Anti-VEGF therapy prolongs recurrence-free survival in patients with glioblastoma but does not improve overall survival. To address this discrepancy, we investigated immunologic resistance mechanisms to anti-VEGF therapy in glioma models. A screening of immune-associated alterations in tumors after anti-VEGF treatment revealed a dose-dependent upregulation of regulatory T-cell (Treg) signature genes. Enhanced numbers of Tregs were observed in spleens of tumor-bearing mice and later in tumors after anti-VEGF treatment. Elimination of Tregs with CD25 blockade before anti-VEGF treatment restored IFNγ production from CD8+ T cells and improved antitumor response from anti-VEGF therapy. The treated tumors overexpressed the glutamate/cystine antiporter SLC7A11/xCT that led to elevated extracellular glutamate in these tumors. Glutamate promoted Treg proliferation, activation, suppressive function, and metabotropic glutamate receptor 1 (mGlutR1) expression. We propose that VEGF blockade coupled with glioma-derived glutamate induces systemic and intratumoral immunosuppression by promoting Treg overrepresentation and function, which can be pre-emptively overcome through Treg depletion for enhanced antitumor effects.

Significance: Resistance to VEGF therapy in glioblastoma is driven by upregulation of Tregs, combined blockade of VEGF, and Tregs may provide an additive antitumor effect for treating glioblastoma.

Introduction

VEGF and its receptor (VEGFR) have been identified as critical mediators of angiogenesis (1). Interaction of VEGF with three different tyrosine kinase (TK) receptors (VEGFR1–3) plays a significant role in physiologic and pathologic angiogenesis, including tumor angiogenesis (2). Evidence suggests that the blockade of VEGF (bevacizumab, a humanized anti–VEGFA mAb) or VEGFR2 (ramucirumab) reduces tumor-induced angiogenesis. Therefore, inhibition of this pathway was thought to be a promising treatment modality for patients with highly vascularized cancers, such as glioblastoma (GBM; ref. 3). However, although several clinical reports using bevacizumab, either alone or in combination with other therapeutic approaches, have shown prolonged recurrence-free survival (RFS), it has failed to improve overall survival outcomes in patients with newly diagnosed GBM (4, 5). This pattern is consistent across tumors. These counterintuitive results suggest that certain critical suppressive pathways might become activated and thus jeopardize the antitumor response mediated by VEGF inhibition. Previous reports have demonstrated that VEGF blockade results in vascular remodeling, reducing perfusion and increasing hypoxia inside treated tumors (6, 7). A dramatic increase in tumor cell invasion into the normal brain has also been observed (8). Despite substantial efforts to understand the underlying mechanism of VEGF blockade treatment in these vasculature-rich tumors, such as GBM, many questions still need to be answered.

The GBM tumor microenvironment is an intricate network (9) composed of dozens of different cellular and soluble components, such as tumor cells, vascular endothelial cells, neural precursor cells, tumor stem cells, stromal cells, residual microglia, infiltrating immune cells, cytokines, and extracellular matrix proteins. Among those components, the vascular endothelial cells and infiltrating immune cells play crucial roles in manipulating the tumor landscape and affecting tumor progression (10–12). Our recent studies on human gliomas suggest that the interconnection between glioma angiogenesis and intratumoral CD4+ and Foxp3+ T cells influences tumor progression in patients with glioma. Activated CD4+ T cells were significantly elevated in bevacizumab-resistant recurrent tumors. Regulatory T cells (Treg) were determined to be an independent risk factor for progression in that patient population (12). Thus, we hypothesized that inhibition of the VEGF/VEGFR pathway might negatively affect the antitumor immunity that contributes to treatment failure.

In this study, our hypothesis was tested in glioma models using two VEGF-blocking agents (anti-VEGFA or anti-VEGFR2). We found that VEGF inhibition enhances Treg-suppressive functions by dysregulating the glutamate/cystine antiporter, and that combinatorial use of Treg blockade can overcome this effect.

Materials and Methods

Murine glioma lines

Murine glioma cell lines, GL-261 or KR-158B, were transduced with firefly luciferase plasmid pLenti CMV Puro LUC, gifted from...
Eric Campeau (Zenith Epigenetics Corp.; Addgene plasmid #17477). After the lentiviral transfection and puromycin (631305, ClonTech) selection, the cells were cultured in a single clone by limiting dilution. The selected cells were then expanded in a DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL); 15140163, Thermo Fisher Scientific), and kept in a 37°C incubator with 5% CO2. All the cell lines used in this study are Mycoplasma negative tested using PlasmoTest Mycoplasma Detection Kit (Invivogen).

GL261-Luc and K1R158B-Luc brain tumor mouse models
Female C57BL/6 mice (6- to 8-week-old) were anesthetized, and 5 × 10^5 GL261-Luc or K1R158B-Luc tumor cells in a 2.5 µL volume were intracranially injected at 2.0-mm lateral to the bregma at a depth of 3.0 mm below the dura mater with a sterile Hamilton syringe fitted with a 25-gauge needle. The tumor-bearing mice were monitored by IVIS imaging on day 10 after implantation, and mice were randomized into treatment groups. The average tumor luciferase intensities were approximately equal among groups. Humanitarian endpoints (also as treatment endpoints) were reached when animals exhibited the following deficits: (i) reluctant to move, (ii) weight loss >20% body weight, (iii) hunched posture, and (iv) lethargy.

In vivo neutralizing and depleting antibody treatments
The doses of antibodies used in this study were based on previous reports (13–16). For tumor-bearing mice, the anti-VEGFA antibody (B20-4.1.1, Genentech) was administered by intraperitoneal injection at 50 µg/animal. VEGFR2 mAb (clone DC101, BE0060, BioXCell) was administered intraperitoneally at 800 µg/dose and the PD-L1 mAb (clone 10F.9G2, BE0101, BioXCell) was used intraperitoneally at 200 µg/dose. For nontumor-burdened mice, the VEGFR2 antibody was administered intraperitoneally at 800 µg/dose every 3 days for a total of 4 doses. Treg depletion was achieved intraperitoneally. Furthermore, 200 µg of anti-CD25 (clone PC-61.5.3, BE0012, BioXCell) was injected on days 10 and 12 after tumor implantation (17).

Isolation of TILs
The tumor-infiltrating lymphocytes (TIL) isolation method has previously been described (18). The brains were removed after PBS perfusion and digested with collagenase (1010357801, Sigma-Aldrich)/DNase-I digestive enzymes (15002007, Worthington Biochemical Corp.). TILs were collected from 37%, and 70% of the tumor tissue of GL261-Luc-bearing mice with or without VEGFR blockade treatment and TaqMan gene expression assays (SLC7A11, SLC3A2, Ki67, and r18s) were purchased from Thermo Fisher Scientific. In addition, r18s was used for the normalization. Relative quantification of the gene expression was carried out via the double delta Ct analysis.

Glutamate concentration assay
GL261-Luc cells (2.0 × 10^3/well) were cultured in a 6-well plate with or without 100 µmol/L of CoCl2 (C8661, Sigma-Aldrich). After 48 hours, glutamate concentrations in the medium were measured using a glutamate assay kit (ab83389, Abcam) according to the manufacturer’s instructions. The tumor pieces were resuspended in 500 µL of PBS and the supernatant was collected after centrifuging. The glutamate concentration of the supernatant was measured using the kit described above.

In vitro GL261-Luc cells xCT expression
GL261-Luc cells (2.0 × 10^5/well) were cultured in a 6-well plate according to the following conditions: (i) the cells were treated with or without 100 µmol/L of CoCl2 (C8661, Sigma-Aldrich), and (ii) the cells were treated with or without 20 µg/mL VEGFR2 blockade (clone DC101, BE0060, BioXCell). After 48 hours, the xCT expression on the cells was determined using the xCT antibody (NB300-318, Novus Biologicals) by flow cytometry.

IHC of xCT
Tumor tissues were resected and frozen, and 5-μm sections were put on slides. The slides were incubated with an anti-xCT antibody (1:100; NB300-317, Novus Biologicals). Goat anti-rabbit IgG (1:100; A-11011, Alexa Fluor 568, Thermo Fisher Scientific) was used as the secondary antibody. Images were taken using Olympus U-HGLGPS microscopes.

Isolation of CD4+CD25+ Tregs and CD4+CD25- T conventional cells
Both CD4+CD25+ Tregs and CD4+CD25- Tconvs from spleens of C57Bl/6 mice were isolated using the Treg isolation kit (130-091-041, Miltenyi Biotec), according to the manufacturer’s instructions.

RNA sequencing and data analysis
Fresh frozen normal brain or tumor tissues from healthy mice and GL261-Luc–bearing mice treated with different doses of anti-VEGFA (50, 100, and 200 µg/dose) were sent to Novogene Corporation for RNA sequencing (RNA-seq). The raw data have been deposited in NCBI’s Gene Expression Omnibus (GEO; ref. 19), accessible through GEO Series accession number GSE107423 (https://www.ncbi.nlm.nih.gov/geo/). Paired-end reads were mapped to muscles UCSC mm10 and assembled using STAR aligner and Cufflinks Pipeline 2 using the Illumina base space sequence hub (https://basespace.illumina.com/) as the default setup. Each transcript expression was estimated as an FPKM (fragments per kilobase million) value. Subsequent two-dimensional principal component analysis (2D PCA) was performed using Subio Platform (Subio). The signature scores of Treg and exhausted CD8+ T cells were evaluated using single sample gene set enrichment analysis (ssGSEA; ref. 20) with gene sets (GSE7832 Treg vs. Tconv up, and GSE9650 exhausted vs. memory CD8+ T-cell up, respectively).

Real-time quantitative reverse transcription-PCR
Total RNA was extracted from Tregs cultured in vitro and from tumor tissue of GL261-Luc–bearing mice with or without VEGFR blockade treatment and TaqMan gene expression assays (SLC7A11, SLC3A2, Ki67, and r18s) were purchased from Thermo Fisher Scientific. In addition, r18s was used for the normalization. Relative quantification of the gene expression was carried out via the double delta Ct analysis.
Treg suppression assay

Violet (C34537, Invitrogen) labeled Tconv cells (3 × 10⁴/well) were cultured with Tregs at different ratios (1:1, 1:0.5:1, 1:0.25, and 1:0.125) in 96-well round-bottom plates in the presence of a T-cell medium containing 0, 175, or 350 μmol/L of γ-glutamate (G8415, Sigma-Aldrich). Cells were then stimulated with Dynabeads Mouse T-Activator CD3/CD28 (3 × 10⁴/well) in the absence of IL2 for 3 days, and a FACS analysis was performed to measure the CellTrace violet dilution. The T-cell medium was Minimum Essential Medium without γ-glutamate and was supplemented with an additional 10% FBS. For the Treg and Tconv proliferation experiment, 1 × 10⁴ Tregs and Tconv cells per well were stimulated with Dynabeads Mouse T-Activator at a 1:1 cell to bead ratio in the absence of IL2, under different concentrations of γ-glutamate (0, 175, or 350 μmol/L), in 96-well round-bottom plates. The absolute cell counts were determined by hemocytometer.

Western blots

Tregs and Tconv cells were lysed in RIPA buffer (PI89900, Thermo Fisher Scientific) containing protease/phosphatase inhibitor cocktail (P17842, Thermo Fisher Scientific). Protein concentration was measured using a BCA assay (23227, Thermo Fisher Scientific). Protein concentration was measured using a BCA assay (23227, Thermo Fisher Scientific). Anti-GluR1 (NB10-82396, Novus Biologicals) was used as the primary antibody, and β-actin (49705, Cell Signaling Technology) was used as the loading control. Blots were detected automatically using the Simple Western System (Wes, Bio-techne).

Statistical analysis

Data are presented as mean ± SEM. Mann-Whitney U tests were used to determine significant differences between two groups. A one-way ANOVA was used to compare more than two groups. Survival was analyzed using Kaplan–Meier survival curves and log-rank Mantel–Cox tests. Statistical analyses were performed using GraphPad Prism 7 software. P values of less than 0.05 were considered significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

Study approval

In our murine experiments, mice were handled in accordance with the animal care policy at the University of Florida (UF); all protocols were approved by UF’s Institutional Animal Care and Use Committee (IACUC). IACUC#201609208: The combination therapies for gliomas (principal investigator: J. Huang).

Results

VEGF blockade alters tumor immune landscape

To gain insight into the impact of VEGF blockade on the immunologic landscape of gliomas, we treated C57BL/6 mice bearing malignant gliomas with various amounts of anti-VEGFA antibody until the treatment endpoint was reached (Fig. 1A). The antitumor response was found to be dose dependent; a higher dose resulted in longer survival benefits as reported previously (21). However, all animals succumbed to their tumors before 50 days post tumor implantation. We resected tumor samples from the mice near the date of median survival from each group (12 total), and tissues from 3 normal brains were also included. RNA samples were sent for sequencing analysis. The data have been deposited in NCBI’s Gene Expression Omnibus (19), accessible using GEO Series accession number GSE107423 (https://www.ncbi.nlm.nih.gov/geo). Next, we assessed the major pathways enriched by VEGF treatment using the data obtained from the RNA-seq analysis. Reactome pathway enrichment was first employed to evaluate overall genetic landscape changes post anti-VEGF therapy. The results indicate that 5 of the top 20 enriched pathways were immune-related (Fig. 1B). Furthermore, we evaluated the previously published microarray analysis of chemo-naïve patients with GBM or patients who had failed bevacizumab treatment (22–24). The results indicate that the bevacizumab-resistant tumors represent nearly identical genetic changes to those we observed in the mouse model. More immune-related pathways were revealed compared with the chemo-naïve tumors (Supplementary Fig. S1A and S1B). These results suggest that VEGF blockade may alter the GBM immune landscape. To test our hypothesis that anti-VEGF therapy alters immunologic infrastructure, a 750 immune-related genaset, a commercially available program (nCounter PanCancer Immune Profiling Panel, NanoString Company) was used. This panel consists of 770 genes (750 immune genes and 20 internal reference genes) from 24 different immune cell types, checkpoints, CT antigens, and genes spanning the adaptive and innate immune response network. A dose-dependent separation/cluster was observed in the anti-VEGF treatment using 2D PCA, whereas the normal brains were distinctly separated from samples treated with anti-VEGF (Fig. 1C). Next, RNA-seq data were enriched using a ssGSEA for immunosuppressive subpopulations (e.g., M1/M2 macrophages and Tregs) based on previous reports that VEGF2 blockade combined with Ang-2 can alter the polarization of macrophages (25). We did not find changes in the M1:M2 ratio after treatment with VEGF blockade alone. However, we did notice a dose-dependent increase in Tregs and exhausted CD8 T-cell gene scores after the anti-VEGF treatment (Fig. 1D and E). Collectively, anti-VEGF therapy is associated with the tumor-immunosuppressive genetic landscape in a dose-dependent manner.

Enrichment of Tregs in spleens and tumors post anti-VEGF treatment

We treated the same tumor-bearing mice with another VEGF blockade agent (due to supply-discontinuation of the anti-VEGF), anti-VEGFR2 (800 μg/dose, i.p. every 3 days until the endpoint, which is equivalent to 100 μg/dose of the anti-VEGF regarding median survival, Supplementary Fig. S2A and S2B). Tumors were, respectively, harvested on D10, D17, and D25 after continuous anti-VEGFR2 administration (Fig. 2A). The tumor size was found to be equivalent to the tumor size in the nontreated (NT) group on D17. Flow cytometry analysis of TILs was then performed, and the gating strategy is shown (Fig. 2B). The frequencies of Tregs (CD4⁺ Foxp3⁺), Tconv (CD4⁺ Foxp3⁻), CD8⁺, and other cells (CD3⁺ CD8⁻ T cells), with or without the treatment in spleen and tumor are shown in pie charts (mean) with statistical significance indicated. On D17 posttumor implantation, the percentage of Tregs in the spleens was approximately 1.7 times greater in a VEGF2-treated animals compared with controls, but no similar change was seen in spleens of healthy mice with the same treatment schedule and dose (Fig. 2C; Supplementary Fig. S3A–S3E). In tumors, the Tregs fraction was decreased by approximately 25% at D17, but then almost tripled by D25 to reach 35.5% (Fig. 2D). Because of the prolonged survival of mice in the anti-VEGF–treated group, the significant increase in the tumor-infiltrating Tregs at a later time might be a result of a cumulative effect. To address this issue we then included a control group (anti-PD-L1) that provides a similar length of survival as the anti-VEGFR2 treatment (Fig. 2E). A significant increase in Treg frequency, Tregs/CD8⁺ T-cell ratio, and Tregs/Tconv ratio were detected in the anti-VEGFR2–treated...
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Anti-VEGFA treatment alters the tumor-immune genetic landscape and enhances Treg signature genes. 

Figure 1.

Anti-VEGFA treatment alters the tumor-immune genetic landscape and enhances Treg signature genes. A, Treatment schedule of anti-VEGFA treatment. C57BL/6 mice were intracranially inoculated with GL-261-Luciferase GBM cells, and after 10 days, the tumor engraftment was confirmed with IVIS imaging of luminescence, and only tumor-bearing mice were randomly divided into 4 groups (9 per group). The animals were then treated with the anti-VEGFA antibody (0, 50, 100, or 200 µg per dose, respectively) every 3 days until they reached the endpoint. B, Pathway enrichment was performed for all genes (total 525 genes) that showed a significant elevation between 0 and 200 µg per dose groups (fold increase >2; P < 0.05) using the Reactome pathway database (murine) based on the ranking of the entities found (https://reactome.org/). Blue, immune-related pathways. C, The RNA-seq results from all 5 groups were also enriched against a 750 immune (Counter Immunology Panel for Mouse)-related geneset. Clusters were circled for different treatment groups, including the normal brain (NB) group. D, Increase in Treg signature genes after the anti-VEGFA treatment. The RNA-seq data were enriched using the Treg to Tconv (GSE7852_treg_vs_tconv_up) gene set provided by GSEA (https://software.broadinstitute.org/software/cprg/). A score was given by GSEA for each tumor sample RNA-seq. A heat map (left) and the comparisons of the signature scores (means ± SEM) among the groups (right) are shown. E, A dose-dependent increase in exhausted to memory (E/M) CD8 signature gene scores using an E/M CD8 (GSE9650_exhausted_vs_memory__cdbg_tcell_up) geneset. An one-way ANOVA was used to determine the significant differences between the means.

Blocking Tregs using anti-CD25 antibody before treatment with VEGF therapy restores antitumor activity mediated by anti-VEGFR2 treatment

To confirm whether Tregs are the predominant factor limiting the efficacy of VEGF blockade, we sought to eliminate Tregs through CD25-targeted inhibition before anti-VEGFR2 treatment. Two doses of anti-CD25 antibody were administered on day 10 and 12, followed by the anti-VEGFR2 treatments every 3 days until the treatment endpoint (Fig. 3A). The animals showed a moderate improvement in survival using anti-VEGFR2 and anti-CD25 antibody as a mono-therapy (compared with untreated animals). A significant benefit was achieved in the combination group (Fig. 3B). We then evaluated CD25 expression on CD4+ T cells and found that the anti-CD25 antibody
eliminates CD25 expression on TILs at D25, which potentially was due to the competition of the blocking and detecting antibodies, as the efficiency of the CD25 blockade is 56% to 77% (26). Nevertheless, these results indicate efficient effects of controlling CD25 on CD4⁺ T cells (no CD8⁺CD25⁺ T cells were detected) by the blockade in vivo (Fig. 3C). IFNγ production was then measured from CD4⁺ and CD8⁺ TILs after a rechallenge with anti-CD3/CD28 beads (Fig. 3D). Interestingly, both anti-CD25 (green dots) and anti-VEGFR2 (red dots), which were used individually, significantly increased IFNγ production from both CD4⁺ and CD8⁺ T cells that may be responsible for the moderate survival benefit shown in Fig. 3B. The anti-VEGFR2 treatment alone produced relatively less cytokine from both T-cell subsets compared with the anti-CD25. When a combinatory strategy was employed, the IFNγ production was only restored (an additive effect, red dots vs. blue dots) in the CD8⁺ cells, not the CD4⁺ cells. The data also suggest that the prolonged survival in the combination treatment may rely on enhanced CD8⁺ T-cell function. In summary, Tregs play a critical role in suppressing the antitumor effects of...
The TILs were collected from tumor samples at the endpoints, and phenotypical analysis was carried out by sequentially gating live cells, CD45

Blocking Tregs using an anti-CD25 antibody during the early phase of anti-VEGF therapy restores some of the antitumor reactivity mediated by the anti-VEGFR2.

Two doses of the anti-CD25 antibody (200 μg/dose) were given intraperitoneally to the tumor-burdened mice on D10 and D12. The anti-VEGFR2 (800 μg/dose) was started on day 11 (D11) and continued every 3 days until the treatment endpoint. The survival of the differentially treated mice was plotted using the Kaplan–Meier curve, and the log-rank test determined a significant difference. The frequency of CD4+CD25+ T cells in the TILs of mice treated with or without the anti-VEGFR2. The TILs were collected from tumor samples at the endpoints, and phenotypical analysis was carried out by sequentially gating live cells, CD45+, and CD3+ T cells. A representative flow cytometry analysis (left) and summary (right) are shown. Adding anti-CD25 therapy before the continuous treatment of anti-VEGFR2 restores the IFNγ production of T cells. The tumors were resected from the mice at each endpoint, and TILs were isolated and challenged using anti CD3/CD28 beads (cell to bead ratio = 1:1). The percentages of CD4+ IFNγ+ and CD8+ IFNγ+ T cells in TILs were measured using flow cytometry, and the representative flow cytometry data (left) and calculated results (right) are shown. Each color represents the corresponding group (NT, black; anti-CD25, green; anti-VEGFR2, red; and combination, blue). The experiments were repeated twice. A Mann–Whitney U test was used to determine the significant difference between the two groups. The graphs show a sum of two repeated experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3.

Blocking Tregs using an anti-CD25 antibody during the early phase of anti-VEGF therapy restores some of the antitumor reactivity mediated by the anti-VEGFR2. A, Treatment strategy. Four groups (n = 7) of 6–to 8-week-old C57BL/6 mice were intracranially inoculated with GL-261-Luc, and 10 days after the tumor intracranial, two doses of the anti-CD25 antibody (200 μg/dose) were given intraperitoneally to the tumor-burdened mice on D10 and D12. The anti-VEGFR2 (800 μg/dose) was started on day 11 (D11) and continued every 3 days until the treatment endpoint. B, The survival of the differentially treated mice was plotted using the Kaplan–Meier curve, and the log-rank test determined a significant difference. C, The frequency of CD4+CD25+ T cells in the TILs of mice treated with or without the anti-VEGFR2. The TILs were collected from tumor samples at the endpoints, and phenotypical analysis was carried out by sequentially gating live cells, CD45+, and CD3+ T cells. A representative flow cytometry analysis (left) and summary (right) are shown. D, Adding anti-CD25 therapy before the continuous treatment of anti-VEGFR2 restores the IFNγ production of T cells. The tumors were resected from the mice at each endpoint, and TILs were isolated and challenged using anti CD3/CD28 beads (cell to bead ratio = 1:1). The percentages of CD4+ IFNγ+ and CD8+ IFNγ+ T cells in TILs were measured using flow cytometry, and the representative flow cytometry data (left) and calculated results (right) are shown. Each color represents the corresponding group (NT, black; anti-CD25, green; anti-VEGFR2, red; and combination, blue). The experiments were repeated twice. A Mann–Whitney U test was used to determine the significant difference between the two groups. The graphs show a sum of two repeated experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4.

Elevation of SLC7A11 gene expression after anti-VEGF treatment is associated with increased intratumoral Tregs

When looking for the increased expression of genes that overlap among the three doses of anti-VEGFA-treated tumors, three genes were revealed: SLC7A11, Gpr141, and Cth (Fig. 4A and B). Using qPCR, we confirmed that the gene expression of SLC7A11 and the xCT-binding partner CD98 (SLC3A2) were increased with anti-VEGFR2 treatment (Fig. 4C and D). The protein expression of xCT in tumors was increased in treated tumors than nontreated tumors (Fig. 4E). Anti-VEGF therapy leads to more hypoxic tumor microenvironment (8), we evaluated the expression of the hypoxia-related gene, HIF1α. A dose-dependent increase in HIF1α gene expression was observed (Fig. 4F). We then evaluated whether conditional hypoxia in vitro could alter the xCT expression using cobalt(II) chloride hexahydrate (CoCl2; ref. 27). The result demonstrates that CoCl2 increases tumor xCT expression and glutamate production. However, no direct effect of anti-VEGFR2 on xCT expression was seen when the tumor cells were cultured with the anti-VEGF (Fig. 4G–I), suggesting an indirect influence instead. Finally, a positive correlation between SLC7A11 gene expression and Treg signature scores was determined in these treatment animals and the mesenchymal subgroup of GBM (Fig. 4J; Supplementary Fig. S5), which has been demonstrated to have more immune infiltrations and higher levels of the endothelial markers CD31 and VEGFR2 than other subgroups (28–30). These data imply that a dysfunctional glutamate/cystine antiporter in tumors may influence the function of Tregs.

Glutamate promotes Tregs function in vitro

Previous studies show that glutamate plays a central role in the malignant progression of glioma via numerous mechanisms (31). We found in this study that anti-VEGF can enhance the expression of glutamate/cystine antiporter, which leads to more glutamate production by the tumors. Thus, we performed a Treg-suppression assay in the presence of L-glutamate in vitro. The results demonstrate a dose-dependent inhibition of Tconv cell proliferation (Fig. 5A–C). A significant decrease in the activation of Tconv cells, measured by CD69 expression on the population, was observed (Fig. 5D). Meanwhile, phenotypic analysis of Tregs revealed that adding glutamate increases the surface expression of early and late T-cell activation markers CD69 and CD154, and Ki67 in a dose-dependent manner (Fig. 5E–G). When glutamate was added to Treg- or Tconv-only cultures, the total cell counts of Tregs increased, and no change was observed in the Tconv cells (Fig. 5H and I), suggesting that the activity...
of glutamate in T cells is limited to the Treg compartment. To test whether these two cell populations expressed glutamate receptors, protein expression of metabotropic and ionotropic glutamate receptors (mGluR1, 4, and AMPA3; ref. 32) on Tregs and Tconv cells were then evaluated. Only mGluR1 was detected, with a dose-dependent enhancement of this receptor corresponding to glutamate exposure in both cell types, and with the Tregs showing a more dramatic enhancement compared with the Tconv cells (Fig. 5I). These results suggest that glutamate promotes an activated Treg phenotype and function.

Discussion

Mounting evidence has shown that tumor angiogenesis and immunosuppression are key features in refractory malignancies. VEGF signaling has been involved in fostering immunosuppression (both systemically and intratumorally), which may be unlocked through the use of antiangiogenic agents (10, 33–36). Blocking the VEGF/VEGFR pathway has been demonstrated to increase survival and decrease tumor immunosuppressive status in animal models, including GBM (37, 38). However, VEGF blockade with bevacizumab prolongs only progression-free survival (PFS), without significant enhancement of overall survival benefit in patients with GBM (4, 5). These findings are likely multifactorial. Our data suggest that immune suppressive mechanisms contribute to anti-VEGF failure. Our recently published study on human gliomas showed that the level of Foxp3high T cells located in the perivascular tumor niche correlates with tumor vascularization and is an independent predictor of shortened PFS (not overall survival) in astrocytic gliomas (12). Moreover, VEGFR2 has been found to be selectively expressed by Foxp3high CD4+ Tregs and can be increased by TGFβ (39), suggesting that the Treg population may be preferentially affected by anti-VEGF treatment. These data suggest interconnections between glioma angiogenesis and inhibitory immune infiltration. In this report, we provide an in-depth understanding of anti-VEGF therapy resistance and a strategy for improving the therapeutic effect of the antiangiogenic agents in GBM.

We observed that at the beginning of the treatment, anti-VEGF showed a moderate reduction of Tregs (4% lower than NT) and enhancement of antitumor response (increased INFγ production from both CD4+ and CD8+ T cells, and more prolonged survival than NT), which reproduced the previous reports that anti-VEGF can help to restore the tumor immune microenvironment (37, 40, 41). However, the response could not be sustained despite continued administration of the drug. Ultimately, the treatment failed and the mice succumbed to the tumor, similar to outcomes from human clinical trials. Thus, we

Anti-VEGF Therapy Promotes Treg Function

Figure 4.
A dose-dependent upregulation of the SLC7A11 gene and its associates with Tregs after the anti-VEGFR2 treatment. A, Identification of genes that were significantly upregulated by anti-VEGFA treatment. The RNA-seq data from the anti-VEGFA treatment experiment shown in Fig. 1B were analyzed. Overlapping genes that were enriched (fold increase >2; P < 0.05), respectively, using the 0-μg group as a reference, from the 50, 100, or 200 μg group are presented using a Venn diagram, and overlapped genes from the three comparisons are shown. B, A dose-dependent increase in SLC7A11 gene expression determined by FPKM was observed after the anti-VEGFA treatment. C and D, SLC7A11 and SLC3A2 gene expression was increased using anti-VEGFR2 therapy. The total RNA was isolated from tumor tissues at the endpoints of 4 tumor-burdened mice in each group (the same treatment schedule as is described in Fig. 2A), and RT-qPCRs were performed. E, Anti-VEGFR2 increases xCT protein expression in vivo. The same tumors described in C and D were also evaluated by fluorescent IHC. The in vivo experiments were repeated three times. F, A dose-dependent increase appeared in HIF1α expression after the anti-VEGFA treatment. The HIF1α expression in a different dose of anti-VEGFA treatment described in Fig. 1B was graphed. G, Hypoxia increases xCT expression on the tumor cells. The GL-261 cells were cultured under hypoxic conditions using 100 or 150 μmol/L of CoCl2 for 48 hours in vitro, and the xCT expression on the cells was measured using flow cytometry. H, Anti-VEGFR2 does not change the xCT expression of tumor cells in vitro. The GL-261 cells were cultured in medium containing 20 μg/mL of the anti-VEGFR2 antibody for 48 hours (13), and the xCT expression on these cells was assessed using flow cytometry. I, Glutamate was induced using conditional hypoxia. The GL-261 cells were cultured in medium with or without 100 μmol/L of CoCl2 for 48 hours, and glutamate concentration in the culture supernatant was assessed using three individual experiments. J, SLC7A11 gene expression was highly correlated with Treg signature scores. An one-way ANOVA was used to determine the significance in B, F, G, and H. A Mann–Whitney U test was used to determine the significant difference in C, D, and I. The association between SLC7A11 and the Treg score was determined using Spearman rank correlation coefficient.
Glutamate dose-dependently enhances Tregs proliferation, activation, and suppressive function by glutamate. The Tregs (CD4+ CD25+ and Tconv cells (CD4+ CD25−)) were freshly isolated from the spleens (n = 5) of 6- to 8-week-old C57BL/6 mice. CellTrace Violet-labeled Tconv cells were cocultured with Tregs and stimulated with anti-CD3/CD28 beads in the presence of 1, 175, and 350 μmol/L of L-glutamate, respectively. B and C, Glutamate dose-dependently enhances Tregs' suppressive function. On day 3, the cocultured cells were analyzed using flow cytometry, and the Treg suppression was assessed by measuring the nondivided population of the Tconv cells. D, Glutamate dose-dependently decreases the CD69 expression on the Tconv cells. The cocultured Tconv cells were also evaluated for the early activation marker, CD69, using flow cytometry. E and F, Glutamate enhances Tregs’ activation by upregulating the surface expression of CD69 and CD154. A flow cytometry analysis was carried out to assess the marker expressions. G, Glutamate enhances Tregs’ proliferation. The Tregs (5 × 10^5/mL) were cultured in media containing different concentrations of L-glutamate in the presence of anti-CD3/CD28 beads (n = 8); the cells were lysed, and qRT-PCR was performed to determine the Ki67 gene expression. H, Glutamate does not directly influence Tconv cell proliferation. The CellTrace Violet-labeled Tconv cells (1.5 × 10^5/mL; n = 5) were stimulated with anti-CD3/CD28 beads and cultured in different doses of glutamate (in the absence of Tregs) for 3 days. The divided cell population was gated (left) and summarized data were graphed (right). I, Glutamate only increases cell counts of Tregs, not of the Tconv cells. The anti-CD3/CD28 bead-stimulated Tregs and Tconv cells (5 × 10^5/mL) were individually cultured with different concentrations of the L-glutamate, and the cell counts were carried out after 3 days (n = 8). All the data are representative of three independent experiments, and values are means ± SEM. An one-way ANOVA was used to determine the significance in each graph. J, Enhanced protein expression of metabotropic glutamate receptor 1 (mGlutR1) on activated Tregs by L-glutamate in vitro. Tregs and Tconv cells were, respectively, isolated and activated by anti-CD3/CD28 beads in the presence of an indicated amount of L-glutamate for 3 days. Western blot analysis was performed to assess the expression of mGlutR1, and β-actin was used as a loading control. The result was representative of two independent experiments.

sought to determine the immunologic manifestations of treatment failure and observed a link between treatment failure and Treg enrichment. We found a dose-dependent increase in Treg signature genes in the tumor after the anti-VEGF treatment and confirmed the overrepresentation of these cells, compared with CD8+ T-cells or CD4+ Tconv cells in spleens and tumors sequentially. These results imply that anti-VEGF treatment leads to a more immunosuppressive phenotype. Interestingly, the anti-VEGF had no similar impact on Tregs in lymphoid tissues of healthy animals, suggesting that the tumor plays a critical role in the immunosuppression induced by anti-VEGF therapy, that is, factors produced by glioma cells may be involved in the promotion of Treg function. Previous animal studies have shown that anti-VEGF inhibits Treg accumulation in mice 2 weeks after the treatment (equivalent to the results we found on D17), and we provide evidence that this effect was short-lived, and enhanced intratumoral Tregs were found in the tumors of failed animals. The VEGF blockade specifically acts on the CD4 compartment by stimulating Tregs and suppressing Tconv functions, which can be overcome through CD25 blockade, a clinically available agent that inhibits Tregs but has demonstrated only modest therapeutic activity against established tumors (42, 43). Because of the potential suppression of activated effector cells by the CD25 blockade, we only administered two doses...
before anti-VEGF treatment. IFNγ-producing CD4⁺ T cells were enhanced by anti-CD25 monotherapy, but this enhancement was diminished by combination with anti-VEGFR2 (to a similar level as the anti-VEGFR2 monotherapy). These data confirm our finding that the anti-VEGFR2 treatment mostly impacts CD4⁺ T cells, offsetting the benefit of Treg depletion in this compartment. In contrast, IFNγ-producing CD8⁺ T cells were enhanced by the combination, which may be responsible for the prolonged survival.

Next, the identification of SLC7A11 gene overexpression with anti-VEGF treatment led us to link between anti-VEGF therapy and its impact on CD4⁺ T cells. It is well known that this gene encodes a crucial glutamate/cystine antiporter, xCT. Increased production of this antiporter has been implicated in enhanced glutamate production in other cancer types (44, 45). In the central nervous system (CNS), xCT controls the exchange between glutamate and cystine. Overexpression of xCT elevates glutamate in the extracellular space, promoting tumor progression, and drug resistance. The knockdown of xCT has been shown to decrease glutamate production in cancer cells (44, 46, 47). Under physiologic conditions, glutamate is an excitatory neurotransmitter that is pivotal for the proper functioning of the CNS (48); however, under pathophysiologic conditions (e.g., autoimmune diseases, neurodegenerative diseases, and cancers), this amino acid has been found to be excessively produced with leakage into the extracellular matrix, where it can act as a neurotoxin that damages neural cells (49, 50). Under hypoxic conditions, gliomas have been shown to overexpress xCT (51), and pathophysiologic glutamate has been detected in the extracellular space. A result of an analysis of 9 patients with glioma indicates that glutamate concentrations were 100-fold higher in the peritumoral versus nontumoral zones, which can drive malignancy by promoting proliferation and invasion (52). High glutamate can also cause glioma-associated seizures (53, 54). The anti-VEGF treatment magnifies this effect by further elevating glutamate production, which potentially leads to increased tumor cell invasion (8), and fatal seizures. We found that the effect of anti-VEGF on xCT overexpression was not direct, and intermediary factor(s) may be involved. Because it has been demonstrated that anti-VEGF induces hypoxia, which elicits HIF-dependent expression of SLC7A11/ xCT (55), we hypothesize that the hypoxic tumor microenvironment induced by the anti-VEGF therapy may be one of the pathways involved in the dysfunction of glutamate/cystine antiporter. As a consequence, glutamate was overproduced by the tumor, promoting the suppressive functions of Tregs and resulting in treatment failure (Fig. 6). More studies focusing on the mechanism proposed in this study are necessary.

Excessive glutamate can act as a neurotoxin and can trigger immunologic involvement in the CNS, as a “protective autoimmune mechanism” (56, 57). This mechanism has been described as a beneficial action for the CNS to protect astrocytes from neurotoxin attacks under pathophysiologic conditions (57, 58). Gliomas may adopt this mechanism to protect themselves and in
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References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Anti-VEGF Therapy Promotes Treg Function


Dysregulation of Glutamate Transport Enhances Treg Function That Promotes VEGF Blockade Resistance in Glioblastoma


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