Original Article
Temozolomide promotes immune escape of GBM cells via upregulating PD-L1

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Abstract: Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor with poor prognosis, and currently effective therapeutic strategies are still limited. Although temozolomide (TMZ) is commonly used for GBM therapy and its mechanism was well characterized, while its side effects were required comprehensive investigation. In the present study, we revealed that TMZ-challenged GBM cells strongly suppressed pro-inflammatory cytokines expression in activated periphery blood mononuclear cells (PBMC), which depended on enhanced transcription of CD274 (encoding PD-L1), but not other immune checkpoints, such as CD276, HVEM and galectin-9. Moreover, abundance of membranous PD-L1 was also increased in TMZ-treated GBM cells. When PD-L1 expression was knocked down by short hairpin RNA (shRNA), inhibitory effect of TMZ-treated GBM cells on PBMC became weakened, suggesting that PD-L1 was crucial for immune inhibition capacity of TMZ-treated GBM cells. Additionally, actinomycin D reduced PD-L1 expression in GBM cells after TMZ challenge, indicating that PD-L1 induction occurred at transcriptional level. The immunoblotting results demonstrated that STAT3 signaling was involved in TMZ-mediated PD-L1 induction, and attenuated expression of PD-L1 was observed using STAT3 inhibitor VI or STAT3 shRNA. Finally, the animal study showed that combination of TMZ and PD-1 antibody therapy strongly inhibited tumor growth and achieved the improved survival rate of GBM mice. Accordingly, this study revealed the classical chemotherapy drug TMZ promoted GBM cells immune escape, even TMZ combine with PD-1 antibody treatment not further improve survival ratio of recurrent GBM patients compared with traditional therapy methods, while our animal study provided evidence that combination of TMZ and PD-1 antibody was a promising way to treat GBM, these contradictory results indicate improving the PD-1 antibody delivery efficiency can exert strong combinational therapy outcomes.

Keywords: GBM, temozolomide, PD-L1, immune escape, combinational therapy

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults. Patients with GBM have traditionally been thought to have terrible prognosis, despite multiple therapies such as surgical resection combined with radiotherapy and chemotherapy using temozolomide (TMZ). Due to growth feature of GBM cells, it is difficult to remove tumor tissue completely through surgery. Surgery concomitant radio- and chemotherapy increased median survival time of the patients to 12-15 months, while 5-year survival rate is still less than 5% [1]. Thus, how to improve the treat-ment outcome in GBM is one of the biggest therapeutic challenges of the modern medicine [2].

TMZ is an oral alkylating agent and regarded as the first-line chemotherapeutic drug to treat GBM after operation in this decade [3]. Anti-tumor effect of TMZ depends on its ability to alkylate/methylate DNA, which commonly occurs at N^7 or O^6 position of guanine residues [4]. This modification damages DNA and induces death of cancer cells. However, this type of DNA damage can be repaired by O6-alklguanine DNA alkyltransferase encoded by O-6-methylguanine-DNA methyltransferase (MGMT) gene

[5]. Therefore, the MGMT expression level is highly correlated with the TMZ therapeutic efficacy on GBM. Yung et al. revealed that TMZ alone also suppressed tumor growth [6]. For improving the GBM patient survival time and the curative effect, physicians combines TMZ with radiotherapy, anti-angiogenesis therapy or immunotherapy to treat GBM, and TMZ concomitant radiotherapy is the most powerful strategy for GBM treatment until now [1, 7-10].

In recent 5 years, several clinical studies showed that immunotherapy achieved good results in some cancers, like lung cancer, melanoma and prostate cancer [11-13]. Moreover, preclinical experimental study suggested that combination of classical chemotherapy with immunotherapy was a new encouraging way to combat GBM [14, 15]. It is well known that chemotherapy can partially induce immune suppression like leucopenia and granulocytopenia, but remaining immune-related cells can exert their functions on tumor killing [16-18]. However, some chemotherapeutic drugs, such as anthracycline, doxorubicin and daunorubicin, are capable to induce activation markers on immune cells and immunogenic markers on tumor cells for further enhancing anti-tumor activity of immune cells [19, 20]. Besides, small amount of chemotherapy agents exhibit immunoactivation effects, whereas some agents upregulate immunosuppressive molecules in cancer cells or other cancer-surrounding cells for blocking the anti-tumor capacity of immune cells [21].

Ishikawa et al. showed that TMZ also has adverse effect as inducing leucopenia during GBM therapy, but whether TMZ can promote or impair immune evasion of GBM cells is not elucidated [22]. In order to have a better understanding of TMZ effects on tumor immune escape, we co-cultured periphery blood mononuclear cells (PBMC) with GBM cells or TMZ-treated GBM cells, and showed that TMZ-treated GBM cells had higher inhibition capacity on proinflammatory cytokines production of PBMC than control GBM cells. Knock-down of PD-L1 impaired TMZ-induced inhibition effect of GBM cells on PBMC, suggesting that PD-L1 was a key immune checkpoint involved in GBM cells immune evasion after TMZ challenge. Additionally, TMZ-mediated PD-L1 expression in GBM cells was depended on the activation of STAT3 signaling. Finally, combination therapy of

TMZ and PD-1 antibody greatly shrank the tumor size and improved survival rate in GBM animal models. In general, these data illustrated that TMZ promotes immune escape of GBM cells via upregulating PD-L1, and combination of TMZ and PD-1 antibody was a promising strategy for GBM patients.

Materials and methods

Reagents, antibodies and immunoblot analysis

The Tubulin antibody (1:5000, T6074) and TMZ were obtained from Sigma-Aldrich (St. Louis, MO). STAT3 inhibitor VI was purchased from EMD Millipore (Billerica, MA). Antibodies against human PD-L1 (1:1000, 13684), STAT3 (1:1000, 9139), STAT3pY705 (1:1000, 9145), HIF-1α (1:1000, 72933) and c-Myc (1:1000, 13987) were purchased from Cell Signaling Technology (Danvers, MA). Cells were lyzed by RIPA lysis buffer (Beyotime, Beijing, China), and the extracts were clarified via centrifugation at 15000 g. Total protein concentration in the cell lysate was determined by BCA (Beyotime), and 50 μg protein was subjected to immunoblot analysis with corresponding antibodies. Each experiment was repeated at least 3 times.

Primers and qPCR

Total RNA isolation, reverse transcription (RT), and real-time PCR were performed as described the previous study [23]. The following primers were used for qPCR: human CD274 forward 5'-CTGCACTTTTAGGAGATTAGATC-3' and reverse 5'-CTACACCAAGGCATAATAAGATG; murine CD274 forward 5'-GTGAAACCCTGAGTCTTATCC-3' and reverse 5'-GACCATTCTGAGACAATTCC-3'; human β-actin forward 5'-CTCCATCCTGGCCTC-GCTGT-3' and reverse 5'-GCTGTCACCTTCACC-GTTCC-3'; murine β-actin forward 5'-ATGGA-TGACGATATCGCTGCGC-3' and reverse 5'-GCA-GCACAGGGTGCTCCTCA-3; human IL-2 forward: 5'-ACTCACCAGGATGCTCACATT-3' and reverse 5'-AGTCCCTGGGTCTTAAGTGA-3'; human IFN-γ forward 5'-CTGCCAGGACCCATATGTAA-3' and reverse 5'-GAGACAATTTGGCTCTGCATT-3'; human CD276 forward 5'-AGCTGTGAGGAGGAG-AATGCA-3' and reverse; 5'-CTTGTCCATCATCTT-CTTTGCTG-3'; human galectin-9 forward 5'-CCTTTTCTGGGACTATTCAAGG and reverse 5'-GAAGTGGAAGGCAATGTCATTTC-3'; human HV-EM forward 5'-CAAAACCGACGTCTTGAGGCT-3' and reverse 5'-CCTCCTTCACACGATAACCTG-3'.

Cell culture, treatment and shRNA

U87, U251, GL261 and luciferase-expressing GL261 (GL261-Luc) cells which were provided by BeNa culture collection (Beijing, China) were maintained in Dulbecco's modified Eagle's medium (DMEM, ThermoFisher Scientific, Grand Island, NY) or DMEM/F12 (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). All the cell lines were tested for mycoplasma. Cells were plated at a density of $4*10^5$ per 60 mm dish or $1*10^5$ per well of 6-well plate for 24 h before treatment. The GBM cells were treated with different dosages of TMZ for various times as described in figure legends. Transfection was performed using lipofectamine 3000 reagent (ThermoFisher Scientific) according to the manufacture's instruction. The following pGIPZ shRNAs (GE Dharmacon, Chicago, IL) were used: control shRNA oligonucleotide, GCTTCTAACA-CCGGAGGTCTT; human STAT3 shRNA oligonucleotide: TACCTAAGGCCATGAACTT; human PD-L1 shRNA oligonucleotide: TTGACTCCATCTTT-CTTCA.

GL261-Luc mouse GBM model and animal experiment

One-month-old male C57BL/6 mice were intracranially injected $1*10^5$ GL261-Luc cells. We divided the mice into 4 groups, 10 mice in each group. 100 mg of PD-1 antibody (RMP1-14, Bio X Cell) or a rat IgG (Bio X Cell) as a control was injected intratumorally on day 3, 6, 9, 12, 15 post GL261-Luc cell inoculation, and TMZ (800 µg in 100 µl PBS) or control PBS was injected intraperitoneally (i.p) on day 3, 7, 11, 15. All the mice were monitored for survival and tumor volume. Survival was determined as the number of days from tumor cell inoculation to the day that animals had to be euthanized due to following symptoms, such as hemiparesis, seizures, loss of weight more than 20%, cannot move and other heavy neurological deficits symptoms. The survival results were analyzed by using Graphpad 5 Prism 5.0 software. For tumor volume determination, 5 mice in each group were pick up randomly and anesthetized with isoflurane and i.p with 150 mg/kg D-luciferin in PBS, the bioluminescence imaging was conducted (IVIS 200, Xenogen; exposure time of 30 seconds, binning of 8, field of view of 15 cm, f/stop of 1, and no filter) after 10 minutes of injection. All of the mice were

housed in the Wenzhou Medical University animal center (Wenzhou, China); All animal experiments were performed according to the guidelines and regulations of Zhejiang Medical Laboratory Animal Management Committee.

Generation of stable cell line, and co-culture experiments

The U87 and U251 were transfected with corresponding PGIPZ shRNA by using lipofectamine 3000 reagent for 48 hours, and the cells were selected with 1 µg/ml puromycin (InvivoGen, San Diego, CA). The blood was collected from healthy donors and stored at anticoagulant tube, the PBMC were isolated from blood by gradient density centrifugation on RosetteSep™ DM-L Density Medium (Stemcell Technologies, Cambridge, MA), and were activated by Anti-CD3/CD28 Dynabeads (ThermoFisher Scientific) according to the instruction for 12 h, meanwhile, the U87 and U251 were treated with or without TMZ for 24 h, then replace with fresh medium and co-cultured activated PBMC for 24 h at a ratio of 1:3 (cancer cell: PBMC). For neutralization experiment, the PD-L1 neutralization antibody (ThermoFisher Scientific, MIH1) or mouse IgG (ThermoFisher Scientific) were added into the cancer cell culture plates prior to adding PBMC. Finally, the supernatants were collected and subjected to enzyme-linked immune-sorbent assay (ELISA) for quantifying IFN-γ and IL-2 producytion (R&D System, Minnesota, USA) according to the instructions.

Flow cytometry analysis

The GBM cells were digested by 0.05% trypsin and washed in PBS for 3 times, then stained for cell surface PD-L1 on ice for 30 min in FACS blocking buffer (0.5% BSA and 2% FBS in PBS). PD-L1 PE-conjugated antibody (ThermoFisher Scientific, MIH1) or IgG1 PE-conjugated antibody (R&D System) were added to the cell suspension solution on ice for 30 min, then the cells were washed with FACS buffer (PBS contained 0.5% BSA) for 3 times and detected by a BD FACSAria III (BD Biosciences) cytometer. The results were analyzed by FlowJo V10.

Statistical analysis

Data in bar graphs represent the mean fold change relative to control groups with s.d. of three independent experiments. A 2 group comparison was conducted using a 2-sided, 2-sam-
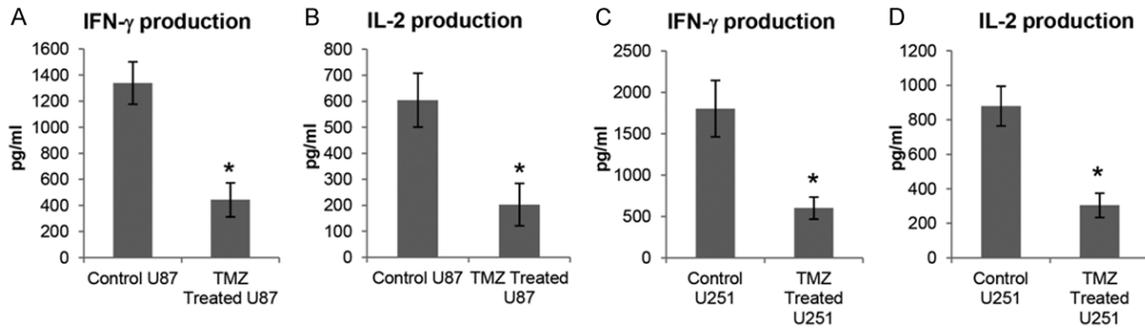
Figure 1. *T*MZ-treated GBM cells strongly impaired expression of pro-inflammatory cytokines. A, B. PBMC were pre-activated with Anti-CD3/CD28 beads for 12 h, and co-cultured with U87 cells which were pretreated with or without 200 µM TMZ. Then the culture medium was collected and subjected to IFN-γ and IL-2 quantification by ELISA after 24 h co-incubation. C, D. The activated PBMC were co-cultured with U251 cells which were challenged with or without 50 µM TMZ. Then IFN-γ and IL-2 protein level in co-culture medium were measured by ELISA after 24 h co-incubation. *$P < 0.05$, on the basis of Student's *t* test.

ple Student's t test. A simultaneous comparison of more than 2 groups was conducted using 1-way ANOVA through SPSS (Ver. 20, SPSS, Chicago, IL). A *P* value <0.05 was considered statistically significant.

Results

TMZ treated GBM cells exhibited higher inhibition capacity on pro-inflammatory cytokines production in activated PBMC than control cells

In order to determine whether TMZ can exacerbate the inhibition capacity of GBM on PBMC activation, we co-cultured the PBMC with GBM cells or TMZ treated GBM cells, then the pro-inflammatory cytokines IL-2 and IFN-γ in co-culture medium were detected in these two groups. As shown in **Figure 1A**, IFN-γ expression was significantly reduced in supernatant which collected from PBMC co-cultured with TMZ treated U87 cells compared to the supernatant collected from PBMC co-cultured with U87 cells. Furthermore, IL-2 production in the medium was also greatly inhibited when PBMC co-cultured with TMZ treated U87 cells (**Figure 1B**). In addition, IFN-γ and IL-2 had similar manners in co-culture medium when PBMC were co-cultured with TMZ treated U251 cells when compared to parallel group (**Figure 1C** and **1D**).

TMZ elevated mRNA levels of CD274, but not other immune checkpoints in GBM cells

To further uncover the underlying mechanism of the strengthened inhibition of GBM cells after TMZ challenge on pro-inflammatory cytokines expression in PBMC, we investigated a serial of immune checkpoints expression in

U87 and U251 after TMZ stimulation. Results showed that CD274 (encoding PD-L1) mRNA was up-regulated in U87 after TMZ challenge, and CD274 mRNA level was increased gradually in a time-dependent manner (**Figure 2A**). However, mRNA levels of other immune checkpoints HVEM, galectin-9 and CD276 were not altered in U87 after TMZ treatment from 0-72 h (**Figure 2B-D**). Additionally, we also determined gene expression of the above immune checkpoints in U251 with or without TMZ treatment, and the same tendencies were observed (**Figure 2E-H**).

The ratio of PD-L1 positive GBM cell is significantly increased after TMZ treatment

As PD-L1 expressed on the surface of tumor cells exerts immunosuppressive effects through binding to PD-1 on activated T cells, it is essential to assess the level of PD-L1 on cell surface or the percentage of PD-L1 positive cells with or without TMZ treatment. PD-L1 protein levels were evaluated in TMZ-stimulated U87 and U251 cells, showing that PD-L1 expression was increased along with time (**Figure 3A** and **3B**). The membranous PD-L1 expression in U87 and U251 were also significantly increased after TMZ challenge which analyzed by flow cytometry (FCM, **Figure 3C**, **3D**).

Depletion of PD-L1 impaired enhanced inhibition of TMZ-treated GBM cells on activated PBMC

As shown in Figure S1A, CD274 mRNA levels in PD-L1 KO U87 were about 10% of that in control cells. Additionally, the PD-L1 depletion effi-
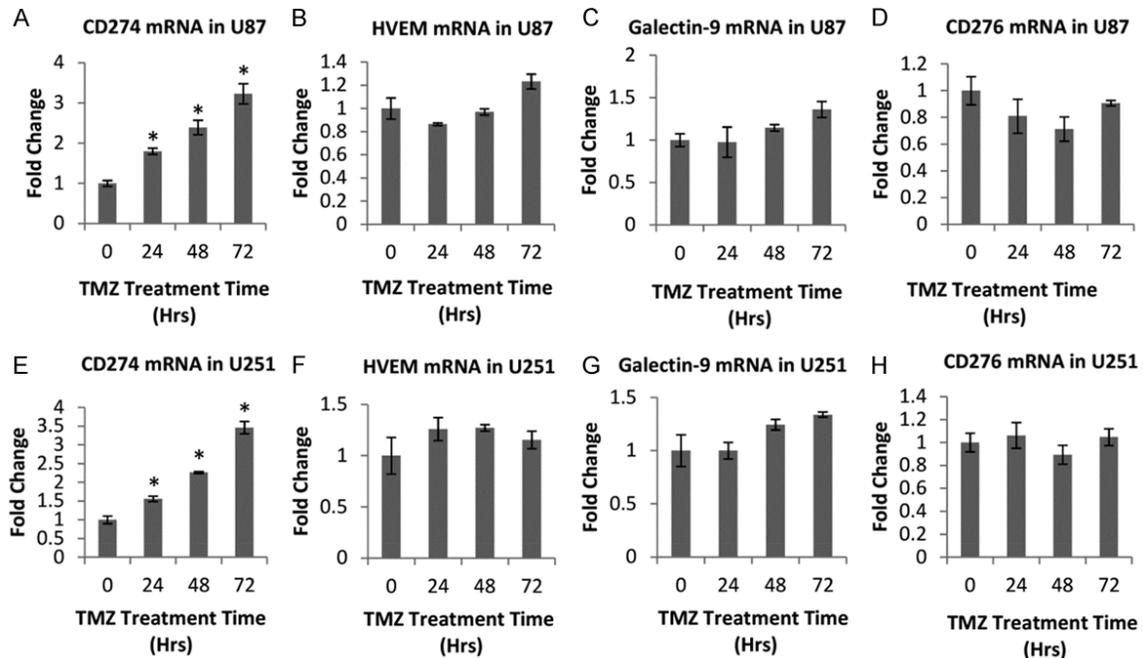
Figure 2. The expression levels of immune checkpoints in GBM cells after TMZ challenge. A-D. U87 cells treated with 200 μM TMZ for different times, were collected for measuring mRNA expression levels of CD274, HVEM, Galectin-9 and CD276 by realtime PCR. E-H. After treated with 50 μM TMZ for 0-72 h, U251 cells were collected for evaluating mRNA expression levels of CD274, HVEM, Galectin-9 and CD276 by realtime PCR. *$P < 0.05$, on the basis of Student's t test.

ciency were also evaluated by FCM (**Figure 4A**). Depletion of PD-L1 greatly impaired inhibition ability of U87 cells on pro-inflammatory cytokines production in medium of PBMC and U87 co-culture system (**Figure 4B**, **4C**). Despite TMZ strengthened inhibition activity of U87 cells, depletion of PD-L1 weakened the inhibitory effect of U87 cells on IFN-γ and IL-2 production in the supernatant of co-culture system (**Figure 4B**, **4C**). Furthermore, the similar results were acquired when PBMC co-cultured with PD-L1 KO U251 after TMZ challenged (**Figures 4D-F, S1B**). In order to further confirm that upregulation of PD-L1 in TMZ treated GBM is a key factor for reduction of IL-2 and IFN-γ expression in co-culture medium, we added PD-L1 antibody into the co-culture medium, showed that neutralization of PD-L1 also weakened the inhibitory effect of U87 on pro-inflammatory cytokine production (Figure S1C, S1D).

STAT3 activation is vital for TMZ-induced PD-L1 expression in GBM cells

To explore whether TMZ-induced PD-L1 expression is regulated at transcriptional level or post-translational modification in cancer cells, we treated U87 and U251 with or without TMZ and actinomycin D. **Figure 5A** and **5B** showed that

actinomycin D attenuated induction effect of TMZ on PD-L1 expression in U87 and U251. Moreover, activation of transcription factors STAT3, HIF-1α and c-Myc could up-regulate CD274 (encoding PD-L1) mRNA level [24-26]. The immunoblotting results revealed that only STAT3pY705 (phosphorylation of tyrosine 705) was highly increased after TMZ treatment from 0.5 h-4 h (**Figure 5C**). To validate whether STAT3 was involved in TMZ-induced PD-L1 expression, our data showed that STAT3 inhibitor VI not only blocked phosphorylation of tyrosine 705 in STAT3, but also ameliorated induction of PD-L1 which triggered by TMZ (**Figures 5D** and S2A). What's more, STAT3 shRNA could block TMZ-mediated PD-L1 induction in U87 cell (**Figures 5E** and S2C). Blockade of STAT3 signaling also attenuated TMZ-enhanced GBM inhibition capacity on pro-inflammatory cytokines production in the co-culture system (Figure S2B, S2C, S2E, S2F).

Combination of TMZ with PD-L1 antibody in GL261 murine GBM models was superior to single therapy

We confirmed that TMZ could induce PD-L1 expression in mouse GBM cell line GL261 as
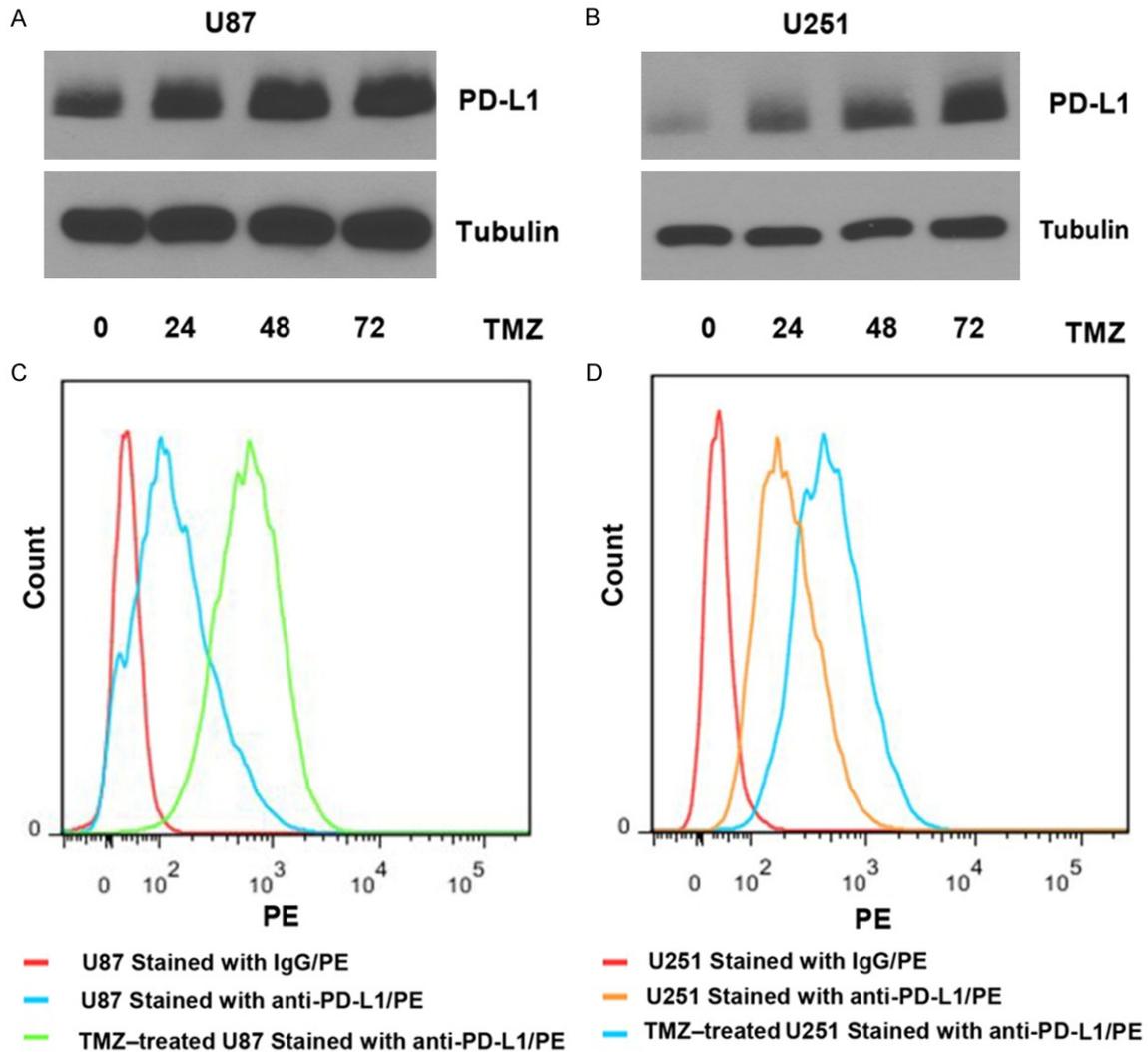
Figure 3. PD-L1 protein expression was dramatically increased in TMZ-treated GBM cells. A, B. U87 and U251 cells were treated with TMZ (200 μM for U87 and 50 μM for U251, similarly hereinafter) for 0-72 h, respectively. Then cells were lyzed by RIPA lysis buffer and immunoblotting analysis was performed with PD-L1 and Tubulin antibodies. C, D. U87 and U251 cells were treated with or without TMZ for 24 h, then the cells were digested and stained with PE-conjugated PD-L1 antibody and were analyzed using a flow cytometer. *$P < 0.05$, on the basis of Student's t test.

well as in human GBM cell line (Figure S3). The GBM mice were divided into four groups, including control group, TMZ group, anti-PD-1 group, and TMZ plus anti-PD-1 group. The drug administration time were indicated at **Figure 6A**. TMZ or PD-1 antibody alone could suppress tumor growth in GBM mice model, furthermore, combinational therapy of TMZ and PD-L1 antibody had stronger inhibitory effect on tumor growth than control group or the other two single treatment groups (**Figure 6B**). In addition, survival curves also demonstrated that combination therapy using TMZ plus PD-1 antibody achieved the best survival proportion through all experimental observation process (**Figure 6C**).

Discussion

In recent years, immunotherapy has attracted considerable attention because of the efficient role played by anti-CTLA-4 and PD-1/PD-L1 blockade therapies for late-stage cancer patients, especially for melanoma and lung cancers patients [23-25]. Encouraged by the good performance of immunotherapy on other types of cancers, the neuro-oncologist also applied this strategy for recurrent GBM treatment. Unfortunately, the prognosis is not as good as scientists and physicians expected [2, 26].

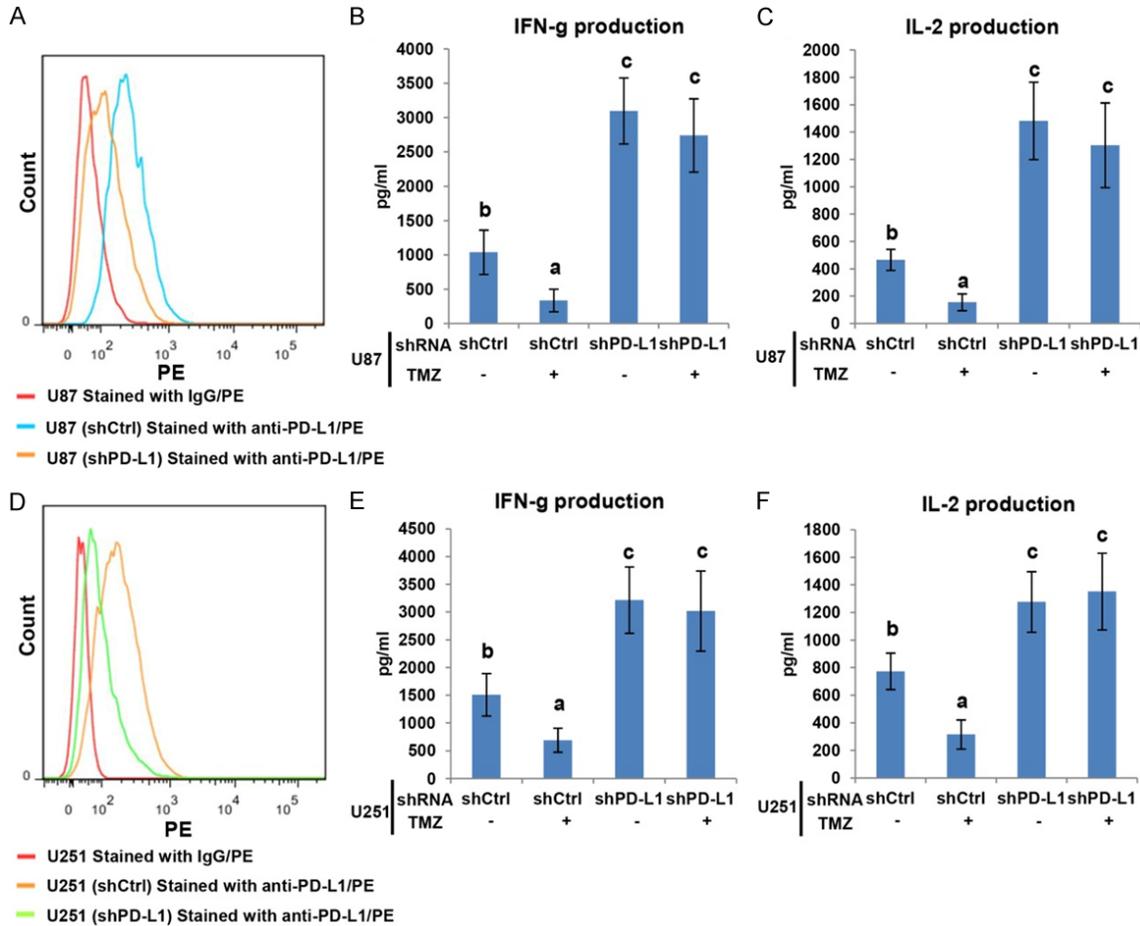Although TMZ has been widely used as the first line chemotherapy drug for gliomas [27], the

Figure 4. PD-L1 was essential for enhanced inhibition of TMZ-challenged GBM cells on activated PBMC. A. U87 cells were transfected with control shRNA or PD-L1 shRNA before collected for detecting PD-L1 expression. B, C. U87 cells which stable express shRNA or PD-L1 shRNA were pretreated with or without 200 μM TMZ, then co-cultured with pre-activated PBMC for 24 h. IFN-γ and IL-2 level in co-culture medium were analyzed using ELISA. D. U251 cells were transfected with control shRNA or PD-L1 shRNA before collected for detecting PD-L1 expression. E, F. U251 cells which stably express shRNA or PD-L1 shRNA were pretreated with or without 50 μM TMZ, then co-cultured with pre-activated PBMC for 24 h. IFN-γ and IL-2 in the supernatant were analyzed using ELISA. The asterisk or different alphabets denote a significant difference at P < 0.05.

mechanisms by which TMZ affects characteristics or capacities of GBM cells remain elusive. In the present study, we revealed that suppression of pro-inflammatory cytokines IL-2 and IFN-γ expression in the medium which collected from PBMC co-cultured with TMZ treated U87 and U251 cells, indicating that TMZ exacerbated the immune evasion of GBM cells. These findings guided us to speculate that TMZ may give effect to the expression of immune checkpoints in GBM cell. To address this hypothesis, we investigated mRNA levels of several immune checkpoints in U87 and U251 cells, and found only CD274 expression level was remarkably enhanced after TMZ challenge,

which was consistent with Derer et al. study that they showed TMZ induced murine PD-L1 expression in GBM cells [28], while whether TMZ could induce human GBM cells PD-L1 expression was not defined. What's more, increase in membranous PD-L1 in GBM cells strengthened positive role of TMZ on immune evasion of GBM cells.

For further demonstrating the pivotal role of PD-L1 in immune escape of GBM cells with TMZ treatment, we knocked down PD-L1 expression by shRNA or neutralized PD-L1 by antibody in GBM cells. Depletion of PD-L1 in TMZ treated GBM cells, effectively rescued expres-
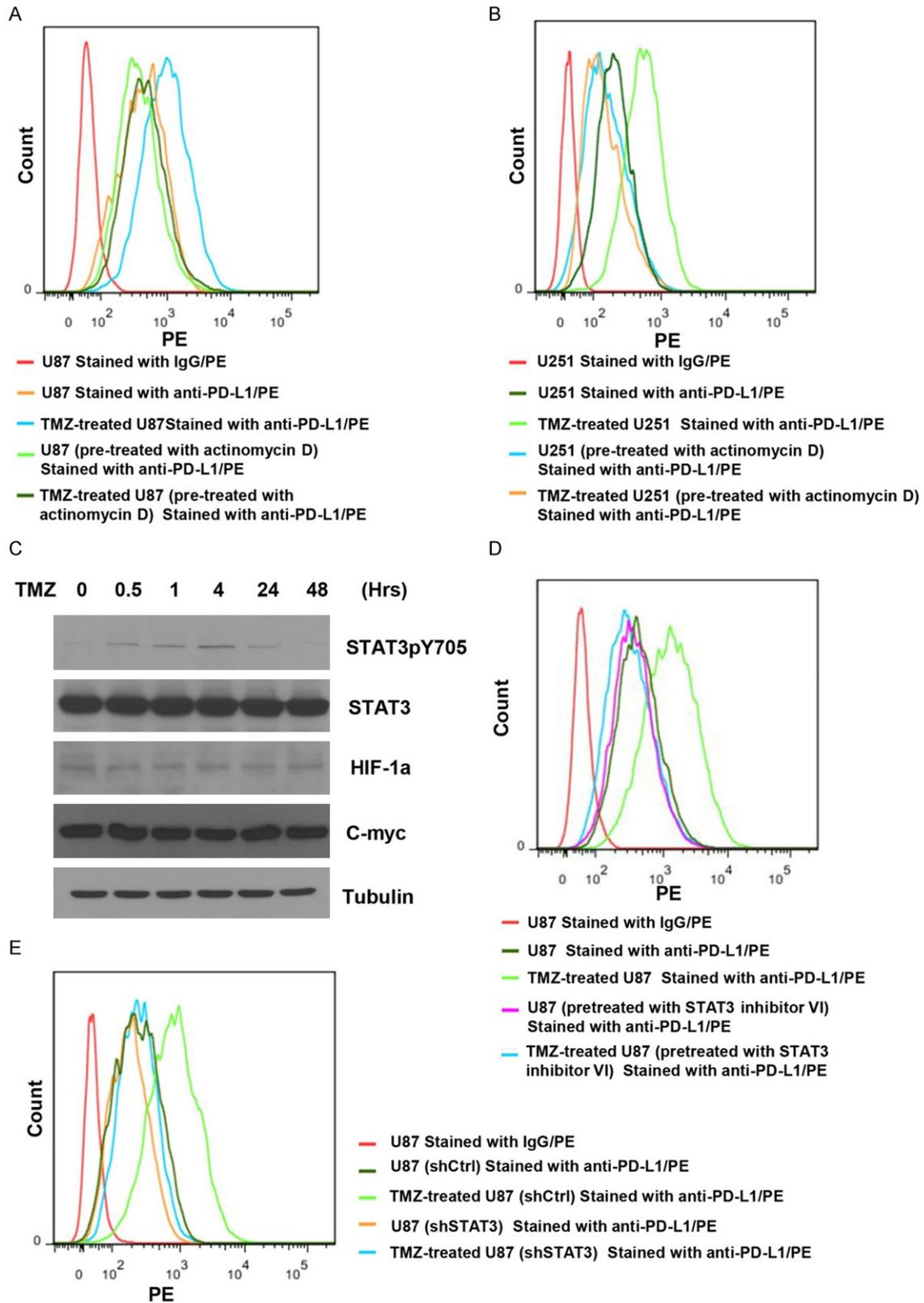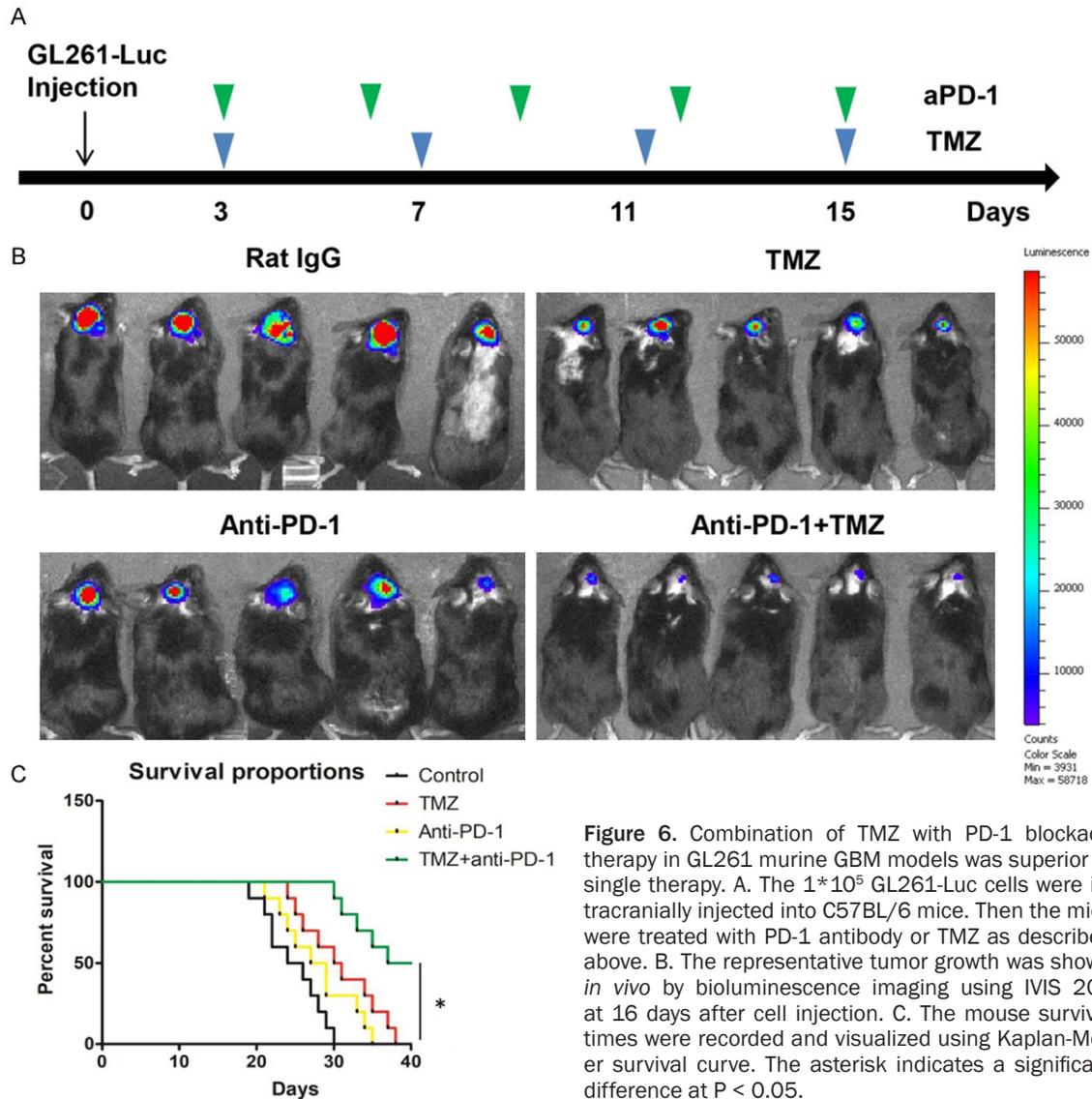
Figure 5. STAT3 signaling is critical for TMZ-induced PD-L1 expression in GBM cells. A, B. U87 and U251 cells were pretreated with 1 μg/ml actinomycin D for 2 h and then challenged with or without TMZ for 24 h. The PD-L1 expression level in GBM cells were determined by FCM. C. U87 cells were treated with 200 μM TMZ for 0-48 h, followed by WB analysis using STAT3pY705, STAT3, HIF-1, c-Myc and Tubulin antibodies. D. U87 cells were pretreated with

or without STAT3 inhibitor VI for 1 h and then treated with or without TMZ for 24 h, followed by FCM analysis using PE-conjugated PD-L1 antibody. E. U87 cells which stably express control shRNA (shctrl) or STAT3 shRNA (shSTAT3) were pretreated with or without TMZ for 24 h, followed by FCM quantification with PE-conjugated PD-L1 antibody.

Figure 6. Combination of TMZ with PD-1 blockade therapy in GL261 murine GBM models was superior to single therapy. A. The $1*10^5$ GL261-Luc cells were intracranially injected into C57BL/6 mice. Then the mice were treated with PD-1 antibody or TMZ as described above. B. The representative tumor growth was shown *in vivo* by bioluminescence imaging using IVIS 200 at 16 days after cell injection. C. The mouse survival times were recorded and visualized using Kaplan-Meier survival curve. The asterisk indicates a significant difference at P < 0.05.

sion of pro-inflammatory cytokines IFN-γ and IL-2 in co-culture medium of PBMC and GBM, suggesting that PD-L1 functions as a key factor for inhibitory capacity of GBM cells after TMZ challenge on PBMC. Subsequently, we demonstrated that PD-L1 expression was induced by TMZ at transcriptional regulation, since actinomycin D could attenuate induction effect of TMZ on PD-L1 expression.

How to avoid immune evasion of GBM cells? Blocking PD-1/PD-L1 interaction with antibody

or inhibiting the pathway involved in PD-L1 induction with inhibitor are effective strategy. But delivery of antibody into brain tumor is difficult due to the existence of blood-brain barrier [29], whereas small chemical inhibitors which can suppress PD-L1 expression in tumor cells may be the best option to counteract immune evasion, because lots of small chemical inhibitors are easier to cross blood-brain barrier. According to the other studies, CD274 which encoding PD-L1, is mainly regulated by STAT3, HIF-1α and c-Myc in several types of cancer

cells [24-26], while our results revealed that only STAT3pY705 was increased after TMZ treatment in U87 cells. We proved that TMZ could trigger STAT3 activation, and STAT3 acted as a key transcription factor which promoted PD-L1 expression, indicating that STAT3 signaling was critical for PD-L1 induction after TMZ challenge. Despite of several experimental application small chemical inhibitors for STAT3 are available commercially, the inhibitors for the clinical use or even in late-stage clinical study are not available yet [30]. Accordingly, PD-1 antibody or PD-L1 antibody is the last option to block the TMZ-mediated immune inhibitory activity of GBM cells.

As we know, the combinational therapy is a popular and efficient way to fight with cancer. Combination of PD-1/PD-L1 blockade and TMZ can suppress tumor growth, but also can impair immune evasion of GBM cell after chemotherapy theoretically. The animal study showed that the combinational therapy is more effective than single therapy, both in inhibiting tumor growth and improving survival ratio.

Taken together, we demonstrated that TMZ contributed to GBM cells immune escape through upregulating PD-L1, which does not facilitate the treatment of GBM. In order to obtain good clinical curative effect, we proposed that combination of TMZ and PD-1 antibody was a promising way to treat GBM, which was strongly supported by our animal experiment. Nevertheless, injection of the PD-1 antibody intracranially as mentioned above, would increase the risk of brain infection, so developing an alternative approach to deliver antibody directly to brain tumor will be the main target of our further work.

Disclosure of conflict of interest

None.

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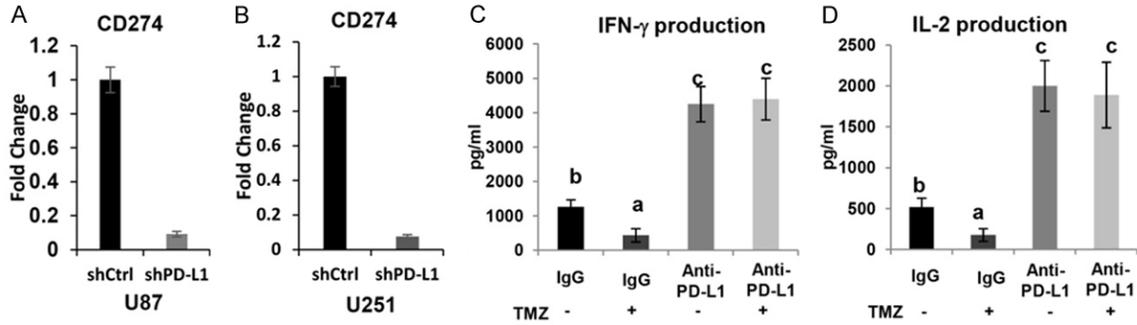
Figure S1. Blockade of PD-L1 rescued pro-inflammatory cytokines production on activated PBMC. A, B. RNA was extracted from GBM cells which stably expressed control shRNA (shCtrl) or PD-L1 shRNA (shPD-L1), and then quantified the CD274 mRNA expression by qRT-PCR. C, D. U87 cells were treated with or without TMZ for 24 h, then isotype IgG or PD-L1 antibody were added to the culture medium. After that, U87 cells were co-cultured with activated PBMC for 24 h, and the supernatants were collected for IFN-γ and IL-2 quantification by ELISA. The different alphabets indicates a significant difference at P < 0.05.
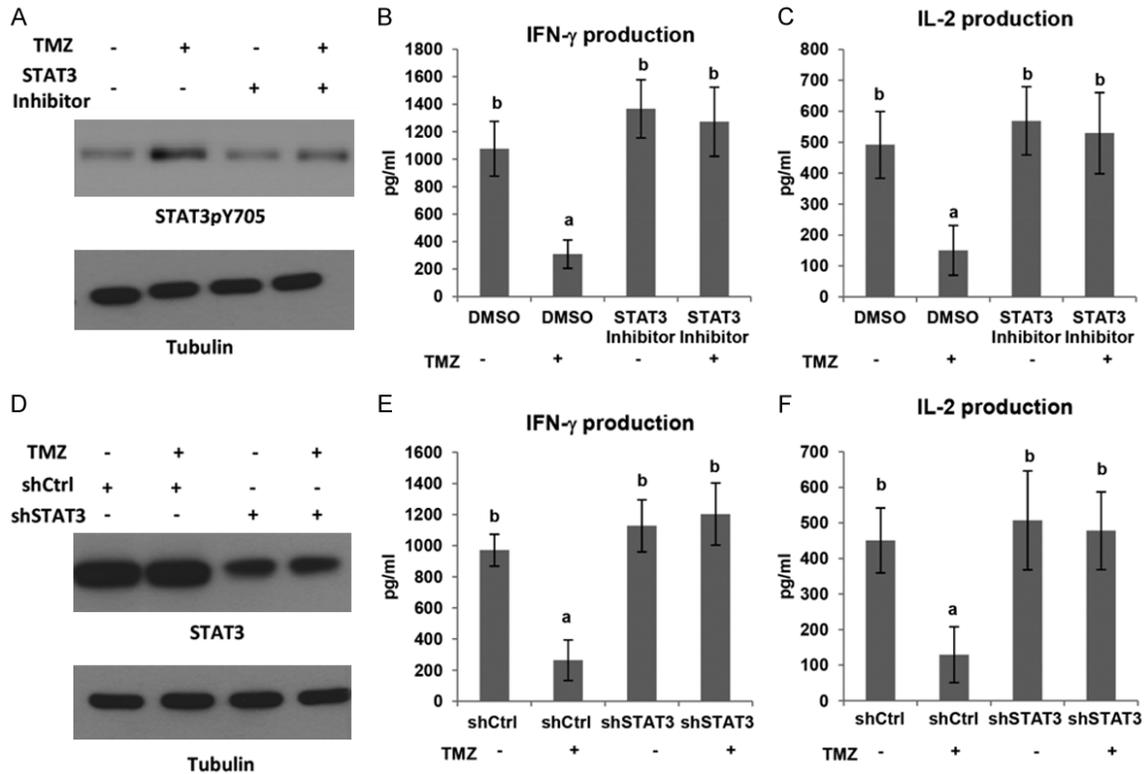

Figure S2. Blockade of STAT3 pathway impaired immune suppression capacity of GBM which enhanced by TMZ. A. U87 cells were pretreated with or without STAT3 inhibitor for 1 h, and then treated with or without TMZ for 24 h, followed by WB analysis using STAT3 pY705 and Tubulin antibodies; B, C. Or the U87 cells were co-cultured with activated PBMC for 24 h and the supernatant were clarified for determining IFN-γ and IL-2 expression by ELISA. D. U87 cells which stably express control shRNA (shCtrl) or STAT3 shRNA (shSTAT3) were treated with or without TMZ for 24 h, and the cells were lyzed and subjected to immunoblotting by using STAT3 and Tubulin antibody; E, F. Or the cells were co-cultured with activated PBMC for 24 h, followed by ELISA analysis for IFN-γ and IL-2 expression in the co-culture medium. The different alphabets indicates a significant difference at P < 0.05.
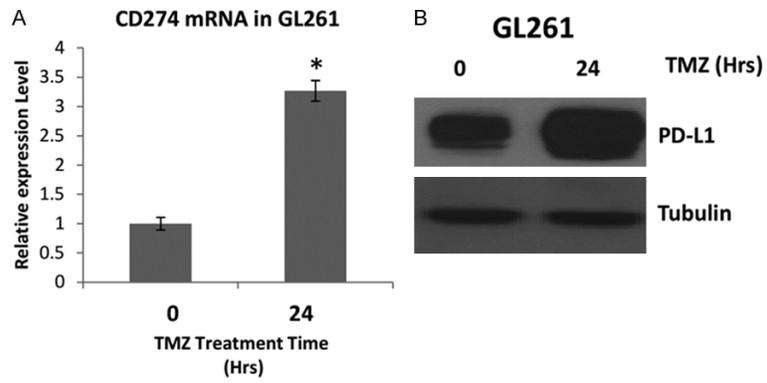
Figure S3. PD-L1 expression was upregulated in GL261 cells after TMZ challenge. A, B. GL261 cells were treated with or without 200 μM TMZ for 24 h, followed by realtime PCR and western blotting for PD-L1 expression. The asterisk indicates a significant difference at $P < 0.05$.