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Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy

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In patients with glioma, the tumor microenvironment can significantly impact pro-inflammatory immune cell functions. However, the mechanisms by which this occurs are poorly defined. Because immunosuppressive regulatory T cells (Treg) are over represented in the tumor microenvironment compared with peripheral blood, we hypothesized that the tumor may have an effect on Treg survival, migration, expansion, and/or induction of a regulatory phenotype from non-Treg conventional CD4+ T cells. We defined the impact of soluble factors produced by tumor cells on Treg from healthy patients in vitro to determine mechanisms by which gliomas influence T cell populations. We found that tumor-derived soluble factors allowed for preferential proliferation and increased chemotaxis of Treg, compared with conventional T cells, indicating that these mechanisms may contribute to the increased Treg in the tumor microenvironment. Conventional T cells also exhibited a significantly increased expression of pro-apoptotic transcripts in the presence of tumor-derived factors, indicating that survival of Treg in the tumor site is driven by exposure to soluble factors produced by the tumor. Together, these data suggest that tumor burden may induce increased Treg infiltration, proliferation, and survival, negating productive anti-tumor immune responses in patients treated with immunotherapies. Collectively, our data indicate that several mechanisms of Treg recruitment and retention in the tumor microenvironment exist and may need to be addressed to improve the specificity of immunotherapies seeking to eliminate Treg in patients with glioma.

Keywords: glioma, immunotherapy, regulatory T cell, tumor microenvironment.

Traditional treatment of patients with glioblastoma multiforme (GBM) using chemotherapy and radiation can have a devastating effect on a patient's quality of life, making immunotherapy an appealing alternative. The major strength of targeting tumor cells using immunotherapy is the potential to specifically target tumor cells without collateral damage to healthy neural cells. Several studies using mouse models have indicated that immune cell responses to tumor cells are critical to the early elimination of tumor cells.[1] Studies using mice deficient in T cells and natural killer (NK) cells or the proinflammatory cytokines that they produce have demonstrated the importance of an intact immune system in tumor surveillance in vivo.[2–4] However, establishment of ex vivo and peripheral immune responses does not guarantee improved clinical outcome.[5–7] Therefore, understanding the mechanisms for poor clinical efficacy of early generation immunotherapies has led researchers to evaluate the impact of the tumor microenvironment on immune cell functions that may inhibit immune responses to the tumor in situ.[8–10]

Among cells known to impair productive anti-tumor immunity in many tumor microenvironments are CD4 + CD25 + FoxP3 + Treg.[11–13] Treg are an endogenous subset of T cells, which play an important role in maintaining peripheral self-tolerance and preventing possible immunopathologies by mitigating potential and ongoing immune reactions. Treg constitute 5%–10% of the peripheral CD4+ T cell population in

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healthy patients and directly counteract the proliferation and cytokine production by conventional T cells.[14] In patients with high-grade glioma, reports demonstrate that up to 60% of the tumor-infiltrating lymphocyte (TIL) population is composed of Treg.[15,16] These studies suggest that immunotherapies relying on the effector functions of conventional T cells may be improved by eliminating this population in the tumor microenvironment. Indeed, depletion of Treg has been shown to increase tumor immunity[17] and improve survival,[18] indicating a role for Treg in preventing immunity to tumors.

Although it has been demonstrated that Treg cell frequencies are vastly overrepresented in the tumor microenvironment, compared with those in circulation,[15] the mechanism of recruitment and expansion and/or survival of these cells is controversial. Previous reports have shown the potential for preferential attraction of Treg by chemokines secreted by various tumor types and that tumor-derived chemokines can increase Treg infiltration;[12,19] however, it is not clear whether these chemokines are necessary or sufficient to attract a Treg population to the tumor. Glioma-derived TGFβ has also been shown to prevent conventional effector T cell migration to the tumor site, which is associated with a poor prognosis in patients with GBM,[20] suggesting a mechanism for Treg overrepresentation in the tumor microenvironment and failure of local anti-tumor responses. More recently, it has been demonstrated that myelomonocytic cells present in the tumor microenvironment of patients with glioma may also promote Treg induction.[21] Together, these studies indicate that a tumor may rely on various and potentially redundant mechanisms to recruit and retain Treg within the tumor microenvironment, preventing productive effector T cell functions.

These studies collectively suggest that Treg play a pivotal role in the failure of immune therapies; therefore, we asked whether Treg preferentially migrate, proliferate, or survive after exposure to soluble factors produced by glioma cells. Furthermore, we examined the possibility that a conventional T cell can be converted to a Treg after stimulation in the presence of these secreted factors, as has been recently demonstrated.[22,23] Our data suggest that local conversion of conventional T cells to Treg does not significantly contribute to the observed increased frequency but that Treg cells in the tumor microenvironment may preferentially migrate, proliferate, and survive in response to factors that are produced by tumor cells. Collectively, our data support the notion that multiple mechanisms contribute to Treg accumulation in the tumor microenvironment in addition to preferential chemoattraction. Therefore, reduction of Treg in the tumor may be more effective if immunotherapies target additional mechanisms, such as preferential survival and proliferation of Treg, potenitally improving the clinical outcome of GBM treated with immunotherapies.

Methods and Materials

Clinical Specimens

Tumor tissue and blood samples were collected from patients with GBM during surgical resection. Blood samples were also collected from patients free of malignant glioma tumor burden during procedures, such as meningioma resection, as negative controls. Meningioma-resected patients were used as negative control subjects for 2 reasons. The first is that blood samples were collected from both patient subsets at the time of surgery, and accordingly, all patients will have been subjected to a standard algorithm of preoperative management including, but not limited to, antibiotic administration, a bolus of decadron, mannitol, and other medicatons, including anti-convulsant drugs. In addition, to obtain TILs used as comparison for Treg tumor infiltration, the patients must undergo intracranial surgery, limiting the availability of healthy control subjects. All patients gave informed consent for sample acquisition under the University of California, San Francisco (UCSF) Internal Review Board–approved Brain Tumor Research Center protocol CHR# 10-01271.

Cell Lines

Glioma cell lines U87, SF767, and U251 were obtained through the UCSF Brain Tumor Research Center. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM H-21) with 10% fetal bovine serum and 1% penicillin-streptomycin.

Tumor Conditioning

Tumor conditioned medium (TCM) was prepared by culturing cell lines in complete T cell medium (RPMI-1640 25 mM Hepes, 2.0 g/L $NaHCO_3$ supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 10 mM nonessential amino acids) when confluent for 48 h. The conditioned medium was diluted 1:1 with fresh complete T cell medium to replenish nutrients and was used in subsequent assays.

TIL and Circulating Lymphocyte Isolation

Peripheral blood lymphocytes (PBL) were isolated from whole blood using a Ficoll-Paque Plus density gradient solution (GE HealthCare). TILs were isolated from tissue samples by mincing tumor samples and treating them with 1mg/mL Collagenase D (Sigma) for 30 min. The resulting slurry was subjected to a Percoll density gradient (Sigma), as previously described.[24] Cells were resuspended in 70% isotonic Percoll (Amersham Biosciences) and centrifuged for 25 min at room temperature at 553 ×*g*on at a 30%:37%:70% Percoll

gradient with densities of ρ = 1.065, ρ = 1.072, and ρ = 1.088, respectively. Treg were isolated from whole PBL using a negative CD4 T cell selection, followed by a CD25 positive selection (Stem Cell Technologies), according to the manufacturer's instructions. Conventional T cells were harvested using the negative fraction remaining from the CD25-positive T cell selection.

Flow Cytometry

T cells were surface stained with anti-human CD4 PerCP (BD Pharmingen) and CD25 APC (eBoscience) on ice for 25 min in PBS/2% BSA. Cells were fixed in 2% paraformaldehyde and permeabilized using CytoPerm (BD Pharmingen). Cells were then intracellularly stained in a saponin-containing buffer with FoxP3 FITC (eBioscience) and TGFβ PE (R&D Systems). Samples were analyzed on FACSCalibur Flow Cytometer using CellQuest Pro (BD Biosciences) and analyzed using FlowJo (Treestar) software.

T Cell Proliferation

Treg were stained using the CellTrace CFSE Cell Proliferation kit (Invitrogen) according to the manufacturer's instructions. In brief, selected Treg were stained at 37°C for 10 min with 10 μM CFSE, quenched with FBS, and washed 3 times with serum-containing media. Conventional CD4+ T cells were stained with PKH26 (Sigma-Aldrich), according to the manufacturer's protocol. In brief, conventional T cells were stained for 5 min at room temperature, quenched with FBS, and washed 3 times with serum-containing media. PKH-labeled T cells were plated alone or with CFSE labeled Treg in a 1:1 ratio for a total cell concentration of 10^6 cells/mL with 5 μg/mL purified anti-CD3 and 2 μg/mL purified anti-CD28 (eBioscience) in the presence of tumor-conditioned media. The cultures were incubated for 72 h at 37°C and 5% CO_2. The cells were harvested and analyzed using flow cytometry, as described above.

T Cell Suppression Assay

Conventional T cells were stained with 10μM CFSE and stimulated with 5 μg/mL purified anti-CD3 and 2 μg/mL purified anti-CD28 in the presence of the indicated number of Treg cells. After 72-h incubation, cells were analyzed using flow cytometry for Treg markers CD3, CD4, CD25, and FoxP3.

T Cell Conversion Assay

Conventional T cells were cultured at 1×10^6 cells/mL in complete T cell medium or U87 TCM and stimulated with 5 μg/mL purified anti-CD3 and 2 μg/mL purified anti-CD28 (eBioscience). Cells were harvested on days 3, 5, 7, and 10; 10 U/mL IL-2 (10^7 U/mg) was added to cells every 48 h. Brefeldin A (BFA, 10 μg/mL; Sigma-Aldrich), Phorbol 12-myristate 13-acetate (PMA; 50 ng/mL), and ionomycin (500 ng/mL) were added to the coculture for the last 5 h of each time point. Cells were stained as described above and analyzed using flow cytometry.

Chemoattraction Assay

CFSE-labeled Treg and unlabeled conventional T cells were plated at 10^5 cells/well in the top chamber in 5-um pore polycarbonate 24-well transwell plates (Corning Incorporated). U87 and SF767 TCM, CCL22 (10 ng/mL or 50 ng/mL), or CCL5 at 30 ng/mL (R&D Systems) were seeded on the bottom chamber of the plates. Concentrations of the negative control, CCL5, were selected based on manufacturer-provided ED_{50} concentrations. CCL22 concentrations were also selected on the basis of manufacturer-provided ED_{50} and were chosen to be 1× (10 ng/mL) and 5× (50 ng/mL) concentrations of those used in previously published work.[19] Twenty-four h later, cells were harvested, stained with anti-human CD45 APC (eBioscience), and analyzed using flow cytometry. Migration was expressed as percentage of total recovered cells found to migrate through pores and normalized to baseline migration.

Quantitative Polymerase Chain Reaction (PCR)

Whole cell mRNA was extracted using the RNeasy Mini Kit (Qiagen). Total RNA was then extracted and converted to cDNA by random hexamer priming and SuperScript III reverse transcriptase (100–250 ng RNA per reaction; Invitrogen). PCR amplification was performed with SYBR green master mix (5–10 ng cDNA per reaction; Applied Biosystems) via an iQ5 Real-Time PCR thermal cycler (BioRad). Reactions were performed in duplicate; C_t values were normalized to expression levels of hypoxanthine phosphoribosyltransferase (HPRT) and displayed as relative expression units (Table 1).

Statistical Analysis

Data for all the figures were collected from at least 2 independent experiments performed in triplicate. Representative raw experimental data are shown to clarify gating strategy and are consolidated in Excel format. Determinations of statistical significance were made on the basis of integrated experimental data. Statistical significance was determined using a 2-tailed Student t test, with $P < .05$. Error bars represent ± standard deviation of the mean of repeated experiments. Quantitative PCR was analyzed using the Pfaffl method, and statistical significance was determined using a nonparametric Wilcoxon 2-group test as described elsewhere.[25]

Table 1. Primer sets used for quantitative polymerase chain reaction

Gene name	Forward Primer	Reverse Primer
BAK	CAACCGACGCTATGACTCAG	AGTGATGCAGCATGAAGTCG
BAX	ACCAAGAAGCTGAGCGAGTG	GTGTGACTGGCCACCTTCTT
BID	CCCGCTTGGGAAGAATAGAG	GTGTGACTGGCCACCTTCTT
BAD	CGGAGGATGAGTGACGAGTT	GGAGTTTCGGGATGTGGAG
BIM	CAGCACCCATGAGTTGTGAC	CAATGCATTCTCCACACCAG
HPRT	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG

Results

Because of the well-established role of Treg in suppressing productive immune responses, we hypothesized that the accumulation of Treg in tumor tissue from patients with GBM may be achieved through multiple mechanisms, including preferential Treg chemoattraction.[12,16] We first confirmed the findings of previous studies that demonstrate an overrepresentation of Treg in the tumor microenvironment, compared with Treg in circulation.[15] Similar to previous reports, we found an increased percentage of Treg in the tumor tissue, compared with that in circulation. Patients with GBM had a mean of 38.5% Tregs, as defined by flow cytometric analysis of CD3, CD4, CD25, and FoxP3 expression at the tumor site ($n = 20$) (Fig. 1A). This population was significantly more than 11.9% in the peripheral blood of the same patients (Fig. 1B). Percentages in the peripheral blood were still higher than those observed in healthy individuals or patients with benign meningioma undergoing intracranial surgery (5%–10%; data not shown), suggesting that GBM tumor burden has both a local and systemic effect on CD4 T cell populations.

Tumors secrete various soluble factors, including chemoattractant molecules that have the potential to attract Tregs from the periphery to the tumor site. We performed a transwell assay to determine whether CFSE-labeled Treg preferentially migrated in response to tumor cell–derived soluble factors, compared with unlabeled conventional T cells (Fig. 2A). Gliomas have been shown to secrete CCL22,[16] and we found that this chemokine preferentially attracted Treg. We also tested a panel of other tumor-derived chemokines, including CCL5, CCL2, CCL10, CXCL10, CCL22, CCL17, CXCL13, and CCL20, and found that, as previously published,[19] only CCL22 had a statistically significant effect on Treg migration. SF767 Tumor TCM, containing a variety of chemokines and cytokines, had 2.5-fold preference for Treg migration over that of conventional T cells, compared with a 1.5-fold preference for the highest dose of CCL22 alone (Fig. 2B). Furthermore, antibody blocking of the CCL22 receptor CCR4 did not reduce Treg migration to the level observed for conventional T cells cultured with TCM (data not shown). Together, these data suggest that soluble factors produced by glioma cells, including CCL22, can induce preferential migration of Treg.

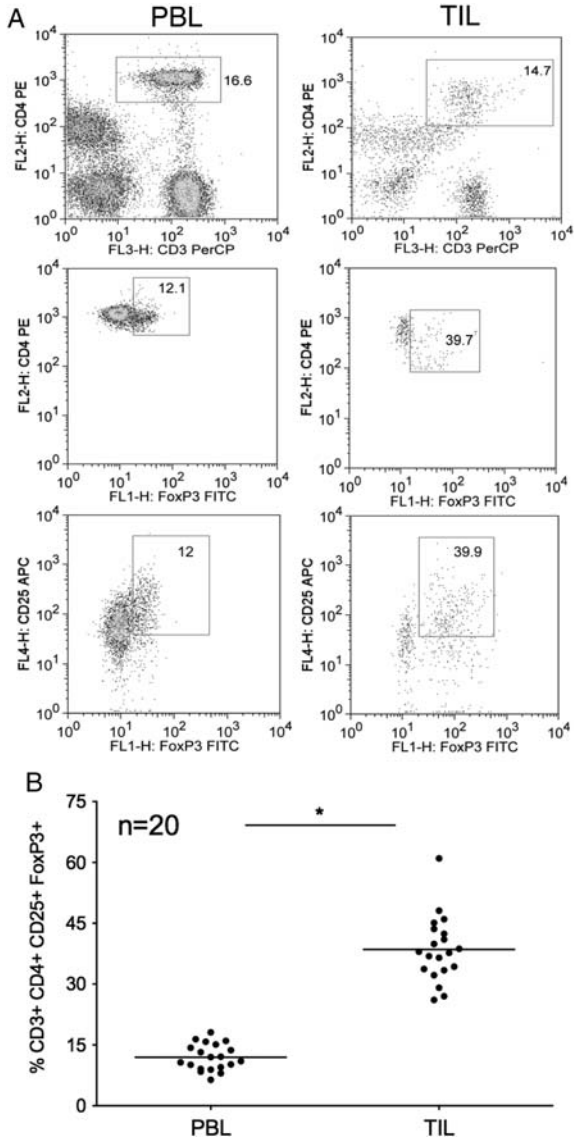

Fig. 1. Patients with GBM have an increased percentage of Treg in circulation relative to healthy controls, and Treg are overrepresented within the tumors. (A) Lymphocytes were isolated from blood and tissue collected during glioblastoma multiforme (GBM) surgical resections. T cells were defined as CD3 + CD4 +, and Tregs were defined as CD3 + CD4 + CD25 + FoxP3 + T cells. Representative FACS plots for gating Treg are depicted. (B) FoxP3 + expression in tumor infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) from patients with GBM is depicted ($n = 20$).

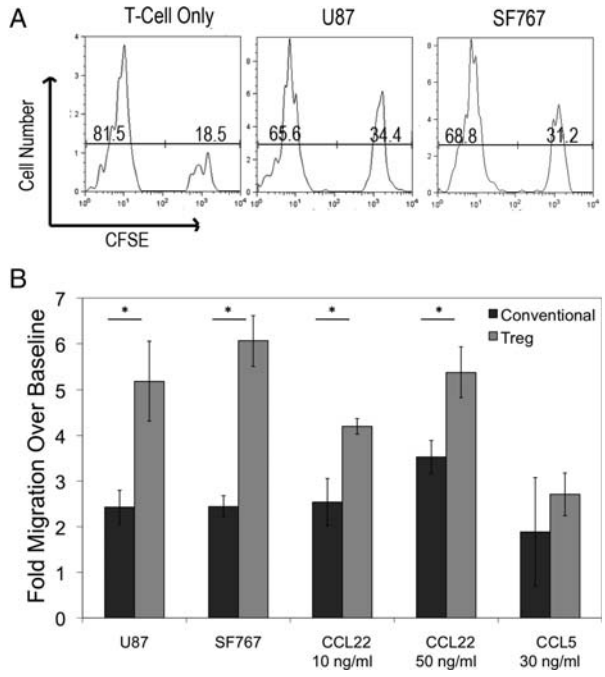

Fig. 2. Tumor-derived soluble factors preferentially attract Treg. (A) 1×10^5 CFSE labeled Treg were cocultured with 1×10^5 unlabeled conventional T cells in 0.5 μm transwell plates. Cells from the bottom chambers were analyzed using flow cytometry after culturing in complete T cell medium alone (left histogram), U87 TCM (center histogram), and SF767 TCM (right histogram). Representative histograms are shown. (B) Fold migration of both Treg and conventional T cells are shown over baseline migration for cells incubated in medium with U87 or SF767 TCM, CCL22 at 10 ng/mL or 50 ng/mL, and CCL5 at 30 ng/mL ($n = 3$).

Because all human T cells will increase expression of the canonical Treg markers CD25 and FoxP3 after stimulation, we first labeled Treg with CFSE and conventional T cells with PKH26—2 fluorescent dyes that are distinguishable by FACS. Once labeled, we subjected the mixed culture to CD3/CD28 stimulation and coculture with TCM. We found that the 2 cell populations were easily detectable, and the dyes diluted as expected after T cell stimulation (Fig. 3A). To demonstrate that the Treg isolated from healthy individuals were functional, we cocultured stimulated conventional T cells and added increasing numbers of Treg. The addition of as few as 2×10^4 Treg significantly reduced conventional T cell proliferation (Fig. 3B). For subsequent coculture experiments, we therefore modified the ratio of Treg to conventional T cells to 1:1.8. In doing so, we sought to minimize Treg-mediated suppression in coculture and to establish a coculture with physiologically relevant ratios based on tumor-infiltrating T cell data from patients with GBM (Fig. 1). We also analyzed the proliferation of conventional T cells in the absence of any Treg after stimulation and found that conventional T cells cultured in TCM in the absence of Treg proliferate after stimulation (mean = 33.5%) (Fig. 3C) , suggesting that soluble tumor-derived factors inhibit conventional T cell proliferation independently. In contrast, when cultured with TCM, Treg proliferation is significantly greater than that observed for conventional T cells (mean = 45.6%). In the tumor microenvironment, where both Treg and conventional T cells are present in ratios as great as 5 Treg to 1 conventional T cell, it is likely that there is an even greater reduction in the ability of conventional T cells to proliferate in response to stimulation (mean = 25.4%).

Many recent studies have focused on T cell plasticity and polarizing conditions, which can conditionally induce Treg phenotypes in conventional T cells. If this mechanism is exploited by GBM, immunotherapies that are predicated on initiating T cell responses and recruitment to the tumor site may instead promote tumor evasion through converted Treg-mediated immune suppression. We therefore examined the effects of malignant gliomas on T cell plasticity to determine whether conventional T cells may be converted to a suppressive Treg after recruitment to the tumor microenvironment. We cultured a population of conventional T cells in the presence of tumor-conditioned medium and observed Treg-associated markers FoxP3 and TGFβ over the incubation (Fig. 4A). Initially, there was a significant increase in the percentage of cells expressing both FoxP3 and TGFβ (Fig. 4A and B). The percentages of conventional T cells expressing TGFβ and FoxP3 after stimulation in the presence of TCM were 3.3 and 2.4 times greater, respectively, than those of conventional T cells stimulated without TCM (Fig. 4C). By day 10, however, these percentages were equivalent, suggesting that conversion to a suppressive Treg phenotype is transient and may be apparent only during peak stimulation (Fig. 4C).

Conventional T cells that were transiently induced to increase expression of FoxP3 and TGFβ after TCM culture (converted Treg) were phenotypically similar to Treg (4B and data not shown), but it was unclear whether they were functionally suppressive. To address this, we cultured with autologous CFSE-labeled conventional T cells with converted Treg and found that, similar to thymically derived Treg, converted Treg suppressed conventional T cell proliferation (Fig. 4D). Our data suggest that soluble factors produced by gliomas can upregulate Treg-associated markers in conventional T cells after stimulation and that these cells are functionally suppressive, even if only transiently. Collectively, our data support the notion that patients with a minimal tumor burden would be preferred candidates for T cell–based immunotherapies to prevent even temporary increases in functional Treg during immune responses.

In cases of some immune cells in the tumor microenvironment, such as myelomonocytic cells, tumor cells may actively promote the survival of immune cells that support tumor immune escape and prevent antitumor immune responses.[26,27] We therefore sought to determine whether tumor-derived soluble factors influenced the survival of Treg, compared with conventional T cells, as determined by changes in the expression of

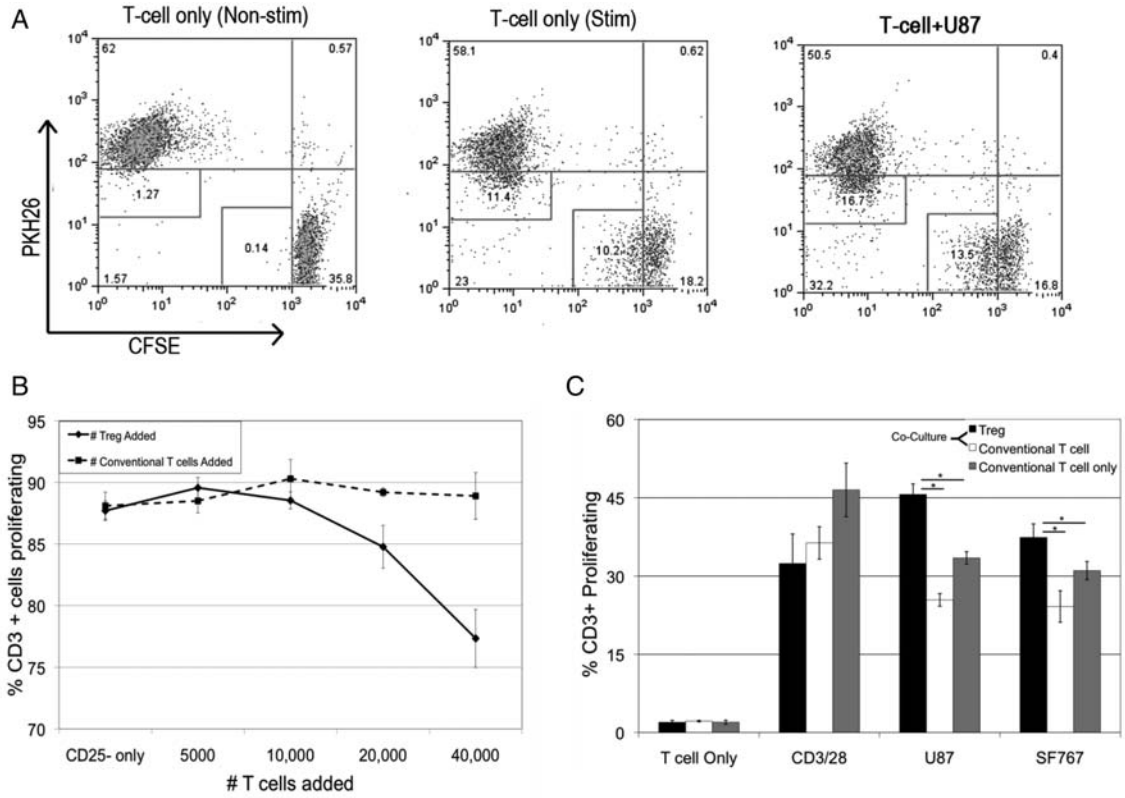

Fig. 3. Soluble factors produced by glioblastoma cells promote Treg proliferation while suppressing conventional T cell proliferation. (A) Isolated conventional T cells, labeled with PKH26, were cultured alone or with CFSE labeled Treg, in either complete T cell medium, U87 TCM, or SF767 TCM (not shown). Treg and conventional T cells were cultured in a 2:1 ratio. Proliferation was measured using flow cytometry. Representative FACS plots are shown for PKH26/CFSE dot plots. (B) Percent of proliferating cells Treg and conventional T cells from the same coculture are shown with the rate of conventional T cells cultured alone. Varying conditions include in complete T cell medium with or without stimulation and in the presence of U87 or SF767 TCM. (C) CFSE-labeled conventional T cells were plated with varying numbers of Treg or conventional T cells ($n = 3$). Proliferation of conventional T cells was measured using flow cytometry as described in the Materials and Methods section.

pro- and anti apoptotic genes. We found that expression of anti-apoptotic genes Bcl2, Bcl-XL, IAP-1, and IAP-2 did not vary significantly after stimulation or in the presence of tumor TCM (data not shown). After stimulation in the absence of TCM, both Treg and conventional T cells decreased transcription of pro-apoptotic genes Bak, Bax, and Bim. The most striking reduction was seen in Bax transcription in both Treg (8.2-fold) and conventional T cells (10.6-fold) (Fig. 5A). After culture with TCM, Treg significantly reduced pro-apoptotic genes Bax, Bak, and Bim, even in the absence of stimulation (Fig. 5A). In contrast, conventional T cells increased expression of Bax, Bak, and Bim, suggesting a possible mechanism of preferential Treg survival in the tumor microenvironment. For example, Treg reduced Bax expression from 31.4 to 12.2 (61.2% reduction) relative to HPRT without stimulation, compared with conventional T cells, which increased Bax expression after TCM culture from 26.9 to 43.1, relative to HPRT. Indeed, tumor TCM coculture increased transcript levels for conventional T cells of several pro-apoptotic genes, with a mean 1.98-fold increase after stimulation in the presence of TCM, whereas Treg expression of these genes significantly decreased, compared with unstimulated Treg (Fig. 5B).

Having demonstrated that preferential migration, proliferation, and survival may contribute to the suppressive tumor microenvironment, we sought to determine whether the effects of tumor-mediated increases in Treg are restricted to the tumor microenvironment or whether the tumor may also exert a systemic effect. Increased survival, trafficking, and proliferation of Treg may in part explain the increase in Treg percentages in the circulation of patients harboring a GBM tumor. Therefore, we sought to determine whether tumor burden is associated with circulating Treg percentages in patients with GBM. To test this, we compared MRIs used to determine tumor burden and analyzed percentages of Treg by flow cytometry in patients within 48 h of the image acquisition. We analyzed a small cohort of patients prior to tumor resection, a mean of 34.8 days after the procedure, and again prior to surgery for tumor recurrence. We observed a significant decrease in circulating Treg

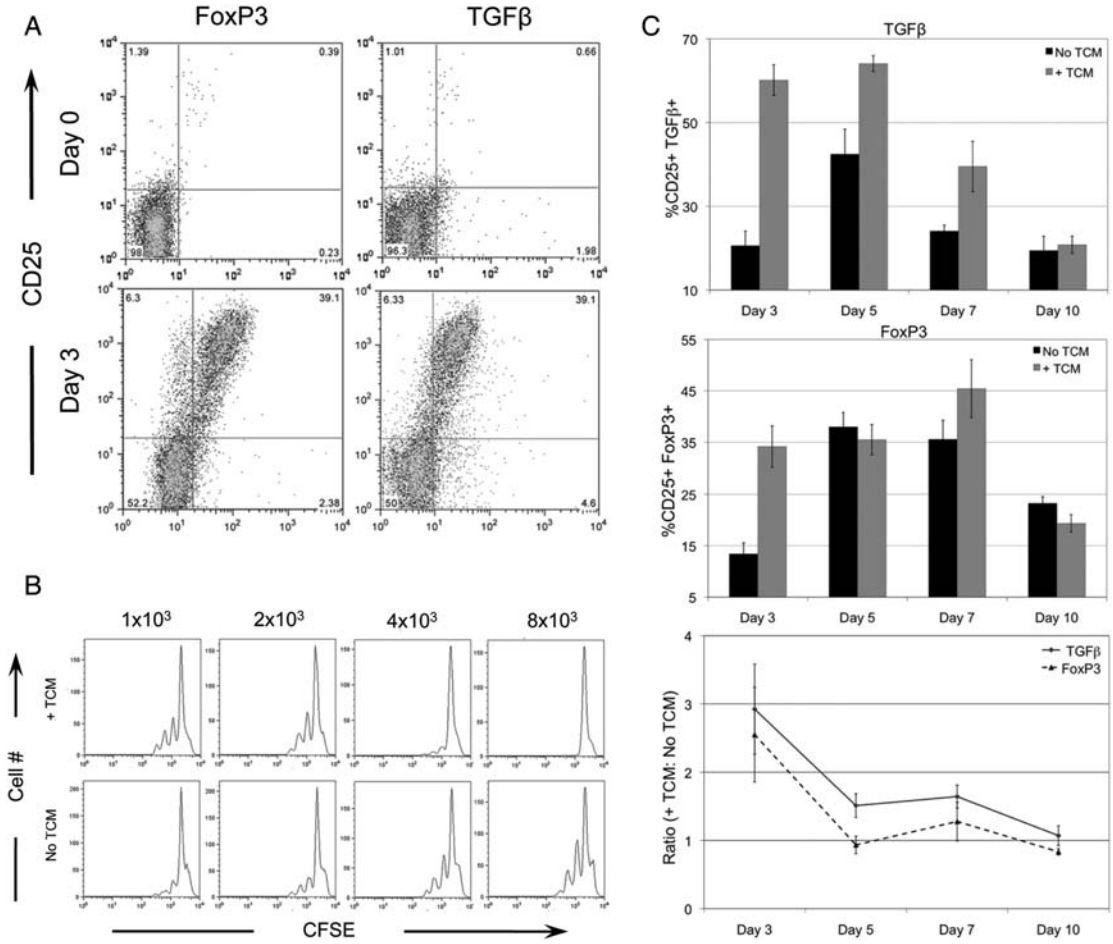

Fig. 4. Conventional T cells transiently increase expression of Treg associated proteins FoxP3 and TGF-β in response to soluble tumor factors and are functionally suppressive. (A) Freshly isolated conventional CD4 + T cells were cultured in complete T cell medium or U87 cultured medium (TCM). Cells were harvested at Day 3, 5, 7, 10 poststimulation and stained for Treg markers, FoxP3 and TGF-β in addition to CD3, CD4, and CD25. Representative FACS plot are shown. (B) Time course of TGF-β (Top) and FoxP3 (Bottom) expression on CD4 + CD25 conventional T cells following stimulation in the presence of TCM ($n = 3$). (C) The ratio of expression of Treg associated markers, FoxP3 and TGF-β, induced by culturing in the presence of TCM: complete T cell media ($n = 3$). (D) T cells cultured in normal T cell media and those cultured in TCM (with tumor) were isolated at Day 7. CD25 + T cells from both conditions were then cultured with freshly isolated and CFSE-labeled, CD3/CD28 stimulated, autologous conventional T cells. Histograms depict the proliferation of CFSE labeled conventional T cells cultured with autologous CD25 + CD4 T cells.

populations after a gross total tumor resection (more than 0%) from 16.1% to 5.97%, which rebounded to 14.5% by the time of recurrence (Fig. 6A). We then examined the trend in a subset of patients opting for repeat surgery at the time of recurrence. Of the 7 patients analyzed, we found that, although not always statistically significant, the percentage of circulating regulatory T cells decreases after gross total resection and increases at the time of surgery for tumor recurrence (Fig. 6B). Together, these data suggest an association between tumor burden in patients with glioma and percentages of circulating Treg. Current experiments seek to evaluate a larger and prospectively analyzed cohort of patients to determine whether the observed trend is reproducible. These data suggest that circulating Treg may be useful as an indicator of tumor burden in patients with GBM.

Discussion

The accumulation of Treg in the tumor microenvironment in patients with GBM has been a focus of those seeking to improve immune therapy because of the influence of tumor-associated Treg on local and systemic immunity.[28] However, the source of Treg in the tumor microenvironment remains controversial. Similar to previous reports, our data suggest that preferential migration of Treg in response to tumor-derived soluble factors contributes to the observed accumulation at the tumor site. Our data indicate that increased proliferation and survival also contribute to Treg accumulation, particularly in the context of T cell activation.

Although increased Treg chemoattraction in response to CCL22 has been demonstrated,[19] our data suggest

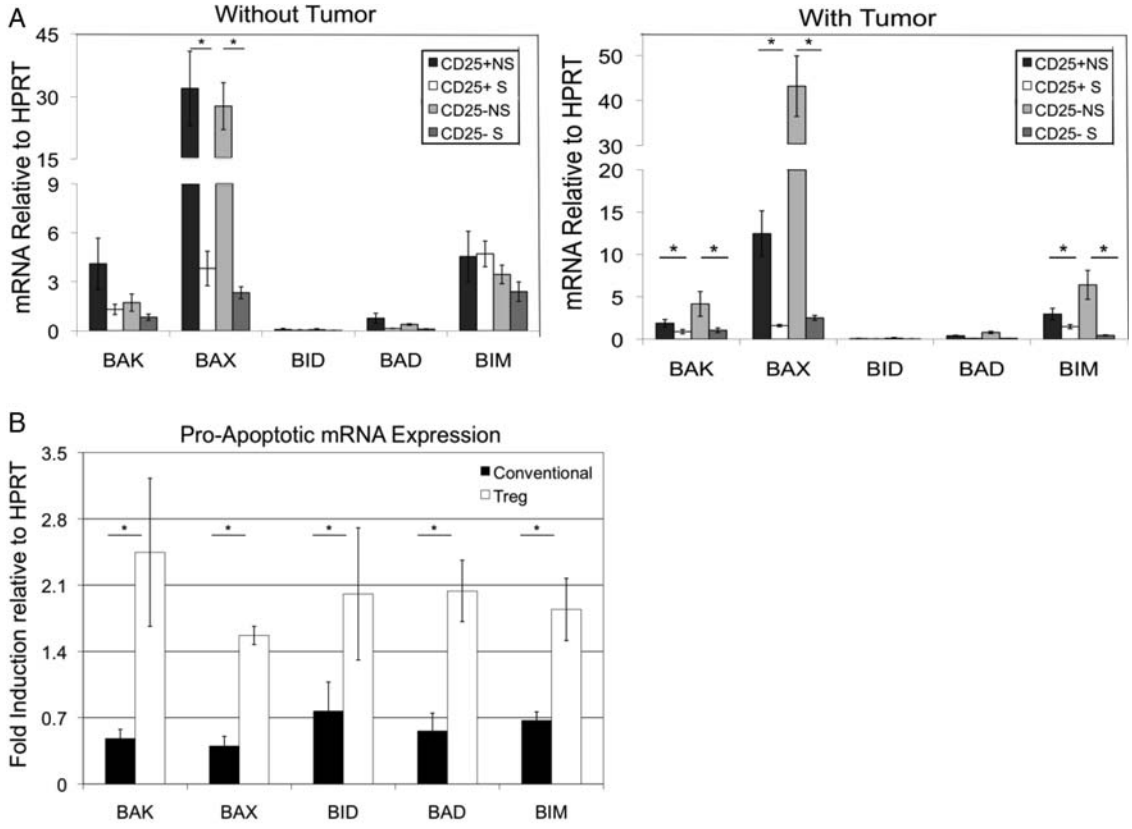

Fig. 5. Conventional CD4 + T cells increase pro-apoptotic mRNA expression in the presence of tumors. Magnetically purified Treg and conventional T cells were cultured in T cell medium (TCM). Following isolation of RNA, whole cell reverse transcription and Quantitative PCR was conducted to measure mRNA levels of known pro-apoptotic proteins BAK, BAX, BID, BAD, and BIM and standardized to HPRT gene expression. (A) Relative mRNA expression for Treg and conventional T cells for pro-apoptotic proteins without tumor conditioning (left) or following coculture with TCM (right). Bar graphs also show expression levels for unstimulated (NS) CD25+ Treg and CD25- conventional T cells and following 72 h CD3/CD28 stimulation (S) ($n = 3$). (B) Transcript expression of Treg and conventional T cells following stimulation in the presence of TCM were normalized to gene expression in unstimulated Treg or conventional T cell subsets ($n = 3$).

that other soluble factors secreted by glioma cells may also contribute to Treg accumulation in the tumor microenvironment, because blocking of CCL22 receptor did not completely abrogate the observation of preferential Treg migration. Future studies are needed to identify additional chemokines responsible for Treg recruitment to and retention in the tumor microenvironment.

Recent work has also examined proliferation rates in response to tumor-derived microvesicles and has shown Treg proliferation in response to microvesicles in head and neck squamous cell carinoma cells.[29,30] Our data support the observation that tumor-derived secreted factors increase proliferation of Treg, compared with conventional T cells. In the present study, we extend the observation to show that this increased proliferation occurs in response to glioma-derived soluble factors and is specific to cells defined as Treg prior to stimulation. Identification of human Treg is difficult after stimulation, because stimulated CD4 cells increase expression of all canonical Treg markers (Fig. 5). Future experiments will examine whether glioma-derived microvesicles originally described by Bastida et al. in 1984[31] have a similar effect on Treg expansion. More recently, Coffelt et al. described a preferential expansion in Treg population in the tumor environment using an angiogenic mammary tumor model,[32] suggesting that this phenomenon may extend to many types of tumors, independent of location.

We also sought to evaluate the potential for stable conversion of conventional T cells to Treg in patients with glioma, which is a highly controversial subject because of demonstration in some animal models.[22,33,34] Although we found a transient induction of the suppressive phenotype after activation, our data did not indicate stable conversion in vitro. One possible explanation is that Treg conversion in vitro may be much less stable because of decreased methylation on the FoxP3 regulatory site.[35] Cell-cell interactions, such as stabilizing interactions from CD103+ dendritic cells and retinoic acid found in vivo, have been shown to enhance FoxP3 induction.[36] Recent data from Wainwright et al. demonstrate that the Treg that predominate the glioma microenvironment are thymically derived rather than stably converted,[37] a finding that is

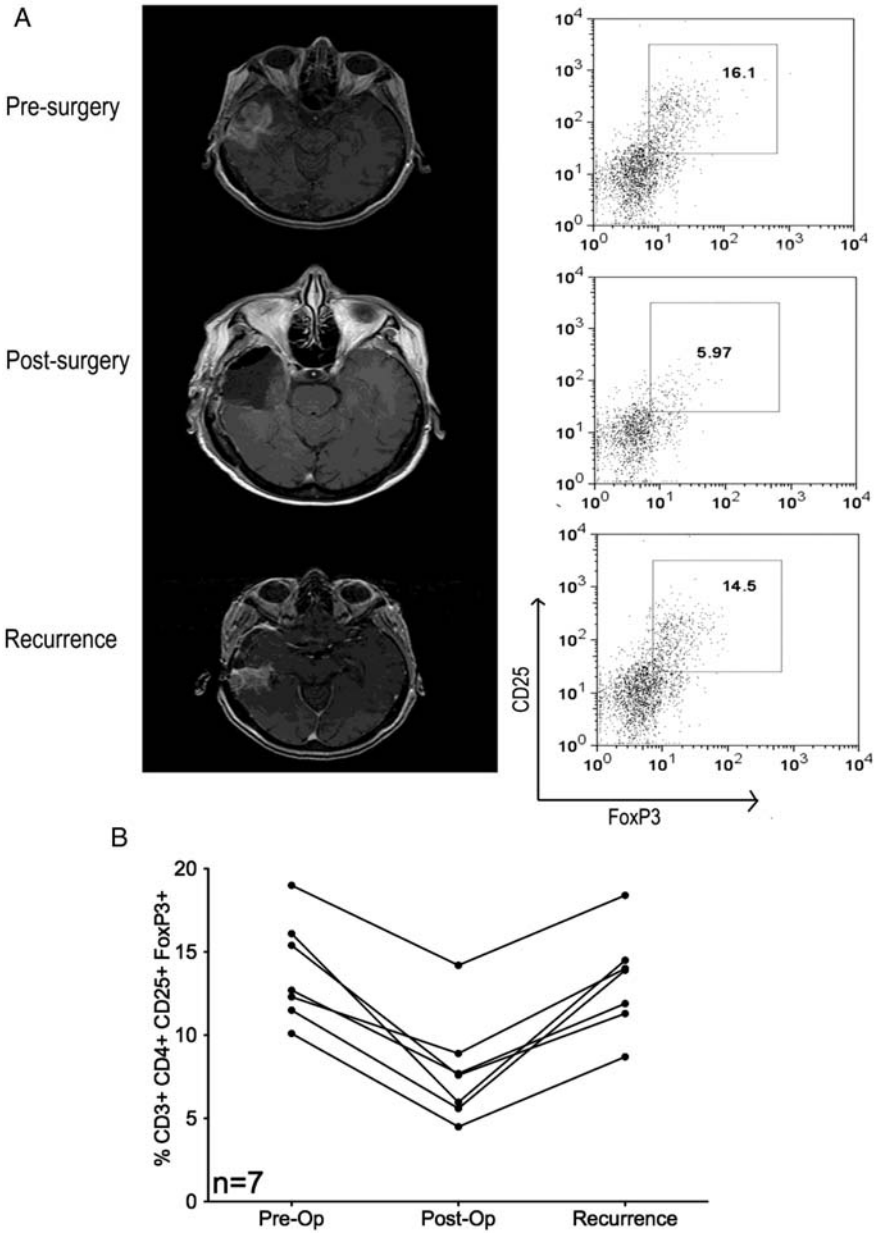

Fig. 6. Treg populations in circulation correlate with tumor burden. Magnetic resonance imaging showing T1 contrast with FLARE indicate the tumor burden at the time of PBL analysis (Left). Percentage of Treg relative to total CD4 + T cells in PBL were measured at 3 time points for patients with high-grade gliomas: prior to tumor resection, following tumor resection, and prior to a subsequent surgery for tumor recurrence. CD25 + FoxP3 + cells were analyzed by flow cytometry and expressed as a percent of total CD4 + CD3 + T cells. (representative patient data) (B) Analysis of patients prior to surgical resection, following gross total resection, and at the time of surgery for tumor recurrence as defined by pathology ($n = 7$).

supported by our data of only temporary Treg conversion after stimulation.

Our data also indicate that, in contrast to conventional T cells, Treg suppress the transcription of pro-apoptotic genes. We previously demonstrated that conventional T cells undergo apoptosis in response to programmed cell death ligand-1 (PD-L1) expression by tumor cells through the interaction with the receptor programmed cell death-1 (PD-1).[38] Recently, Terawaki et al. demonstrated that IFN-α mediates the expression of the PD-1 gene by T cells.[39] Taken with the increase in pro-apoptotic proteins shown here, these data suggest that soluble factors in the tumor microenvironment during therapy may therefore preferentially reduce the number of conventional T cells in the microenvironment relative to Treg by increasing conventional T cell apoptosis.

To date, significant improvements in prognosis have not been established with traditional or experimental therapies for patients with malignant glioma. Our data

suggest that immune therapies may be improved if the effects of tumors on the local immune environment are improved. In support of this hypothesis, Pellagrata et al. recently showed that improved antigen presentation in the tumor microenvironment and pro-inflammatory cytokine production improve survival in mouse models of glioma, specifically through the reduction of Treg in the microenvironment.[40]

The data presented in this manuscript are not without limitations, and future experiments are necessary to determine whether our findings are consistent with mechanisms of Treg recruitment and retention in the tumor microenvironment in patients. Although the recent use of well-characterized glioma cell lines serves as a reproducible system for the study of tumor initiation,[41] tumor cell survival,[42] angiogenesis,[43] invasion,[44] and influence on immune cells both in vitro[45] and in vivo,[46] it should be noted that these cell lines have been cultured for several years and may not be representative of tumor cell behavior in vivo. For example, the system may be improved by the use of autologous tumor samples grown in a manner that better sustains the heterogeneity found in tumor masses.[47] This is of particular interest because it relates to the variability of tumor cell properties, such as oncogenic pathway activation,[48] epigenetic abnormalities,[49] immune evasion[50,51] and resistance to therapies.[52] The use of autologous human serum, rather than serum of bovine origin, may provide further insight into mechanisms of our findings. Although no xenogeneic effects of bovine serum have been specifically demonstrated in this context, soluble factors produced in each patient that cross the blood-brain barrier and influence circulating T cells would remain throughout in vitro culture and may therefore influence Treg function. Finally, we seek to validate our data by prospectively analyzing a larger cohort of patients to determine whether or not Treg percentage may indeed serve as a peripheral biomarker of glioblastoma tumor burden.

Our data indicate that glioma cells rely on a variety of methods mediated by soluble, tumor-derived factors to recruit, expand, and promote the survival of Treg in the microenvironment and in circulation. A better understanding and prevention of these mechanisms may improve the efficacy of future immune therapies for patients with GBM. Therefore, we propose that a better understanding of Treg recruitment and their cross-talk with tumor cells and the soluble factors that they produce will improve our ability to design more effective immune therapies for patients with GBM.

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