

Received: 2016.12.29
Accepted: 2017.01.20
Published: 2017.07.24

Combined Blockade of T Cell Immunoglobulin and Mucin Domain 3 and Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 Results in Durable Therapeutic Efficacy in Mice with Intracranial Gliomas

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF G 1 **Jinhu Li**
CG 1 **Xiaodong Liu**
BC 2 **Yijun Duan**
AE 1 **Yueting Liu**
FG 1 **Hongqin Wang**
BCD 1 **Shizhong Lian**
BCD 3 **Guotao Zhuang**
AG 1 **Yimin Fan**

1 Department of Neurosurgery, The First Hospital of Shanxi Medical University, Taiyuan, Shanxi, P.R. China
2 Department of Immunology, Shanxi Provincial Cancer Hospital, Taiyuan, Shanxi, P.R. China
3 Department of Neurosurgery, General Hospital of Datong Coal Mine Group, Datong, Shanxi, P.R. China

Corresponding Author: Yimin Fan, e-mail: blueskyfan@126.com

Source of support: This study was supported by the National Natural Science Foundation of China (grant no. 81470115), the Science and Technology Research Program of Shanxi Province (grant no. 2015025), the Basic Research Program of Shanxi Province (grant no. 201601D011102), and the Graduate Student Education Innovation Program of Shanxi Province (grant no. 2016BY081)

Background: Glioblastoma multiforme (GBM) evades immune surveillance by inducing immunosuppression via receptor-ligand interactions between immune checkpoint molecules. T cell immunoglobulin and mucin domain 3 (Tim-3) is a key checkpoint receptor responsible for exhaustion and dysfunction of T cells and plays a critical role in immunosuppression. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) has been recently identified as a heterophilic ligand for Tim-3.

Material/Methods: We established an intracranial GBM model using C57BL/6 mice and GL261 cells, and treated the mice with single or combined monoclonal antibodies (mAbs) against Tim-3/CEACAM1. The CD4⁺, CD8⁺, and regulatory T cells in brain-infiltrating lymphocytes were analyzed using flow cytometry, and the effector function of T cells was assessed using ELISA. We performed a rechallenge by subcutaneous injection of GL261 cells in the "cured" (>90 days post-orthotopic tumor implantation) and naïve mice.





Results: The mean survival time in the control, anti-Tim-3, anti-CEACAM1, and combined treatment groups was 29.8, 43.4, 42.3, and 86.0 days, respectively, with 80% of the mice in the combined group becoming long-term survivors showing immune memory against glioma cells. Infiltrating CD4⁺ and CD8⁺ T cells increased and immunosuppressive Tregs decreased with the combined therapy, which resulted in a markedly elevated ratio of CD4⁺ and CD8⁺ cells to Tregs. Additionally, plasma IFN- γ and TGF- β levels were upregulated and downregulated, respectively.

Conclusions: Our data indicate that combined blockade of Tim-3 and CEACAM1 generates robust therapeutic efficacy in mice with intracranial tumors, and provides a promising option for GBM immunotherapy.

MeSH Keywords: **Costimulatory and Inhibitory T-Cell Receptors • Glioma • Immunotherapy, Active**

Abbreviations: **GBM** – glioblastoma multiforme; **Tim-3** – T cell immunoglobulin and mucin domain 3; **CEACAM1** – carcinoembryonic antigen-related cell adhesion molecule 1; **PD-1** – programmed death-1; **CTLA-4** – cytotoxic T lymphocyte antigen-4; **mAb** – monoclonal antibody; **BIL** – brain-infiltrating lymphocyte; **Treg** – regulatory T cell; **ELISA** – enzyme-linked immunosorbent assay

Full-text PDF: <http://www.medscimonit.com/abstract/index/idArt/903098>

 3337  —  5  43



Background

Glioblastoma multiforme (GBM) is the most prevalent and lethal primary brain tumor in adults [1,2]. Although patients with GBM undergo aggressive and multimodality treatment, such as standard-of-care surgery combined with chemoradiotherapy, the median survival period is only about 15 months post-diagnosis [3,4]. Therefore, it is imperative to search for more effective therapeutic strategies for GBM. Recently, immunotherapy has garnered increasing attention as an attractive treatment modality for GBM, owing to the precision and memory of antitumor immunologic cytotoxicity [5].

Several solid tumors, including glioma, have been shown to form an immunosuppressive environment for impairing immunosurveillance and evading immunologic pressure by coordinating with negative immune checkpoint molecules expressed on tumor-infiltrating lymphocytes, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), T cell immunoglobulin and mucin domain 3 (Tim-3), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) [6–11]. Based on these data, the development of monoclonal antibodies (mAbs) that block checkpoint-ligand binding has been proven to be a major advance in tumor immunotherapy. Indeed, mAbs that block PD-1 and CTLA-4 are already showing clinical success, as they enhance the prognosis and survival of patients with advanced cancers [12–14]. Thus, it is crucial to look at novel immune checkpoint regulators with the hope that they can control tumor growth and increase the frequency of immune responses.

Tim-3, originally identified as a cell surface molecule, is selectively expressed on interferon- γ (IFN- γ)-producing CD4⁺ T helper 1 and CD8⁺ T cytotoxic 1 T cells [15]. Growing evidence has further characterized Tim-3 as a key checkpoint regulator responsible for the exhaustion and dysfunction of T cells, which arises in chronic infections and malignant tumors. Tim-3-expressing T cells fail to proliferate and produce cytokines in response to antigens, which mediates immunologic tolerance and immune evasion of tumors [16–18]. Previous studies have shown that Tim-3 expression is elevated on circulating or tumor-infiltrating T cells from patients with various tumors, and is associated with progression and poor prognosis of cancers such as prostate cancer, renal cell carcinoma, osteosarcoma, colorectal cancer, and glioma [19–23]. The proliferation of mammary cancer was found to be suppressed in Tim-3-deficient mice, and targeting Tim-3 blockade with mAbs inhibits the growth of CT26 colon adenocarcinoma and WTMCA2 fibrosarcoma [24]. These data suggested to us that Tim-3 is a promising target for reversing immune tolerance and increasing tumor-specific immune responses within tumor microenvironments.

CEACAM1 is considered as a specific biomarker that correlates with tumor progression, metastasis, and poor prognosis [25,26]. CEACAM1 has been identified as an immune checkpoint regulator that plays a crucial role in regulating immune responses [27,28]; it can also inhibit cytotoxicity and attenuate antitumor immunity in natural killer (NK) cells, and CEACAM1-silenced tumor cells exhibit greater sensitivity to the cytotoxic effects of NK cells [29]. Furthermore, it has been shown that antitumor effects can be enhanced in malignant melanoma using mAbs to block the inhibitory CEACAM1 pathway [11]. Recently, CEACAM1 has also been proven to be a new heterophilic ligand for Tim-3, and the interactions between the 2 molecules play a critical role in suppressing antitumor immunity. Further studies have suggested that the antitumor effect is enhanced with the co-blockade of Tim-3 and CEACAM1 with antibodies in colorectal cancer [30]. These exciting findings have revealed the great potential of combined blocking of Tim-3 and CEACAM1 for immunotherapy in cancers.

Therefore, the present study aimed to determine the effect of dual blockade with anti-Tim-3 and anti-CEACAM1 mAbs on the antitumor immune response and survival in a murine orthotopic GBM model. Our findings suggest that combined blocking of Tim-3 and CEACAM1 with mAbs provides a novel method in immunotherapy for patients with GBM.

Material and Methods

Cells and mice

The experiments in this study were authorized by the Clinical Research Ethics Committee of Shanxi Medical University, Taiyuan, Shanxi, P.R. China. All experimental procedures were performed in accordance with the criteria of the international guidelines for laboratory animals. GL261-Luc mouse glioma cells (a kind gift of Yijun Duan, Department of Immunology, Shanxi Provincial Cancer Hospital, Taiyuan, Shanxi, P.R. China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Boster, WuHan, HuBei, China) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA) and 1% penicillin-streptomycin at 37°C in a humidified incubator (Thermo Electron, Waltham, MA) in 5% CO₂. C57BL/6 mice (female, 6–8 weeks old) were purchased from the Shanxi Medical University Animal Center and maintained in pathogen-free environments at the Shanxi Medical University Animal Center.

Antibodies

Therapeutic antibodies were as follows: Anti-Tim-3 mAb (Clone RMT3-23; Catalog#: BE0115; BioXcell, West Lebanon, NH, USA), Rat IgG2a isotype control (Clone 2A3; Catalog#: BE0089; BioXcell), anti-CEACAM1 mAb (Clone MAb-CC1; Catalog#:

134504; Biolegend, Santa Cruz, CA, USA), and Mouse IgG1 isotype control (Clone MOPC-21; Catalog#: 400153; Biolegend). Antibodies for flow cytometry were as follows: anti-mouse CD3e (Catalog#: 15-0031-82; eBioscience, San Diego, CA, USA), anti-mouse CD4 (Catalog#: 12-0041-82; eBioscience), anti-mouse CD8a (Clone 53-6.7; Catalog#: 11-0081-82; eBioscience), anti-mouse CD25 (Catalog#: 25-0251-82; eBioscience), and anti-mouse Foxp3 (Catalog#: 11-5773-82; eBioscience). Antibodies for depletion were as follows: anti-CD4 (Clone GK1.5; Catalog#: BE0003-1; BioXcell) and anti-CD8 (Clone 2.43; Catalog#: BE0061; BioXcell).

Intracranial tumor induction and treatment

In our experiments, to establish intracranial gliomas, 4×10^6 GL261-Luc cells were stereotactically injected into the right caudatum of the C57BL/6 mice (10 mice per group) over a period of 2 min at the following coordinates: 2 mm lateral, 1 mm posterior from right bregma, and 4 mm deep from the skull. The growth of the gliomas was measured using luciferase imaging on day 10 after the injection, and the mice were randomly allocated into 4 groups. The groups received either 250 μ g of anti-Tim-3 mAbs, anti-CEACAM1 mAbs, a combination of anti-Tim-3 and anti-CEACAM1 mAbs, or control mAbs in 200 μ L sterile PBS on days 12, 15, and 18. The animals were weighed weekly and observed daily for clinical symptoms and evidence of toxicity by evaluating their eating, mobility, weight loss, hair loss, and hunched posture. According to the predetermined signs, the mice were killed when they showed weight loss of more than 20%, hunched posture, failure to eat or ambulate, or lethargy. Survival was recorded and the overall survival was calculated for each mouse. All experiments were performed in duplicate.

Flow cytometry

For flow cytometry, 5 randomly selected mice in each group were killed and their brains were removed on day 24 after implantation. Brain-infiltrating lymphocytes (BILs) were isolated from the fresh brain tissues. The brain tissues were minced with sterile tissue scissors and then digested with collagenase IV (Invitrogen Life Technologies, Carlsbad, CA, USA), hyaluronidase (Seebio Biological Technology, Shanghai, China), and DNase I (Ontores, Shanghai, China) in sterile PBS (Boster, WuHan, HuBei, China) at 37°C for 45 min. The digested tissues were filtered through 100- μ m stainless steel filters, and the resulting cells were separated by centrifugation using Percoll solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 800 \times *g*. The BILs obtained were then incubated for 30–40 min at 4°C in a dark room with the mAbs. The cells were then collected and analyzed using BD FACSCanto™ II (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Histopathological analysis

To confirm the pathological type of the intracranial tumors in the mice, the brains were fixed with formalin, embedded in paraffin, and cut into 3–4- μ m-thick sections. The sections were stained with hematoxylin and eosin and viewed under the Aperio Digital Pathology Slide Scanner (Aperio Technologies, Vista, CA, USA).

CD4 and CD8 depletion

For the depletion of T cells, the mice treated with a combination of anti-Tim-3 and anti-CEACAM1 mAbs were injected with 250 μ g of either anti-CD4, anti-CD8, anti-CD4 and anti-CD8, or control mAbs on days 7–9 after tumor implantation (another independent experiment). The levels of CD4 and CD8 T cells in the mice were then measured on day 11 by flow cytometry, and >90% depletion was confirmed. On day 16, the mice were additionally injected with a dose of depleting antibodies.

Enzyme-linked immunosorbent assay (ELISA)

The venous blood was collected from the eye socket vein of the mice, and centrifuged for 8 min at 800 \times *g*. The concentrations of interferon- γ (IFN- γ , pg/mL) and transforming growth factor- β (TGF- β , pg/mL) were then measured using an ELISA kit (catalog#EK0373; Boster, WuHan, HuBei, China) and TGF- β kit (catalog#EK0515; Boster), according to the manufacturers' instructions.

Tumor rechallenge

To assess the development of immune memory, the "cured" long-term survivors (survival for more than 90 days post-intracranial tumor implantation) that had received the combined anti-Tim-3 and anti-CEACAM1 mAb treatment and the control mice (naïve mice matched by age) were subcutaneously injected with 1×10^6 GL261-Luc cells (in 200 μ L sterilized PBS) in the left groin. The tumors were then observed using luciferase imaging on day 20 after the subcutaneous injection. The tumors were studied every 2 to 3 days, and the tumor volumes were calculated as $1/2 \times (\text{length}) \times (\text{width})^2$.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as means \pm SEM. Student's *t*-test and Kaplan-Meier survival curves were used to analyze the differences and survivals between the various study groups. *P* < 0.05 was considered statistically significant.

Results

Tim-3 and CEACAM1 blockade in mice with orthotopic gliomas showed durable survival benefit

We tested the hypothesis that combined blockade of Tim-3 and CEACAM1 by using mAbs could mediate a synergistic antitumor effect in an intracranial glioma model. We therefore transplanted 4×10^6 GL261-luc cells into C57BL/6 mice under a stereotaxic apparatus (day 0) and observed the orthotopic tumor engraftment by luciferase imaging (day 10). Histopathological analysis confirmed the intracranial glioma. The anti-Tim-3 and anti-CEACAM1 mAbs were intraperitoneally injected (i.p.) into the tumor-bearing mice on days 12, 15, and 18 (Figure 1A). The controls received isotype antibody treatment. We then reassessed the tumor progression by luciferase imaging on day 24. Representative sample images are shown in Figure 1B. The bioluminescent signals in the different groups were almost equal on day 10 (before treatment). However, the signals of the control group were strongest and the combined treatment group showed no signal on day 24 (after treatment). The survival analyses data corroborated the trend of tumor growth observed using bioluminescent imaging. A single injection of anti-Tim-3 or anti-CEACAM1 mAbs slightly prolonged the survival period of the animals, but the combined treatment markedly prolonged the survival time after tumor implantation (Figure 1E, $P < 0.001$). The mean survival times (MSTs) of the control, anti-Tim-3, and anti-CEACAM1 groups were 29.8 ± 1.6 , 43.4 ± 3.9 , and 42.3 ± 2.4 days, respectively, thus indicating that treatment with anti-Tim-3 or anti-CEACAM1 mAbs improved the MST ($P < 0.05$). However, the group treated with a combination of anti-Tim-3 and anti-CEACAM1 mAbs showed an MST of 86.0 ± 4.0 days (Figure 1F, $P < 0.001$), with 80% of the mice becoming long-term survivors (survival time of more than 90 days post-implantation). These results showed that combined blockade of Tim-3 and CEACAM1 had a synergistic antitumor effect and resulted in a significant improvement in the survival of the mice with intracranial gliomas.

Combination therapy increased the ratios of CD4⁺/CD8⁺ T cells to Tregs

To determine the potential mechanisms underlying the synergistic effect of Tim-3 and CEACAM1 blockade, we euthanized some mice in each group and removed their brains. We then analyzed the effects of combined or single mAb treatment on the BILs of the mice by using flow cytometry. Our data revealed that the proportions of CD4⁺ and CD8⁺ T cells in the BILs of the mice receiving the single therapies were higher than those in the control mice; this proportion was further increased in the combined treatment group (Figure 2A, 2B). We also measured the percentages of Tregs using CD4⁺CD25⁺FoxP3⁺ markers and found that the mice receiving anti-Tim-3 + anti-CEACAM1 mAbs

showed significantly decreased levels of Tregs in their brains (Figure 2C), which resulted in the significantly higher CD4⁺ and CD8⁺ T cells to Treg ratios in this group (Figure 2D, 2E). Together, these results suggested that the influx of CD4⁺ and CD8⁺ T cells was the mechanism underlying the effect of the combined blockade of Tim-3 and CEACAM1. To test this hypothesis, we depleted the CD4/CD8 cells before anti-Tim-3 + anti-CEACAM1 therapy.

The survival benefit of the combination therapy was dependent on the CD4⁺ and CD8⁺ T cells

We utilized antibody-mediated T cell depletion in the mice and found that the survival benefit of the combination therapy with anti-Tim-3 and anti-CEACAM1 mAbs was abrogated when the mice were depleted of CD4⁺ and CD8⁺ T cells. The data showed that mice with CD4 or CD8 depleted had a marginal survival benefit, but the treatment effect on the mice with both CD4 and CD8 cells depleted was completely abrogated (Figure 3A, 3B).

Combined blockade of Tim-3 and CEACAM1 might exert its effect by regulating the production IFN- γ and TGF- β

IFN- γ is secreted by activated immune cells and plays a vital role in antitumor immunity. Therefore, we analyzed the plasma IFN- γ levels of the mice in the various groups with gliomas using ELISA. Our data indicated that the plasma IFN- γ levels were significantly upregulated in the combined treatment group (Figure 4A, $P < 0.01$). TGF- β , one of the major immunosuppressive molecules, can inhibit antitumor immunoreaction via multiple signaling pathways. We therefore also assessed the serum TGF- β levels of the mice in the various groups and found a lower level of TGF- β in the combined treatment group compared with that in the control group (Figure 4B, $P < 0.01$). These data indicated that the combined blockade of Tim-3 and CEACAM1 might exert a significant effect on antitumor immunoreaction by regulating the production of the related cytokines, IFN- γ and TGF- β .

Long-term survivors receiving the combination therapy showed immunologic memory

To test the long-term survivors for durable immune memory against glioma cells, we performed a rechallenge by subcutaneous injection of GL261-luc cells in the "cured" (surviving for more than 90 days post-orthotopic tumor implantation) and naïve mice. All the naïve mice developed subcutaneous tumors, and the tumor size reached 1 cm³ by day 30 post-implantation. In contrast, all the long-term surviving mice remained tumor-free on day 60, as confirmed using luciferase imaging on day 20 after the rechallenge (Figure 5A, 5B).

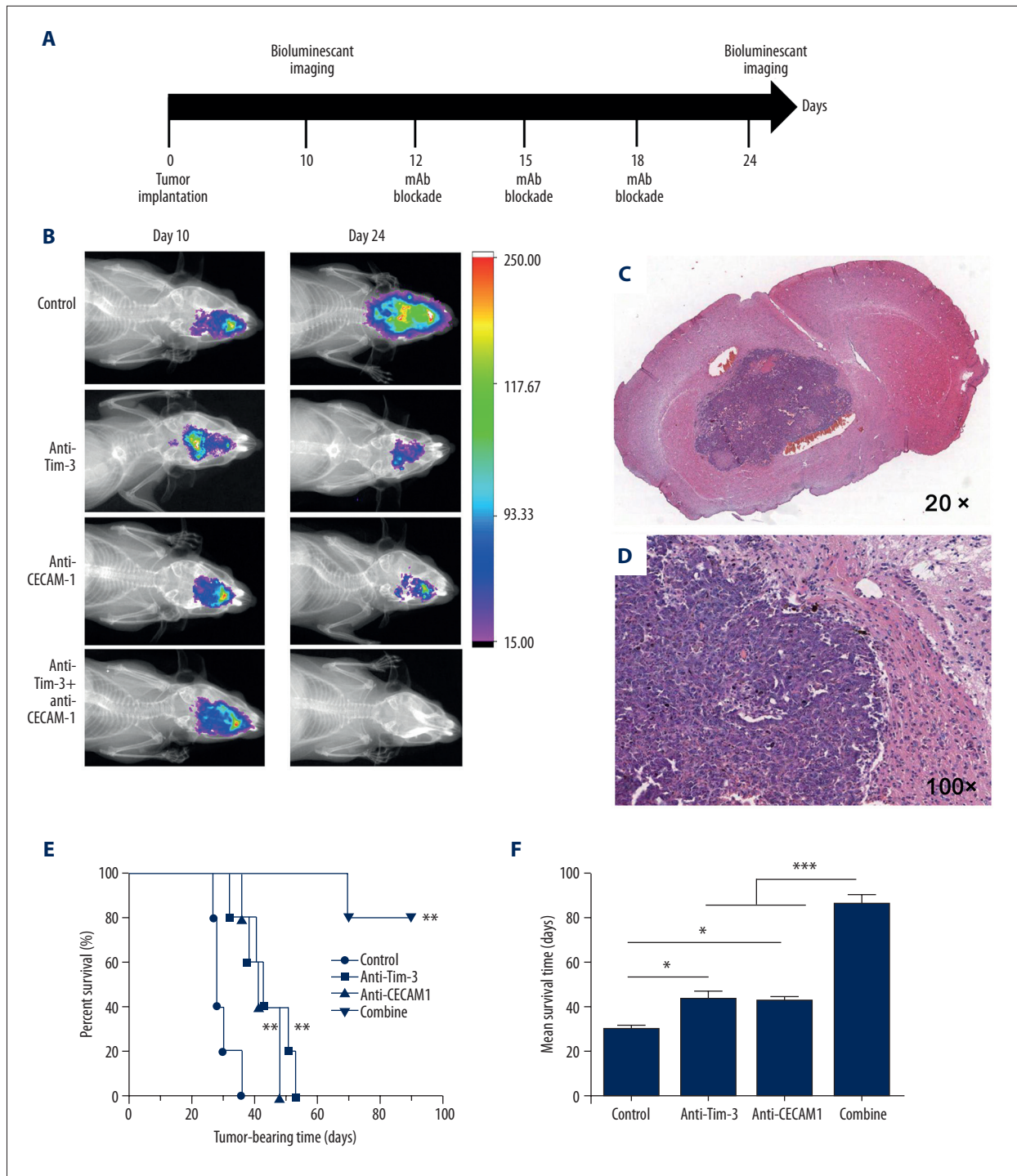


Figure 1. Therapeutic efficiency of combined treatment with anti-Tim-3 and anti-CEACAM1 mAbs in a murine intracranial glioma model. Mice were intraperitoneally injected with anti-Tim-3 and/or anti-CEACAM1 mAbs or control mAbs on days 12, 15, and 18 after tumor implantation. Luciferase imaging was performed before (day 10) and after (day 24) treatment. **(A)** Timeline of antibody blockade after intracranial injection (i.c.) of 4×10^6 GL261-Luc cells in C57BL/6 mice. **(B)** Representative luciferase imaging of mice, individually matched on days 10 and 24, in the various treatment groups. All images are at the same scale. **(C, D)** Histopathological analysis of the brain and intracranial glioma (stained with hematoxylin and eosin; magnification, $40 \times$ and $100 \times$, respectively). **(E)** Survival analysis of mice in the various treatment groups. **(F)** The mean survival times of the control, anti-Tim-3, anti-CEACAM1, and anti-Tim-3/anti-CEACAM1 groups were 29.8 ± 1.6 , 43.4 ± 3.9 , 42.3 ± 2.4 , and 86.0 ± 4.0 days, respectively. All experiments performed in duplicate. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, compared with control group.

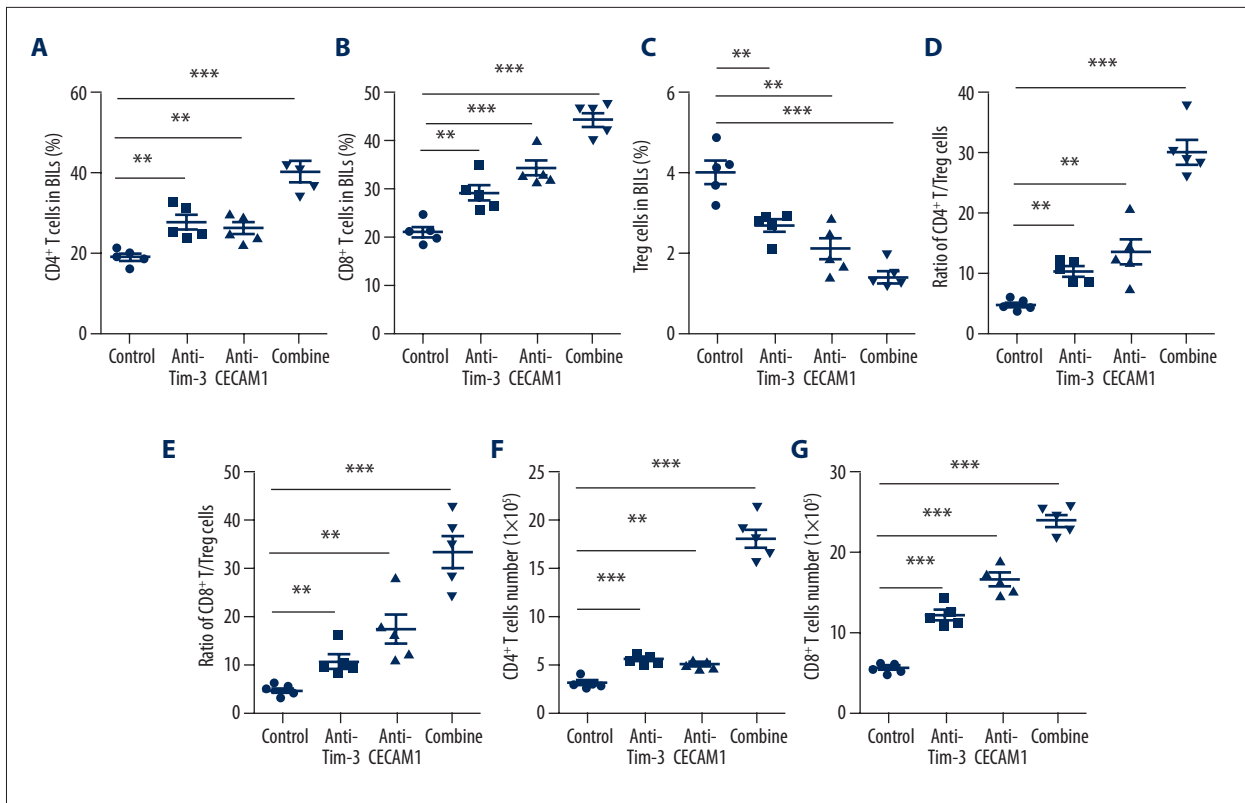


Figure 2. Analysis of brain-infiltrating lymphocytes of mice. Mice were intraperitoneally injected with anti-Tim-3 and/or anti-CEACAM1 mAbs or control mAbs on days 12, 15, and 18. On day 24, the BILs of mice in the different groups were analyzed by flow cytometry. **(A)** The proportion of CD4⁺ T cells in the brains of mice. **(B)** The percentage of CD8⁺ T cells in the brains of mice. **(C)** The proportion of CD4⁺CD25⁺FoxP3⁺ Treg cells in the brains of mice. **(D, E)** Ratios of CD4⁺ and CD8⁺ T cells to Tregs. **(F, G)** The absolute numbers of CD4⁺, CD8⁺ T cells in the brains of mice. All experiments performed in duplicate. *** *P* < 0.001; ** *P* < 0.01, compared with control mice.

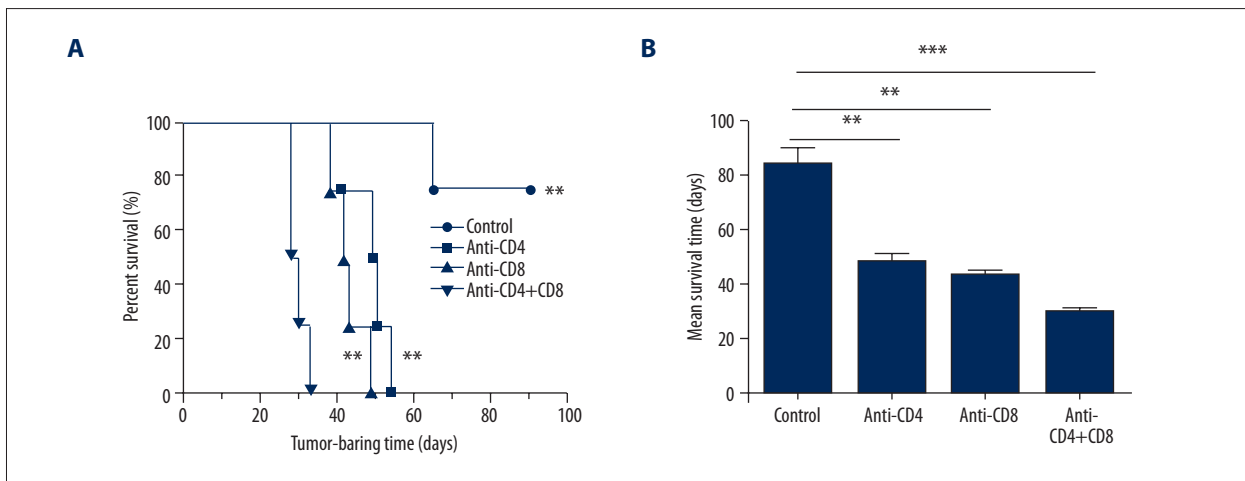


Figure 3. Combined treatment of mice depleted of CD4⁺ and CD8⁺ T cells showed no survival benefit. Mice treated with a combination of anti-Tim-3 and anti-CEACAM1 mAbs were injected with 250 μg of anti-CD4, anti-CD8, anti-CD4/anti-CD8, or control mAbs on days 7–9 after tumor implantation (another independent experiment). On day 14, we additionally injected a dose of depleting antibodies. **(A)** Survival analysis of mice in various groups. **(B)** Mean survival time of mice. *** *P* < 0.001; ** *P* < 0.01, compared to control mice.

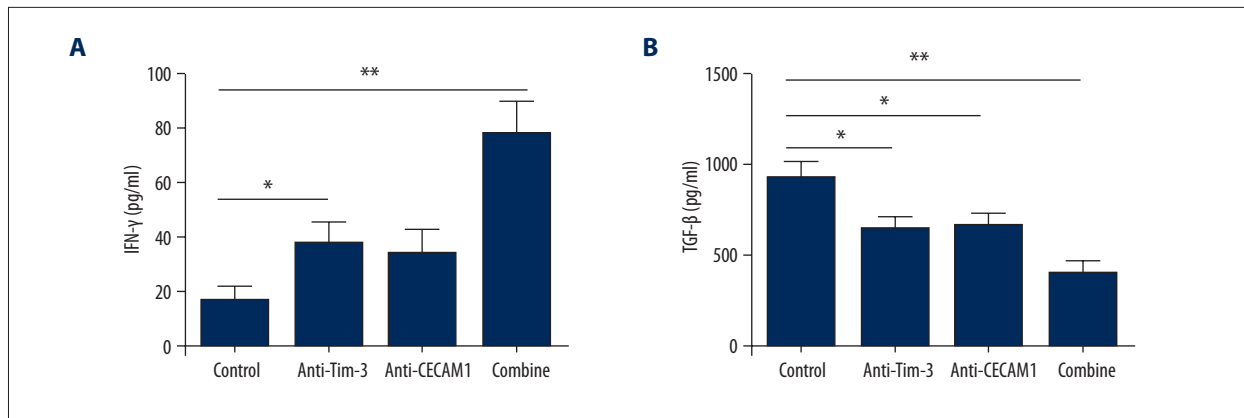


Figure 4. Plasma levels of IFN- γ and TGF- β in the mice belonging to the various groups. **(A)** Plasma IFN- γ levels in mice receiving combined treatment were markedly increased. **(B)** Plasma TGF- β levels were decreased in the combined treatment group. * $P < 0.05$; ** $P < 0.01$, compared with control mice.

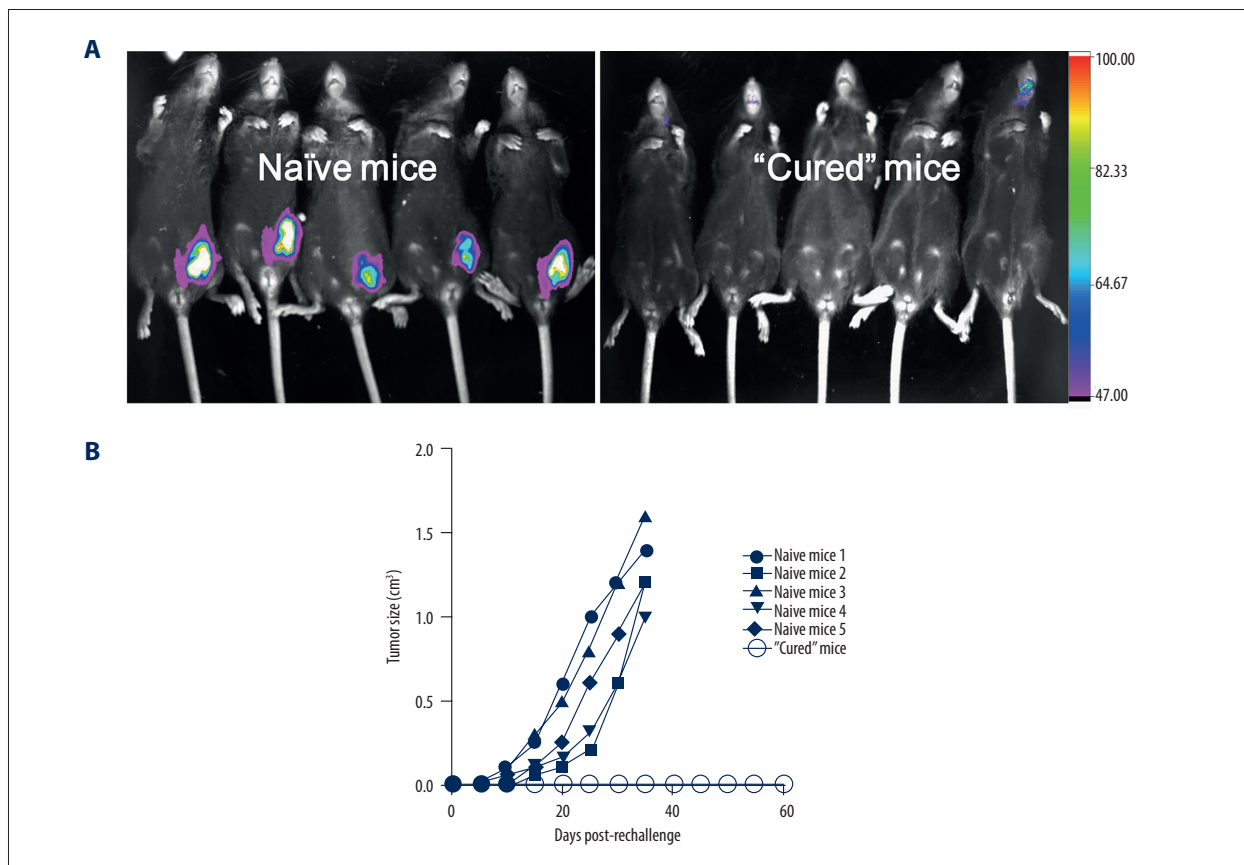


Figure 5. Long-term surviving mice show durable immune memory. The mice that were “cured” by day 90 post-implantation were rechallenged with 1×10^6 GL261 cells in the left groin and compared with naïve control mice. **(A)** Luciferase imaging of the rechallenged mice on day 20 after subcutaneous injection. **(B)** Subcutaneous tumors in the naïve control mice reached a size of 1 cm^3 by day 30, but none of the “cured” mice grew tumors by day 60.

These results indicated that the combination therapy resulted in durable systemic immune memory against GL261-luc glioma cells in the mice.

Discussion

Inhibitory immune checkpoint molecules interact with their ligands to suppress the proliferative capacity and effector functions of T cells, which results in immune escape and development of cancer. Tim-3 is considered a key immune checkpoint molecule and plays a critical role in downregulating immune responses. In experimental models of both hematologic and solid cancers, as well as in humans, Tim-3 marks a dysfunctional phenotype of the T cells [31,32]. The blockade of only Tim-3 has been found to be effective in multiple preclinical cancer models such as those of CT26 and MC38 colon carcinoma and Wilms tumor-3 sarcoma and in mouse prostate C-1 transgenic adenocarcinoma models. However, the combined blockade of Tim-3 and PD-1 has been shown to be significantly more effective, as the tumor suppression is more complete compared to that achieved with blockade of either Tim-3 or PD-1 alone [33]. CEACAM1 is another important inhibitory checkpoint regulator expressed on activated immune cells that interacts homophilically with CEACAM1 or heterophilically with CEACAM5 [34,35]. Interestingly, in a recent study, CEACAM1 has been identified as a novel heterophilic ligand for Tim-3, and it can regulate Tim-3-mediated immune tolerance and exhaustion of T cells [30]. In addition, co-blockade of Tim-3 and CEACAM1 has been found to result in a synergistic therapeutic effect in mouse colorectal cancer models [30]. Collectively, these studies strongly support the potential of co-blocking Tim-3 and CEACAM1 for immunotherapy in cancer.

In the present study, our survival analysis showed that 80% of the mice treated with a combination of anti-Tim-3 and anti-CEACAM1 mAbs became long-term survivors, while those treated with either mAb alone only showed a marginal survival benefit. In addition, the MST of mice in the combined treatment group was twice that of the mice receiving a single injection of either mAb. Flank tumor rechallenge of all long-term survivors showed that the "cured" mice could reject GL261-luc cells, suggesting that these animals possessed durable immune memory. This phenomenon is similar to the results of treatment for glioma with a combination of anti-PD-1 blockade and stereotactic radiation [36]. These findings suggested that combined treatment with anti-Tim-3 and anti-CEACAM1 mAbs significantly suppresses the growth of intracranial gliomas, thus resulting in durable therapeutic efficacy and immune memory. Given that glioma is characterized by high tumor recurrence rates, which lead to high mortality, our results provide an attractive therapeutic method for patients with glioma.

Further, we found that the survival benefit conferred by dual blockade with anti-Tim-3 and anti-CEACAM1 mAbs was abrogated when the mice were depleted of T cells. Our data are concordant with the data from a previous study showing that the combined targeting of Tim-3 and CD137 has a long-lasting therapeutic effect in mice with ovarian cancer [37].

To understand the potential immunologic mechanisms underlying the antitumor effects of the combined blockade of Tim-3 and CEACAM1, we next determined the changes in the levels of CD4⁺ and CD8⁺ T lymphocyte subsets infiltrating the brains of the mice. The proportion of both CD4⁺ and CD8⁺ T cells in the BILs of the combined treatment group was markedly higher than that of the other groups, which suggested that blockade with anti-Tim-3 and anti-CEACAM1 mAbs can promote the proliferation of T cells. The ratio of T cells to Tregs has been shown to be a marker of favorable therapeutic effect in tumor immunotherapy [38], and a high ratio of T cells to Treg has been found to indicate improved survival in clinical studies of ovarian cancer [39]. Accordingly, we additionally assessed the percentage of Tregs in the BILs of the mice and analyzed the ratio of T cells to Tregs. Our data showed that the T cell to Treg ratio in the mice belonging to the combined treatment group was significantly elevated.

IFN- γ is secreted by activated immune cells and plays a critical role in antitumor immunity [40]. However, tumors can evade immunosurveillance by producing the immunosuppressive cytokine TGF- β , which suppress cytotoxic lymphocyte (CTL) function through transcriptional repression [41]. Therefore, to observe the effector functions of T cells in mice after treatment, we analyzed the plasma levels of IFN- γ and TGF- β using ELISA. Our results showed that the plasma IFN- β and TGF- β levels were strongly upregulated and downregulated, respectively, in the combined treatment group. Based on these data, we believe that the potent immunologic response achieved using the combined blockade of Tim-3 and CEACAM1 with mAbs likely stems from the reversal of T cell exhaustion combined with the deprogramming of Tregs in GBM.

In our experiments, we found no obvious toxic adverse effects such as hair and weight loss in the mice receiving combined treatment with anti-Tim-3 and anti-CEACAM1 mAbs or in those receiving antibodies against either one of the regulatory molecules. This is largely because the expression of Tim-3 is primarily in tumor-infiltrating lymphocytes or IFN- γ -producing T cells rather than in all T cells [15,42]. Moreover, unlike PD-1-deficient or CTLA-4-deficient mice, Tim-3-deficient mice do not exhibit autoimmune-like toxicities [43]. Taken together, our results indicate that Tim-3 is an advantageous therapeutic target for tumor immunotherapy and that targeting Tim-3 is less likely to result in autoimmune-like adverse effects compared to blockade of either CTLA-4 or PD-1. Furthermore, treatment with anti-Tim-3

mAbs in combination with anti-CEACAM1 mAbs leads to reliable suppression of intracranial GL261 gliomas in the long-term surviving mice, revealing that blockade with mAbs may generate immunologic memory against GL261 cells.

Conclusions

To the best of our knowledge, this is the first study to show potent antitumor activity against intracranial glioma using a novel combination of anti-Tim-3 and anti-CEACAM1 mAbs.

References:

- Porter KR, Mccarthy BJ, Freels S et al: Prevalence estimates for primary brain tumors in the United States by age, gender, behavior, and histology. *Neuro-Oncology*, 2010; 12: 520–27
- Xiao Y, Zhang L, Song Z et al: Potential diagnostic and prognostic value of plasma circulating MicroRNA-182 in human glioma. *Med Sci Monit*, 2016; 22: 855–62
- Stupp R, Mason WP, van den Bent MJ et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*, 2005; 352: 987–96
- Yang H, Xiang W, Feng D et al: MiRNA-323-5p promotes U373 cell apoptosis by reducing IGF-1R. *Med Sci Monit*, 2015; 21: 3880–86
- Thomas AA, Brennan CW, Deangelis LM, Omuro AM: Emerging therapies for glioblastoma. *JAMA Neurol*, 2014; 71: 1437–44
- Parsa AT, Waldron JS, Panner A et al: Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med*, 2007; 13: 84–88
- Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*, 2012; 12: 252–64
- Peggs KS, Segal NH, Allison JP: Targeting immunosuppressive cancer therapies: Accentuate the positive, eliminate the negative. *Cancer Cell*, 2007; 12: 192–99
- Topalian SL, Drake CG, Pardoll DM: Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol*, 2012; 24: 207–12
- Liu Z, Han H, He X et al: Expression of the galectin-9-Tim-3 pathway in glioma tissues is associated with the clinical manifestations of glioma. *Oncol Lett*, 2016; 11(3): 1829–34
- Ortenberg R, Sapir Y, Raz Z et al: Novel immunotherapy for malignant melanoma with a monoclonal antibody that blocks CEACAM1 homophilic interactions. *Mol Cancer Ther*, 2012; 11: 1300–10
- Hodi FS, O'Day SJ, McDermott DF et al: Improved survival with ipilimumab in patients with metastatic melanoma. *New Engl J Med*, 2010; 363: 711–23
- Hamid O, Robert C, Daud A et al: Safety and tumor responses with lambrolizumab (Anti-PD-1) in melanoma. *New Engl J Med*, 2013; 369: 134–44
- Topalian SL, Hodi FS, Brahmer JR et al: Safety, activity, and immune correlates of Anti-PD-1 antibody in cancer. *New Engl J Med*, 2012; 366: 2443–54
- Monney L, Sabatos CA, Gaglia JL et al: Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*, 2002; 415: 536–41
- Fourcade J, Sun Z, Benallaoua M et al: Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*, 2010; 207: 2175–86
- Yang ZZ, Grote DM, Ziesmer SC et al: IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Investigation*, 2012; 122: 1271–82
- Granier C, Dariane C, Combe P et al: Tim-3 expression on tumor-infiltrating PD-1+CD8+T cells correlates with poor clinical outcome in renal cell carcinoma. *Cancer Res*, 2016 [Epub ahead of print]
- Piao YR, Jin ZH, Yuan KC, Jin XS: Analysis of Tim-3 as a therapeutic target in prostate cancer. *Tumor Biology*, 2014; 35: 11409–14
- Cai C, Xu YF, Wu ZJ et al: Tim-3 expression represents dysfunctional tumor infiltrating T cells in renal cell carcinoma. *World J Urol*, 2016; 34: 1–7
- Liu H, Zhi L, Ning D, Su P: Abnormal expression of Tim-3 antigen on peripheral blood T cells is associated with progressive disease in osteosarcoma patients. *Febs Open Bio*, 2016; 6: 807–15
- Xu B, Yuan L, Gao Q et al: Circulating and tumor-infiltrating Tim-3 in patients with colorectal cancer. *Oncotarget*, 2015; 6: 20592–603
- Han S, Feng S, Xu L et al: Tim-3 on peripheral CD4+ and CD8+ T cells is involved in the development of glioma. *DNA Cell Biol*, 2014; 33: 245–50
- Dardalhon V, Anderson AC, Karman J et al: Tim-3/galectin-9 pathway: Regulation of Th1 immunity through promotion of CD11b+Ly-6G+ myeloid cells. *J Immunol*, 2010; 185: 1383–92
- Kiriyama S, Yokoyama S, Ueno M et al: CEACAM1 long cytoplasmic domain isoform is associated with invasion and recurrence of hepatocellular carcinoma. *Ann Surg Oncol*, 2014; 21: 505–14
- Dupuis ML, Fiori V, Soriani A et al: The human antibody fragment DIATHIS1 specific for CEACAM1 enhances natural killer cell cytotoxicity against melanoma cell lines *in vitro*. *J Immunother*, 2014; 38: 357–70
- Markel G, Sapir Y, Mandel I et al: Inhibition of the novel immune checkpoint CEACAM1 to enhance anti-tumor immunological activity. *J Clin Oncol*, 2016 ASCO Annual Meeting (June 3–7, 2016). 2016; 34
- Rovituso D M, Scheffler L, Wunsch M et al: CEACAM1 mediates B cell aggregation in central nervous system autoimmunity. *Sci Rep*, 2016; 6: 29847
- Hosomi S, Chen Z, Baker K et al: CEACAM1 on activated NK cells inhibits NKG2D-mediated cytolytic function and signaling. *Eur J Immunol*, 2013; 43: 2473–83
- Huang YH, Zhu C, Kondo Y et al: CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. *Nature*, 2015; 517: 386–90
- Sakuishi K, Apetoh L, Sullivan JM et al: Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med*, 2010; 207: 2187–94
- Zhou Q, Munger ME, Veenstra RG et al: Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood*, 2011; 117: 4501–10
- Ngjow SF, Von SB, Akiba H et al: Anti-TIM3 antibody promotes T cell IFN- γ -mediated antitumor immunity and suppresses established tumors. *Cancer Res*, 2011; 71: 3540–51
- Watt SM, Teixeira AM, Zhou GQ et al: Homophilic adhesion of human CEACAM1 involves N-terminal domain interactions: Structural analysis of the binding site. *Blood*, 2001; 98: 1469–79
- Markel G, Gruda RH, Katz G et al: The critical role of residues 43R and 44Q of carcinoembryonic antigen cell adhesion molecules-1 in the protection from killing by human NK cells. *J Immunol*, 2004; 173: 3732–39
- Zeng J, See A P, Phallen J et al: Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol Biol Phys*, 2013; 86(2): 343–49
- Guo Z, Cheng D, Xia Z et al: Combined TIM-3 blockade and CD137 activation affords the long-term protection in a murine model of ovarian cancer. *J Transl Med*, 2013; 11: 773–76
- Waitz R, Solomon SB, Petre EN et al: Potent induction of tumor immunity by combining tumor cryoablation with anti-CTLA-4 therapy. *Cancer Res*, 2012; 72: 430–39

The combined treatment blocked the 2 key inhibitory immune checkpoints in tumors, thus restoring multiple functions of exhausted T cells and decreasing the ratio of Tregs. Given that the GBM is characterized by rapid proliferation, invasion, and high tumor recurrence rates, our results provide an attractive therapeutic method for patients with GBM.

Acknowledgements

We would like to thank Editage [www.editage.cn] for English language editing.

39. Sato E, Olson SH, Ahn J et al: Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci USA*, 2005; 102(51): 18538–43
40. Hong B, Li H, Yong L et al: USP18 is crucial for IFN- γ -mediated inhibition of B16 melanoma tumorigenesis and antitumor immunity. *Mol Cancer*, 2014; 13: 1–12
41. Thomas DA, Massagué J: TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell*, 2005; 8: 369–80
42. Gao X, Zhu Y, Li G et al: TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS One*, 2012; 7: e30676
43. Anderson AC: Tim-3: An emerging target in the cancer immunotherapy landscape. *Cancer Immunol Res*, 2014; 2: 393–38