Systemic Inhibition of Transforming Growth Factor-β in Glioma-Bearing Mice Improves the Therapeutic Efficacy of Glioma-Associated Antigen Peptide Vaccines

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Abstract

Purpose: A variety of cancers, including malignant gliomas, overexpress transforming growth factor-β (TGF-β), which helps tumors evade effective immune surveillance through a variety of mechanisms, including inhibition of CD8+ CTLs and enhancing the generation of regulatory T (Treg) cells. We hypothesized that inhibition of TGF-β would improve the efficacy of vaccines targeting glioma-associated antigen (GAA)–derived CTL epitopes by reversal of immunosuppression.

Experimental Design: Mice bearing orthotopic GL261 gliomas were treated systemically with a TGF-β–neutralizing monoclonal antibody, 1D11, with or without s.c. vaccinations of synthetic peptides for GAA-derived CTL epitopes, GARC-1 (77-85) and EphA2 (671-679), emulsified in incomplete Freund’s adjuvant.

Results: Mice receiving the combination regimen exhibited significantly prolonged survival compared with mice receiving either 1D11 alone, GAA vaccines alone, or mock treatments alone. TGF-β neutralization enhanced the systemic induction of antigen-specific CTLs in glioma-bearing mice. Flow cytometric analyses of brain-infiltrating lymphocytes revealed that 1D11 treatment suppressed phosphorylation of Smad2, increased GAA-reactive/IFN-γ-producing CD8+ T cells, and reduced CD4+/FoxP3+ Treg cells in the glioma microenvironment. Neutralization of TGF-β also upregulated plasma levels of interleukin-12, macrophage inflammatory protein-1α, and IFN-inducible protein-10, suggesting a systemic promotion of type-1 cytokine/chemokine production. Furthermore, 1D11 treatment upregulated plasma interleukin-15 levels and promoted the persistence of GAA-reactive CD8+ T cells in glioma-bearing mice.

Conclusions: These data suggest that systemic inhibition of TGF-β by 1D11 can reverse the suppressive immunologic environment of orthotopic tumor-bearing mice both systemically and locally, thereby enhancing the therapeutic efficacy of GAA vaccines.

Malignant gliomas represent the most common primary central nervous system tumors and exhibit a dismal prognosis despite recent advances made in surgical, radiological, and chemotherapeutic approaches (1). Development of novel, multimodal therapeutic approaches is thus critical to improve the outcome of these deadly tumors (2). Among these approaches, immunotherapy can be promising for glioma treatment if we are able to induce potent immune responses directed against glioma-associated antigens (GAA; refs. 3–5). Indeed, several immunologic strategies have been evaluated in early phase clinical trials; however, modest clinical benefit in glioma immunotherapy trials calls for further improvement of these strategies (6). Transforming growth factor-β (TGF-β) is a highly pleiotropic cytokine that plays key regulatory roles in many aspects of immunity (7). It directly inhibits cytolytic activity of natural killer cells, macrophages, and CD8+ cytotoxic T cells (8, 9). It can also inhibit instruction, activation, and expansion of tumor-specific helper and cytotoxic T-cell populations (10) and enhance the generation of immunosuppressive regulatory T (Treg) cells.
Translational Relevance

Overexpression of transforming growth factor-β (TGF-β) by gliomas contributes to systemic immunosuppression and may be a major factor limiting the efficacy of current immunotherapy strategies. This study shows that systemic administration of anti-TGF-β antibody induces a dynamic change of the immunologic environment of glioma-bearing mice and improves the efficacy of vaccinations against glioma-associated antigens (GAA). Neutralization of TGF-β led to promotion of vaccine-induced type-1 adaptive immune response systemically and reduction of regulatory T cells in the tumor. The current study is the first to evaluate the effects of neutralizing TGF-β in combination with vaccines targeting GAA in a syngeneic murine glioma model. Given our experience conducting clinical trials of GAA-targeted vaccines in glioma patients, these data provide support for development of vaccine clinical trials in combination with a monoclonal antibody against human TGF-β.

(11). Thus, the presence of TGF-β in the tumor microenvironment is predicted to disable effective immune surveillance by multiple mechanisms. Indeed, many human tumors, including malignant gliomas, overexpress TGF-β, and elevated expression frequently correlates with tumor progression and poor prognosis (12). The mammalian TGF-β isoforms β1, β2, and β3 are produced by various malignant glioma cells (13, 14). Indeed, in a recent phase I clinical trial of dendritic cell (DC)-based vaccines in glioblastoma multiforme, resection of post-vaccine tumors showed that TGF-β/β2 expression within the tumors was inversely correlated with both the intensity of tumor infiltration by cytotoxic T cells and clinical survival (15). Furthermore, data from preclinical studies suggests that antagonism of TGF-β can suppress tumorigenesis by enhancing and/or restoring effective antitumor immune surveillance (16–18). These observations led to the hypothesis that inhibition of TGF-β would reverse TGF-β–induced immunosuppression and improve the efficacy of vaccines targeting GAA-derived CTL epitopes.

In the current study, administration of the TGF-β–neutralizing monoclonal antibody (mAb), 1D11, can promote systemic and local CTL responses against GAA-derived CTL epitopes, and reverse the suppressive immunologic environment of orthotopic gliomas, and thereby enhance the therapeutic efficacy of GAA vaccines. This study provides strong support for the development of novel combinatorial strategies using anti–TGF-β mAb in anti-glioma vaccine clinical trials.

Materials and Methods

Reagents. RPMI 1640, fetal bovine serum, L-glutamine, sodium pyruvate, β-mercaptoethanol, nonessential amino acids, and antibiotics were obtained from Invitrogen Life Technologies. Recombinant human interleukin (IL)-2 was obtained from PeproTech. The following peptides were synthesized by the automated solid-phase peptide synthesizer in the University of Pittsburgh Peptide Synthesis Facility with >95% purity as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis: H-2Dβ–binding EphpA2(671-679) (FSHHINIHL), H-2Dβ–binding GARC–1(177-185) (AALINKLYA), I-Aβ–binding HBV core128–140 (TTPAYRPNNPIL), H-2Dβ–binding human/mouse gp100 (h/mgp100) 25–33 (KVPREQIDWNL), and H-2Kβ–binding ovalbumin (OVA) 257–264 (SIINFEKL).

Cell culture. The TAP2–/– RMA S mouse thymoma cell line (H-2b) was kindly provided by Dr. Walter J. Storkus (University of Pittsburgh, Pittsburgh, PA). GL261 mouse glioma cells (H-2b) kindly provided by Dr. Robert Prins (University of California Los Angeles, Los Angeles, CA), express mgp100, EphpA2, and GARC–1 as GAA s (4, 19, 20). All cells were maintained in mouse complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 μmol/L l-glutamine (Life Technologies Invitrogen)) in a humidified incubator in 5% CO2 at 37°C. Culture supernatants were harvested, filtered, and concentrated 10-fold using an Amicon Ultra Filter (Millipore). GL261-conditioned medium (GL261-CM) was prepared by mixing the concentrated medium and fresh medium at 1:9 ratio.

Animals. C57BL/6 mice (H-2b) were obtained from Taconic Farms. Animals were handled in the Animal Facility at the University of Pittsburgh per an Institutional Care and Use Committee–approved protocol.

Antibodies and tetramers. The anti-murine TGF-β mAb, 1D11, which neutralizes all three isoforms of TGF-β (21), and a murine IgG1 mAb against Shigella toxin, 13C4, which serves as an isotype control, were obtained from Genzyme Corporation. The following antibodies were obtained from CAILTAC Laboratories: phycoerythrin (PE)–conjugated anti–IFN-γ, TC-conjugated anti-CD4, TC-conjugated anti-CD8, and PE-isotype-matched controls. FITC-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD25 (PC61) were obtained from BD Biosciences. Anti-Smad2 and anti-phosphorylated Smad2 (pSmad2) antibodies were purchased from Cell Signaling Technology. APC-conjugated anti-CD62L (Mel-14) and PE–cy7–conjugated anti-CD44 (IM7) were purchased from BioLegend. PE-conjugated anti-FoxP3 (NRRF-30) antibody was obtained from ebioScience. PE-conjugated H-2Dβ/EphpA2671–679 tetramer (EphpA2 tetramer) and PE-conjugated H-2Dβ/GARC–177–85 tetramer (GARC–1 tetramer) were produced by the National Institute of Allergy and Infectious Disease tetramer facility within the Emory University Vaccine Center (Atlanta, GA).

Flow cytometry. The procedure used in the current study has been described previously (22). Briefly, single-cell suspensions were surface stained with fluorescent dye–conjugated antibodies. For intracellular staining, cells were surface stained, washed, fixed, permeabilized with Cytotox/Cytoperm buffer (BD Biosciences), and intracellularly stained. All stained cells were compared with those stained with isotype-matched control antibodies. Samples were examined by Coulter EPICS cytometer or CyAn ADP (Beckman Coulter) and data were analyzed by the WinMDI software or Summit software (Dako Colorado, Inc.).

Intracranial injection of GL261 glioma cells. The procedure used in the current study has been described previously (22–25). Briefly, using a Hamilton syringe (Hamilton Company), 1 × 106 GL261 cells in 2 μL PBS were stereotactically injected through an entry site at the bregma, 3 mm to the right of sagittal suture, and 4 mm below the surface of the skull of anesthetized mice by using a stereotactic frame (Kopf).

Treatment of intracranial tumor-bearing mice with anti–TGF-β antibody and GAA peptide–based vaccines. Beginning 3 d following tumor cell inoculation, 25 mg/kg of anti–TGF-β antibody (1D11) or isotype control antibody (13C4) was administered i.p. every 2 d for a total of 12 doses. The animals received s.c. vaccinations with HBV core128–140 and GAA peptides, including EphpA2(671–679) and GARC–1(177–185) (100 μg each peptide), emulsified in incomplete Freund’s adjuvant (IFA; Difco Laboratories) on days 3, 13, and 23 after tumor inoculation. In some experiments, symptom-free survival was monitored as the

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primary end point; in other experiments, treated mice were sacrificed on indicated days to evaluate immunologic end points such as effects on brain-infiltrating lymphocytes (BIL).

**BIL isolation.** The procedure used in the current study has been described previously (25). Briefly, mice were sacrificed by CO₂ asphyxiation and immediately perfused with PBS through the left cardiac ventricle. Brain tissues were mechanically minced, resuspended in 70% Percoll (Sigma-Aldrich), overlaid with 37% and 30% Percoll, and centrifuged for 20 min at 500 × g. Enriched BIL populations were recovered at the 70% to 37% Percoll interface.

**Cytokine and chemokine release assay.** Peripheral blood samples were collected from the tail vein of mice, centrifuged, and the resulting supernatants were pooled as plasma samples. The cytokine and chemokine levels in plasma samples were evaluated by specific ELISA kits. The following ELISA kits for murine cytokines and chemokines were purchased from commercial vendors: IL-12p70 (eBioscience); TGF-β1, IFN-inducible protein-10 (IP-10), and IL-15 (R&D Systems). Mouse cytokines and chemokines were also analyzed using a 20-plex kit from Invitrogen/Biosource for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, IP-10, IFN-γ, monokine induced by IFN-γ, macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α, and monocyte chemotactic protein-1 by the University of Pittsburgh Cancer Institute Luminex Core Facility. Analyses of mouse plasma were done in a 96-well microplate format according to manufacturers’ protocols as previously described (26). Data were presented as the mean ± SD ng/mL of protein.

**In vitro cytolytic assay.** The procedure used in the current study has been described previously (5). Briefly, target GL261 or peptide-loaded RMAS cells (1 × 10⁴ cells in 100 μL) labeled with 50 μCi of Na₂¹⁵⁹C₁₄O₂ (¹⁵⁹Cr) were added to wells containing 100 μL of varying numbers of effector cells using U-bottomed 96-well plates (Corning). After a 4-h incubation at 37°C, 30 μL of supernatants were harvested from each well and transferred to wells of a LumaPlate-96 (Packard, Inc.). The amount of ¹⁵⁹Cr in each well was measured in a Micro Plate Scintillation Counter (Packard, Inc.). The percentage of specific lysis (% specific lysis) was calculated using triplicate samples as follows: percentage lysis = (cpm experimental release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) × 100.

**Statistical analysis.** The statistical significance of differences between groups was determined by one-way ANOVA with Holm’s post hoc test. Survival data were analyzed by log-rank test. We considered differences significant when P value is <0.05. All data were analyzed by SPSS version 14.0 (SPSS) and Statcel 2 (OMS Publishing, Inc.).

**Results**

**Systemic inhibition of TGF-β improves the therapeutic efficacy of vaccinations targeting GAA-derived CTL epitopes.** To evaluate the therapeutic benefit of neutralization of TGF-β in combination with a vaccine therapy, mice were treated with 1D11 in combination with s.c. vaccinations targeting GAA-derived CTL epitopes EphaA263-71-679 and GARC-171-85, beginning 3 days after intracranial (i.c.) injection of GL261 glioma cells. Histologic evaluations confirmed that i.c. injected GL261 cells form solid and vascularized tumors in the brain of syngeneic mice on day 3 following stereotactic inoculation (Fig. 1A). Mice receiving the combinatorial therapy of 1D11 and GAA vaccines exhibited significantly improved survival with 6 of 10 mice treated with the combination regimen surviving longer than 100 days, whereas only 2 of the 10 mice treated with GAA vaccines and the isotype control antibody, 13C4, survived longer than 100 days (Fig. 1B). Treatment with either 1D11+IFA or 13C4+IFA did not provide significant therapeutic benefit in this model. These results indicate that the therapeutic effects of GAA vaccines can be significantly enhanced by i.p. administration of 1D11.

**Effects of 1D11 on the systemic induction of GAA-specific CTL responses.** The impact of 1D11 administration on the systemic induction of GAA-specific CTL responses was evaluated using splenocytes from glioma-bearing mice treated with the solo or combinatorial therapies (Fig. 2). 1D11 treatment significantly elevated the levels of vaccine-induced CTL activity in the spleen and draining lymph nodes against RMAS cells loaded with EphaA2571-679, at all effector/target ratios. Although modest, significant increases of the vaccine-induced CTL activity against GL261 cells and GARC-171-85–loaded RMAS cells were observed at an effector/target ratio of 60:1 (Fig. 2). No increase of CTL activity was observed against RMAS cells loaded with an irrelevant peptide, as expected.

**Systemic administration of 1D11 leads to decreased TGF-β signaling in the glioma microenvironment.** Despite the remarkable improvement of the therapeutic efficacy in
glioma-bearing mice (Fig. 1), 1D11 treatment only modestly enhanced the systemic CTL activities induced by GAA vaccines (Fig. 2), suggesting that 1D11 treatment might promote antitumor immunity in the i.c. glioma microenvironment. To address this, the TGF-β signaling status (Fig. 3A) and phenotye (Fig. 3B and C) of BILs was evaluated. TGF-β binding to its cell surface receptors results in phosphorylation of the receptor leading to subsequent phosphorylation and activation of downstream signal transducers called Smads (27). pSmad2 and phosphorylated Smad3 associate with Smad4 then translocate to the nucleus to initiate transcription of TGF-β-mediated genes (28). Therefore, 1D11-mediated inhibition of Smad2 phosphorylation in vitro and in vivo was investigated. Secretion of TGF-β by GL261 glioma cells was confirmed through ELISA analysis of conditioned media of GL261 cells grown in culture as these cells secrete 163 ± 33 pg/10^6 cells/24 hours. Furthermore, conditioned media from GL261 cells could induce phosphorylation of Smad2 in splenocytes (SPC) from naive C57BL/6 mice and treatment with 1D11 inhibited Smad2 phosphorylation (Supplementary Fig. S1), demonstrating secretion of active TGF-β by GL261 cells and confirming functional blockade of the TGF-β signaling by 1D11.

BILs were isolated from C57BL/6 mice bearing i.c. GL261 tumors that were treated with GAA peptides and either 1D11 or 13C4. Treatment with 1D11 and the GAA vaccine resulted in decreased levels of pSmad2 in CD4- or CD8-gated BILs (Fig. 3A), suggesting that systemically administered 1D11 may penetrate i.c glioma, thereby inhibiting the TGF-β signaling in the tumor microenvironment.

Treatmetn with 1D11 results in increased infiltration of type-1 anti-GAA CTLs and decreased infiltration of T_reg cells in gliomas. BILs were analyzed by flow cytometry for the presence of CD8+ CTLs versus CD25+/FoxP3+ T_reg cells. GAA vaccines alone increased the infiltration of GAA-reactive CD8+ T cells and IFN-γ-producing CD8+ T cells into the brain when compared with mice receiving control therapies, and treatment with 1D11 further increased the infiltration of GAA-reactive and IFN-γ-producing CD8+ CTLs into glioma (Fig. 3B). Conversely, treatment with 1D11 significantly reduced the number of CD4+/CD25+ and CD4+/FoxP3+ T_reg cells in the glioma microenvironment (Fig. 3C). These data indicate that the systemic 1D11 administration significantly impacts the i.c. glioma microenvironment by promoting a type-1 (i.e., IFN-γ expressing) CD8+ T-cell response and reducing infiltration of immunosuppressive T_reg cells.

Neutralization of TGF-β with 1D11 promotes systemic type-1 cytokine/chemokine profiles. Tumor antigen–specific CD4+ and CD8+ T lymphocytes, especially IFN-γ–producing type-1 helper T (Th1) and type-1 cytotoxic T cells, play crucial roles in tumor eradication (29). Because TGF-β blockade promoted type-1 function of vaccine-stimulated T cells within the i.c. tumor microenvironment (Fig. 3B and C), the cytokine/chemokine profiles in plasma samples obtained from treated glioma-bearing mice were determined. Treatment with 1D11 seems to upregulate plasma levels of IL-12 (Supplementary Fig. S2; Fig. 4A), IP-10 (Fig. 4B), and MIP-1α (Fig. 4C) regardless of whether or not the mice were concurrently treated with GAA vaccines. GAA vaccines by themselves did not elevate plasma levels of these cytokines and chemokines at significant levels. Although IFN-γ was upregulated in CD8+ BILs derived from mice receiving the combination therapy (Fig. 3B), measurable levels of IFN-γ were not detected in the plasma samples from these mice (data not shown). Nevertheless, these results suggest that neutralization of TGF-β may promote type-1 cytokine/chemokine production profiles systemically in the glioma-bearing mice, thereby inducing a strong antitumor immunity.

Neutralization of TGF-β with 1D11 promotes the persistence of GAA-reactive CD8+ T cells in association with systemically upregulated IL-15. IL-15 has shown to be essential for primary expansion and generation of memory CD8+ T cells in vivo (30). Therefore, the possibility that neutralization of TGF-β by 1D11 could improve the efficacy of GAA vaccines associated with systemic upregulation of IL-15 and the
persistence of antigen-specific memory CD8+ T cells was evaluated. Plasma and SPCs were harvested from GL261 glioma-bearing mice on day 35 after the tumor inoculation (12 days following the last vaccination). Administration of GAA vaccines significantly elevated plasma levels of IL-15 compared with mice treated with IFA, and the addition of 1D11 further enhanced the circulating levels of IL-15 (Fig. 5A).

Next, flow cytometry was used to characterize the GAA-specific effector/memory CD8+ T-cell phenotype of SPCs derived from the glioma-bearing treated mice. With regard to proportions in CD8+ cells, 1D11 treatment alone slightly but significantly increased the proportion of EphA2671-679-reactive/CD8+ cells compared with controls, as did GAA vaccines alone though to a stronger degree (Fig. 5B). The combination of GAA vaccines and

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**Fig. 3.** Systemic 1D11 promotes type-1 anti-GAA CTLs while reducing Treg cells in the i.c. glioma tissue. Mice bearing orthotopic gliomas were treated with 1D11 and GAA vaccines as described previously. **A,** left, on day 25, BILs were isolated, and intracellular pSmad2 and Smad2 levels were evaluated in CD8- or CD4-gated populations by flow cytometry. Open and shaded histograms, cells stained for Smad2 or pSmad2, respectively. **Right,** relative expression levels of pSmad2 in CD8-gated (black column) or CD4-gated (white column) population of the BILs calculated as relative mean fluorescence intensity values of pSmad2 to those of Smad2 in corresponding cell populations. BILs were isolated from treated mice, and flow cytometric analyses were done for **B** surface expression of CD8 and GARC-177-85 tetramer, EphA2671-679 tetramer or intracellular IFN-γ, and **C** surface expression of CD4 and CD25 or intracellular FoxP3 in lymphocyte-gated populations. For flow cytometric analyses of BILs (**B** and **C**), because of the small number of BILs obtained per mouse (4 x 10^6 cells/mouse), BILs obtained from five mice per group were pooled, and evaluated for the relative number and phenotypes between groups. Numbers in each dot plot, the percentage of double-positive cells in **B** CD8-gated or **C** CD4-gated BILs. One of two representative experiments with similar results is shown.
1D11 further enhanced the frequency of these cells. The same effects were observed in absolute numbers of the central memory cell subset (CD44\(^{\text{high}}\) and CD62L\(^{\text{high}}\)) and the effector memory cell subset (CD44\(^{\text{high}}\) and CD62L\(^{\text{low}}\); Fig. 5C). Although the short survival time of glioma-bearing mice did not allow for evaluation of the development of memory cells with longer observation periods, these data suggest that neutralization of TGF-\(\beta\) with 1D11 promotes persistence of vaccine-induced GAA-specific effector and central/effector memory CD8\(^{+}\) T cells in hosts associated with upregulation of plasma IL-15.

**Discussion**

In the current study, the focus was on the development of combinational strategies using anti–TGF-\(\beta\) mAb with GAA peptide–based vaccines for glioma immunotherapy, and evaluation of immune mechanisms of action underlying the therapeutic effect of this combination regimen.

Cancer vaccines elicit a clinical benefit by catalyzing a number of critical events including: (a) systemic induction of antigen-specific effector and memory CTL responses; (b) traffic of the effector cells to the tumor site; and (c) the execution of antitumor effects by the tumor-infiltrating effector cells. In this study, neutralization of TGF-\(\beta\) with a TGF-\(\beta\) mAb improved the therapeutic efficacy of GAA vaccines (Fig. 1) by improving both systemic induction of GAA-specific CTL responses (Fig. 2) and homing of GAA-reactive and IFN-\(\gamma\)-producing CD8\(^{+}\) T cells into i.c. gliomas (Fig. 3B).

Recent studies have suggested that production of TGF-\(\beta\) by gliomas may have a significant role in the induction of glioma-associated T\(_{\text{reg}}\) cells (31, 32). In a separate study with a non–central nervous system tumor model, 1D11 has been shown to inhibit tumor-induced conversion of naïve CD4\(^{+}\)CD25\(^{+}\) T cells into CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\) T\(_{\text{reg}}\) cells (33). In the current study, systemic administration of 1D11 reduced TGF-\(\beta\) levels and T\(_{\text{reg}}\) cells within the i.c. glioma microenvironment (Fig. 3). Furthermore, inhibition of Smad2-mediated signaling in BIL in the tumor microenvironment suggests that, in addition to its systemic effects, i.p. administration of 1D11 may directly inhibit the protumorigenic effects of TGF-\(\beta\) in the glioma microenvironment. With regard to the cell types producing TGF-\(\beta\), GL261 glioma cells cultured in vitro produce active TGF-\(\beta\) as shown by ELISA and cell-based assays (Supplemental Fig. S1). Recently, a study by Umemura et al. (34) showed that the major source of TGF-\(\beta\) in the i.c. GL261 glioma model is likely to be glioma-infiltrating myeloid-derived suppressor cells rather than GL261 glioma cells. Given the potential role of myeloid-derived suppressor cell–derived TGF-\(\beta\) in gliomas, it would be intriguing to evaluate the effects of 1D11 treatment on myeloid-derived suppressor cells in future studies.

Neutralization of TGF-\(\beta\) with 1D11 promoted systemic type-1 immunity as evidenced by increased IFN-\(\gamma\)-expressing CD8\(^{+}\) T cells in BILs and upregulated plasma levels of IL-12 and IP-10. TGF-\(\beta\) has been shown to downregulate DC-derived IL-12 (35), which is a potent inducer of IFN-\(\gamma\)-producing Th1 cells (36). Moreover, TGF-\(\beta\) contributes to the shift toward a type 2 helper T cell responses through direct and IL-10–mediated pathways and dampens the type-1 cytotoxic T-cell population in tumor-bearing mice (37). These data suggest that treatment with 1D11 in the current study may have triggered type-1 cytokine/chemokine production cascades by promoting IL-12 production from antigen-presenting cells in glioma-bearing hosts. Although macrophage- or DC-derived MIP-1\(\alpha\) is crucial in controlling leukocyte activation and recruitment of Th1 cells into the tissues at inflammatory sites (38), it remains to be determined how blockade of TGF-\(\beta\) promotes production of the Th1 chemokine MIP-1\(\alpha\).

TGF-\(\beta\) blockade by 1D11 also increased the number of GAA-specific central/effector memory CD8\(^{+}\) T cells, with a concomitant elevation in IL-15 levels (Fig. 5). One of the mechanisms by which TGF-\(\beta\) negatively regulates CD8\(^{+}\) memory T-cell homeostasis is by opposing the positive effects of IL-15, which is involved in primary expansion of antigen-specific memory.
CD8+ T cells (30). TGF-β has been shown to counteract IL-15 by modulating expression of the β-chain of the IL-15 receptor (39), and it is hypothesized that TGF-β neutralization in these studies counteracted TGF-β–mediated suppression of IL-15Rβ resulting in elevated systemic IL-15. Central memory CD8+ T cells confer efficient and persistent antitumor immunity in preclinical tumor models (40), and TGF-β inhibition may have contributed to the upregulation of GAA-specific central/effector memory CD8+ T cells in GL261-bearing mice, thereby improving the therapeutic efficacy of GAA vaccines. Future studies evaluating the blockade of IL-15 (e.g., by use of IL-15–specific blocking mAb or IL-15 receptor–deficient mice) would directly determine the significance of IL-15 induction to the observed therapeutic effect.

It has been well documented that overexpression of TGF-β by gliomas contributes to systemic immunosuppression and may be a major factor responsible for the failure of current immunotherapy strategies (13, 14, 41). Therefore, TGF-β neutralization might result in reduced tumor-induced immunosuppression and enhanced immunotherapeutic modulation. Indeed, specific blockade of TGF-β expression (42), signaling (16, 18) and ex vivo blockade by antibody (43) have all been shown to result in a beneficial effect on lymphocyte function and have resulted in enhanced immune responses in a variety of models, including glioma. With regard to the use of 1D11 in previous studies, 1D11 treatment reduced the number of lung metastases in a mouse metastatic breast cancer model (44), suppressed tumor growth through downregulation of IL-17 in tumor cells (45), and reduced tumor burden in a renal cell cancer model through inhibiting tumor conversion of naïve CD4+CD25+ T cells into CD4+CD25+FoxP3+ Treg cells (33). The current study is the first evaluating the effects of 1D11 in combination with vaccines targeting GAA.

Concomitantly with our present study, Terabe et al. (46) have studied the effects of 1D11 in combination with tumor antigen–specific vaccines in a murine TC1 s.c. lung tumor model (this study appears in the same issue). Although their study provides unique mechanistic insights that are not addressed in our study, such as the contribution of NK-T cells, one major difference from ours is that they did not observe any significant reduction of Treg cells by 1D11 treatment. This may be at least partially due to the lower dose of 1D11 administration in their study (5 mg/kg) than that in our study (25 mg/kg), and potentially different levels of TGF-β elaboration by different tumor

![Fig. 5.](image-url) Elevated plasma IL-15 levels are associated with increased GAA-reactive CD8+ T cells in mice treated with 1D11 and GAA vaccines. On day 35, peripheral blood samples and SPCs were collected from glioma-bearing treated mice. A, plasma IL-15 levels were evaluated by ELISA. B and C, (B) proportions of EphA2671-679 tetramer+/CD8+ cells in lymphocyte-gated CD8+ cells, and (C) absolute numbers of EphA2671-679 tetramer+/CD8+/CD44high/CD62Llow (central memory) or EphA2671-679 tetramer+/CD8+/CD44high/CD62Llow (effector memory) cells per 1 × 10^5 SPCs were analyzed by flow cytometric analyses. Columns, mean of samples from three mice per group; bars, SD. *, P < 0.05; **, P < 0.01 for indicated comparisons.
models. Nevertheless, both studies show therapeutic benefits of the combination approach with tumor vaccines and TGF-β blockade by 1D11.

Few studies have investigated the effect of combining TGF-β inhibition and vaccine therapy to enhance antitumor immunity. Tumor-bearing rats receiving administration of TGF-β2 anti-sense oligonucleotides combined with systemic vaccination using irradiated tumor cells exhibited significantly prolonged survival compared with rats without any treatment, although little was addressed with regard to the underlying immune mechanisms in this approach (47, 48). Administration of an antibody against TGF-β (2G7) enhanced the ability of an intratumorally injected DC vaccine to inhibit the growth of established mouse breast cancer cells (49). Recent studies with an orally available TGF-β receptor I kinase inhibitor (SM16) have shown that when combined with adenovirus-based vaccines (50) or adoptive T-cell therapy (51), SM16 treatment potentiated the efficacy of both immunotherapies. The authors showed that this was due to changes in the tumor microenvironment, including an increase in antitumor CTLs, type-1 cytokines and chemokines, and endothelial adhesion molecules with a decrease in arginase mRNA expression. Although their studies and our current study commonly show the favorable alteration of tumor microenvironment by systemic TGF-β blockade, novelty of our current study includes the demonstration of decreased CD4+/CD25+ FoxP3+ Treg cells as well as increased IFN-γ producing CD8+ T cells within the central nervous system tumor microenvironment.

Although all these studies point to the significance of developing clinically applicable TGF-β blockade strategies in combination with other forms of immunotherapy, our current study has identified a novel mechanism by which 1D11 treatment induces potent and persistent type-1 adaptive antigen presentation in combination with GAAs vaccines, providing a strong basis for developing novel therapeutic strategies that combine immunotherapy with TGF-β neutralization in the treatment of glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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