Original Research

Tripartite motif-containing 25 facilitates immunosuppression and inhibits apoptosis of glioma via activating NF-κB

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Impact Statement

Tripartite motif-containing 25 (TRIM25) without any glioma-related studies is an important cancer-associated E3 ligase. Our investigation revealed that glioma patients with high expression of TRIM25 had an unfavorable prognosis and malignant progression. Bioinformatic analyses determined that TRIM25 may have potential regulatory effects on apoptosis and tumor immunity. Furthermore, TRIM25 regulated the transcriptional activity of NF-κB through affecting its nuclear translocation, which contributed to the immune suppression via NF-κB/PD-L1 axis. Based on the above effect, TRIM25 ablation also triggered mitochondria-induced apoptosis and suppressed the proliferation of glioma cells, indicating that TRIM25 may be a novel prognostic biomarker of gliomas.

Abstract

As a crucial tumor type of the central nervous system, gliomas are characterized by a dismal prognosis. Tripartite motif-containing 25 (TRIM25), an essential E3 ubiquitin ligase, participates in various biological processes. This study sought to demonstrate its functional role in gliomas. Data obtained from publicly available databases – including The Cancer Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA), and the Repository for Molecular Brain Neoplasia Data (REMBRANDT) – were employed. TRIM25 expression pattern and its association with different clinical characteristics were analyzed. Kaplan–Meier analysis was utilized to compare different TRIM25 expressions with glioma patients' survival. Subsequently, we performed bioinformatic analyses to investigate the biological functions of TRIM25, which were further validated by *in vitro* experiments, CIBERSORT algorithm, and ESTIMATE evaluation. TRIM25 expression was upregulated in glioma patients and can predict an unfavorable prognosis. Bioinformatic results indicated the involvement of TRIM25 in apoptosis and immune regulation. TRIM25 was associated with programmed death-ligand 1 (PD-L1) related and macrophage-induced immune suppression in gliomas. Meanwhile, silencing TRIM25 promoted apoptosis in glioma cells, which is attributed to its regulation of NF-κB. Therefore, TRIM25 contributed to the glioma malignant progression and suppressive immune microenvironments via NF-κB activation, which may play a therapeutic role in gliomas.

Keywords: TRIM25, glioma, immune suppression, apoptosis, NF-κB

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Introduction

As a class of intractable intracranial tumors, gliomas are notorious for dismal prognosis.[1] According to their histopathological characteristics, gliomas are classified into four grades (I–IV). Grade II and III are defined as low-grade glioma (LGG). Aggressive glioblastoma multiforme (GBM) is grouped as grade IV. There is a low overall survival (OS) rate for most patients with gliomas even after chemoradiotherapy and surgical resection. Only 5% of patients survive beyond five years.[2,3] Nowadays, multiple novel strategies – including targeted therapy, antiangiogenesis, or immunotherapy – have attracted substantial attention and are hoped to improve the treatment status of gliomas.

Tripartite motif-containing 25 (TRIM25) has been identified as an important cancer-associated protein.[4] The expression of TRIM25 was upregulated in lung,[5] breast,[6] and prostate[7] cancers, while decreased in endometrial[8] cancer. Recent studies have indicated that TRIM25 led to epirubicin resistance via promoting the ubiquitination of phosphatase and tensin homolog (PTEN) in hepatocellular carcinoma.[9] TRIM25 can also interact with G3BP2 and regulate p53 activity, enhancing prostate cancer cell proliferation and survival.[7] Despite TRIM25 participating in numerous tumorigenic processes, its clinical and prognostic significance in gliomas still needs to be documented.

In this study, the functional significance of TRIM25 in glioma patients was dug out based on the data from The Cancer

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Genome Atlas (TCGA), the Chinese Glioma Genome Atlas (CGGA), and the Repository for Molecular Brain Neoplasia Data (REMBRANDT) databases. Since the immune microenvironment was of great significance in the progression of gliomas, the immune-related analyses, including ESTIMATE and CIBERSORT algorithms, were applied to display the pertinence between TRIM25 and immune infiltration. We have also conducted relevant cell biology experiments to further verify the above results and clarify the potential mechanism of TRIM25 in gliomas.

Materials and methods

Data mining from public databases

The expression profile of TRIM25 among 33 cancer types and paired normal samples were acquired from the TCGA (https://portal.gdc.cancer.gov) and Genotype-Tissue Expression (GTEx) (https://www.gtexportal.org) databases. Subsequently, the clinical and RNA-seq data of glioma patients from the TCGA, CGGA,[10] and REMBRANDT databases were obtained from the above website, http://www.cgga.org.cn/, and GlioVis (http://gliovis.bioinfo.cnio.es/), respectively.

Survival analysis

According to the median expression value of TRIM25 in gliomas, patients were classified as either low expression ($TRIM25^{low}$) or high expression ($TRIM25^{high}$). To compare the survival times between these two groups, Kaplan–Meier analysis was implemented.

Bioinformatics and immune-related analyses

TRIM25 relevant genes were screened out by Pearson's correlation analysis using the above three data sets. Gene Ontology (GO), Reactome Pathway, and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analyses were performed on TRIM25 relevant genes via clusterProfiler R package. The top 5 GO analysis terms and top 15 TRIM25-related pathways were selected for plotting histograms or bubble charts.

An analysis of TRIM25 expression in gliomas using the CIBERSORT analytical tool[11] was conducted in order to see if there was an association with the abundance of immune cells. The ESTIMATE algorithm was applied to infer the status of stromal and immune cells infiltration in tumor tissues and evaluate tumor purity.[12]

Glioma cell culture and treatment

U87 cells were purchased from EK-Bioscience Biotechnology (Switzerland) (CC-Y1526). The cells are kept up in Dulbecco's Modified Eagle Medium (DMEM) high-glucose medium (containing 10% fetal bovine serum and 1% antibiotics) and grown at 37°C in a humidified atmosphere with 5% CO_2. 50 nM TRIM25 siRNAs (SIGS0008362-1, RiboBio, China) were transiently transfected into U87 cells utilizing Lipofectamine 2000 reagent (11668019; Invitrogen, Waltham, MA, USA). After transfection, these cells were continued to culture for 48 h and then collected for further analysis.

Quantitative real-time polymerase chain

Total RNA of U87 cells was extracted using TRIzol (Invitrogen), and 1 μg RNA was reversely transcribed by the First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by SYBR® Premix Ex Taq™ II (RR820Q, TaKaRa, Japan) using the LightCycler 480 System (Roche). The fold change of target mRNA expressions was calculated by equation $2^{-\Delta\Delta Ct}$ with respect to *GAPDH*. The primer sequences are listed in Supplementary Table 1.

Western blots

U87 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (P0013C; Beyotime, Haimen, China), and protein concentrations were determined by BCA Protein Assay (Beyotime, P0012). Equal amounts of lysate were mixed with 2× sodium dodecyl sulfate (SDS) loading buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to the polyvinylidene difluoride (PVDF) membrane. After blocking in the 5% skim milk, membranes were reacted with primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies and detected using a ProteinSimple FluorChem HD2 imaging system (Bio-Rad, Hercules, CA, USA). Mitochondrial and cytosolic fractions of U87 cells were isolated using the Cell Mitochondria Isolation Kit (Beyotime, C3601). The fractions were subjected to immunoblot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a cytosolic or total protein reference and cytochrome c oxidase IV (COX IV) served as a mitochondrial reference. Primary antibodies against programmed death-ligand 1 (PD-L1) (66248-1-Ig), TRIM25 (67314-1-Ig), and NF-κB (66535-1-Ig) were obtained from ProteinTech (Rosemont, IL, USA). Primary antibodies against cytochrome c (sc-13156) and COX IV (sc-376731) were purchased from Santa Cruz (Dallas, TX, USA). Primary antibodies against GAPDH (#5174), cleaved Caspase-3 (#94530), and cleaved poly (ADP-ribose) polymerase (PARP) (#5625) were bought from Cell Signaling Technology (CST; Danvers, MA, USA).

Cell Counting Kit-8 and colony formation assays

Both negative control and si-TRIM25 transfected U87 cells were plated in 96-well plates (5×10^3 cells/well). Cells without any treatment were served as control. After incubating for one to five days, CCK8 reagent (Beyotime, C0038) was added to wells daily, and a wavelength of 450 nm was set to measure absorbance. The first day's measurements were regarded as the baseline, and growth curves were plotted based on multiples of the following four days. U87 cells with the same treatment were spread in six-well plates (1×10^3 cells/well) and cultured for 10 days. Cell colonies were washed with phosphate-buffered saline (PBS) three times, fixed with 4% paraformaldehyde for 15 min, and stained with 0.1% crystal violet solution for 15 min. A gel imager captured visible colonies under white light.

Flow cytometry assay

After different treatments, U87 cells were collected and washed three times with cold PBS. Following resuspension in 100 μL 1× Annexin V binding solution, the cells were stained with 5 μL Annexin V-fluorescein isothiocyanate (FITC) conjugate plus 5 μL propidium iodide (PI) solution (AD10; Dojindo, Rockville, MD, USA) for 30 min in darkness. Eventually, these cells were added with 400 μL Annexin V binding solution. The apoptotic rates were analyzed using flow cytometry (BD, FACSCalibur, USA) within 1 h.

Mitochondrial membrane potential detection

U87 cells treated with si-TRIM25 were rinsed with PBS and incubated with JC-1 (10 μM) at 37°C for 20 min (M8650; Solarbio, Beijing, China). Following washing three times with JC1 buffer solution, the cells were kept in culture medium and mitochondrial membrane potential (MMP) was analyzed by fluorescent microscope ($\lambda = 530$ nm for monomers; $\lambda = 590$ nm for aggregates).

Statistical analyses

Quantitative data were presented as the mean value ± standard derivation (SD). Differences between two groups were examined by Wilcox's test, while differences among more than three groups were examined using one-way analysis of variance (ANOVA) with Dunnett's test. The P value was set at 0.05.

Results

Pan-cancer expression landscape of TRIM25

The TRIM25 expression profile was determined using the Oncomine database to evaluate its prevalence in different cancers[13] (Figure 1(A)). Compared to paired normal specimens, TRIM25 was over-expressed in the brain and central nervous system (CNS) and breast cancer, whereas downregulated in breast, colorectal cancer, and lymphoma. Moreover, we analyzed RNA-seq data from the TCGA database to assess TRIM25 expression in pan-cancer. A total of 26,455 mRNA expression profiles from 30 cancer types were obtained. There was a broad spectrum of TRIM25 expression among various cancer types. Compared with adjacent normal tissues, the expression of TRIM25 was significantly higher in 14 cancers, including breast invasive carcinoma (BRCA), cholangio carcinoma (CHOL), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), GBM, head and neck squamous cell carcinoma (HNCS), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain LGG, pancreatic adenocarcinoma (PAAD), rectum adenocarcinoma (READ), sarcoma (SARC), stomach adenocarcinoma (STAD), and uterine corpus endometrial carcinoma (UCEC). Moreover, decreased TRIM25 expression was also observed in 10 cancers, including adrenocortical carcinoma (ACC), lymphoid neoplasm diffuse large B-cell lymphoma (DLBC), kidney chromophobe (KICH), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), skin cutaneous melanoma (SKCM), testicular germ cell tumors (TGCTs), thyroid carcinoma (THCA), and uterine carcinosarcoma (UCS) (Figure 1(B)).

The clinical and survival significances of TRIM25 expression in gliomas

To determine whether elevated TRIM25 expression contributed to the advanced progression of gliomas, associations between TRIM25 mRNA expression and numerous clinicopathological parameters were analyzed. The results determined that patients with World Health Organization (WHO) grade II/III and isocitrate dehydrogenase (IDH) mutation have lower levels of TRIM25 expression. On the contrary, high TRIM25 expression was relevant to an unfavorable prognosis, higher WHO grades, aggressive glioma subtype (GBM), and wild-type IDH (Figures 1(C) and 2(A)). On the basis of Kaplan–Meier analysis, TRIM25 expression exhibited an opposite trend to the OS in the CGGA data (27.10 months for $TRIM25^{high}$ vs 43.20 months for $TRIM25^{low}$; $P = 0.0002$, log-rank test), which was further verified in the TCGA (38.90 months for $TRIM25^{high}$ vs 67.50 months for $TRIM25^{low}$; $P = 0.0004$, log-rank test) and REMBRANDT (15.10 months for $TRIM25^{high}$ vs 30.40 months for $TRIM25^{low}$; $P < 0.0001$, log-rank test) cohorts (Figure 1(D)). We next included the CGGA, TCGA, and REMBRANDT data for meta-analysis to systematically assess whether TRIM25 expression was associated with OS in glioma patients. As displayed in Figure 2(B), the pooled RR (risk ratio) with 95% CI was 1.26 (1.16, 1.37). There was no significant heterogeneity among these three data sets ($P = 0.259$, $I^2 = 26.10\%$). Consequently, it is conceivable that elevated TRIM25 expression may be an adverse factor in the malignant progression of gliomas.

Biological signatures and signaling pathways associated with TRIM25 in gliomas

To gain insight into the functional role of TRIM25 in gliomas, we filtered its related genes through Pearson's correlation analysis among these three data sets. Three hundred seventy-seven genes were generated by intersecting the above data (Figure 3(A) and Supplementary Table 2). GO, Reactome Pathway, and KEGG Pathway analyses were performed on TRIM25-relevant genes. The results indicated that TRIM25 with ubiquitin-protein transferase and protein-binding activities was enriched in the nucleus or endoplasmic reticulum (ER), which was involved in immune-related biological processes (Figure 3(B)). Reactome Pathway analysis pointed to the regulatory role of TRIM25 in immunity, especially in interferon and NF-κB signaling pathways (Figure 3(C)). Similarly, KEGG Pathway analysis also showed that TRIM25 was closely implicated with protein processing in ER, apoptosis, PD-L1 expression and PD-1 checkpoint pathway in cancer, and NF-κB signaling pathway (Figure 3(D)). Therefore, the main biological functions of TRIM25 in gliomas can be attributed to the following two categories, namely, tumor immunoregulation and apoptosis.

Immune-related analyses of TRIM25 in gliomas

It has been reported that glioma with immunosuppressive nature always mediates antitumor immune responses.[14]

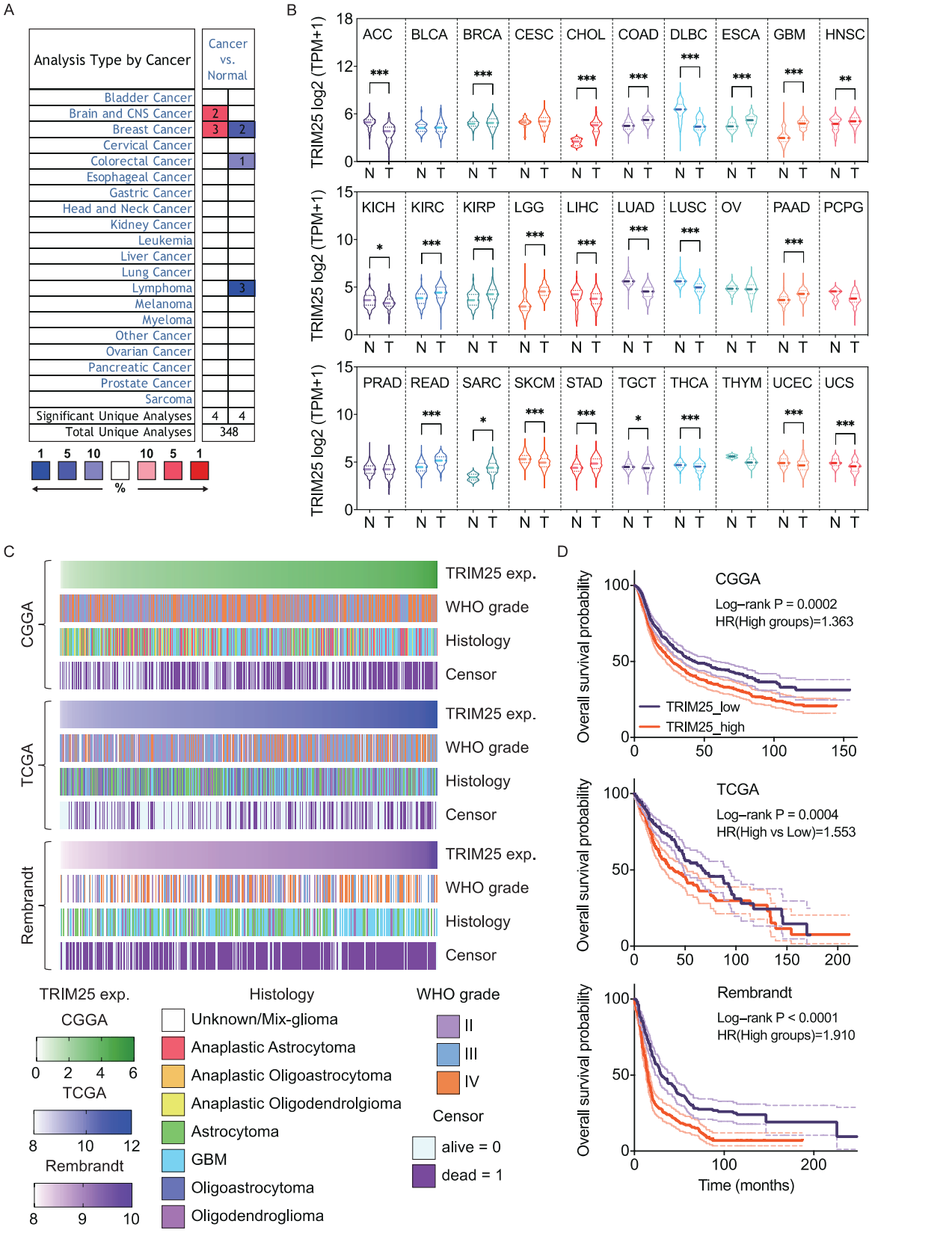

Figure 1. (A) Searching the Oncomine database to investigate TRIM25 expression in various cancer data sets versus normal tissues. (B) The distribution of TRIM25 in normal or tumor tissues was obtained from TCGA or GTEx databases. Data for acute myeloid leukemia (LAML), mesothelioma (MESO), and uveal melanoma (UVM) should be excluded due to the lack of matched normal data. The remains were used for pan-cancer analysis. The results were displayed in violin plots, in which the horizontal and vertical axis represents tumor types and the gene expression of TRIM25, respectively. (C) Patients' clinicopathological parameters of CGGA, TCGA, and REMBRANDT data sets were listed in ascending order of TRIM25 expression. (D) Kaplan–Meier survival curves were depicted in $TRIM25^{low}$ and $TRIM25^{high}$ groups based on the above data sets. (A color version of this figure is available in the online journal.)
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ significantly different from tumor groups.

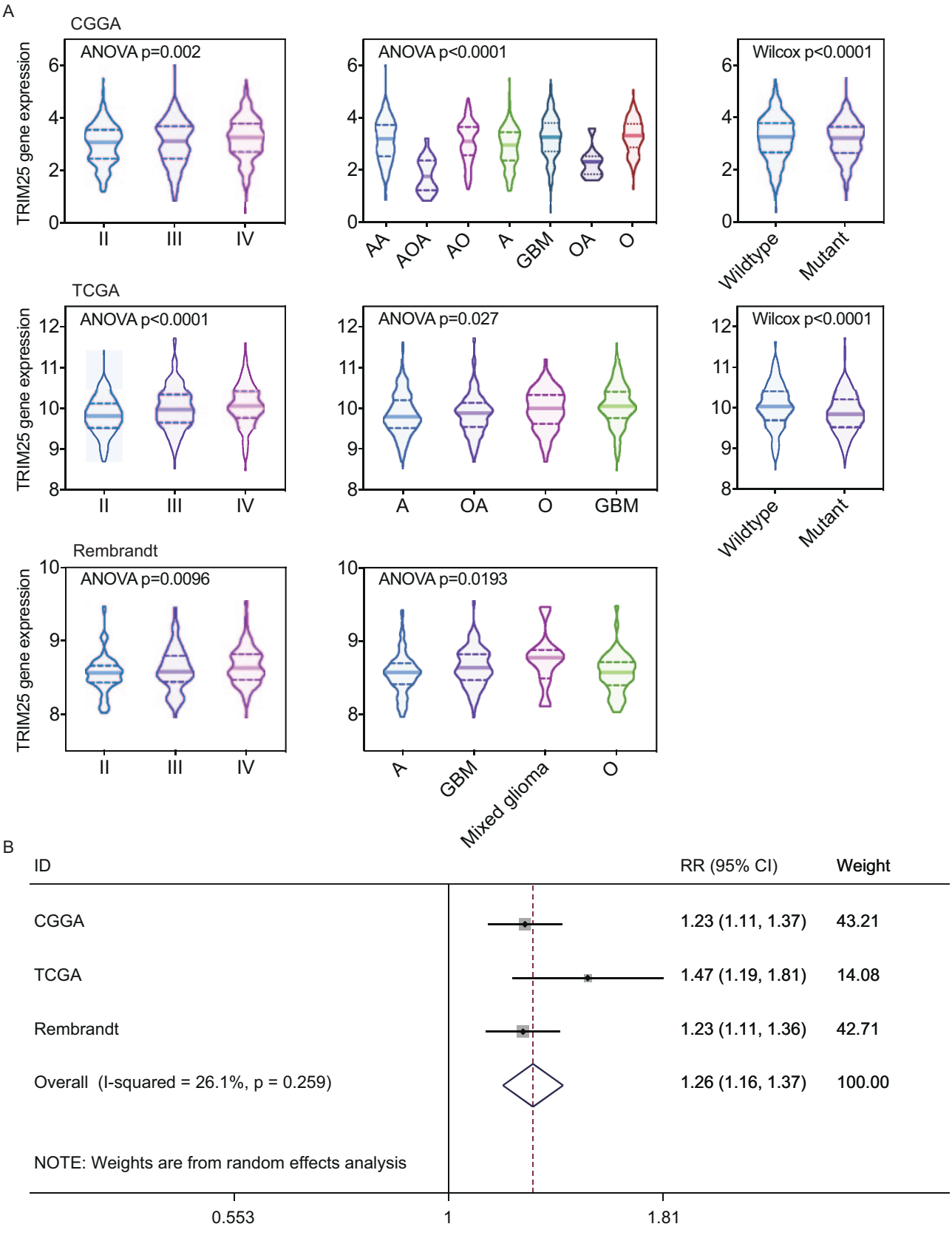

Figure 2. (A) Clinical characteristics of glioma patients (including WHO grade, histological cancer type, and IDH mutation) were stratified according to TRIM25 expression in the CGGA, TCGA, and REMBRANDT data sets. (B) Forest plot of higher TRIM25 expression with worse overall survival (OS) in glioma patients based on these three cohorts. (A color version of this figure is available in the online journal.)

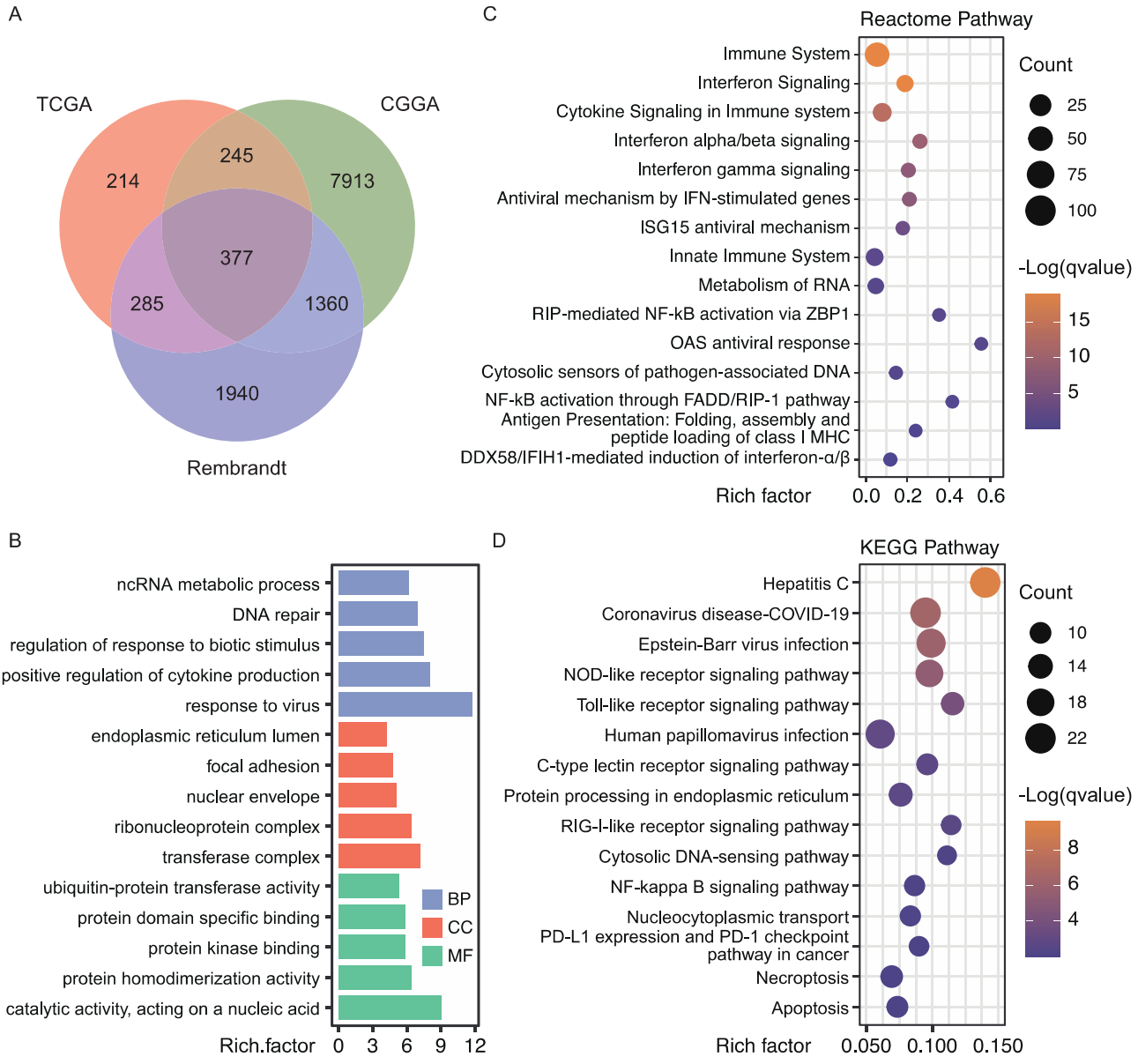

Figure 3. TRIM25-related biological signatures and signaling pathways in gliomas. (A) TRIM25 relevant genes were selected from CGGA, TCGA, and REMBRANDT cohorts by Pearson's correlation analysis. The absolute value of relevant indicators is greater than or equal to 3 as inclusion criteria. Finally, 377 intersected genes were screened out. (B) Gene Ontology (GO), (C) Reactome Pathway, and (D) KEGG Pathway analyses were performed on these genes, which results were displayed in a histogram and bubble diagrams. (A color version of this figure is available in the online journal.)

As shown in Figure 4(A), ESTIMATE[12] evaluation pointed out that the TRIM25^{high} population had higher ESTIMATE, stromal, and immune scores, as well as lower tumor purity than those in the TRIM25^{low} group. Immune checkpoint molecules (programmed death receptors and their ligands) can block the body's ability to mount effective antitumor immune responses. The expression profiles of TRIM25 and immune checkpoints (including *SIGLEC15*, *CD274*, *PDCD1LG2*, *TIGIT*, *PDCD1*, *HAVCR2*, *LAG3*, and *CTLA4*) were extracted from these three data sets, and correlation analyses were conducted. In the CGGA and TCGA cohorts, TRIM25 expression was positively linked with the PD-1/PD-L1 axis, and HAVCR2 and LAG3 in the REMBRANDT data (Figure 4(B)). Subsequently, we used the CIBERSORT analytical tool to comprehensively evaluated intratumoral immune cell infiltration in TRIM25^{low} and TRIM25^{high} groups to see if it governs the immune microenvironment of gliomas. The results were relatively consistent across these three data sets. M2 macrophages, the predominant immune cells in gliomas,[15] were mainly enriched in the TRIM25^{high} group (Figure 5). As reported previously, M2-like macrophages can facilitate tumor growth through mediating the immune repressive microenvironment, normally deemed tumor-associated macrophages (TAMs).[16] TRIM25^{high} group also exhibited significantly higher expression of M2 macrophage and TAM markers, as well as some M1 macrophage markers, possibly because TAM does not exactly follow the M1 and/or M2 phenotypes[17] (Figure 6(A) to (C)). Based on the above results, high TRIM25 expression may be associated with PD-L1-related

Figure 4. (A) Heatmap of TRIM25 expression and microenvironment signatures in glioma patients after analyzing by ESTIMATE algorithm. (B) The expressions of immune checkpoint relevant transcripts were extracted from CGGA, TCGA, and REMBRANDT data sets. Subsequently, correlation analyses between TRIM25 and these genes were performed. (A color version of this figure is available in the online journal.)

and macrophage-induced immune suppression in gliomas, leading to the failure of immune checkpoint blockade (ICB) therapy.

According to the results in Figure 3 and previous reports,[18] TRIM25 positively regulates the transcription factor activity of NF-κB. In U87 cells, siRNAs were applied to silence TRIM25. TRIM25 knockdown attenuated NF-κB translocating into the nucleus and downregulated PD-L1, a pivotal downstream gene of the NF-κB pathway[19] (Figure 6(D) to (F)). Immunofluorescence more visually demonstrated that TRIM25 inhibition can reduce PD-L1 expression (Figure 6(G)). Furthermore, we investigated whether TRIM25 mediated TNF-α-induced NF-κB activity. Knocking down TRIM25 inhibited the import of NF-κB into the nucleus and also downregulated PD-L1 expression after TNF-α stimulation (Supplementary Figure 1(A)). Hence, TRIM25 may contribute to the immune repressive microenvironment of gliomas by regulating NF-κB/PD-L1 axis.

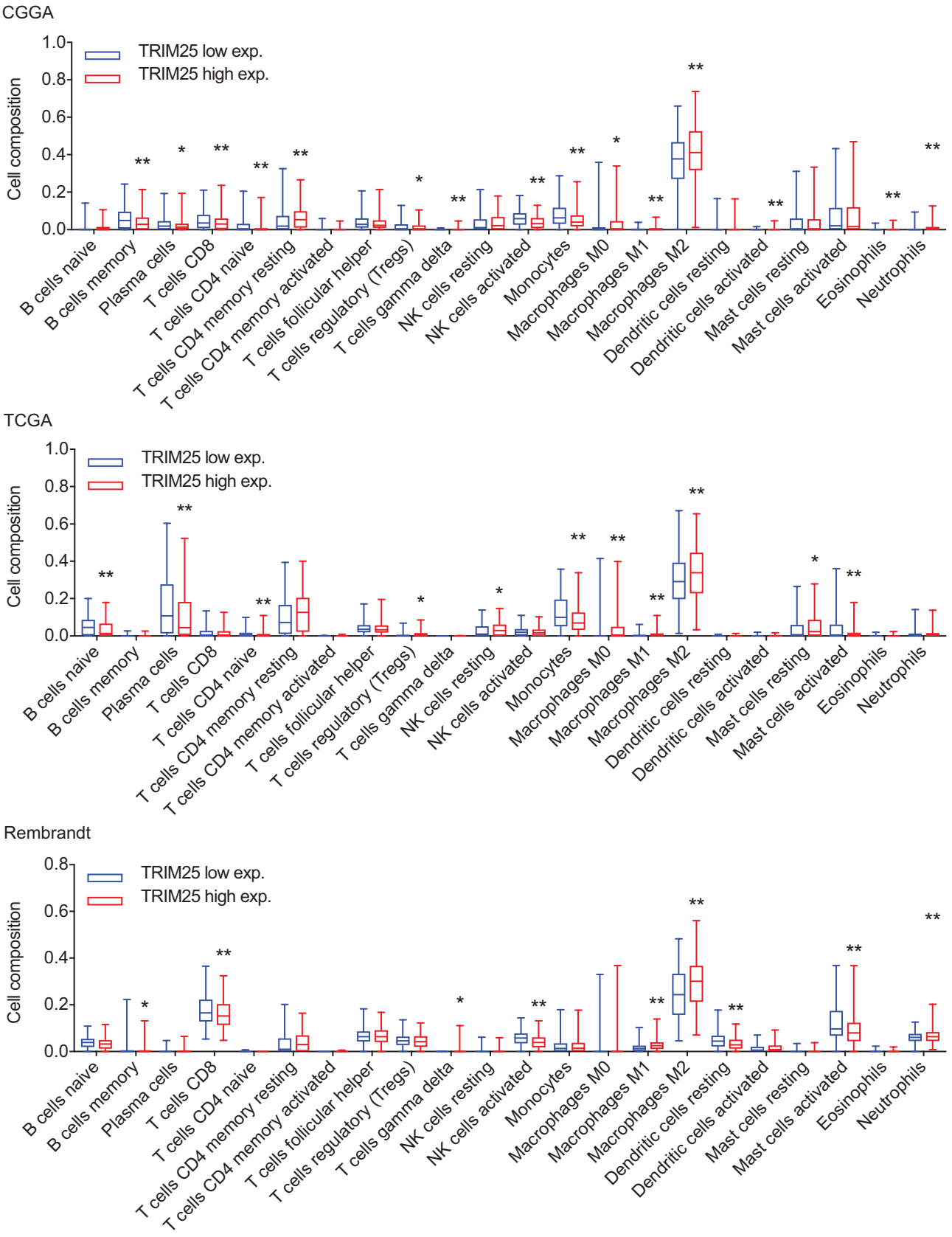

Figure 5. TRIM25 influenced immune cells infiltration. The discrepancies of immune cell infiltration in TRIM25^{low} and TRIM25^{high} groups were analyzed using the CIBERSORT algorithm. Data in each boxplot were represented as 1–99 percentile. (A color version of this figure is available in the online journal.)
*$P < 0.05$; **$P < 0.01$ indicated significant differences between these two groups.

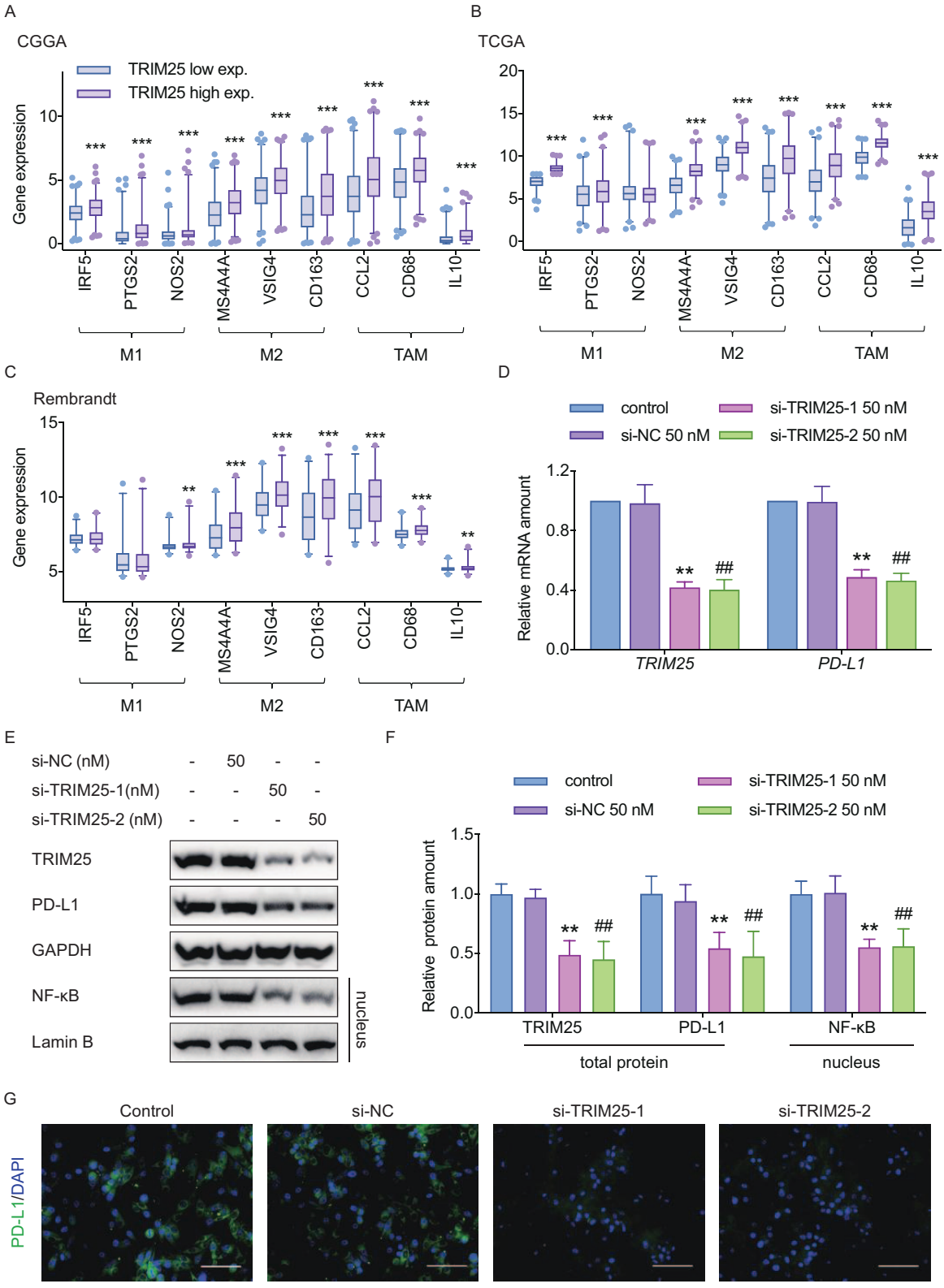

Figure 6. The distribution of macrophages (M1, M2, and TAMs) polarization markers in TRIM25^{low} and TRIM25^{high} groups based on (A) CGGA, (B) TCGA, and (C) REMBRANDT data sets. (D) After treating with si-TRIM25 in U87 cells, real time-PCR was used to detect the mRNA levels of TRIM25 and PD-L1. (E) The protein expression of TRIM25, nuclear NF-κB, and PD-L1 in si-TRIM25-treated U87 cells was measured by immunoblotting. GAPDH and Lamin B served as loading control of total protein and nucleus samples, respectively. (F) Gray statistics of these proteins were displayed in the histogram. Data were represented as mean value ± SD of three independent experiments. (G) Immunofluorescent staining of PD-L1 in si-TRIM25-treated U87 cells (scale bar: 100 μm). (A color version of this figure is available in the online journal.)
$^{**}P < 0.01$; $^{***}P < 0.001$ versus TRIM25^{low} group (a to c).
$^{**}P < 0.01$; $^{\#\#}P < 0.01$ indicated significant differences between the control group and si-TRIM25-1 or si-TRIM25-2 groups (d and f).

TRIM25 ablation attenuated the survival and growth of glioma cells

Except associated with tumor immunosuppression, NF-κB is also regarded as a key regulator of tumor cell survival. Combined with the previous bioinformatics results, we conducted a series of experiments to verify that TRIM25 knockdown can induce apoptosis and repress the proliferation of glioma cells. After treating with si-TRIM25, CCK-8 and clone formation assays denoted that TRIM25 depletion suppressed the growth and proliferation of U87 cells (Figure 7(A) and (B)). Numerous proliferation marker genes (including *PCNA*, *XIAP*, *CCNB1*, *CCND1*) were downregulated in si-TRIM25-treated U87 cells, which suggested that TRIM25 positively controlled cell proliferation (Figure 7(C)). Following the same treatment as described above, the protein expression of cleaved PARP and Caspase-3 was also obviously elevated, triggering the pro-apoptotic program in U87 cells. Since NF-κB inhibition can stimulate mitochondria-mediated apoptosis,[20,21] we isolated the mitochondrial fractions of U87 cells and observed a reduction of cytochrome c, while its elevated release to the cytoplasm was detected (Figure 7(D) to (F)). Flow cytometry analysis revealed that increased apoptotic rates were observed in si-TRIM25 groups compared to the control group (Figure 7(G)). Furthermore, we applied JC-1 assay to examine MMP in si-TRIM25-treated U87 cells. Collapsed MMP was detected after TRIM25 ablation as indicated by the decreased ratio of red/green fluorescence intensity (Figure 7(H)). In agreement with these findings, silencing TRIM25 can also suppress TNF-α stimulated the upregulation of NF-κB downstream genes, including *COX2*, *IL-8*, and *c-IAP1*, which served as an important modulators of cell survival and apoptosis (Supplementary Figure 1(B)). These results indicated that TRIM25 inhibition can induce U87 cell apoptosis by regulating NF-κB signaling pathway and exhibited potential in treating gliomas.

Discussion

Gliomas with extremely high mortality rates represent the most common primary CNS neoplasms, distinguished by the rapid proliferation and extensive invasion among brain tissues.[22] Currently, treatment options for gliomas are still scarce. Some patients are also under risk of tumor recurrence even with aggressive therapy. Exploring novel therapeutic targets and developing corresponding drugs may blaze a trail of improving glioma patients' prognoses. TRIM25 has been declared to accelerate cell growth and proliferation in numerous cancer types. However, its regulatory role in gliomas remains unclear. To verify the effects of TRIM25 on gliomas and explore its underlying mechanisms, a battery of analyses and experiments was conducted. We identified TRIM25 as a potential therapeutic target for gliomas, based on the following observations: (1) TRIM25 over-expression was observed in glioma patients; (2) high TRIM25 expression is a poor prognostic factor for glioma patients; (3) high TRIM25 expression contributed to immune suppression of gliomas via NF-κB/PD-L1 axis; (4) TRIM25 knockdown resulted in mitochondrial dysfunction and promoted apoptosis of glioma cells.

Further bioinformatic analyses identified that TRIM25 was mainly enriched in immune-relevant biological processes and signaling pathways. Among them, PD-L1 is upregulated in multiple tumor types and responsible for tumor immune escape. However, many PD-L1 positive tumors are not susceptible to ICB therapy, exhibiting significant heterogeneity. Although TLX over-expression[15] and PTEN deficiency[23] have been determined to mediate PD-L1 expression, it is still attractive to uncover its novel regulatory mechanisms. This study demonstrated a positive association between the expression of TRIM25 and PD-L1. The transcription of PD-L1 can be induced by NF-κB activation[24] and TRIM25 positively regulates the NF-κB pathway. Combined with our results that depleting TRIM25 downregulated PD-L1, it is speculated that the TRIM25/NF-κB/PD-L1 axis regulated the glioma microenvironment (GME) and benefited for immunotherapy. GME comprises multiple non-cancerous cell types, including fibroblasts, pericytes, immune, and endothelial cells,[25] which provide a communication platform for their dynamic interactions. Glioma cells also secrete various chemokines and cytokines, which attract immune cells to infiltrate.[26] On this platform, immune cells create a distinctive tumor niche and accelerate tumor growth, leading to treatment failure. As the majority of immune cells in glioma, macrophages have been implicated in tumor angiogenesis and metastasis, which depletion therapies result in a survival advantage.[27] Novel immune-oncology strategies were anchored in intervening TAMs and revised their conversion to the pro-tumoral M2 phenotype. Activated M2 macrophages that secrete anti-inflammatory cytokines and attract T regulatory cells (Tregs) can block antitumor immunoreaction and facilitate immune tolerance.[28] Meanwhile, PD-1 was reportedly distributed in not only activated T cells but also TAMs. Over-expression of PD-1 on TAMs can impair their phagocytosis, while tumorous PD-L1 removal can rescue TAMs' function and overcome ICB resistance.[29] This study showed that upregulation of TRIM25 attracted more M2 macrophages infiltrating and was accompanied by elevated expression levels of their markers. Therefore, TRIM25 may affect macrophage infiltration by regulating PD-L1 and reducing tumor burden. However, this study only focused on the regulatory effects of TRIM25 on tumorous PD-L1 expression. Since PD-L1 can be also detected in TAMs apart from tumor cells,[30] further investigations are still required to elucidate whether TRIM25 can modulate PD-L1 expression in TAMs and their polarization.

Otherwise, we observed that TRIM25 knockdown can induce apoptosis in glioma cells based on bioinformatics analyses and *in vitro* experiments. TRIM25 is an important cancer-associated E3 ubiquitin ligase that recognizes ubiquitin and ISG15 and transfers them to its substrates.[31] TRIM25 has been reported to promote the poly-ubiquitination of TRAF2 and subsequently potentiate NF-κB signaling.[18] As an important linker between inflammation and tumor, NF-κB constitutively activated in various tumor types and the corresponding microenvironment can result in over-proliferation, apoptosis suppression, and angiogenesis.[32] Herein, we identified that TRIM25 ablation triggered mitochondria-related apoptotic programs through inhibiting NF-κB, showing potent antitumor activity. Another investigation demonstrated that TRIM25 disrupted the poly-ubiquitination of p53 while promoting its sumoylation, which impaired

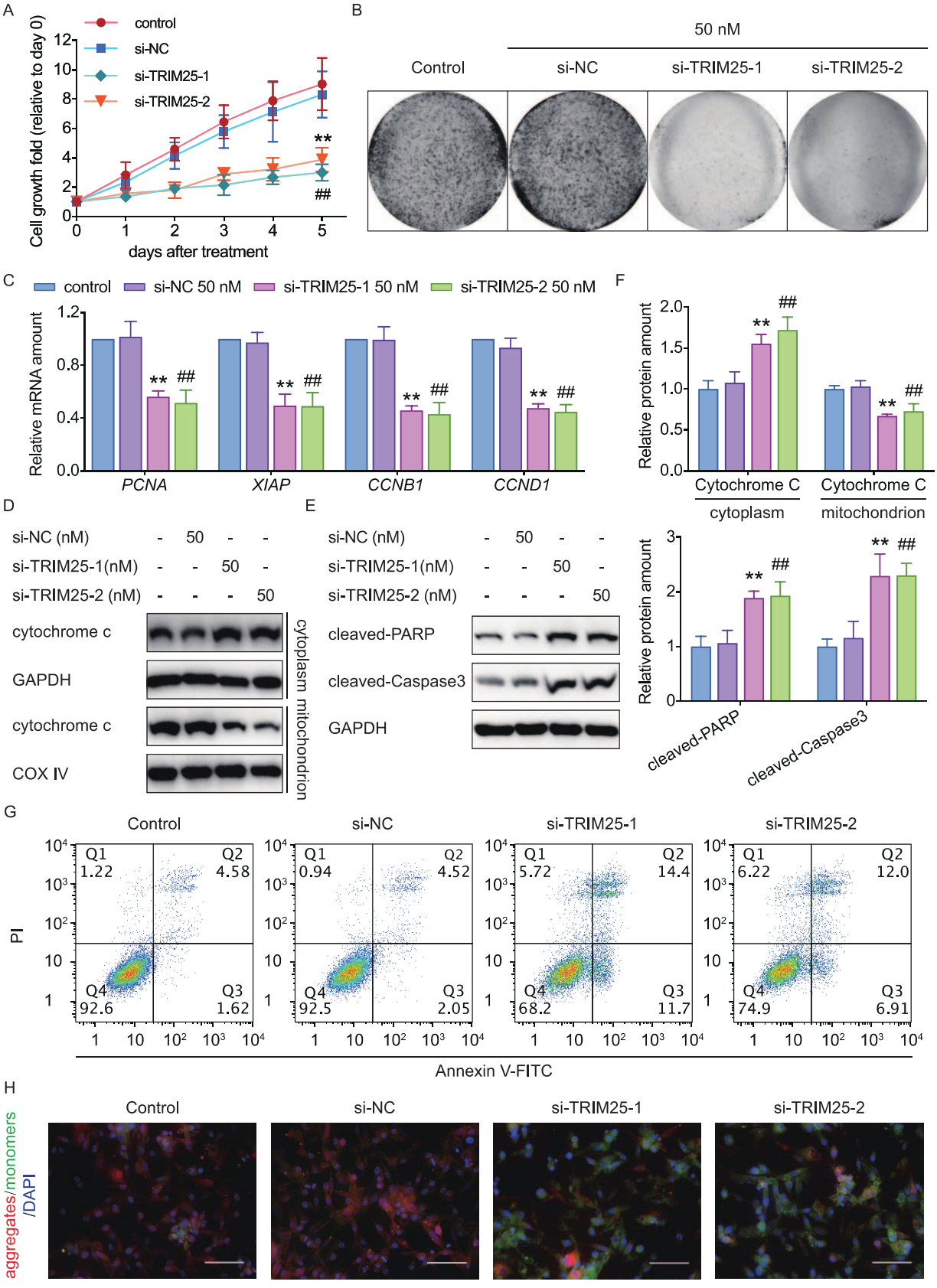

Figure 7. After treating with si-TRIM25, U87 cells continued to be cultured. CCK-8 (A) and clone formation assays (B) were performed. (C) mRNA levels of proliferation markers (*PCNA*, *XIAP*, *CCNB1*, *CCND1*) in si-TRIM25-treated U87 cells. (D) The mitochondrial and cytoplasmic fractions of U87 cells were isolated, and the distribution of cytochrome c was analyzed by immunoblotting. (E) The protein levels of cleaved PARP and Caspase-3 were detected by immunoblotting in U87 cells following the same treatment. (F) Gray statistics of the above proteins were displayed in the histogram. Data were represented as mean value ± SD of three independent experiments. (G) Flow cytometric analysis of si-TRIM25-induced apoptosis in U87 cells was measured by Annexin V-FITC/PI double staining. (H) U87 cells were treated with si-TRIM25 for the indicated time and subsequently stained with JC-1. MMPs were detected by fluorescent microscopy. (A color version of this figure is available in the online journal.) $^{**}P < 0.01$; $^{\#\#}P < 0.01$ indicated significant differences between the control group and si-TRIM25-1 or si-TRIM25-2 groups.

p53 activity and functioned to accelerate cell proliferation.[7,33] TRIM25 also served as an ER stress regulator and degraded Keap1 through the ubiquitin-proteasome pathway, which activated Nrf2, and finally consolidated antioxidant defense. TRIM25 depletion significantly increased the mRNA level of GRP78 and deteriorated ER stress.[34] TRIM25 can post-transcriptionally regulate the levels of tumor-associated proteins (such as NF-κB, p53, and Keap1) mainly through its E3 ligase activity and facilitate tumor cell survival under stress. Whether there are other regulatory mechanisms of TRIM25 on tumorigenesis and malignant progression remains to be further confirmed by subsequent studies.

In summary, we clarified that tumorous TRIM25 has predictive value for the poor survival of gliomas, which facilitated immunosuppression and inhibited mitochondria-mediated apoptosis via the NF-κB signaling pathway, indicating a potential prognostic and therapeutic role of TRIM25 in gliomas.

AUTHORS' CONTRIBUTIONS

Y.S. and M.G. designed the study. D.L. and M.G. mainly acquired and analyzed data of this study. M.G. completed the related experiments. M.G., D.L., and Y.S. wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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