

**Human Cancer Biology**

**Effector T-Cell Infiltration Positively Impacts Survival of Glioblastoma Patients and Is Impaired by Tumor-Derived TGF-β**

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**Abstract**

**Purpose:** In glioma—in contrast to various other cancers—the impact of T-lymphocytes on clinical outcome is not clear. We investigated the clinical relevance and regulation of T-cell infiltration in glioma.

**Experimental Design:** T-cell subpopulations from entire sections of 93 WHO II–IV gliomas were computationally identified using markers CD3, CD8, and Foxp3; survival analysis was then done on primary glioblastomas (pGBM). Endothelial cells expressing cellular adhesion molecules (CAM) were similarly computationally quantified from the same glioma tissues. Influence of prominent cytokines (as measured by ELISA from 53 WHO II–IV glioma lysates) on CAM-expression in GBM-isolated endothelial cells was determined using flow cytometry. The functional relevance of the cytokine-mediated CAM regulation was tested in a transmigration assay using GBM-derived endothelial cells and autologous T-cells.

**Results:** Infiltration of all T-cell subsets increased in high-grade tumors. Most strikingly, within pGBM, elevated numbers of intratumoral effector T cells (Teff, cytotoxic and helper) significantly correlated with a better survival; regulatory T cells were infrequently present and not associated with GBM patient outcome. Interestingly, increased infiltration of Teff cells was related to the expression of ICAM-1 on the vessel surface. Transmigration of autologous T cells in vitro was markedly reduced in the presence of CAM-blocking antibodies. We found that TGF-β molecules impeded transmigration and downregulated CAM-expression on GBM-isolated endothelial cells; blocking TGF-β receptor signaling increased transmigration.

**Conclusions:** This study provides comprehensive and novel insights into occurrence and regulation of T-cell infiltration in glioma. Specifically, targeting TGF-β1 and TGF-β2 might improve intratumoral T-cell infiltration and thus enhance effectiveness of immunotherapeutic approaches. *Clin Cancer Res; 17(13); 4296–308. ©2011 AACR.*

**Introduction**

Glioblastoma (GBM) is the most malignant type of brain tumor. More than 90% of GBMs are diagnosed de novo (primary GBM, pGBM) whereas others are thought to arise from malignant transformation of lower-grade astrocytic and oligodendrogial tumors (secondary GBM, sGBM; ref. 1). Despite intensive therapy including surgery followed by radio- and chemotherapy, GBMs are still incurable. This is mainly due to the high propensity of GBM cells to invade the surrounding normal brain, which prevents a complete tumor resection (2). Therefore, investigation of complementary approaches is urgently needed to eliminate persisting glioma cells.

Triggering the immune response is theoretically an attractive treatment method because even scattered tumor cells can be selectively targeted without damaging the normal brain (3). That immunotherapy can be efficient and safe was shown by a number of clinical trials aiming at a specific antiglioma T-cell activation. These approaches include adoptive transfer of tumor-reactive T cells, *ex vivo* tumor antigen-primed dendritic cells, or vaccination with peptides, inactivated autologous tumor cells and gene-modified tumor cells (4). However, despite beneficial observations patients were not cured and some of them even did not respond to antiglioma immunotherapy. This is largely due to the location and special properties of all glial brain tumors.

Gliomas develop in an organ that is normally shielded from the immune system by the blood–brain barrier.
T-Cell Infiltration Improves Survival

Translational Relevance

In most cancers, the type and density of intratumoral T cells have been shown to have strong prognostic significance. However, in glioma, studies of the clinical relevance of T-cell infiltration have yielded incongruent results. We have discovered that T-effector (Teff) cell infiltration positively affects glioblastoma (GBM) patient survival. Thus, Teff-cell infiltration might serve as a tool to monitor the responses of patients to immunotherapy treatment. We found that the glioma cytokine TGF-β is an important negative regulator of T-cell transmigration through GBM-derived endothelium. Further, we identified a novel immunosuppressive mechanism of TGF-β in glioma: the downregulation of CAM expression, which consequently reduces T-cell transmigration. Accordingly, our results predict that the effectiveness of glioma immunotherapy could be improved by simultaneously inhibiting TGF-β signaling within tumors.

(BBB), thereby limiting the transmigration of immune cells (5). However, T cells can cross an intact BBB if they are in an activated state (6). Moreover, relative to low-grade gliomas, GBMs are characterized by a compromised BBB due to breaks in tight junctions, augmented fenestrations and decreases in BBB-associated pericytes (7, 8). Hence the success of immunotherapy for the treatment of gliomas might be limited by the BBB, but to a variable extent.

Antiglioma immune responses are impaired by the tumor itself through expression of transforming growth factor-beta (TGF-β) and interleukin-10 (IL-10; ref. 9). For example, TGF-β inhibits T-cell activation and proliferation, represses production of lytic enzymes and drives development of naive T cells into regulatory T cells (Treg; ref. 10, 11). Under normal physiological conditions Treg cells are necessary to protect against autoimmune diseases but in GBM patients they were suggested as a major contributor to depressed cellular immunity (12). Moreover, tumor-secreted cytokines that are involved in the angiogenic process may add to the immunosuppressive microenvironment. Basic fibroblast growth factor (bFGF) and VEGF, considered being key mediators of glioma angiogenesis, act as inhibitors of cell adhesion molecule (CAM) expression in nonglioma animal models and in experiments on endothelial cells derived from normal tissues (13–19). Among these CAMs, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are regarded as the most important anchorage molecules for T cells on the blood vessel surface during the transmigration process (20–22).

Despite all these hurdles for T-lymphocytes to reach the tumor site, many investigators have observed the presence of glioma T-cell infiltrates in patient tissues, implying the generation of spontaneous antitumor immune responses (23). Whether these T-cell responses in therapy-naive patients are linked to their clinical outcome is still not clear. Investigations of intratumoral T cells were done over 20 years ago and identified immune infiltrates by leukocyte cuffing, a morphological criterium that does not discriminate among leukocyte types; their results are inconsistent however, reporting a positive correlation to clinical outcome (24, 25), a negative correlation (26) and no correlation (27). By this method T cells were not clearly identified by specific markers and no discrimination was made among the contents of immunosuppressing and effector T-cell (Teff) subpopulations. More recent studies have not analyzed Teff populations or failed to find a WHO grade-independent correlation of intratumoral Treg cells in glioma patients (28–30). And to date, a comprehensive analysis of different T-cell subsets in glioma has not been done on native tumor tissues.

The role of intratumoral T-cell subsets in gliomas may be of critical importance for the design of future immunotherapies. Therefore we investigated T-cell infiltrates in gliomas of different WHO grades with a special emphasis on the quantitative analysis of effector and inhibitory T cell subsets by co-staining of several lymphocyte markers in complete sections of native tumor tissues. By using a novel FACS-like quantitative method, we found that Teff cells particularly had a positive influence on GBM patient survival. We then established a functional model using GBM-derived endothelial cells and Teff cells isolated from the peripheral blood of the same patient. With this assay we investigated the influence of tumor-expressed cytokines on the T-cell transmigration process. We found that transmigration is mediated by both ICAM-1 and VCAM-1 and reduced in the presence of TGF-β1 and TGF-β2. Strikingly, on GBM-derived endothelial cells in culture, we observed a strong downregulation of ICAM-1 and VCAM-1 by recombinant TGF-β1 and TGF-β2.

Our data indicates that blocking both TGF-β1 and TGF-β2 promotes T-cell infiltration by restoring CAM expression. It suggests that targeting TGF-β is a potentially useful strategy to boost effectiveness of multi-modal glioma treatments by overcoming immune resistance mechanisms.

Material and Methods

Tumor material

Glioma specimens of different WHO grades were gathered from patients at the Department of Neurosurgery at Heidelberg University, Germany. Informed consent was obtained from each patient according to the research proposals approved by the Institutional Review Board at the Heidelberg Medical Faculty. Samples used for immunostainings were immediately snap frozen after surgery and stored at -80°C until processing. Intratumoral localization and WHO grading was confirmed by an experienced neuropathologist. Clinical data of the respective patients are summarized in Supplementary Table 1. A total of 93 glioma samples representing WHO II-IV were examined (study sample A). For survival analysis (study sample B, n = 44, newly diagnosed WHO IV gliomas) only those
patients were included who had received current standard therapies and experienced a tumor-related death.

**Multicolor immunostainings and evaluation**

Acetone-fixed cryostat sections (5–7 μm) were used for the double and triple immunostaining techniques. T-cell subpopulations were distinguished by combined antibody staining against CD3, CD8 and Foxp3. In all staining series human tonsil were used as internal positive control for comparable identification of T-cell subpopulations. Expression of ICAM-1 and VCAM-1 was determined together with endothelial marker CD31. Evaluation was done by TissueFAXS. Advantages of this method compared to manual evaluation of native tissue are the computerized quantification of multiple markers in large tissue areas. Leakiness of blood vessels was examined by staining of fibrinogen together with CD31 and CD8. For staining procedures, quality controls and image analysis see Supplementary Methods and Supplementary Fig. S1.

**Growth factor ELISA**

Frozen tumor pieces of approximately 0.5 mm³ size were homogenized in 1 mL PBS containing 25 μL protease inhibitor (Roche). Lysates were purified from cell debris by a 13,000 rpm centrifugation for 30 min at 4°C and filtering through a 22-μm sterile filter. VEGF, HGF, bFGF, TGF-β1, TGF-β2, PDGF-AB, PDGF-BB, G-CSF and GM-CSF in the homogenates were measured by a commercial sandwich ELISA (R&D Systems) as described (31). Assessed cytokine levels were normalized to total protein content as determined by Bradford assay (BioRad).

**Flow cytometry**

Single cell suspensions of glioma-derived endothelial cells stained with monoclonal mouse antibodies ICAM-1 (1:1,000; Acris) and VCAM-1 (1:100; Acris) in PBS containing 0.5% BSA and 2 mM EDTA for 1 h at 4°C, then washed and stained with PE-conjugated anti-mouse mAb (1:100; Dianova). Flow-cytometric analyses were performed using FACSCalibur (BD Biosciences). All samples were evaluated with appropriate isotype controls and analyzed using FlowJo software (TreeStar).

**In vitro transmigration assay**

Isolated GBM-derived endothelial cells were allowed to grow as a monolayer on gelatin-coated culture plate inserts (ThinCerts, 24-well, pore size 3.0 μm, Greiner Bio-One) in MV2 medium (Promocell) for 4 to 6 days. Tightness of the cell layer was confirmed by Evans blue dye exclusion. Ten microliters of 0.25% Evans blue dye (Sigma) were added to the medium of the upper chamber. Wells that were leaky were excluded from the assay. Transwell chambers were washed twice with cytokine-free MV2 basal medium (MV2 Kit except supplement components VEGF, IGF, bFGF, and EGF; Promocell) and placed into new 24-well plates. To prove influence of tumor-derived factors on transmigration of T-cells, transwells were incubated with VEGF, HGF, bFGF, TGF-β1 or TGF-β2 (ReliaTech), 50 μg/mL autologous tumor lysate, 50 μg/mL autologous lysate plus 1 μM SD-208 (kindly provided by W. Wick, University Hospital Heidelberg, Germany), or basal medium as a control. After 48 h, medium was renewed and supplemented with 100 ng/mL SDF-1α at the lower chamber as a chemoattractant. CD25-negative- or CD25-enriched T cells (3 × 10⁶/well) were added to the upper chamber. The number of transmigrated T cells was measured after 24 h in duplicates and detected by characteristic T-cell scatter profiles using FACSAarray (BD Biosciences). For detailed descriptions of isolation, cultivation and characterization of glioma-derived endothelial cells and T cells see Supplementary Methods and Supplementary Figs. S8 and S10.

**Statistical analysis**

Data were analyzed with 2-sided Student’s t-test, using GraphPad Prism 5.02 software. A P < 0.05 was defined as statistically significant. Kaplan–Meier estimates were used to visualize the survival curves and log-rank test was performed to compare overall survival between patients of different groups. Cut-offs for CD3⁺ T-cell populations were set at the median T-cell infiltration, cut-offs for CD8⁻ and CD8⁺ T-cell populations were set according to their mean proportion on the CD3⁺ population. The Spearman’s correlation test was used to determine the strength of correlation between different parameters analyzed within the same patient tissue.

**Results**

**Tumoral T-cell infiltration increases during malignant progression but correlates with better survival in GBM patients**

T-lymphocyte infiltration was determined in 93 gliomas of different WHO grade (Supplementary Table S1) by staining with CD3 and CD8 antibodies (Fig. 1A). Direct identification of the T-helper subset by CD4 was impractical because of high background staining in cryopreserved tissue produced by all CD4 antibodies tested (n = 3). Therefore, CD3 and CD8 served as indirect marker combination to distinguish between putative T-helper (CD3⁺/CD8⁺) and cytotoxic (CD3⁺/CD8⁻) subpopulations. Suitability of this approach was confirmed by FACS analysis on freshly dissociated glioma samples where the CD8-negative T-cell population was CD4-positive in >93% of CD3⁺/CD8⁻ cells (see Supplementary Fig. S2A).

Infiltrates of CD3⁺ cells (T cells), CD3⁺/CD8⁻ cells (T-helper cells) and CD3⁺/CD8⁺ cells (cytotoxic T cells) were observed in all samples; however, measured numbers of T cells varied markedly between samples analyzed. In WHO IV mean counts of all T-cell populations increased 9–10-fold as compared to WHO II and III tumors, whereas between pGBMs and sGBMs no significant difference was measurable (Fig. 1B). Nevertheless, even within the subgroup of WHO IV tumors considerable variances in T-cell counts were observed.

Next we addressed whether the sharp increase of T-cell infiltration from WHO II and III to WHO IV tumors...
might arise from a disturbed BBB. Therefore we stained tissues of different WHO grade for the plasma protein fibrinogen, which can diffuse into the tumor stroma only if the vessel wall becomes leaky. Indeed, significant staining of fibrinogen around leaky tumor-supplying blood vessels was only found in WHO IV (see Supplementary Fig. S3).

Further, infiltrating T cells were most commonly seen in WHO IV fibrinogen-positive tumor areas. This supports our hypothesis that leaky vessels, which typically occur in GBM with the onset of angiogenesis, facilitate T-cell transmigration as compared to lower grade tumors.

In summary, we found a WHO-grade-dependent increased T-cell infiltration. Second, in WHO IV tumors, elevated T-cell numbers were associated with BBB leakiness.

**Occurrence of effector T cells is associated with GBM patient survival**

Next, we asked if T-cell subtypes vary among tumor grades and if the observed survival differences could be attributed to the preferential infiltration of a specific T-cell subtype. This is especially important because some T-cell subtypes have been implicated in the suppression of immune-mediated destruction of tumor cells (12). CD4+ and CD8+ regulatory T cells are considered the most prominent and tumor-relevant subtypes suppressing the activity of Teff cells (32). Therefore, our investigation of T-cell infiltration of gliomas was extended using the Treg marker Foxp3. Because Foxp3 was shown to be also expressed by tumor cells (33), we additionally stained lymphocyte markers CD3 and CD8 (see Supplementary Fig. S4). To prove the validity of this marker combination to distinguish Teff and Treg subpopulations, we did flow cytometry analysis of glioma single cell suspensions with markers CD3, CD4, CD8, Foxp3, CD25, CD127 according to a recent publication on gliomas, which defined Tregs as Foxp3+CD25highCD127low (30; see Supplementary Methods and Supplementary Fig. S2B). This analysis confirmed that Foxp3 was not present on CD127+ but on
CD25+ cells. Thus CD3/CD8/Foxp3 is a suitable marker to distinguish Teff and Treg cells. Further, nearly all CD8- cells are CD4+ (see Supplementary Fig. S2A), constituting to define CD3+CD8+ Foxp3- cells as T-helper cells and CD3+CD8+ Foxp3+ cells as cytotoxic T-cells in our study.

Foxp3+CD3+ infiltrates were found in only 36% of the sGBMs and 43% of the pGBMs analyzed (Fig. 2A). Further, the proportion of the immunosuppressive T cells was very low: immunosuppressive T cells represented <1% of the total T cells identified in GBMs (Fig. 2A). This perhaps explains why we did not detect any immunosuppressive T cells in WHO II tissues, which generally had very low T-cell infiltration.

To confirm sensitivity of the Foxp3 antibody we used sections of human tonsil served as positive control (see Supplementary Fig. S5). With a proportion of 3% Foxp3+ cells within the CD3+CD8+ population, our data are in good agreement with a previous study reporting a content of 1.5% (34).

To determine the respective influences of effector and immunosuppressive T-cell subpopulations on clinical outcome, we compared their amount to the overall survival time of newly diagnosed WHO IV glioma patients (study sample B, n = 44). We observed a significant survival correlation with helper T cells (CD3+CD8+Foxp3- ; P = 0.01) and cytotoxic T cells (CD3+CD8+Foxp3+ ; P = 0.02) as well as both effector T-cell subtypes together (CD3+Foxp3- ; P < 0.01; Fig. 2B) but not for age, extent of resection and adjuvant treatment (Supplementary Table S2). In contrast, we observed neither a correlation between survival and the infiltration of Treg cells (Fig. 2B) nor ratios of Treg/Teff populations (data not shown).

Collectively, our analysis suggests that infiltration of GBM tumors with effector T cells in particular has a positive impact on patient survival.

T-cell infiltration is associated with CAM expression

Before transmigration, T cells must attach to the luminal side of the tumor-supplying endothelium. This process requires endothelial expression of adhesion molecules such as ICAM-1 and VCAM-1 (21, 22). Indeed, we found that ICAM-1 and VCAM-1 colocalized with the endothelial cell marker, CD31, in GBM tissues of study sample A (see Supplementary Table S1 and Fig. 3A). Approximately 20% to 30% of CD31-positive endothelial cells from both sGBM and pGBM coexpressed ICAM-1 and VCAM-1 (Fig. 3B).

To study the relationship between T-cell infiltration and endothelial CAM expression, we did statistical correlation analysis on data obtained from 65 GBM tumor tissues. A significant positive correlation was revealed for ICAM-1-expressing endothelial cells and all T-cell subpopulations analyzed except Treg cells (Fig. 4). We note that even on low ICAM-1-expressing endothelia remarkable T-cell infiltration was still observed, indicating that other factors (e.g., leaky vessels) might also influence the infiltration process. Nonetheless, ICAM-1 negative endothelium did not correlate with T-cell infiltration (see Supplementary Fig. S6), pointing to an influence of ICAM-1 on T-cell transmigration. Regarding VCAM-1-positive endothelial cells no significant relationship with T-cell infiltration could be proven (see Supplementary Fig. S7). That this is caused by the limited case number and the generally lower VCAM-1 expression as compared to ICAM-1-expression cannot be excluded.

In summary, we found that a substantial portion of tumor-derived endothelial cells expressed CAMs and that their expression of ICAM-1, but not VCAM-1, was positively associated with T-cell infiltration.

Proangiogenic cytokines are frequently present in glioma tissues

Gliomas secrete angiogenesis-promoting factors that are suspected of altering adhesion molecule expression (14–18, 31). To determine the expression levels of 9 known angiogenic cytokines, we measured their respective concentrations relative to total protein in WHO II (n = 14), III (n = 16), and IV [sGBM (n = 7); pGBM (n = 16)] tumor lysates (Fig. 5). Except for bFGF, cytokine expression levels were significantly higher in WHO IV gliomas (P < 0.05). Cytokines bFGF, VEGF and HGF were detected in all samples from WHO IV tumors (pGBM and sGBM) and were the most highly expressed cytokines in these tumors. Cytokines TGF-β1, TGF-β2, PDGF-AB, and PDGF-BB were also detected in all WHO IV tumor samples, but were expressed at lower concentrations than bFGF, VEGF, and HGF. G-CSF and GM-CSF showed an infrequent expression pattern.

Our results indicate that, with few exceptions, angiogenic cytokine expression is ubiquitous but WHO grade-dependent, peaking in grade IV gliomas.

TGF-β1 and TGF-β2 downregulate ICAM-1 and VCAM-1 expression on cultured GBM endothelial cells

We then evaluated the capacity of glioma-derived cytokines investigated above to regulate endothelial cell expression of ICAM-1 and VCAM-1. Similar experiments using umbilical vein endothelial cells (HUVECs) previously found that VEGF and bFGF were inhibitory (16, 18). Yet endothelial cells from different sources have been known to respond differently to the same stimulus (14, 16). Given our interests in and access to GBM, we used endothelial cells extracted from fresh GBM tissues (n = 3). For each sample, endothelial cells were positively identified by their characteristic uptake of acetylated low-density protein (AcLDL; see Supplementary Fig. S8). Cells were then incubated with the cytokines most consistently and highly expressed in GBM samples: VEGF, HGF, bFGF, TGF-β1, and TGF-β2 (Fig. 5). Flow cytometry analysis revealed that VEGF, bFGF, and HGF had only a slight effect on ICAM-1 and VCAM-1 expression (Fig. 6A). In contrast, both TGF-β1 and TGF-β2 reduced ICAM-1 and VCAM-1 expression down to approximately 42% and 8% of control levels, respectively (Fig. 6A). This result suggested that TGF-β signaling plays an important role in...
Figure 2. Effector T cells, but not immune-inhibitory T cells, were associated with patient survival. A, quantification of infiltration by immune-inhibitory (Foxp3\(^+\)) and effector (Foxp3\(^-/\)) T cells among gliomas of different WHO grade (\(n = 93\), Study Sample A). Asterisks indicate significant differences (**, \(P < 0.01\); *, \(P < 0.05\); upper row). CD8\(^-\) and CD8\(^+\) regulatory T cells were detected infrequently and not at significantly different levels between tumor grades (lower row). B, comparison of infiltration by different T-cell populations with GBM patient survival (\(n = 44\)).
regulating adhesion molecule expression. To address whether TGF-β signaling is biologically relevant to GBM, we confirmed that TGF-β receptors TGF-βRI and TGF-βRII were both expressed on endothelial cells derived from each GBM sample (see Supplementary Fig. S9).

In summary, we found that treatment with TGF-β1 and TGF-β2, but not VEGF, HGF or bFGF, markedly lowers adhesion molecule expression on cultured GBM endothelial cells.

**Effector T-cell transmigration is reduced with exogenous TGF-β1 and -β2 treatment and increased when TGF-β receptor signaling is blocked**

Finally, to study the effects of TGF-β signaling on T-cell function, we established a transmigration assay consisting of GBM endothelial cells and autologous T cells (n = 6). T cells were isolated from the peripheral blood of donors and then enriched for subpopulations of Teff and Treg cells using the Treg-cell surface marker, CD25 (see Supplementary Fig. S10). However, remarkably, both T-cell populations were found to do similarly in control transmigration experiments (20% of T cells transmigrated; Fig. 6B).

To test the functional importance of CAM molecules on T-cell transmigration, endothelial cells were first incubated with neutralizing antibodies to ICAM-1 and VCAM-1. We observed a significant decrease in T-cell transmigration efficiency in the presence of either antibody, an effect even more pronounced when they were used simultaneously (Fig. 6C).

The transmigration assay provided a means to test whether TGF-β-induced reduction of CAM expression on GBM endothelial cells could affect T-cell function. We found that incubation of the endothelial monolayer with TGF-β1 and TGF-β2, but not bFGF, VEGF or HGF, markedly impaired T-cell transmigration efficiency (Fig. 6D). The transmigration assay also permitted us to address the converse experiment: whether inhibiting TGF-β signaling in GBM endothelial cells could positively effect T-cell transmigration. We used the TGF-β receptor kinase inhibitor, SD-208, for this purpose (35). We found that incubation of the endothelial monolayer with SD-208 and GBM tumor lysate increased the efficiency of T-cell transmigration 1.5-fold over incubation with tumor lysate alone (Fig. 6E).

We conclude from these data that T-cell transmigration is directly mediated by CAM molecules expressed on GBM endothelial cells and that T-cell transmigration is specifically inhibited by TGF-β signaling activity within GBM endothelial cells.

**Discussion**

The aims of this study were to determine whether T-cell infiltration of gliomas was clinically relevant and to identify molecules that regulate glioma infiltration. Using a novel FACS-like method for the objective quantification of T-cell subtypes in large tumor sections, we found that, relative to low-grade gliomas, high-grade gliomas had significantly larger populations of effector T cells and immunosuppressive T cells. Our studies revealed that Teff cells in particular were associated with increased GBM patient survival; immunosuppressive Treg cells were found to not affect GBM patient outcome. To investigate mechanisms regulating T cell infiltration, we first established a
transmigration assay that more accurately models the process in vivo by using glioma-derived endothelial cells and autologous T cells. We discovered that treating tumor tissue with the glioma cytokines TGF-β1 and TGF-β2 caused suppressed T-cell transmigration. Further, treating with tumor lysates together with a TGF-β-signaling inhibitor caused enhanced transmigration. Considering these results, we hypothesize that TGF-β signaling antagonizes T-cell infiltration of gliomas.

Peculiar to glioma is the paradoxical relationship between T-cell infiltration and tumor grade. Many reports describe a WHO grade-dependent increase of T cells generally (this study, 29, 36, 37) and Treg cells specifically (this study, 38, 29, 30). This relationship implies that T-cell infiltration negatively correlates with patient survival. If true, this would contrast with the positive correlation identified in various other cancers such as skin, breast, prostate, and colorectal cancers (39). Studies of the relationship between glioma T-cell infiltration and patient survival have yielded conflicting results (24–27). In recent studies, Treg cells were found to correlate with a worse patient outcome (29, 30).

However, when GBM is considered separately from lower grade gliomas, differences in patient survival have not correlated with Treg-cell infiltration (this study, 29, 30). In this regard, one must consider the unique environment of gliomas. The brain is normally shielded from inflammatory cells by the BBB. However, as in other cancers, tumor blood vessels of higher-grade gliomas likely become hyperpermeable, causing a breach in the BBB and providing an opening for inflammatory cells and plasma blood proteins to more easily enter the tumor (40). Indeed, consistent with a grade-dependent hyperpermeability in glioma, we detected the plasma protein fibrinogen in samples of GBM tissue but not lower grade tissue. Given these considerations and observations, we suggest that grade-dependent hyperpermeability provides a valid explanation for the grade-dependent infiltration of T cells in glioma. Therefore, we propose that the most accurate method of evaluating the relationship between glioma T-cell infiltration and patient survival will account for differences in tumor blood vessel permeability among the different WHO grades. That is, it is important to maintain a distinction between tumor
grades and restrict comparisons of survival to patients falling within the same tumor grade category. By focusing on GBM, we have identified a subpopulation of glioma-infiltrating T cells, Teff-cells, that positively affect patient survival.

Teff-cell subsets have not been specifically accounted for in previous glioma studies and represent the first example of that T-cell subtype to have a role in glioma. This discovery is complemented by earlier morphology-based analyses of T-cell-containing cuffs in GBM tissues. These cuffs were predominantly found in long-term surviving GBM patients (41) and were associated with an improved patient outcome (24, 42). Further support comes from a very recent publication describing higher numbers of infiltrating CD8+ T cells occurring in GBM patients with a prolonged survival (43).

Regulatory T cells represented less than 1% of the T cells identified in our GBM samples in contrast to a recent publication reporting a Treg content of 14% on total CD4+ T cell population as analyzed by FACS on glioma single cell suspensions (30). To exclude that this low Treg content is due to low sensitivity of our staining procedure, human tonsil served as internal positive control in all staining series confirming that observed frequencies of Foxp3+ cells were in line with the current literature (34). To further investigate whether these contrasting results might be caused by the use of different methods, we repeated their FACS experiment on 4 WHO-grade III–IV single cell suspensions. We found Foxp3+ cells represented 10.5% of the total CD4+ population (see Supplementary Fig. 2B), which was much higher than we observed by TissueFAXS. This supports

Figure 5. Angiogenic growth factor concentrations generally increase with glioma tumor grade. A significant WHO grade-dependent concentration increase was found for VEGF, HGF, TGF-β1, TGF-β2, PDGF-AB, PDGF-BB, G-CSF, and GM-CSF. Values are mean concentrations of growth factors in tumor lysates of gliomas of different WHO grade (n = 52). Asterisks indicate significant differences (P < 0.05) between grades. t.p. = total protein.
the idea that the cause of these different results might be technical in nature. In the approach used by Jacobs et al.—the analysis of suspensions—cells derived from peritumoral areas cannot be excluded. This is a likely and important point because for other tumor entities like metastatic melanoma (44) or hepatocellular carcinoma...
(45), it has been shown that T-cell levels are greater peritumorally than intratumorally. Further, we have made the same observation in large paraffin sections containing invasion zone/tumor border in addition to the tumor center (N. Höll and C. Herold-Mende, unpublished data).

Although we did not find $T_{\text{reg}}$-cells to influence the survival of GBM patients, in animal models $T_{\text{reg}}$-cell depletion has been associated with enhanced immune responses and improved survival (12, 46, 47). Therefore, in an immunotherapeutic setting it might also be important to control immune-inhibitory T-cell subsets. Nevertheless, our data supports the hypothesis that to improve survival of GBM patients, it would be beneficial to increase infiltration of $T_{\text{eff}}$ cells.

T-cell infiltration depends on the expression of several adhesion molecules on the endothelial cell surface, including ICAM-1 and VCAM-1 (21, 22). Blocking these molecules can substantially decrease adhesion and transmigration of T cells (22). Indeed, our pretreatment of GBM-derived endothelial cells with neutralizing antibodies against both CAMs reduced transmigration, providing evidence of their functional relevance to T-cell infiltration. However, that adhesion molecules were only found to be expressed on ~20% to 30% of GBM-derived endothelial cells and with a large intertumoral heterogeneity points to the need to learn more about their regulation in tumor-derived vessels.

We hypothesized that the low frequency of CAM-expressing endothelial cells in GBM tissue might be due to the presence of tumor-secreted cytokines that are known to downregulate these adhesion molecules during neoangiogenesis (13–19). Quantification of 9 well-known angiogenic cytokines in our study sample revealed that 5 of them were constitutively present in glioma lysates; these included: VEGF, HGF, bFGF, TGF-$\beta$1, and TGF-$\beta$2. Furthermore, except for bFGF, the cytokines were detected in highest amounts in GBM tissues. It has been reported that VEGF and bFGF can downregulate ICAM-1 and VCAM-1 in extracranial tumors (16, 17). However, in GBM-derived endothelial cells, we found that only TGF-$\beta$1 and TGF-$\beta$2 resulted in a strong and reliable decrease of CAM expression. This suggests that glioma-derived blood vessels regulate CAM expression by a mechanism distinct from the endothelium in other organs.

In glioma, TGF-$\beta$s have a multifaceted role in tumor promotion. They function as proliferative, proangiogenic and proinvasive factors by inducing the expression of VEGF, matrix metalloproteinases and integrins, especially in high-grade gliomas (48). TGF-$\beta$s also stifle the host immune response to glioma, for example, by inhibiting $T_{\text{eff}}$-cell proliferation and inducing their apoptosis, and by facilitating the transition of naive T cells into immunosuppressive $T_{\text{reg}}$ cells (9–11). Thus, blocking TGF-$\beta$ signaling would seem to be a useful strategy in glioma treatment. In animal models of glioma, treatment with the TGF-$\beta$ receptor small molecule inhibitor, SD-208, caused increased animal survival and increased immune cell infiltration (35, 49). Consistent with these results, we found that treating glioma-derived endothelial cells with SD-208 significantly enhanced the transmigration of autologous T cells. Thus, collectively, our data suggests an additional mechanism by which TGF-$\beta$ asserts its immunosuppressive effect in glioma: the downregulation of CAM expression on tumor-supplying endothelium, which thereby hinders T cells from overcoming the vessel barrier.

There are immediate applications of our discovery that $T_{\text{eff}}$-cell infiltration positively affects GBM patient survival and that the glioma cytokine TGF-$\beta$ is an important negative regulator of T-cell transmigration through GBM-derived endothelium. For example, as a prognostic marker for patient outcome, $T_{\text{eff}}$-cell infiltration might serve as a tool to monitor the responses of patients to immunotherapy treatment. Our results also predict that the effectiveness of glioma immunotherapy could be improved by simultaneously inhibiting TGF-$\beta$ signaling within tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Effector T-Cell Infiltration Positively Impacts Survival of Glioblastoma Patients and Is Impaired by Tumor-Derived TGF-β

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