Dysregulation of glutamate transport enhances Treg function that promotes VEGF blockade resistance in glioblastoma

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Running Title: Anti-VEGF therapy promotes Treg function

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Abstract

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 prior to anti-VEGF treatment restored IFN-γ production from CD8+ T cells and improved antitumor response from anti-VEGF therapy. The treated tumors overexpressed the glutamate/cystine antiporter SLC7A11/ xCT which 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 GBM is driven by upregulation of Tregs, combined blockade of VEGF and Tregs may provide an additive antitumor effect for treating GBM.
Introduction

Vascular endothelial growth factor (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, VEGFR2, and VEGFR3) plays a significant role in physiological and pathological angiogenesis, including tumor angiogenesis (2). Evidence suggests that the blockage of VEGF (bevacizumab, a humanized anti-VEGF-A monoclonal antibody) 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)(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, residential 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 impacting 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 glioma patients. Activated CD4+ T cells were significantly elevated in bevacizumab-resistant recurrent tumors. Tregs 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-VEGF-A 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 (Addgene plasmid # 17477). After the lentiviral transfection and puromycin (631305, Clontech) selection, the cells were cultured in a single clone by limiting the dilution. The selected cells were then expanded in a Dulbecco-modified Eagle medium supplemented with 10% Fetal Bovine Serum, 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 KR158B-Luc brain tumor mouse models
Female C57BL/6 mice (6–8 weeks old) were anesthetized, and $5 \times 10^4$ GL261-Luc or KR158B-Luc tumor cells in a 2.5 µl volume were intracranially injected (i.c.) 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: 1) reluctant to move, 2) weight loss >20% body weight, 3) hunched posture, and 4) 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-VEGF-A antibody (B20-4.1.1, Genentech) was administered by intraperitoneal injection (i.p.) at 50 µg, 100 µg, or 200 µg/dose. VEGFR2 mAb (clone DC101, BE0060, BioXCell) was administered by i.p. at 800 µg/dose, and the PD-L1 mAb (clone 10F.9G2, BE0101, BioXCell) was used by i.p. at 200 µg/dose. For non-tumor-burdened mice, the VEGFR2 antibody was administered via i.p. at 800 µg/dose every three days for a total of 4 doses. Treg depletion was achieved by i.p. 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 tumor-infiltrating lymphocytes**

The tumor-infiltrating lymphocytes (TILs) isolation method has previously been described (18). The brains were removed after PBS perfusion and digested with collagenase (10103578001, Sigma-Aldrich)/DNase-I digestive enzymes (LS002007, Worthington Biochemical Corp.). TILs were collected from 37%, and 70% of the interface of Percoll (17-5445-01, GE Healthcare) gradient, centrifuged at 500 g for 20 min and washed twice.

**Flow cytometry and analysis**
Fluorescence-activated cell sorting (FACS) was performed using LSRII (BD Biosciences). The viability of TILs was determined using the Live/Dead Cell Violet Viability kit (L34964, ThermoFisher Scientific). Cell surface staining antibodies, anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7cd), anti-CD25 (7D4), anti-CD45 (30-F11), anti-LAG-3(C9B7W), anti-CD69 (H1.2F3), and anti-CD154 (MR1), were purchased from BD Biosciences. Anti-PD-1 (J43) and anti-Tim3 (RMT3-23) were purchased from eBioscience and BioLegend, respectively. The levels of Foxp3+ and IFN-γ+ T cells were determined by intracellular staining using a Foxp3 / Transcription Factor Staining Buffer Set, anti-IFN-γ (XMG1.2), and anti-Fop3 (FJK-16s) (all from eBioscience). Appropriate isotype controls were used. All FACS data were analyzed using FlowJo 10.3 software (FlowJo, Ashland, OR, USA).

RNA-Seq and data analysis
Fresh frozen normal brain or tumor tissues from healthy mice and GL261-Luc-bearing mice treated with different doses of anti-VEGF-A (50 µg, 100 µg, and 200 µg/dose) were sent to Novogene Corporation for RNA-Seq (Chula Vista, USA). The raw data have been deposited in NCBI’s Gene Expression Omnibus (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, Tokyo, Japan). The signature scores of Treg and exhausted CD8+ T cells were evaluated using ssGSEA (20) with gene sets (GSE7852 Treg vs. Tconv up, and GSE9650 exhausted vs. memory CD8+ T cell up, respectively).

Real-time quantitative reverse transcription-PCR (RT-qPCR)
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 ThermoFisher 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 (2.0x10^5/well) were cultured in a 6-well plate with or without 100 μM of CoCl₂ (C8661, Sigma Aldrich). After 48 hrs, glutamate concentrations in the medium were measured using a glutamate assay kit (ab83389, Abcam) according to the manufacturer’s instructions. For the in vivo assay, the brains that contained implanted tumors were resected from mice, weighed, and cut into small pieces. 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 (2.0x10^5/well) were cultured in a 6-well plate according to the following conditions: 1) the cells were treated with or without 100 μM of CoCl₂ (C8661, Sigma Aldrich), and 2) the cells were treated with or without 20 µg/ml VEGFR-2 blockade (clone DC101, BE0060, BioXCell). After 48 hrs, the xCT expression on the cells was determined using the xCT antibody (NB300-318, Novus Biologicals) by flow cytometry.

**Immunohistochemistry 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). Goat anti-Rabbit IgG (1:100; A-11011, Alexa Fluor 568, ThermoFisher 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- Tconv cells from spleens of C57Bl/6 mice were isolated using the Treg isolation kit (130-091-041, Miltenyi Biotech), according to the manufacturer’s instructions.

Treg suppression assay

Violet (C34557, Invitrogen, USA) labeled Tconv cells (3x10^4/well) were cultured with Tregs at different ratios (1: 1, 1: 0.5, 1: 0.25 and 1: 0.125) in 96-well round-bottom plates in the presence of a T cell medium containing 0 μM, 175 μM, or 350 μM of L-glutamate (G8415, Sigma-Aldrich). Cells were then stimulated with Dynabeads™ Mouse T-Activator CD3/CD28 (3x10^4/well) in the absence of IL-2 for 3 days, and a FACS analysis was performed to measure the CellTrace™ violet dilution. The T cell medium was Minimum Essential Medium without L-glutamate and was supplemented with an additional 10% Fetal Bovine Serum. For the Treg and Tconv proliferation experiment, 1x10^5 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 IL-2, under different concentrations of L-glutamate (0 μM, 175 μM, or 350 μM), 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 (PI78442, Thermo Fisher Scientific). Protein concentration was measured using a BCA assay (23227, Thermo Fisher Scientific). Anti-GluR1 (NBP1-82396, Novus) was used as the primary antibody, and β-actin (4970S, Cell Signaling) was used as the loading control. Blots were detected automatically using the Simple Western System (Wes™, Bio-techne).

Statistics

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 (PI: Huang, J.).

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-VEGF-A 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 previously reported (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-sequencing (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 GBM patients or patients who had failed bevacizumab treatment (22-24).
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 (Fig. S1A-B). These results suggest that VEGF blockade may alter the GBM immune landscape. To test our hypothesis that anti-VEGF therapy alters immunological infrastructure, a 750 immune-related geneset, 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-A treatment using 2D PCA, while the normal brains were distinctly separated from samples treated with anti-VEGF-A (Fig. 1C). Next, RNA-Seq data were enriched using a Single Sample Gene Set Enrichment Analysis (ssGSEA) for immunosuppressive subpopulations (e.g. M1/M2 macrophages and Tregs) based on previous reports that VEGFR2 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-A treatment (Fig. 1D-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-A), anti-VEGFR2 (800μg/dose, i.p. every 3 days until the endpoint, which is equivalent to 100μg/dose of the anti-VEGF-A regarding median survival, Fig.S2A-B). 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 non-treated (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+CD4-CD8-T cells), with or without the treatment in spleen and tumor are shown in pie charts (mean) with statistical significance indicated. On D17 post tumor i.c., the percentage of Tregs in the spleens was ~1.7 times greater in aVEGFR2 treated animals compared to controls, but no similar change was seen in spleens of healthy mice with the same treatment schedule and dose (Fig. 2C and Fig. S3 A-E). In tumors, the Tregs fraction was decreased by ~25% at D17, but then almost tripled by D25 to reach 35.5% (Fig. 2D). Due to 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 tumors on D25, compared to the anti-PD-L1-treated tumors sampled at the same time point. No significant increase was observed in these parameters between the NT (D17, the endpoint) and the anti-PD-L1 (D25) groups (Fig. 2F). These data led us to predict that these differences between anti-VEGFR2 and anti-PD-L1 groups (~ > 2-fold higher) were the consequence of anti-VEGFR2 treatment. To replicate the finding we observed in the GL-261 tumor model, a second glioma model was tested. KR-158B is an invasive murine glioma line that was derived from spontaneous gliomas in Trp53/Nf1 double-mutant mice in the C57BL/6 background. Similar results were found in this model (Fig. S4 A-G). These results suggest that the tumor plays a critical role in the anti-VEGF associated Treg accumulation.

**Blocking Tregs using anti-CD25 antibody prior to 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 monotherapy (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 56–77% (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). IFN-γ production was then measured from CD4+ and CD8+ TILs after a re-challenge with anti-CD3/CD28 beads (Fig. 3D). Interestingly, both anti-CD25 (green dots) and anti-VEGFR2 (red dots) (which were used individually) significantly increased IFN-γ 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 IFN-γ 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 anti-tumor effects of anti-VEGF treatment, which can be overcome through targeted inhibition of Tregs. 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-VEGF-A treated tumors, three genes were revealed: SLC7A11, Gpr141, and Cth (Fig. 4A-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-D). The protein expression of xCT was higher in treated tumors than non-treated tumors (Fig. 4E). Since anti-VEGF therapy leads to a 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) (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 and 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-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 (32) (mGluR1, 4, and AMPA3) 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 to the Tconv cells (Fig. 5J). 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 GBM patients (4,5). These findings are likely multi-factorial. Our data suggest that immune suppressive mechanisms contribute to anti-VEGF failure. Our recently published study on human gliomas showed that the level of Foxp3+ T cells located in the perivascular tumor niche correlates with tumor-vasculature 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 Foxp3$^{\text{high}}$ CD4+ Tregs and can be increased by TGF-β (39), suggesting the Treg population may be preferentially impacted 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 INF-γ 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 sought to determine the immunological 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 to 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, i.e., 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 aminals. 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). Due to the potential suppression of activated effector cells by the CD25 blockade, we only administered two doses prior to anti-VEGF treatment. IFN-γ 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, IFN-γ 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 physiological conditions, glutamate is an excitatory neurotransmitter that is pivotal for the proper functioning of the CNS (48); however, under pathophysiological 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 pathophysiological glutamate has been detected in the extracellular space. A result of an analysis of 9
glioma patients indicates that glutamate concentrations were 100-fold higher in the peri-tumoral versus non-tumoral 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. Since 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 over-produced 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 autoimmunity mechanism”(56,57). This mechanism has been described as a beneficial action for the CNS to protect astrocytes from neurotoxin attacks under pathophysiological conditions (57,58). Gliomas may adopt this mechanism to protect themselves and in doing so, turn the neurotoxin into an immunosuppressive inducer (i.e. the elevated glutamate levels in vivo may act in a paracrine manner to activate glutamate receptors on the Tregs and enhance their immunosuppressive function). A potential solution might be to remove the excessive glutamate using an xCT inhibitor or through glutamate starvation. It is known that cystine starvation can potentially induce xCT expression as an adaptive response (59,60). However, to our knowledge, there have been no prior studies examining the effect of glutamate starvation on xCT expression. Investigating whether xCT loss of function restores susceptibility to anti-VEGF therapy will be an important study for further
Nevertheless, since glutamate plays a crucial role in the excitatory network of the CNS, blocking this axis may impair the function of normal brains. An FDA–approved drug, sulfasalazine that blocks xCT and inhibits glutamate release, has been shown to have no clinical effect but induced grade 4 toxicity in phase I/II trial in recurrent WHO grade 3 and 4 astrocytic gliomas in adults (61). In summary, because Tregs act as downstream of the xCT/glutamate axis in anti-VEGF treatment resistance, combining VEGF blockade with early Treg depletion represents a potentially safe and effective therapeutic strategy for patients with GBM.

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Figure Legends

Fig. 1 Anti-VEGF-A treatment alters the tumor-immune genetic landscape and enhances Tregs signature-genes. (A) Treatment schedule of anti-VEGF-A treatment. C57BL/6 mice were intracranially inoculated (i.c.) 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-VEGF-A 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 µg 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/](https://reactome.org/)). The immune-related pathways are shown in blue. (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-VEGF-A treatment. The RNA-Seq data were enriched using the Treg to Tconv (GSE7852_treg_vs_tconv_up) gene set provided by Gene Set Enrichment Analysis (GSEA) ([https://software.broadinstitute.org/software/cprg/](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 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_cd8_tcell_up) geneset. A one-way ANOVA was used to determine the significant differences between the means.

**Fig. 2** A enrichment of Tregs in spleen and tumors by anti-VEGF-A or anti-VEGFR2 treatment. (A) The animal experiment design to test the tumor-infiltrating Tregs and other T cell subsets using anti-VEGFR2 antibody in vivo. Six- to eight-week-old C57BL/6 mice (7-8 mice/group) were i.c. inoculated with 4x10^4 of GL-261-Luc tumor cells on day 0 (D0); the IVIS images were taken on day 10 (D10) to confirm the tumor engraftment. The tumor-burdened mice were then randomly separated into 2 groups, followed by the administration of either saline only (non-treated group, NT) or anti-VEGFR2 (800 μg/dose) antibody, every 3 days until the treatment endpoint. Tumors were respectively harvested on D10, D17 (the NT group endpoint) and D25 (the treated group endpoint) post-i.c., and the sample collection and analysis were finished within 3 days after these dates. (B) The gating strategy. (C-D) Post treatment kinetic analysis of % of Tregs, Tconv, CD8+ T and others (CD3+CD4-CD8-) cells in the spleen (C) and tumor
(D) summarized in the pie (Mean) chart with statistical analysis. (E) Experimental design. Three groups of GL-261-Luc tumor-burdened mice (10 mice/group) were treated on D10 after the IC with either saline, anti-VEGFR2 (800 μg/dose), or anti-PD-L1 (200 μg/dose), every 3 days until the treatment endpoints. The tumors were resected and analyzed at D17 for the NT groups, and D25 for both anti-VEGFR2 and anti-PD-L1 groups. The survival curves and proportions of indicated T cell subsets in those resected tumors are shown. (F) Intratumoral % of Tregs, Tregs/CD8+ T cells, or Tregs/Tconv ratio are shown. The data represent mean ± SEM, the survivals are shown using Kaplan–Meier survival curves, and the log-rank test was used to determine the significance. The data are representative of 2–3 independent experiments. A Mann-Whitney U test was used to determine the significant difference between the two groups.

Fig. 3 Blocking Tregs using an anti-CD25 antibody during the early phase of anti-VEGF therapy restores some of the antitumor reactivates mediated by the anti-VEGFR2. (A) Treatment strategy. Four groups (n = 7) of 6-to–8 week-old C57BL/6 mice were i.c. inoculated with GL-261-Luc, and 10 days after the tumor i.c., two doses of the anti-CD25 antibody (200 μg/dose) were given by i.p. to the tumor-burdened mice on D10 and D12. The anti-VEGFR2 (800 μg/dose) was started on 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 prior to the continuous treatment of anti-VEGFR2 restores the INF-γ 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^+ IFN-γ and CD8^+ IFN-γ 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.

**Fig. 4** A dose-dependent up-regulation of the SLC7A11 gene and its associates with Tregs after the anti-VEGFR2 treatment. (A) Identification of genes that were significantly up-regulated by anti-VEGF-A treatment. The RNA-Seq data from the anti-VEGF-A 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 3 comparisons are shown. (B) A dose-dependent increase in SLC7A11 gene expression determined by FPKM was observed after the anti-VEGF-A treatment. (C-D) SLC7A11 and SLC3A2 gene expression were 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. 2C), and RT-qPCRs were performed. (E) Anti-VEGFR2 increases xCT protein expression *in vivo*. The same tumors described in (C-D) were also evaluated by fluorescent immunohistochemistry. The *in vivo* experiments were repeated three times. (F) A dose-dependent increase appeared in HIF-1α after the anti-VEGF-A treatment. The HIF-1α expression in a different dose of anti-VEGF-A 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 μM or 150 μM of CoCl2 for 48 hrs *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 hrs (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 μM of CoCl₂ for 48 hrs, and glutamate concentration in the culture supernatant was assessed using three individual experiments. (J) SLC7A11 gene expression was highly correlated with Treg signature scores. A 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’s rank correlation coefficient.

**Fig. 5** A dose-dependent enhancement of Tregs proliferation, activation, and suppressive function by glutamate in vitro. (A) A representative flow cytometry analysis of Tregs functional analysis in the presence of L-glutamate in vitro. The Tregs (CD4⁺CD25⁺) and Tconv cells (CD4⁺CD25⁻) were freshly isolated from the spleens (n = 5) of 6-8-week-old C57BL/6 mice. CellTrace™ Violet labeled Tconv cells were co-cultured with Tregs and stimulated with anti-CD3/CD28 beads in the presence of 1 μM, 175 μM, and 350 μM of L-glutamate, respectively. (B-C) Glutamate dose-dependently enhances Tregs’ suppressive function. On day 3, the co-cultured cells were analyzed using flow cytometry, and the Treg suppression was assessed by measuring the non-divided population of the Tconv cells. (D) Glutamate dose-dependently decreases the CD69 expression on the Tconv cells. The co-cultured Tconv cells were also evaluated for the early activation marker, CD69, using flow cytometry. (E-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 (5x10^5/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 RT-qPCR was performed to determine the Ki67 gene expression. (H) Glutamate does not directly influence Tconv cell proliferation. The CellTrace™ Violet labeled Tconv cells (1.5x10^5/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 (5x10^5/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. A 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 (left) 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.

Fig. 6 Schema: anti-VEGF therapy enhances Tregs presentation and activity in glioma, dampening the antitumor efficacy of the treatment. Dysfunctional glutamate/cystine antiporter, SLC7A11/xCT, induced by the therapy, enhances the suppressive function of the Tregs via elevated tumor-derived glutamate.
Figure 1

A. Tumor i.c.

B. Up-regulated 525 genes

E. Treg Signature Score

D. Treg Signature Score

C. PC1 and PC2 scatter plots

- NBs
- 0 µg
- 50 µg
- 100 µg
- 200 µg

- CD8 E/M Score
Figure 2

A

D0  D10  D17  D25
Tumor i.c  IVIS  aVEGFR2  NT

B

D0  D10  D17  D25

C

D0  D10  D17  D25

D

D0  D10  D17  D25

E

D0  D10  D17  D25

F

Days after tumor implantation

Percent survival

% Tregs

Tregs /CD8+ T cells

Tregs /Tconv cells

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Figure 3

A) Tumor i.c. CD25 mAb (d10 & d12) VEGFR2-mAb (Start on d11, every 3 days)

B) Percent survival over days after tumor implantation:
- NT
- CD25 mAb (green)
- VEGFR2-mAb (red)
- VEGFR2/CD25 (blue)

C) Flow cytometry analysis of CD4 and CD25:
- CD4+CD25+ percentages:
  - NT (black)
  - CD25 (green)
  - VEGFR2 (red)
  - VEGFR2/CD25 (blue)

D) IFN-γ expression:
- CD4+IFN-γ+ percentages:
  - NT (black)
  - CD25 (green)
  - VEGFR2 (red)
  - VEGFR2/CD25 (blue)

Day 0-Endpoint

% CD4+CD25+:
- NT: 38.6
- CD25: 0.7
- VEGFR2: 56.6
- VEGFR2/CD25: 1.8

% CD4+IFN-γ+:
- NT: 6.4
- CD25: 10.8
- VEGFR2: 19.8
- VEGFR2/CD25: 35.6

% CD8+IFN-γ+:
- NT: 2.6
- CD25: 4.1
- VEGFR2: 22.4
- VEGFR2/CD25: 17.1

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Figure 4

A. Venn diagram showing the overlap of genes SLC7A11, Gpr141, and Cth.

B. Scatter plot showing the expression of SLC7A11 (FPKM) with aVEGF-A Concentration.

C. Scatter plot showing the expression of SLC7A11 with aVEGFR2 Concentration.

D. Scatter plot showing the expression of SL3A2 with aVEGFR2 Concentration.

E. Microscopy images comparing NT and aVEGFR2 conditions.

F. Scatter plot showing the expression of HIF1α (FPKM) with aVEGF-A Concentration.

G. Scatter plot showing the expression of xCT MFI with aVEGF-A Concentration.

H. Scatter plot showing the expression of xCT MFI with CoCl2 Concentration.

I. Scatter plot showing the expression of Glutamate with aVEGFR2 Concentration.

J. Scatter plot showing the expression of SLC7A11 (FPKM) with Treg Score.
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