An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme

Abdeljabar El Andaloussi and Maciej S. Lesniak

Section of Neurosurgery, The University of Chicago, Chicago, IL 60637, USA

The subpopulation of CD4+CD25+ immunoregulatory T (Tr) cells constitutes 5%–10% of CD4+ cells in humans. These cells play a crucial role in the control of tumor immune response. In this study, we evaluated the distribution of Tr cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme and examined the difference between the brain and autologous blood with respect to Tr cells. Glioma samples from 10 patients were classified as WHO grade IV astrocytoma. Control samples were obtained from patients undergoing resection of a seizure focus. The samples were analyzed by flow cytometry to determine the frequency of Tr cells and by real-time PCR for forkhead box P3 (FOXP3) expression. We then examined the expression of CD62L, CD45RO, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and assessed the functionality of Tr cells in vitro. There was a significant difference in the number of FOXP3-expressing CD4+CD25+ T cells within glioma-infiltrating lymphocytes as compared to controls (P < 0.01). This difference was further observed in studies of autologous patient blood and control blood. The expression level of FOXP3 mRNA was high in Tr cells and weak in CD4+CD25– T cells. Moreover, the expression of CD62L and CTLA-4 was elevated in glioma Tr cells as compared to that in the controls. These cells were also CD45RO positive. Functional assays confirmed the suppressive activity of Tr cells in patients with glioma. The expression of CD4+CD25+FOXP3+ T cells was significantly higher in patients with glioblastoma multiforme than in controls. This increase in the frequency of Tr cells that display suppressive activity might play a role in modulation of the immune response against glioma. In light of these findings, Tr cells may represent a potential target for immunotherapy of malignant brain tumors. Neuro-Oncology 8, 234–243, 2006 (Posted to Neuro-Oncology [serial online], Doc. 05-105, May 24, 2006. URL www.dukeupress.edu/neuro-oncology; DOI: 10.1215/15228517-2006-006)

Keywords: glioma, immunotherapy, regulatory T cells, tumor-infiltrating lymphocyte

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It has been shown that Tr cells can be generated in vitro or in vivo from human CD4+CD25− T cells under various stimulation conditions and that generation of these cells correlates with induction of FOXP3 expression (Fontenot et al., 2003, 2005). These results suggest that FOXP3 is a specific molecular marker of Tr cells for discrimination between Tr cells and activated regulatory T cells. The absence of CD4+CD25+FOXP3+ T cells is associated with severe autoimmunity (Sakaguchi et al., 2001). It has been proposed that mechanisms underlying autoimmunity and tumor immunity are linked (Turk et al., 2002). Experimental tumor models have shown that depletion of CD25+ T cells changes the immune response to tumors both in vitro and in vivo. The mechanism by which Tr cells regulate responses in vivo is unknown, and the contribution of Tr-cell-derived cytokines in disease regulation remains controversial. Tr cells are exceptional because their main role seems to be suppression of the function of other cells; hence they are called suppressor cells (Jiang and Chess, 2004). The suppressive mechanisms of Tr cells are not clear, but there is evidence that cell-cell contact is required and that expression of the inhibitory costimulatory molecule CTLA-4 (also called CTLA antigen 4) might be involved. Recently, CTLA-4 was implicated in regulatory T-cell development and was shown to account for the link between polymorphisms at this locus and the biological outcome of adaptive immune responses to self and to pathogens (Atabani et al., 2005). To date, an increased population of Tr cells has been reported in patients with gastric cancer (Ichihara et al., 2003; Wolf et al., 2003), colorectal cancer (Hickey, 1999), pancreatic cancer (Liyanage et al., 2002; Sasada et al., 2003), ovarian cancer, and lung cancer (Woo et al., 2001).

In humans, the presence of tumor-infiltrating lymphocytes (TILs) may be predictive of improved clinical outcomes (Woo et al., 2002), which supports the concept that tumors are antigenic and immunogenic and that the immune system is alert against cell transformation. In this study, several markers known to be expressed on Tr cells were investigated in the context of malignant brain tumors, and we report the first characterization of Tr cells from TILs of glioma patients, compared to peripheral blood of these patients and to controls. Our data show an increase of Tr cells in glioma and peripheral blood and suggest that these cells may downregulate the antitumor response in the CNS.

**Materials and Methods**

**Tissue Specimens**

Glioma samples were obtained from 10 patients undergoing a craniotomy for tumor resection at the University of Chicago. Control brain tissue was obtained from six patients undergoing a temporal lobectomy for seizures (Table 1). All tumor specimens were classified as grade IV glioblastoma multiforme. Peripheral blood was obtained during the time of the surgery from each of the groups. Of note, the study group and the control group both received perioperative steroids and underwent otherwise similar anesthesia. This study was approved by the Institutional Review Board of the University of Chicago.

**Collection of Peripheral Blood Mononuclear Cells**

Peripheral venous blood (10–20 ml) was collected into heparinized tubes. The samples were hand carried to the laboratory and immediately centrifuged on Ficoll-Hypaque (Amersham Biosciences, Piscataway, N.J.). Peripheral blood mononuclear cells (PBMCs) were recovered, washed in staining buffer (PBS, 3% fetal calf serum), and used immediately for experiment.

**T-Cell Isolation from Tumor**

Cell suspensions of tumor were prepared by mechanical disruption of the tissue with a 16-gauge needle. The cells were then passed through a cell strainer and washed with PBS. Peripheral blood lymphocytes were isolated by the Institutional Review Board of the University of Chicago.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of Patients†</th>
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<tr>
<td>A. Patients with glioblastoma multiforme</td>
<td></td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>6</td>
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<tr>
<td>Female</td>
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<tr>
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<tr>
<td>Male</td>
<td>52 (range, 30–62)</td>
</tr>
<tr>
<td>Female</td>
<td>60 (range, 47–69)</td>
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<tr>
<td>Location of tumor</td>
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</tr>
<tr>
<td>Frontal</td>
<td>2</td>
</tr>
<tr>
<td>Parietal</td>
<td>3</td>
</tr>
<tr>
<td>Temporal</td>
<td>4</td>
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<td>Occipital</td>
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<tr>
<td>Prior history of craniotomy, radiotherapy, and chemotherapy</td>
<td>5</td>
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<tr>
<td>B. Controls*</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
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<tr>
<td>Average age</td>
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</tr>
<tr>
<td>Female</td>
<td>43 (range, 37–51)</td>
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<tr>
<td>Location of seizure focus</td>
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<tr>
<td>Right</td>
<td>5</td>
</tr>
<tr>
<td>Left</td>
<td>1</td>
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</table>

†Age is shown as an average value.

*Control tissue was obtained from six patients undergoing temporal lobectomy for seizures.
by centrifugation on Ficoll gradient, washed in staining buffer, and used immediately for analysis by flow cytometry.

**Multicolor Flow Cytometry Analysis**

Tumor and peripheral blood lymphocytes (1 × 10⁶ in 50 µl) were stained with 2 µl of antibody and incubated for 45 min at 4°C in the wells of round-bottom 96-well microtiter plates (BD Biosciences, San Jose, Calif.) to determine their immunophenotype. The antibodies used were anti-CD25-PE (1 µg/ml), anti-CD4-fluorescein isothiocyanate (2 µg/ml), anti-CD3-PE (1 µg/ml), anti-CD62L (5 µg/ml), anti-CD45RO (5 µg/ml), and anti-CTLA-4 (5 µg/ml) (BD Biosciences). The intracellular FOXP3 was stained as described by the manufacturer (eBioscience, San Diego, Calif.). Flow cytometry was performed on a FACSCalibur (Becton Dickinson, St. Louis, Mo.). Primers were purchased from Invitrogen (Carlsbad, Calif.). cDNA was made with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif.) and amplified by PCR using Red Taq polymerase (Sigma, St. Louis, Mo.). Primers were purchased from Invitrogen. The following primers were used to amplify FOXP3 target-gene cDNA: human GAPDH (forward: 5'-agtgcagtatgggctgtat-3'; reverse: 5'-aggagaggctagttggggt-3') and FOXP3 (forward: 5'-cctgttcgtccacatcctcct-3'; reverse: 5'-cctgttgccatcctctct-3').

Quantitative analysis of cDNA amplification was assessed by incorporation of SYBR Green into dsDNA. PCR reactions containing 1 µg of cDNA template, 0.5 µM each of the primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif.) were performed in a total volume of 25 µl under the following conditions: 15-min hot start at 95°C, 15-s denaturation at 95°C, 20-s annealing of primers at 54°C, and 15-s elongation at 72°C, for 40 to 60 cycles. Gene expression was analyzed with the ABI Prism 7700 sequence detector (Applied Biosystems) and ABI Prism SDS software version 1.9.1 (Applied Biosystems). Samples were normalized by dividing the quantity of the FOXP3 gene by the value of the endogenous reference gene (GAPDH). Quadruplicate reactions were done using all cDNA samples. Relative expression was calculated for each gene by using the ΔCt (threshold cycle) method.

**Statistical Analysis**

Paired or unpaired groups were compared by using the appropriate Student’s t-tests. A P value of <0.05 was defined as statistically significant. All statistical analyses were performed with the SPSS statistical software package (SPSS 12.0 for Windows; SPSS Inc., Chicago, Ill.).

**Results**

**Frequency of Lymphocytes in Brain and Blood of Patients with Glioma**

T cells isolated from TILs were first gated on total lymphocytes via their forward and side scatter properties. As shown in Fig. 1, the frequency of TILs was significantly increased (P < 0.01) in brain tumors (mean, 17.7% ± 2.7%; range, 12.1%–21.4%) versus controls (mean, 0.5% ± 0.28%; range, 0.0%–0.85%). Similarly, we observed a significant (P < 0.01) increase in total autologous blood lymphocytes (mean, 16.8% ± 1.9%; range, 13.6%–20%) obtained from tumor patients as compared to peripheral blood (mean, 10.3% ± 1.5%; range, 7.8%–12.4%) from controls.

**Frequency of Tr Cells in TILs of Glioma Patients and Control Blood**

The frequency of Tr cells was defined as the frequency of cells that express a high level of CD25. The analysis of CD4+CD25+ T cells is shown in Fig. 2 as a percentage of total CD3+ cells evaluated in TILs of brain by flow cytometry analysis with triple-color staining. In our subset of 10 patients with glioma, the mean proportion of Tr cells in TILs was 24.7% ± 6.0% (range, 19.1%–27%) (Fig. 2). Tr cells were absent from control brain. Moreover, the frequency of Tr cells in glioma patients’ blood was increased when compared with the frequency observed in control blood (8.56% ± 2.46% [range, 6.4%–12.4%] vs. 0.48% ± 0.13% [range, 0.26%–0.7%]; P < 0.05) (Fig. 3). These data are summarized in Table 2.
FOXP3 Expression in CD4+ T Cell Population

The intracellular expression of FOXP3 in patients with grade IV glioma was analyzed with flow cytometry and triple color. We found that 55.1% ± 1.88% (range, 51%–62.4%) of CD4+ cells in tumor infiltrates versus 33.4% ± 1.95% (range, 29.7%–38.4%) of CD4+ cells in autologous blood versus 15.6% ± 0.76% (range, 13.2%–17.1%) in control blood express FOXP3 (P < 0.01) (Fig. 4A–C). The expression of FOXP3 confirmed the presence of regulatory T cells in TILs. Although Tr cells in both blood and tumor infiltrates coexpressed FOXP3 and CD25+, the expression of FOXP3 was more pronounced in the CD4+CD25+ subset (Fig. 4D).

Table 2. Percentage of regulatory T cells in total CD3+ T cells in brain and in peripheral blood of glioma patients and controls

<table>
<thead>
<tr>
<th>Source</th>
<th>CD3+ T cells %</th>
<th>CD4+CD25- %</th>
<th>CD4+CD25+ %</th>
</tr>
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<tbody>
<tr>
<td>A. Brain</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TILs in glioma</td>
<td>25.1 ± 2.08</td>
<td>18.9 ± 1.56</td>
<td>6.2 ± 0.51</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B. Peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glioma</td>
<td>64.8 ± 3.14</td>
<td>59.22 ± 2.87</td>
<td>5.54 ± 0.26</td>
</tr>
<tr>
<td>Control</td>
<td>83.2 ± 2.76</td>
<td>82.78 ± 2.74</td>
<td>0.4 ± 0.10</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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Abbreviation: TILs, tumor-infiltrating lymphocytes.
We further analyzed FOXP3 mRNA levels in either sorted CD4+CD25– or CD4+CD25+ cells obtained from patients with glioma versus the blood of controls. The real-time PCR data shows that the level of FOXP3 mRNA was fivefold to sixfold higher in CD4+CD25+ cells than in CD4+CD25– cells of glioma patients (Fig. 5A). This difference was significant with respect to control (\( P < 0.05 \)). The result of semiquantitative PCR confirms the previously observed data, where the level of FOXP3 was increased in CD4+CD25+ cells (Fig. 5B).

Expression of CD62L, CD45RO, and CTLA-4 in CD4+CD25+ T Cells

The phenotype of CD4+ T cells was analyzed for CD62L expression in different subsets of CD4+ T cells expressing CD25 (Fig. 6). The prevalence of Tr was compared in autologous PBMCs and control donors. Figure 6 shows an expansion of CD25+CD62L– subsets in glioma patient blood (mean, 75.2% ± 4.03%; range, 70%–81%) compared to control blood (mean, 63.6% ± 3.74%; range, 58%–69%) (\( P < 0.01 \)). The same expansion of CD25+CD62L+ was seen in glioma patients (mean, 90.3% ± 2.83%; range, 88%–97%) versus controls (mean, 83.5% ± 4.34%; range, 77%–88%) (\( P < 0.01 \)). Moreover, the Tr-cell phenotype was membrane positive for CD45RO (Fig. 7). In autologous blood of glioma patients, the pool of CD4+ T cells was CD45RO positive (mean, 41.5% ± 1.73%; range, 38.5%–43%), but the expression level of CD45RO was less than in control donors (mean, 54% ± 2.45%; range, 50%–57%) (\( P < 0.02 \)) (Fig. 7A and B). Mean CTLA-4 expression on CD4+CD25+ regulatory T cells in autologous blood and control blood was 76.8% ± 2.1% (range, 74%–80.5%) versus 50% ± 1.8% (range, 47.7%–52%), respectively (Fig. 7C and D) (\( P < 0.01 \)).

Functional Activity of CD4+CD25+ T Cells

CD4+CD25+ and CD4+CD25– cells obtained from glioma were sorted as shown in Fig. 8A. The functional capacity of CD4+CD25+ cells was then analyzed in a dose-dependent manner by incubating the cells with autologous CD4+CD25– T cells. As shown in Fig. 8B,
Fig. 5. Level of FOXP3 expression in CD4+ T cell subsets. FOXP3 quantification by real-time PCR (A) and semiquantitative PCR (B) in CD4+ T cells. The real-time PCR data shows that the level of FOXP3 mRNA was fivefold to sixfold higher in CD4+CD25+ cells (A). This difference was significant with respect to control (P < 0.05). Lines 1 and 2 consisted of CD4+CD25– and CD4+CD25+ sorted from autologous blood, and lines 3 and 4 consisted of CD4+CD25– and CD4+CD25+ sorted from control blood donor. CD4+CD25– and CD4+CD25+ were sorted separately from PBMCs with more than 97% purity.

Fig. 6. Regulatory T cells show suppressor phenotype. The expression of CD62L after gating of CD4+CD25+ or CD4+CD25– cells in autologous blood of patients (A) and control blood (B). The left panels of rows A and B show, respectively, an expansion of CD25–CD62L– subsets in glioma patient (mean, 75.2% ± 4.03%; range, 70%–81%) compared to control blood (mean, 63.6% ± 3.74%; range, 58%–69%) (P < 0.01). The same expansion of CD25+CD62L+ was seen in glioma patients (mean, 90.3% ± 2.83%; range, 88%–97%) versus controls (mean, 83.5% ± 4.34%; range, 77%–88%) (P < 0.01). The cells were stained with three-color fluorescence and analyzed by fluorescent flow cytometry.

Fig. 7. Expression of CD45RO and CTLA-4 on CD4+ T cells. Flow cytometric analysis of CD45RO and CTLA-4 (CD152) on CD4+CD25+ T cells. A and C. Control blood. B and D. Autologous patient blood. In autologous blood of glioma patients, the pool of memory CD4+CD45RO+ T cells was CD45RO positive (mean, 41.5% ± 1.73%; range, 38.5%–43%), but the expression level of CD45RO was less than in control donors (mean, 54% ± 2.45%; range, 50%–57%) (P < 0.02) (A and B). The CTLA-4 expression on CD4+CD25+ T cells in autologous blood and control blood was 76.8% ± 2.1% [range, 74%–80.5%] versus 50% ± 1.8% [range, 47.7%–52%], respectively (C and D) (P < 0.01).
these CD4+CD25+ T cells show suppressive activity, a function that is consistent with the function of Tr cells.

Discussion

The CNS has long been regarded as an immunologically privileged site. The mechanisms limiting immunoreactivity in the CNS are incompletely understood. The absence of naive T cells in the brain, low major histocompatibility complex expression in the brain parenchyma, and lack of adequate lymphatics are all factors that likely contribute to the lack of immune responses in the brain (Hickey, 1999; Hickey et al., 1991). However, there is increasing evidence that T lymphocytes and major histocompatibility complex antigens are detectable in the CNS during illness and disease (Horwitz et al., 1999). In fact, the brain can be the site of an aberrant immune response, as in the case of multiple sclerosis (Baranzini et al., 2005; Haller et al., 2005; O’Connor et al., 2001). Moreover, the presence of TILs within high-grade gliomas has prompted a renewed interest in the role of T cells in primary brain tumors (Giometto et al., 1996; Kaluza et al., 1993; Perrin et al., 1999).

Glioblastomas are highly infiltrative tumors that do not generally metastasize outside the brain. Immunohistological and molecular analyses of human malignant astrocytoma have shown that T-cell infiltration occurs frequently (Giometto et al., 1996; Kaluza et al., 1993; Perrin et al., 1999), but it has only occasionally been correlated with a favorable prognosis (Brooks et al., 1978). Elimination of a solid brain tumor by immune effector cells is a formidable challenge. Not only are these tumors protected from the immune system by the presence of the blood-brain barrier, but many of them secrete soluble factors that directly inhibit an active immune response (Giometto et al., 1996; Hishii et al., 1995; Kaluza et al., 1993; Sasaki et al., 1995; Schweitzer et al., 2001).

The published data regarding suppressive activities of Tr in patients with tumors (Dunn et al., 2002; Liyanage et al., 2002; Wolf et al., 2003) provide a rationale for analyzing this subset of T cells within glioblastomas. The localization of Tr cells to tumor site in glioma has not been previously investigated. Our results reveal that Tr cells are increased in glioma infiltrates and autologous blood as compared with tumor-free brain and control blood. Moreover, we have shown an expansion of the CD4+CD25+CD62L+ subset in peripheral blood of patients with glioma. Within the Tr-cell population, CD62L+ Tr cells have been described as more efficient suppressors of T-cell proliferation than are CD62L– Tr cells (Kohm and Miller, 2003). Similar results were seen in the context of CTLA-4, a cell surface receptor that behaves as a negative regulator of the proliferation and the effector function of T cells (von Boehmer, 2005). Our functional study further confirms the suppressive function of Tr cells in patients with glioma, as CD4+CD25+ cells inhibited the proliferative response of CD4+CD25– T cells upon T-cell receptor stimulation. Taken together, our results suggest that the expansion of Tr with a suppressive activity in glioma patients may downregulate the antitumor response in the CNS.

When we compared the frequency of Tr cells in patient autologous PBMCs and in control donors, the frequency of Tr cells was elevated in patients with tumors. This suggests that, under normal circumstances, CD4+CD25– may suppress CD4+CD25+ in the periphery. Evidence for this comes from published literature, which indicates that the maintenance of CD25 expression by
CD4+CD25+ cells depends on interleukin 2 secreted by cotransferred CD4+CD25– or by antigen-stimulated T cells in peripheral lymphoid organs (Curotto de Lafaille et al., 2004). In addition, CD4+CD25– cells can produce a number of immunosuppressive cytokines such as interleukin-10 and transforming growth factor β, which have been shown to down-regulate Tr cells (Dieckmann et al., 2005; Noma et al., 2005). The degree of such suppression is likely to be critical, since the loss of CD4+CD25+ cells is also associated with cases of autoimmune disease as well as graft-versus-host disease induction (Hoffmann et al., 2002; Kohm et al., 2003; Lee et al., 2004).

The forkhead/winged helix transcription factor FOXP3 is a master gene for Tr-cell function (Fontenot et al., 2005) and dominant tolerance (Rudensky, 2005). We have shown that the prevalence of FOXP3+ Tr cells is significantly elevated both in tumors and in autologous blood of patients. This further supports the immunosuppressive nature of Tr cells in patients with malignant glioma. One critical question that remains unanswered is how FOXP3 causes T cells to become regulatory. Choi et al. (2005) have shown that FOXP3 can induce heme oxygenase-1 (HO-1) expression and, subsequently, regulatory phenotypes such as the suppression of non-transfected cells in a cell-cell contact-dependent manner, as well as impaired proliferation and production of cytokines upon stimulation in Jurkat T cells. Moreover, the authors confirmed that the suppressive function of the cells was relieved by the inhibition of HO-1 activity. These findings may have important implications for glioma, where the expression of HO-1 has been shown to be elevated and responsible for tumor progression and angiogenesis (Deininger et al., 2000; Nishie et al., 1999).

As a functional consequence of an increased proportion of Tr cells, it has been shown that Tr cells can suppress immune responses of other CD4+ and CD8+ cells (Albers et al., 2005; Woo et al., 2001). It has also been shown that CD4+CD25+ Tr cells suppress the proliferation, cytokine secretion, and cytotoxic activity of Vα24+ natural killer T cells (Azuma et al., 2003). Thus, one of the explanations for impaired cell-mediated immunity in cancer-bearing hosts is the increased prevalence of Tr cells. More studies are needed to address the roles of the two types of Tr cells (natural and inducible) and to understand which one of them elicits a greater antitumor immune response. In line with these results, recent preclinical studies suggest that these cells are likely to play a key role in the development of tumor immunotherapy, because tumor immunity can be induced in nonresponding animals through the depletion of CD4+CD25+ cells (Casares et al., 2003; Shimizu et al., 1989). It is therefore possible that depletion of Tr cells via intravenous injection of anti-CD25 mAb can achieve vigorous, cytotoxic T-lymphocyte-mediated immune rejection of a cerebral malignancy, a finding that we are currently attempting to corroborate and one that could be translated to the clinic.

Conclusions

In conclusion, our results show simultaneous increase of Tr cells in the tumors and peripheral blood of patients with glioblastoma. These results represent the first report of Tr cells in malignant glioma. Effective anti-tumor responses in individuals with cancer depend on the presence and function of immune cells that can recognize and eliminate tumor cells (Javia and Rosenberg, 2003; Marshall et al., 2004; Woo et al., 2001). However, immunotherapy for brain tumors must overcome some of these protective mechanisms to achieve the rejection of glioma cells without disturbing the delicate balance that protects against autoimmune disease of the central nervous system.

References


El Andaloussi and Lesniak: Regulatory T cells in malignant glioma


