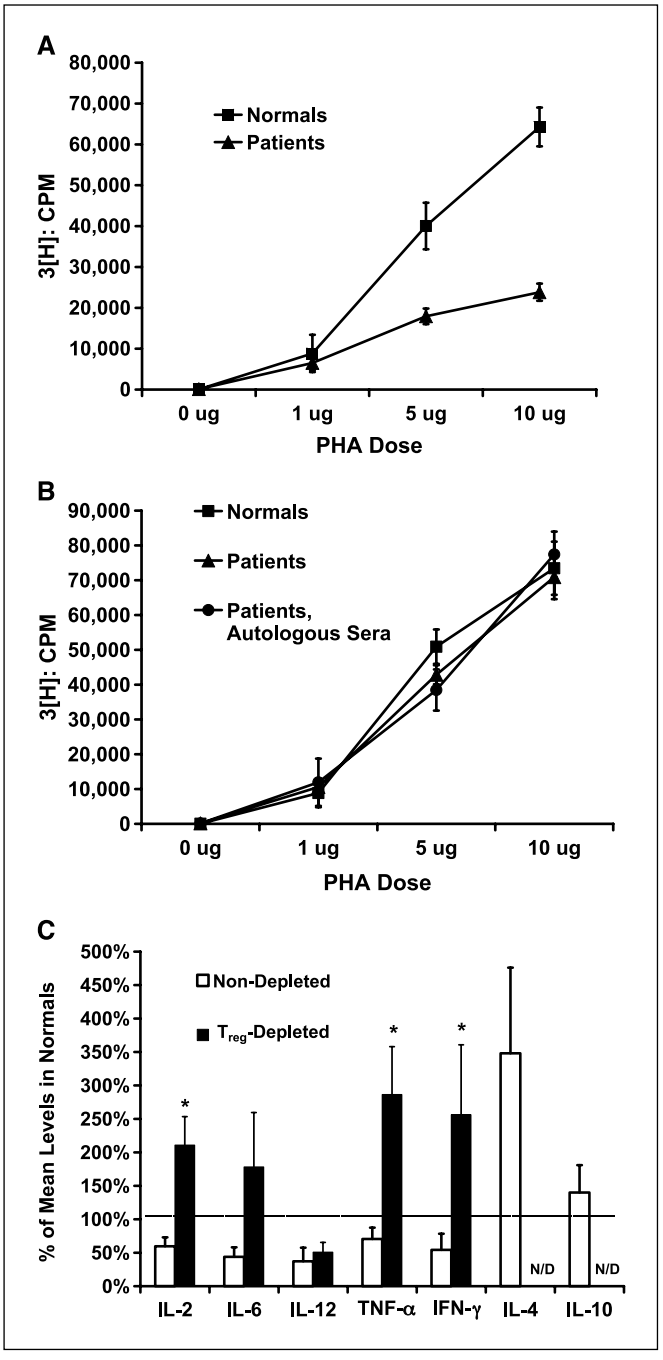

Figure 5. Patient $CD4^+$ T cells function normally in the absence of T_{regs}. *A,* levels of $CD4^+$ T-cell proliferation were assessed among cells from five patients with GBM possessing CD4 proliferative defects and from three healthy controls. $CD4^+$ T cells were cultured in equal numbers in the presence of varying concentrations of PHA, OKT3, or ConA and proliferation was assessed by [^{3}H]thymidine incorporation. Patient $CD4^+$ T cells were cultured in medium containing FCS. Cumulative results of seven experiments with PHA as stimulator. Results were similar across all stimulators. *B,* assays in (*A*) were repeated following removal of $CD25^+$ cells. Differences in proliferation among patients and controls were erased ($P = 0.689$) even when patient cells were cultured in the presence of autologous serum. *C,* T_{reg} depletion relieves T_H2 shift in cytokine production. $CD4^+$ T cells were purified from patients with GBM ($n = 5$) and healthy controls ($n = 3$). Cells left nondepleted or depleted of $CD25^+$ cells were cultured in equal numbers using PHA as stimulator. Supernatants were collected and analyzed in duplicate for levels of IL-2, IL-4, IL-6, IL-10, IL-12p70, TNF-α, and IFN-γ. Levels of production of each cytokine by patients were expressed as percentages of that by normal $CD4^+$ T cells to depict differences in profiles. Cumulative results from three experiments. Before depletion, patient $CD4^+$ T cells produce diminished levels of the T_H1 cytokines IL-2, IL-6, IL-12p70, TNF-α, and IFN-γ, but increased levels of the T_H2 cytokines IL-4 and IL-10. This skew is almost completely eliminated upon depletion of $CD25^+$ cells (*P* values for shifts in production following depletion: IL-2, 0.0002; IL-6, 0.0974; IL-12p70, 0.3264; TNF-α, 0.0017; IFN-γ, 0.0049; IL-4, N/A; IL-10, N/A). Postdepletion data is not available for IL-4 and IL-10 as levels of production were frequently below the level of detection (*N/D*) in both patients and volunteers following depletion (three of three experiments for IL-4 and two of three experiments for IL-10).

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production by patients were expressed as percentages of production by normal $CD4^+$ T cells to permit depiction of relative differences in cytokine profiles. As seen in Fig. 5*C*, before depletion, $CD4^+$ T cells from patients with GBM elaborated reduced quantities of the T_H1 cytokines IL-2, IL-6, IL-12p70, TNF-α, and IFN-γ, but above-normal quantities of the T_H2 cytokines IL-4 and IL-10, when compared with controls.

Among T-cell samples from patients with GBM that were depleted of $CD25^+$ cells, however, production of the T_H1 cytokines IL-2, IL-6, TNF-α, and IFN-γ came generally to match or exceed those levels produced by control $CD4^+$ T-cells, similarly depleted (Fig. 5*C*). As with proliferation, shifts in T_H1 cytokine production among $CD4^+$ T cells from controls were moderate, whereas more substantial changes were seen among $CD4^+$ T cells from patients. The postdepletion shifts in the production of IL-2, IFN-γ, and TNF-α by T cells from patients were significant ($P = 0.0002$, 0.0499, and 0.0017, respectively). IL-12p70 was the only T_H1 cytokine tested for which a deficiency remained apparent (predepletion, 37.23% of normal; postdepletion, 50.96% of normal). Deficits were likewise erased when cells were cultured in the presence of autologous sera (data not shown).

Interestingly, postdepletion shifts in the production of T_H2 cytokines proved dramatic for both patients and controls. Whereas IL-4 and IL-10 were initially elaborated in higher quantities by patient $CD4^+$ T cells, depletion of $CD25^+$ cells brought their levels in both patients and controls down to the limit of detection, such that no statistical comparisons could be made between the two populations (Fig. 5*C*). The net results of depletion, then, were elimination of T_H2 cytokine production by both patient and control $CD4^+$ T cells and the normalization of patient T_H1 cytokine production.

Together, these data show that elevated fractions of T_{regs} undermine the proliferative capacities and cytokine elaboration profiles of $CD4^+$ T cells from patients with GBM. The data also show that these cells are not actually intrinsically defective but instead are capable *in vitro* of essentially normal function when not influenced externally by an elevated T_{reg} fraction.

T_{reg} depletion *in vivo* leads to glioma rejection. We verified that T_{regs} in VM/Dk mice possess the appropriate traits reported for T_{regs} in *in vitro* functional assays. As CD45RO is not a murine phenotypic marker, T_{regs} were defined for this purpose as $CD4^+CD25^+GITR^+$ T cells. Isolated $CD4^+CD25^+GITR^+$ T cells from these mice expressed Foxp3, failed to respond to stimulation, and exhibited the expected dose-dependent capacity to suppress the proliferative responses of surrounding $CD4^+CD25^-$ cells (data not shown). We also established our ability to successfully deplete T_{regs} from VM/Dk mice. A single dose of 0.5 mg anti-CD25 (PC61) delivered i.p. successfully depleted $CD25^+$ cells from mice, and these levels did not recover for ~30 days (data not shown).

To assess the effects of T_{reg} depletion when effected before any disruption of the blood-brain barrier, T_{reg} depletion was initially evaluated in an SMA-560 tumor challenge model. SMA-560 is a malignant astrocytoma cell line derived from tumors that originated spontaneously in inbred VM/Dk mice. All mice ($n = 20$) were challenged with 10,000 SMA-560 cells, placed intracerebrally under stereotactic guidance. Four days before tumor challenge, 10 mice were depleted of T_{regs} with a single injection of PC61, whereas the remaining 10 mice received an isotype control antibody. Despite the systemic (i.p.) route of delivery, anti-CD25 significantly extended survival ($P = 0.0002$) and proved capable of rejecting an intracranial tumor entirely in 50% of treated mice (Fig. 6*A*).

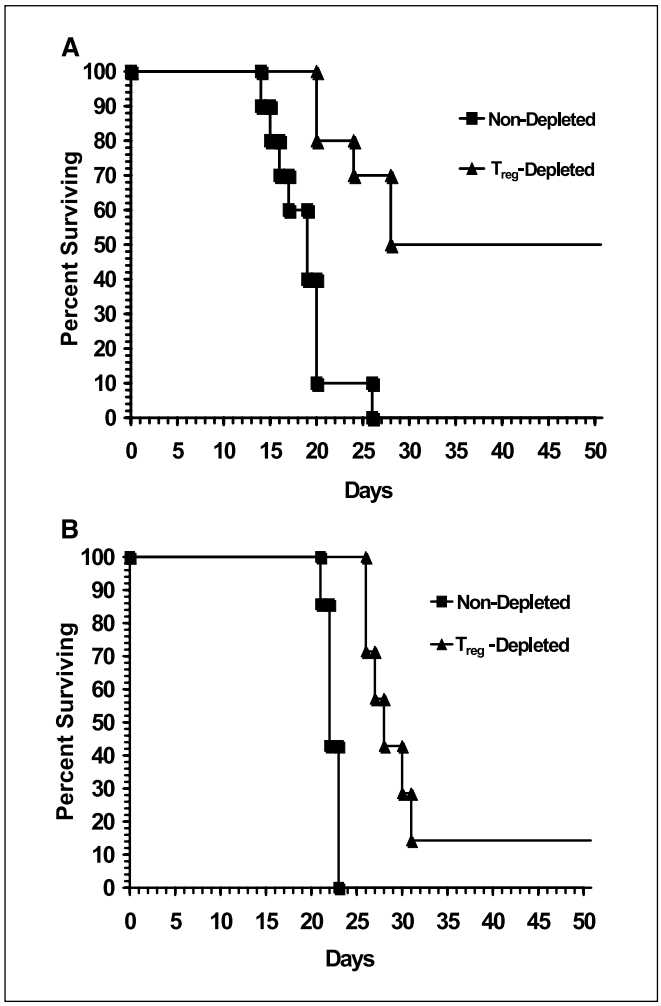

Figure 6. T_{reg} depletion *in vivo* extends survival and permits tumor rejection in a murine model of intracranial glioma. *A,* VM/Dk mice ($n = 10$ per group) were left nondepleted or were depleted of T_{regs} with i.p. PC61 on day −4. On day 0, all mice were challenged intracranially with 10,000 syngeneic SMA-560 astrocytoma cells. T_{reg} depletion produced 50% long-term survivors with no evidence of tumor. Differences in survival curves are significant ($P = 0.0002$). *B,* effects of T_{reg} depletion in a therapeutic model. Depleted mice ($n = 7$) received i.p. PC61 3 days following tumor implantation, when tumor was established. T_{reg} depletion significantly extended median survival ($P = 0.0003$) and produced nearly 15% long-term survivors with no evidence of tumor.

The ability of T_{reg} depletion to extend survival was next evaluated in a more stringent therapeutic model of established tumor. Again, all mice ($n = 14$) were implanted intracranially with 10,000 SMA-560 cells. In this instance, however, 50% of the mice were depleted of T_{regs} with PC61 delivered in a single systemic dose 3 days pursuant to tumor implantation, after tumor had become established. Despite the stringency of the model, median survival was extended nearly 30% ($P = 0.0003$), and ~15% of mice survived long-term with no subsequent evidence of tumor (Fig. 6*B*). Taken with the results of the tumor challenge experiments, these data imply that a reasonable physiologic response against intracranial tumor exists but may fail in the presence of T_{regs}.

Discussion

The initial descriptions of impaired cellular immunity in patients with malignant glioma date to the 1970s (4, 24–26). Lymphocyte

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dysfunction has since been progressively narrowed to the $CD4^+$ T-cell subset (6), which exhibits substantial defects when cultured *en masse*. Attempts to understand and reverse such dysfunction have continued unremittingly.

It is a therefore dramatic finding that upon removal of its T_{reg} constituent, the remaining $CD4^+$ compartment of patients with GBM functions normally *in vitro*. This shows that "intrinsic" $CD4^+$ T-cell functional deficits are neither cell inherent nor irreversible. Instead, they appear to be the product of an elevated T_{reg} fraction and may be abrogated simply via depletion of T_{reg} cells. We have not overlooked the effects of factors present in patient serum; rather, we have focused on and uncovered the source of those substantial defects that remain in their absence. Whereas these intrinsic defects remained erased when T_{reg}-depleted cells were cultured in autologous sera, such sera were not extensively characterized and were retrieved from patients relatively early in their disease course. It remains possible that sera obtained from patients at later stages of disease would have had more substantial effects.

To our knowledge, absolute T_{reg} counts have not previously been reported in patients with malignancy. The increased T_{reg} levels reported in similar studies (18–21) more truly represent what we have termed here the T_{reg} fraction. Consequently, it is unclear whether the elevated T_{reg} levels previously reported among other cancer populations represent a veritable expansion of this compartment *in vivo*. We show, however, that T_{regs} substantially compromise T-cell function despite being reduced in absolute number *in vivo*. What appears of more pertinence than T_{reg} numbers, then, is the fraction that they represent of the surrounding CD4 compartment. In our patients, this fraction is significantly increased, and this increase is what correlates with T-cell dysfunction.

We are also the first to report profoundly reduced $CD4^+$ T-cell counts in preresection patients with GBM. These patients exhibited mean CD4 counts below 200 cells/μL, the demarcation for progression to AIDS in HIV-infected individuals. The severity of this lymphopenia is perplexing, given the overwhelming absence of systemic disease in this population. Another group has reported diminished CD4 counts in patients with primary brain tumors who were already undergoing radiotherapy (27). As cranial radiotherapy is a known contributor to myelosuppression (28), this previous study was examining the incidence of opportunistic infections in patients subjected to long-term steroids and radiation. We do not overlook the potential contributions of preoperative steroids in our study, but such therapy was newly initiated, and we have not seen such drops in CD4 counts in patients receiving identical steroid regimens before surgery for other conditions. The etiology of this $CD4^+$ T-cell lymphopenia therefore remains a logical direction for future research. The goal of this study was to examine the etiology of "intrinsic" T-cell defects, but such a severe lymphopenia is clearly a contributor to depressed cellular immunity in patients with GBM.

Also of interest is why T_{regs} might be differentially susceptible to mechanisms reducing $CD4^+$ T-cell numbers, leading to increased T_{reg} fractions. We have already ruled out the contribution of steroids (Fig. 2*A*). Potential differences in rates of emergence from a dysfunctional thymus (29) or in susceptibility to apoptosis are under investigation. Another potential source that deserves attention, however, is transforming growth factor-β (TGF-β). TGF-β is elaborated by gliomas and is one of the key immunosuppressive factors often present in patient serum (30–32). Importantly, it has a proven role in facilitating the development and elaboration of T_{regs} (33, 34), including *in vivo* (35). These studies suggest TGF-β may be an "upstream" participant in a cascade of events leading to T_{reg}-mediated immunosuppression.

A potential shortcoming of our findings might be a failure to rectify the results of earlier studies demonstrating that inducible IL-2R expression is hindered in lymphocytes from patients with GBM (36). These findings would initially seem to contradict our observation of an increased fraction of T_{regs}, whose most common identifying marker is the α chain of the high-affinity IL-2R (CD25). Upon closer examination, however, these previous studies report only IL-2R expression on lymphocytes following mitogenic stimulation, and no data are offered concerning resting levels on freshly isolated cells. As CD25 is also a T-cell activation marker, it is these preactivation levels that more accurately depict T_{regs}. Interestingly, one study in patients with malignant glioma, published before the current T_{reg} literature, measured CD25 levels on freshly isolated TIL and reported that these levels were markedly elevated (9).

Our results translate into a potential for therapeutic benefit, as T_{reg} depletion in mice proves permissive for glioma rejection in the intracranial compartment even in instances of established tumor. This rejection is notably effected following systemic delivery of antibody and in the absence of additional therapeutic intervention. A salient implication is that in the absence of T_{regs}, the physiologic immune response possesses some capacity to reject tumor located in the immunologically privileged central nervous system. Our data suggest that a systemic immune response to tumors in the central nervous system occurs and that peripheral T_{regs} are important in attenuating this response. Tumors situated intracranially are, in turn, clearly capable of modulating systemic immunity and may derive some of their success in this endeavor from alterations elicited in the T_{reg} fraction.

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