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Clinical characterization and immunosuppressive regulation of CD161 (KLRB1) in glioma through 916 samples

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Abstract

Background: Glioblastoma is a paradigm of cancer-associated immunosuppression, limiting the effects of immunotherapeutic strategies. Thus, identifying the molecular mechanisms underlying immune surveillance evasion is critical. Recently, the preferential expression of inhibitory natural killer (NK) cell receptor CD161 on glioma-infiltrating cytotoxic T cells was identified. Focusing on the molecularly annotated, large-scale clinical samples from different ethnic origins, the data presented here provide evidence of this immune modulator's essential roles in brain tumor biology.

Methods: Retrospective RNA-seq data analysis was conducted in a cohort of 313 patients with glioma in the Chinese Glioma Genome Atlas (CGGA) database and 603 patients in The Cancer Genome Atlas (TCGA) database. In addition, single-cell sequencing data from seven surgical specimens of glioblastoma patients and a model in which patient-derived glioma stem cells were cocultured with peripheral lymphocytes, were used to analyze the molecular evolution process during gliomagenesis.

Results: CD161 was enriched in high-grade gliomas and isocitrate dehydrogenase (IDH)-wildtype glioma. CD161 acted as a potential biomarker for the mesenchymal subtype of glioma and an independent prognostic factor for the overall survival (OS) of patients with glioma. In addition, CD161 played an essential role in inhibiting the cytotoxicity of T cells in glioma patients. During the process of gliomagenesis, the expression of CD161 on different lymphocytes dynamically evolved.

Di Wang and Wenhua Fan contributed equally to this work.

Conclusion: The expression of CD161 was closely related to the pathology and molecular pathology of glioma. Meanwhile, CD161 promoted the progression and evolution of gliomas through its unique effect on T cell dysfunction. Thus, CD161 is a promising novel target for immunotherapeutic strategies in glioma treatment.

KEYWORDS
CD161, glioma, immunotherapy, neuro-immunity, prognosis, T cell evolution

1 | INTRODUCTION

Glioma is the most common and lethal brain tumor in adults.[1] According to the revised 2016 world health organization (WHO) classification of central nervous system tumors, glioblastoma (WHO IV) accounts for about 50% of glioma cases.[2] Most diffuse gliomas have shown resistance to all current standard therapy interventions, including surgery, radiotherapy, and systemic chemotherapy. Although patients with glioma typically undergo all the traditional therapies, most eventually relapse.[3] Therefore, novel and curative treatment strategies are urgently needed.

Many cancers are successfully treated with immunotherapy.[4] Antibodies targeting inhibitory immune checkpoints and chimeric antigen receptor (CAR) T cell therapy are arguably the most significant advances in cancer treatment.[5] However, although these therapies made considerable success in melanoma, lung cancer, renal cell carcinoma, and Hodgkin's lymphoma,[6] there are no reliable randomized clinical trials to provide evidence for distinct advantages of such immune-based approaches in treating patients with glioblastomas.[7] Furthermore, the unique immunosuppressive microenvironments and T cell depletion are presumed to dampen immunotherapies in glioblastomas significantly.[8] Thus, more research on the mechanisms underlying the immunosuppression of glioma-associated T cells, dissecting intervention points to heat up immunological cold tumors, is urgently needed to improve outcomes of neuro-oncological immunotherapies.

Killer cell lectin-like receptor subfamily B member 1 (KLRB1), which encodes the inhibitory T cell receptor called CD161, was identified in glioma-infiltrating cytotoxic T cells.[9] In vitro and in vivo experiments showed that CD161 plays an essential role in inhibiting the cytotoxicity and cytokine secretion of glioma-infiltrating T cells.[9,10] Thus, CD161 is expected to be a novel potential immunotherapeutic target in diffuse gliomas.

Currently, there are no comprehensive reports on CD161 in patients with glioma. Using the Chinese Glioma Genome Atlas (CGGA)[11] and The Cancer Genome Atlas (TCGA), we analyzed the CD161 expression profile in different gliomas and further explored whether CD161 expression was associated with clinicopathological characteristics and survival in patients with glioma. Furthermore, we analyzed the dynamic alteration of CD161 expression in different glioma subgroups infiltrating lymphocytes at a single-cell level during gliomagenesis. Our results provided a basis for investigating CD161 in the current and future design of immunotherapies against glioma.

2 | METHODS

2.1 | Patients and samples

The collection of samples and clinical information in this study was approved by the Institutional Review Board (IRB) of the Beijing Tiantan Hospital, and written informed consent was obtained from each patient. At least two neuropathologists diagnosed each sample in this study as diffuse glioma (WHO II-IV). The detection of isocitrate dehydrogenase (IDH) mutation, 1p/19q codeletion, and O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation of samples was completed at the Molecular Pathology Diagnostic Center of Beijing Neurosurgical Institute. The patients were followed up every 3 months by phone calls or clinical visits. All patients' clinical and follow-up information in this study is available on the CGGA website (http://www.cgga.org.cn/). In addition, the clinical and follow-up data of the validation database were downloaded from a public database (https://tcgadata.nci.nih.gov/tcga/tcgaDownload.jsp).

2.2 | Transcriptome sequencing

Surgically removed tumor samples were quickly immersed in liquid nitrogen for storage. Only models with a pathological diagnosis of diffuse glioma were used for transcriptome sequencing. Total RNA was extracted using the RNAperp Pure Tissue Kit (TIANGEN) following the manufacturer's instructions. Transcriptome sequencing data of glioma samples were generated using the Illumina Hiseq platform.

2.3 | Functional enrichment analysis

The most relevant genes of CD161, or a characteristic gene list of the cell cluster, were uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID, v6.8). The official gene symbol was selected as an identifier, and *Homo sapiens* was selected as species. Finally, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis enrichment results were obtained. The top five results in ascending order of *P*-value ($P < .05$) were displayed in this study.

2.4 | Gene set variation analysis (GSVA)

The gene list of immune processes was obtained from the AmiGO 2 portal (http://amigo.geneontology.org/amigo). The functional enrichment score of each glioblastoma multiforme (GBM) sample was calculated using the given package (R environment) under default parameters. The heatmap of the enrichment results was drawn with the *pheatmap* package (R environment). The correlation between CD161 and immune processes was determined by Pearson correlation analysis.

2.5 | Single-cell sequencing

Single-cell RNA-seq libraries of the GBM samples were constructed following the STRT-seq protocol that was previously described. The libraries were sequenced on the Illumina platform (HiSeq 4000). Single-cell RNA-seq libraries of coculture modes were constructed following the 10× Genomics protocol that was previously described. The libraries were sequenced on the Illumina platform (HiSeq 2000) to generate 150-bp paired-end reads.

2.6 | Cell clustering

The Seurat package performed cell clustering in GBM patients and coculture models (version 4.0). Standard preprocessing workflow of single-cell sequencing results was performed as previously reported. The results of cell clustering have been shown in a previous study. Cell clusters with high CD45 expression were defined as lymphocytes and extracted for further analysis in this study.

2.7 | Similarity analysis of cell clusters

The similarity of cell clusters was defined by the count of the same characteristic genes in different clusters. The higher the number of the same genes, the higher the similarity of cell clusters. The results of similarity analysis were displayed in a Sankey Diagram. The percentage of the same genes of each cluster was also used to define the similarity of clusters. These results were displayed by heatmap.

2.8 | Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA intensity was assessed using a 2100 Bioanalyzer (Agilent Technologies). The ABI 7500 Real-time PCR System analyzed expression levels of target genes. Transcript statistical analysis levels of the GAPDH gene were used for normalization. The relative mRNA expression levels of target genes were calculated by the comparative CT method.

Statistical analyses and visualization were performed in R (version 4.0.0) and IBM SPSS Statistics (version 25.0). An unpaired *t* test tested the significance of the difference between the two groups. One-way ANOVA tested the significance of the difference between more than two groups. The significance of the correlation between the two groups was tested by Pearson correlation analysis. Kaplan-Meier curves evaluated the prognostic value. A log-rank test tested the significance of the prognostic value. A *P*-value of $<.05$ was considered statistically significant.

2.9 | Transient transfection of T cells with siRNA and plasmid

One negative control, three KLRB1(CD161) siRNAs (Si-RNA-371, Si-RNA-503, Si-RNA-608), and one overexpression plasmid (Phs-avc-1442) were purchased from Beijing Syngentech Co., LTD. The T cells were then transfected with siRNA according to the instructions. The growth medium was changed 12 hours after transfection. Forty-eight hours after transfection, qRT-PCR and Western blotting were used to evaluate silencing and overexpression efficiency.

2.10 | Lactate dehydrogenase (LDH) cell cytotoxicity assay and ELISA

Cells were seeded in 96-well plates at a density of 2×10^3 cells per well and starved for 24 hours. They were then treated with indicated concentration of T cells for 24 hours. The RPMI-1640 medium containing 10% Fetal Bovine Serum and 1 ng/mL IL-7, 1 ng/mL IL-15 was used in the co-culture model. The LDH cytotoxicity assay was performed according to the manufacturer's guidelines (Donjido Molecular Technologies INC.). Interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) concentrations were determined according to the manufacturer's guidelines (Quanticyto). Both experiments were repeated three times.

2.11 | Apoptosis assay by Annexin V-FITC and propidium iodide (PI) double staining

The apoptotic and necrotic cell distributions were analyzed by the Annexin V/PI apoptosis detection kit (4A Biotech). Briefly, 1×10^5 U87 or LN229 cells were cocultured with 2×10^5 transfected T cells in six-well plates for 72 hours, respectively. Cells were harvested, washed once with phosphate buffer saline, and resuspended in 200 μL binding buffer. Then, 5 μL of Annexin V-FITC and 10 μL of PI were added, and cells were incubated in the dark at room temperature for 15 minutes. Next, 400 μL of binding buffer was added to each sample, and the cells were immediately analyzed by flow cytometry in 1 hour. The data analysis was performed with FlowJo 10.6.2 software. Positioning of quadrants on Annexin V/PI plots was performed to distinguish intact (Annexin V−/PI−), early apoptotic

(Annexin V+ /PI−), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V−/PI+) cells.

3 | RESULTS

3.1 | Expression of CD161 is enriched in gliomas carrying molecular markers predictive of malignancy

Patients with varying expression levels of CD161 showed distinct patterns of clinical and pathological characteristics. Increases in CD161, MGMT promoter methylation status, 1p/19q codeletion status, IDH mutation status, WHO grade, and histology diagnosis showed asymmetric distributions in the CGGA and TCGA datasets (Figure 1A,B). Comparative analysis was conducted with different groups of these samples. In the CGGA database, CD161 was highly enriched in higher-grade gliomas (Figure 1C) and IDH-wildtype gliomas (Figure 1D). Moreover, samples without 1p/19q codeletion showed a higher expression of CD161 (Figure 1E). The above results were validated in the TCGA database (Figure 1G-I). CD161 was highly expressed in samples without MGMT promoter methylation in the TCGA database (Figure 1J). The expression of CD161 had the same trend in the CGGA database, although this difference was not statistically significant (Figure 1F). Overall, these results show that gliomas with higher malignancy are enriched for CD161.

As a marker of natural killer (NK) and T cells, CD161 was considered to be enriched in high-grade gliomas, resulting in a statistical correlation between CD161 and molecular pathological factors of glioma. To test this hypothesis, immunohistochemical staining of clinical samples of gliomas of all grades was performed. The results showed that both CD8+ T cells and CD161+ cells were enriched in higher-grade gliomas (Figure S1). In short, CD161 was enriched in immune cells, and immune cells were enriched in high-grade gliomas. High-grade gliomas have specific molecular pathological characteristics.

3.2 | CD161 is a potential biomarker for the mesenchymal subtype of gliomas

Transcriptional subtyping of gliomas is a globally accepted method of molecular diagnostics.[12] We explored the distribution of CD161 in different transcriptome subtypes defined by the TCGA network. Compared with other TCGA transcriptome subtypes, CD161 was significantly enriched in the mesenchymal subtype ($P < .0001$) in the CGGA and TCGA databases (Figure 2A,C). The receiver-operating characteristic (ROC) curve was performed to evaluate the expression specificity of CD161 in the mesenchymal subtype of gliomas. As expected, the area under the curve (AUC) was up to 80.6% in the CGGA database ($P < .0001$) (Figure 2B), while the AUC equaled 86.8% in the TCGA database ($P < .0001$) (Figure 2D). These results suggested that CD161 was explicitly enriched in the mesenchymal subtype and may function as a potential mesenchymal biomarker of gliomas. As mesenchymal glioblastomas are particularly aggressive, these results supported our prior identified association of CD161 to the high malignancy of brain tumors.

3.3 | Tumor cell CD161 regulates the immune response in a T cell receptor–dependent manner

To explore the biological functions related to CD161, the genes most related to CD161 were screened out by Pearson correlation analysis ($|R| > .5$, $P < .05$) in the TCGA and CGGA databases. GO and KEGG analysis based on the above gene sets were performed. In the CGGA database, biological processes most related to CD161 include immune response, regulation of immune response, and T cell costimulation (Figure 3A). Moreover, CD161's most related cellular components were an external side of the plasma membrane (Figure 3B). The molecular functions were MHC class II receptor activity and peptide antigen binding (Figure 3C). CD161's most related signaling pathway is a T cell receptor signaling pathway, including cell adhesion molecules and antigen processing and presentation (Figure 3D). The CD161-related biological processes, cellular components, molecular functions, and signaling pathway in the TCGA database were similar to those in the CGGA database (Figure 3E-H). These findings suggested that CD161 on glioma cells probably plays an essential role in immune response and regulation of the disease, namely as a receptor inhibitor in the T cell microenvironment.

3.4 | Tumor cell CD161 is negatively correlated with T cell–mediated immune response to tumor cells

During the process of immunogenic cancer cell death, the activation of lymphocytes, including NK, T, and B cells, and the release of chemokines and cytokines mount the apoptosis or necroptosis of cancer cells.[13] Therefore, we tested the effects of CD161 activation on immune pathways and cytokine signatures. Gene set variation analysis (GSVA) in the CGGA and TCGA databases was used to determine the enrichment score of the immune process. The correlation analysis between the enrichment score and the expression of CD161 showed that CD161 expression was positively correlated with most immune functions, except for the T cell–mediated immune response to tumor cells in the CGGA database (Figure 4A). The above results were validated in the TCGA database (Figure 4B). These results suggested a linkage between CD161 and the suppressed activity of T cells in the glioma immune microenvironment.

3.5 | Activation of established cancer immune checkpoint inhibitors and inflammatory dysregulations are positively correlated with high CD161 expression

CD161 was found to play a role in tumor immunosuppression; therefore, we investigated the relationship between CD161 and

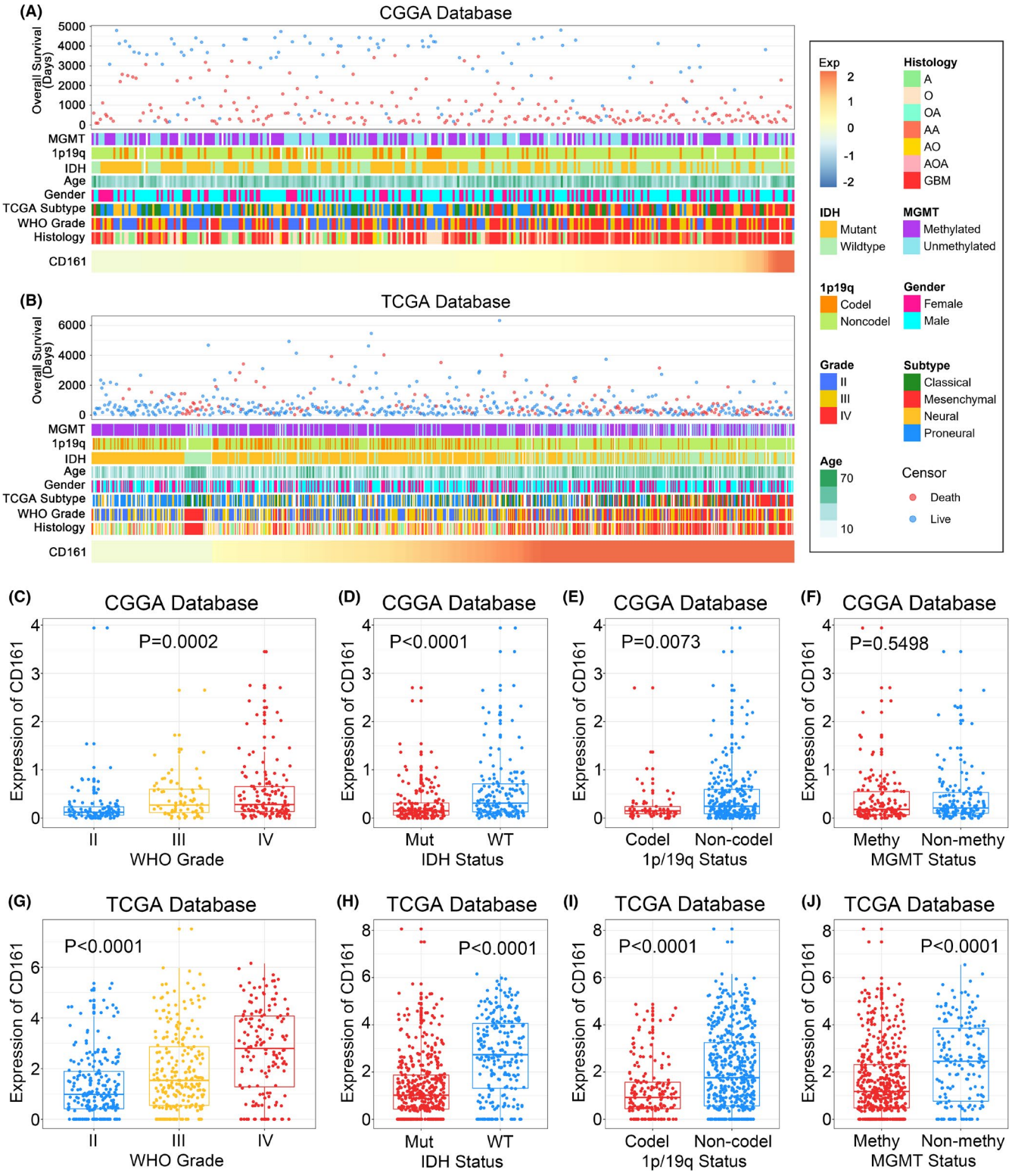

FIGURE 1 Association between CD161 and clinicopathological characteristics of gliomas. A, The landscape of CD161-related clinicopathological features of gliomas in the Chinese Glioma Genome Atlas (CGGA) database. B, The landscape of CD161-related clinicopathological features of gliomas in the The Cancer Genome Atlas (TCGA) database. C and G, CD161 was significantly increased in higher-grade gliomas in the CGGA and TCGA databases. The significance of the difference was tested by one-way ANOVA. D and H, CD161 was significantly increased in gliomas without isocitrate dehydrogenase (IDH) mutation in the CGGA and TCGA databases. The significance of the difference was tested with an unpaired *t* test. E and I, CD161 was significantly increased in gliomas without 1p/19q codeletion in the CGGA and TCGA databases. The significance of the difference was tested with an unpaired *t* test. F and J, CD161 was increased in the O^6-methylguanine-DNA methyltransferase (MGMT) promoter–unmethylated gliomas. This difference was statistically significant in the TCGA database, not in the CGGA database. The significance of the difference was tested using an unpaired *t* test

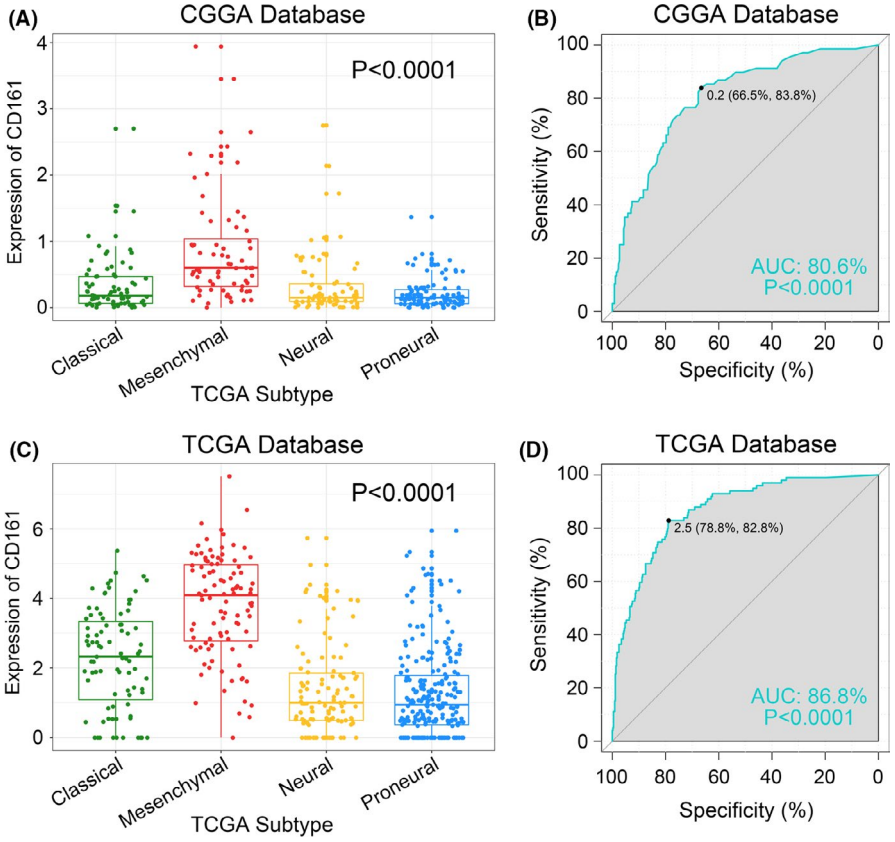

FIGURE 2 CD161 is specifically enriched in the mesenchymal subtype of gliomas. A and C, CD161 was enriched in the mesenchymal subtype of gliomas in the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases. The significance of the difference was tested by one-way ANOVA. B and D, The receiver-operating characteristic (ROC) curve showed the high-expression specificity of CD161 in the mesenchymal subtype of gliomas in the CGGA and TCGA databases. AUC, area under the curve

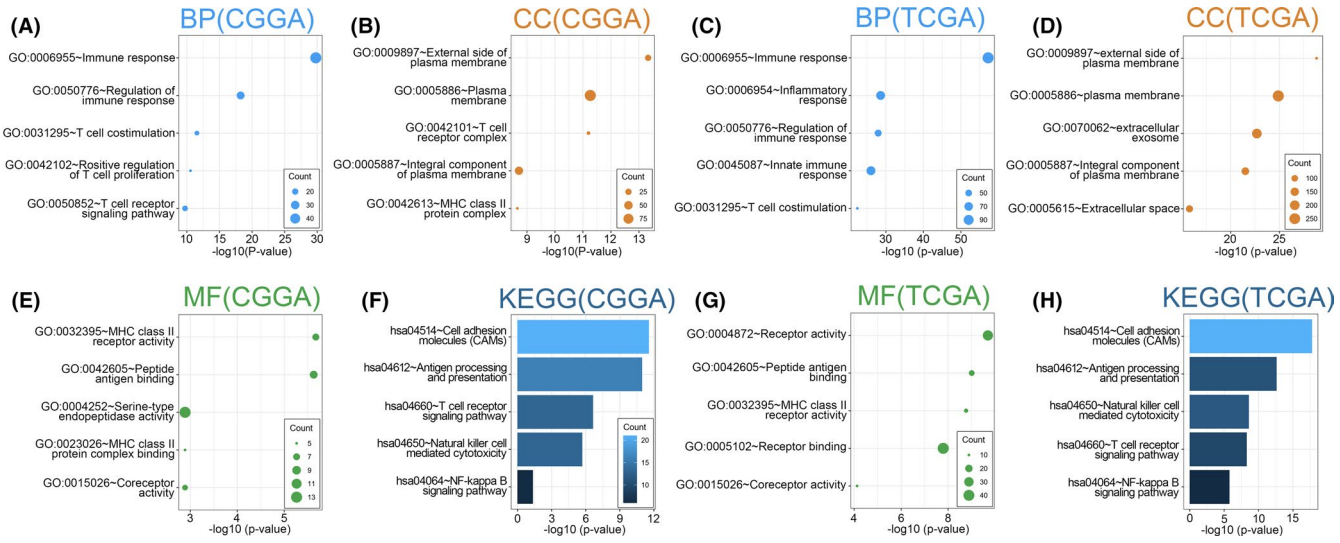

FIGURE 3 CD161 is closely associated with immune process regulation in gliomas. A-C, Biological processes (BP), cellular components (CC), and molecular functions are mostly related to CD161 in the Chinese Glioma Genome Atlas (CGGA) database. D, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of CD161 in the CGGA database. E-G, Biological processes (BP), cellular components (CC), and molecular functions are mostly related to CD161 in the CGGA database. H, KEGG pathway analysis of CD161 in the CGGA database

known inhibitory immune checkpoints, including HVEM, TIM-3, PD-1, PD-2, TIGIT, CD200R1, CTLA-4, and CD47 in the CGGA and TCGA databases (Figure 5A). CD161 showed a strong positive relationship with these inhibitory immune checkpoints, which lead to the suppression of immune response to gliomas. Moreover, we chose seven immune system–related metagene clusters as markers for immunological states,[14-16] including hemopoietic cell kinase (HCK), lymphocyte-specific kinase (LCK), major histocompatibility complex (MHC), signal transducer and activator of transcription 1/2 (STAT1/2), interferon, and IgG.[17,18] Corrgrams showed that CD161 and seven metagene clusters were significantly positively correlated in the CGGA and TCGA databases (Figure 5A). Furthermore, as shown in Figure 5B,C, the expression of CD161 exhibited incredibly positive correlations with most inflammatory

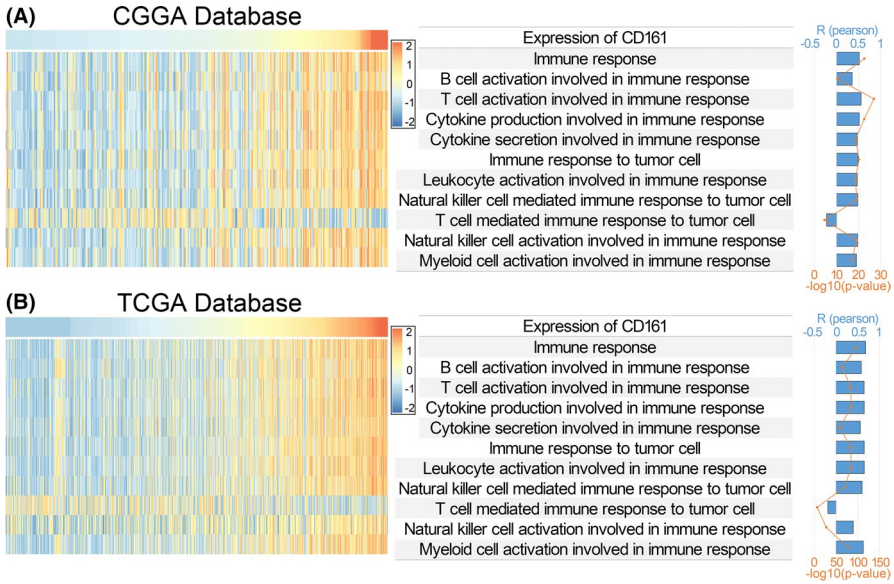

FIGURE 4 Correlation analysis between CD161 expression and immune function enrichment scores. A and B, The heatmap showed the expression of CD161 and the enrichment scores of immune functions of each patient in the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases. The samples were arranged in ascending order of the expression of CD161. The column graph and line graph on the right showed the *R*-value and *P*-value of the correlation analysis

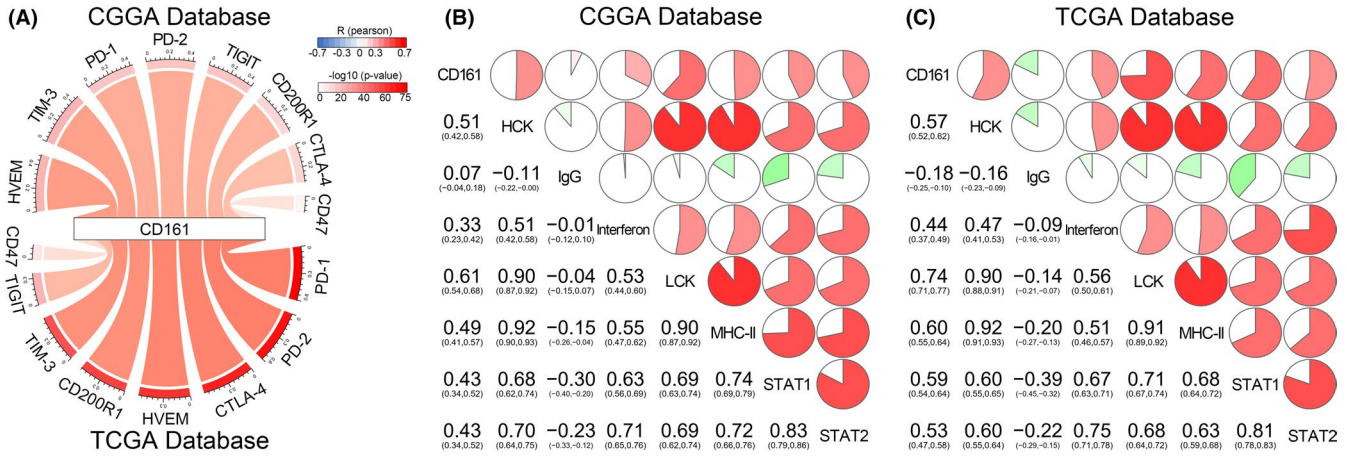

FIGURE 5 The correlation between CD161 expression and T cell immunity and inflammatory activities in the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases. A, Pearson correlation between CD161 and inhibitory immune checkpoints. The width of the band represented the *R*-value. The color of the band represented the *P*-value. The correlation was tested by Pearson correlation analysis. B and C, Correlation matrix of CD161 and inflammatory-related metagenes. The bottom left showed the correlation coefficient. The correlation coefficients were demonstrated as the proportion of the pie charts. The red parts represented a positive correlation, while the green parts represented a negative correlation. The correlation was tested by Pearson correlation analysis

responses of gliomas. These results supported the hypothesis that CD161 and other inhibitory immune checkpoints may be upregulated due to the interaction between tumor cells and T cells, contributing to T cell depletion and the glioma's inhibitory immune microenvironment.[19]

3.6 | CD161 expressed on different subtypes of T cells at distinct stages of glioblastoma genesis

In the previous study, we constructed a coculture model to simulate the early stage of glioblastoma genesis.[20] Lymphocytes from the single-cell sequencing data of the coculture model and glioblastoma samples were extracted for subsequent analysis (Figure S2A,G). In the coculture model, lymphocytes can be divided into seven clusters (Figure 6A). According to the expression of cell markers, clusters 1, 3, and 7 were defined as CD4+ T cells, and clusters 2, 4, 5, and 6 were defined as CD8+ T cells (Figure S2). CD161 was enriched in some CD4+ T cells (cluster 1) and CD8+ T cells (cluster 5). We also found that cytotoxicity genes (GZMA, GZMB, PRF1, and GNLY) were highly expressed in both cell clusters (Figure 6B and Figure S3A-C). In this current study of glioblastoma patient samples, lymphocytes were divided into six clusters based on their unique expression characteristics (Figure 6C). According to the expression of cell markers, clusters I, II, III, IV, and V were defined as CD4+ T cells, and cluster VI was defined as CD8+ T cells (Figure S3). CD161 was enriched in CD8+ T cells (cluster VI). Cytotoxicity genes (GZMA, GZMB, PRF1, and GNLY)

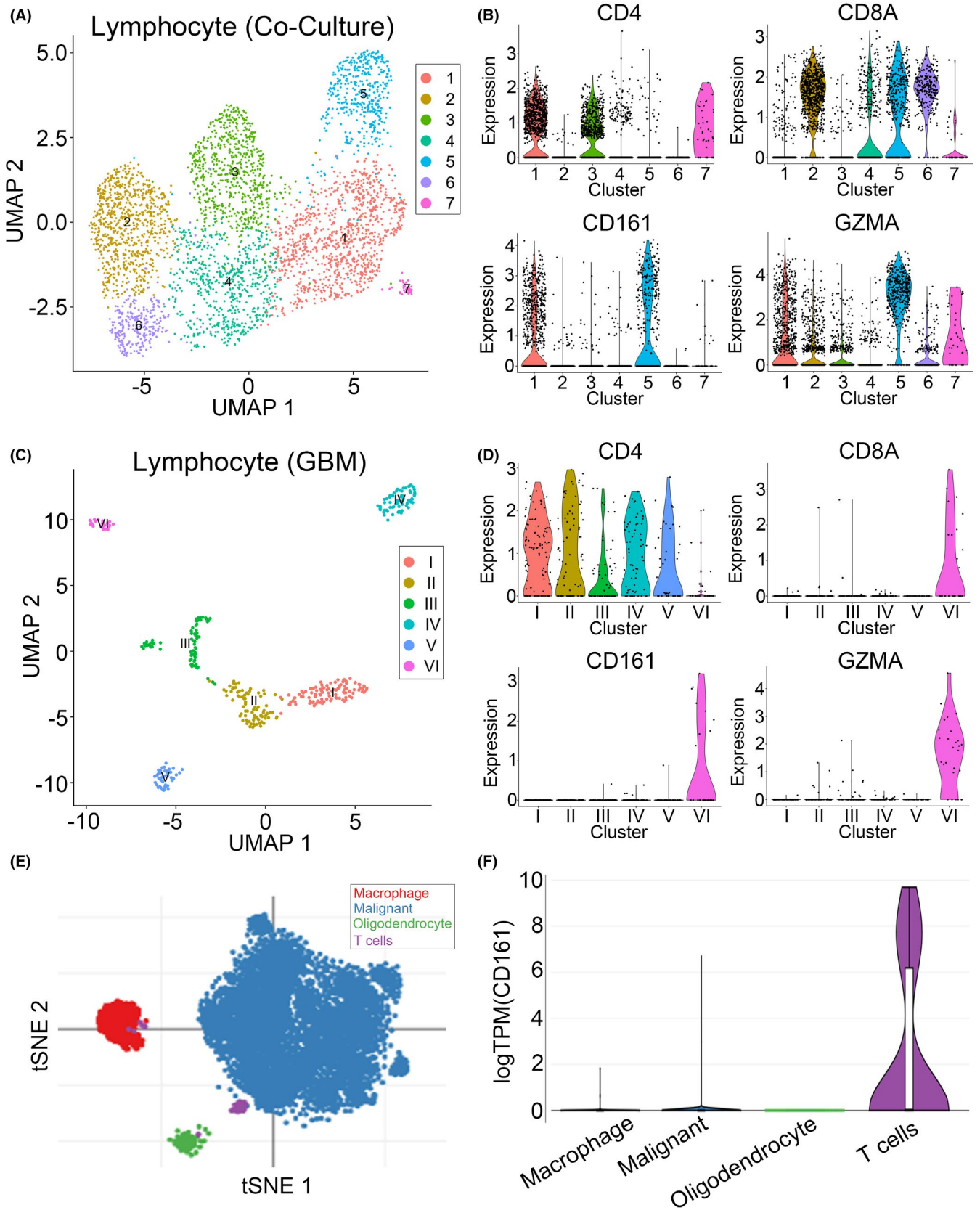

FIGURE 6 The expression pattern of CD161 in lymphocytes. A, Subtypes of lymphocytes from the coculture model. B, The expression of CD161 and other T cell markers in different subtypes of lymphocytes from the coculture model. C, Subtypes of lymphocytes from the surgical samples of glioblastoma patients. D, The expression of CD161 and other T cell markers in different subtypes of lymphocytes from the surgical specimens of glioblastoma multiforme (GBM) patients. E, Subtypes of cells from isocitrate dehydrogenase (IDH)-wildtype glioblastomas in a public database (GSE131928). F, The expression of CD161 in different cells from IDH-wildtype glioblastomas in a public database (GSE131928)

were also highly expressed in this cluster of cells (Figure 6D and Figure S3D-F). Considering that the differences in cell cycle stages may influence the transcriptome features, we quantified cell cycle stages for each cell cluster using supervised analyses with known cell cycle stage–specific markers. As shown in Figure S3, there were no significant differences in cell cycle stages among these lymphocyte clusters. Moreover, the enrichment of CD161 in T cells was further validated with the public single-cell sequencing database (GSE131928). Cells from these glioblastoma samples were mainly grouped into four clusters (Figure 6E). Consistently, CD161 was enriched in T cells rather than macrophages and oligodendrocytes (Figure 6F).

3.7 | Expression of CD161 dynamically evolved during the GBM genesis

In both CD4+ and CD8+ lymphocytes in the coculture model, we found a cluster of cells with a high concentration of CD161. However, only one corresponding cluster was identified in glioma patient specimens. This indicated the dynamic evolution of CD161 expression in tumor cells and the immune microenvironment during glioblastoma genesis. The transcriptome similarity in the lymphocyte clusters from both the coculture model and the patient tissue group was compared to explore this phenomenon further. As shown in Figure 7, each of the CD4+ and CD8+ lymphocyte clusters from the glioblastoma samples possessed a considerable quantity of genes coexpressed in the lymphocyte clusters from the coculture model. Especially, cluster VI in GBM samples, which highly expressed CD161, shared most coexpressed genes with cluster 5 (CD161 highly expressed). The proportion of coexpressed genes is shown in Figure S5. However, the count and proportion of coexpressed genes of cluster 1 from the coculture model and clusters in glioblastoma samples were unremarkable (Figure S5). These results suggested that CD8+ T cells with high levels of CD161 expression are transcriptomically the most similar cells between both models. Further exploration of whether the cell clusters with high CD161 expression in GBM samples are derived from the increasing proportion of CD161 high-expressing cell clusters or other CD161 low-expressing clusters is needed.

3.8 | The expression of CD161 on CD8+ lymphocytes gradually increased during glioblastoma genesis

The biological functions of different CD161-expressing lymphocyte clusters were examined to determine the impact of CD161 expression on the biological functions of immune cells and the possible origin of CD161–high-expressing clusters in glioblastoma patients' samples. CD4+ and CD8+ lymphocyte clusters with high CD161 expression showed similar biological functions and pathway activation (Figure 8A,C and Figures S6A,C, S7A,C). The biological functions and pathway activation of CD4+ and CD8+ lymphocyte clusters, dependent on their CD161 expression status (high vs. low vs. absence), differ significantly (Figure 8 and Figures S6, S7). CD161–high-expressing clusters in glioblastoma patient samples and CD161–low-expressing clusters in the coculture model shared similar biological functions and pathway activation (Figure 8D,E and Figures S6D,E, S7D,E). The above results indicated that the expression of CD161 significantly affects the biological functions of T cells, and the expression of CD161 on CD8+ lymphocytes gradually increases during glioblastoma genesis.

3.9 | CD161 is an independent prognostic factor for the overall survival of patients with glioma

To explore the prognostic prediction value of CD161 in glioma patients, we conducted Kaplan-Meier and Cox proportional hazard model analyses based on the CGGA and TCGA databases. Patients with higher expression of CD161 had significantly shorter overall survival (median survival: 484 days) compared with those with lower CD161 expression (median survival: 1812 days) in the CGGA database (Figure 9A). In addition, the prognostic value of CD161 was verified in the TCGA database (Figure 9B). CD161 expression was a prognostic factor, independent of known prognostic factors, in the Cox regression analysis, including WHO grade, age at diagnosis, IDH mutation, 1p/19q codeletion, and MGMT promoter methylation. These findings revealed that CD161 is an independent prognostic factor in the CGGA and TCGA databases (Tables 1 and 2).

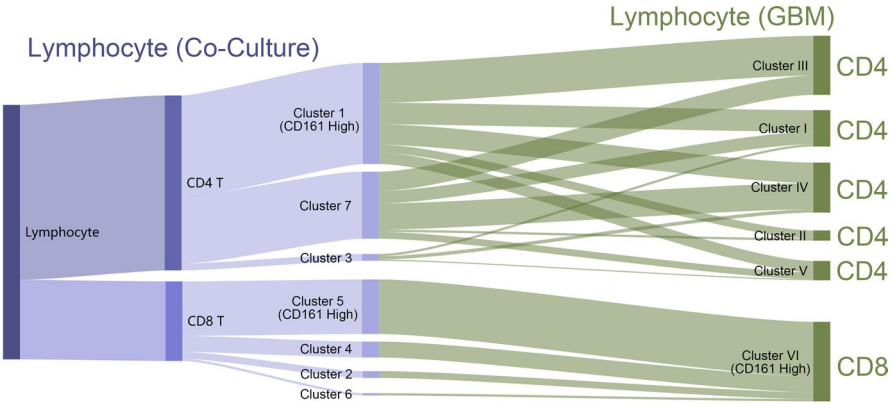

FIGURE 7 Similarity analysis of T cell subgroups between the coculture model and GBM surgical samples. The width of the band represented the counts of genes simultaneously expressed in different subtypes of lymphocytes

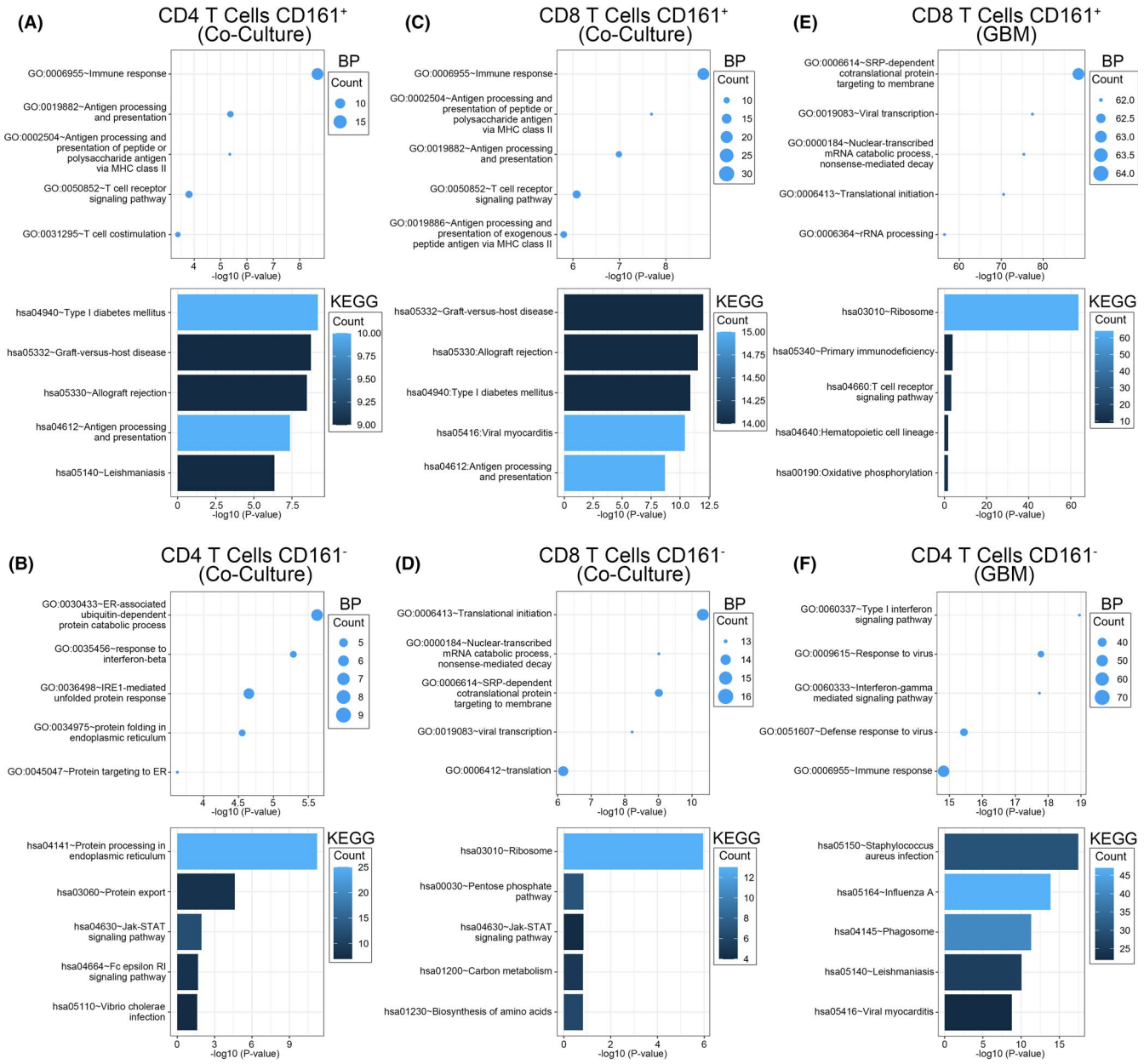

FIGURE 8 Biological processes and pathway activation of lymphocyte subtypes from the coculture model and glioblastoma multiforme (GBM) surgical samples. A and B, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of CD4+ lymphocyte subtypes with high or low expression of CD161 from the coculture model. C and D, GO and KEGG analysis of CD8+ lymphocyte subtypes with high or low expression of CD161 from the coculture model. E and F, GO and KEGG analysis of CD4+ and CD8+ lymphocyte subtypes from the GBM surgical specimens

3.10 | CD161 expression level significantly affected the cytotoxicity and cytokine secretion of T cells

As a supposed immune checkpoint, how CD161 affects the survival of tumor cells by regulating the function of T cells does need to be experimentally verified. The expression of CD161 was knocked down and overexpressed in patient-derived T cells by a plasmid-based transfection system. Three different siRNAs (SI-3), an overexpression (OE), and a negative control (NC) plasmid were transfected into CD3&CD27-activated T cells, respectively. CD161 expression of the transfected cells was detected by real-time PCR and Western blot test. The results showed that CD161 expression was significantly suppressed in the SI-3 group and overexpressed in the OE group compared with the NC group (Figure 10A,B).

Coculture models of T cells and two glioblastoma cell lines (U87 and LN229) were used to test the effect of CD161 expression on T cell functions. After a 48-hour coculture, tumor cell cytotoxicity of T cells was determined by LDH activity in culture medium.

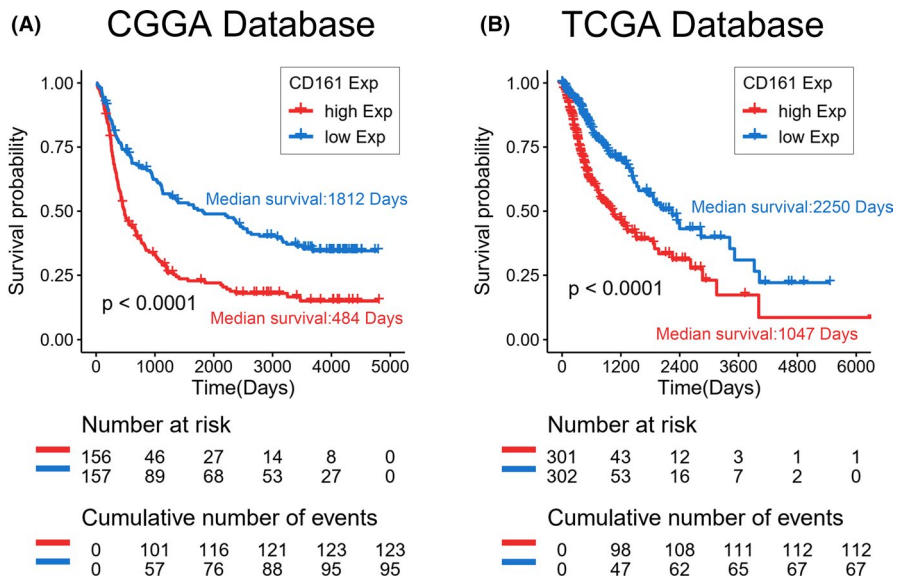

FIGURE 9 Kaplan-Meier analysis of CD161 expression in the Chinese Glioma Genome Atlas (CGGA) and The Cancer Genome Atlas (TCGA) databases. A and B, The cutoff of the group is the median expression of CD161. The significance of the prognostic value was tested by a log-rank test

TABLE 1 Univariate and multivariate analysis of prognostic parameters in the Chinese Glioma Genome Atlas (CGGA) database overall survival (OS)

	Univariate analysis		Multivariate analysis	
Variable	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
CD161 expression	2.229 (1.679-2.959)	2.93e-8	1.455 (1.082-1.957)	.013
WHO grade	2.911 (2.416-3.507)	2.70e-29	2.361 (1.926-2.894)	1.34e-16
Age	1.033 (1.020-1.046)	2.66e-7	1.013 (1.001-1.025)	.039
IDH status	0.355 (0.269-0.468)	2.62e-13	1.188 (0.847-1.666)	.319
1p/19q Codel	0.170 (0.104-0.277)	1.25e-12	0.268 (0.159-0.451)	7.55e-7
MGMT status	0.830 (0.632-1.089)	.178		

Abbreviations: CI, confidence interval; HR, hazard ratio; IDH, isocitrate dehydrogenase; WHO, world health organization.

TABLE 2 Univariate and multivariate analysis of prognostic parameters in The Cancer Genome Atlas (TCGA) database overall survival (OS)

	Univariate analysis		Multivariate analysis	
Variable	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
CD161 expression	2.823 (2.089-3.813)	1.36e-11	1.476 (1.034-2.106)	.032
WHO grade	4.901 (3.822-6.285)	5.17e-36	1.830 (1.327-2.524)	2.26e-4
Age	1.075 (1.063-1.088)	4.28e-34	1.056 (1.040-1.072)	2.90e-12
IDH status	0.091 (0.064-0.129)	2.19e-40	0.384 (0.216-0.681)	.001
1p/19q Codel	0.220 (0.130-0.375)	2.31e-8	0.484 (0.261-0.898)	.021
MGMT status	0.312 (0.225-0.433)	2.96e-12	0.914 (0.623-1.340)	.644

Abbreviations: CI, confidence interval; HR, hazard ratio; IDH, isocitrate dehydrogenase; WHO, world health organization.

For the LDH assay, killing percentages of tumor cells were calculated as: (experimental LDH activity)/(maximum LDH activity). The results demonstrated that the tumor cell cytotoxicity of CD161–knocked down T cells was significantly increased, while the cytotoxicity of CD161-overexpressed T cells was significantly decreased (Figure 10C,D).

Previous studies revealed the critical roles of IFN-γ and TNF-α in the apoptosis of glioblastoma cell lines induced by CD8+ and CD4+ T cells. Therefore, the concentration of IFN-γ and TNF-α in the supernatant of the coculture models were tested. The results showed that the concentration of IFN-γ and TNF-α were significantly increased in the coculture models of CD161–knocked down T cells

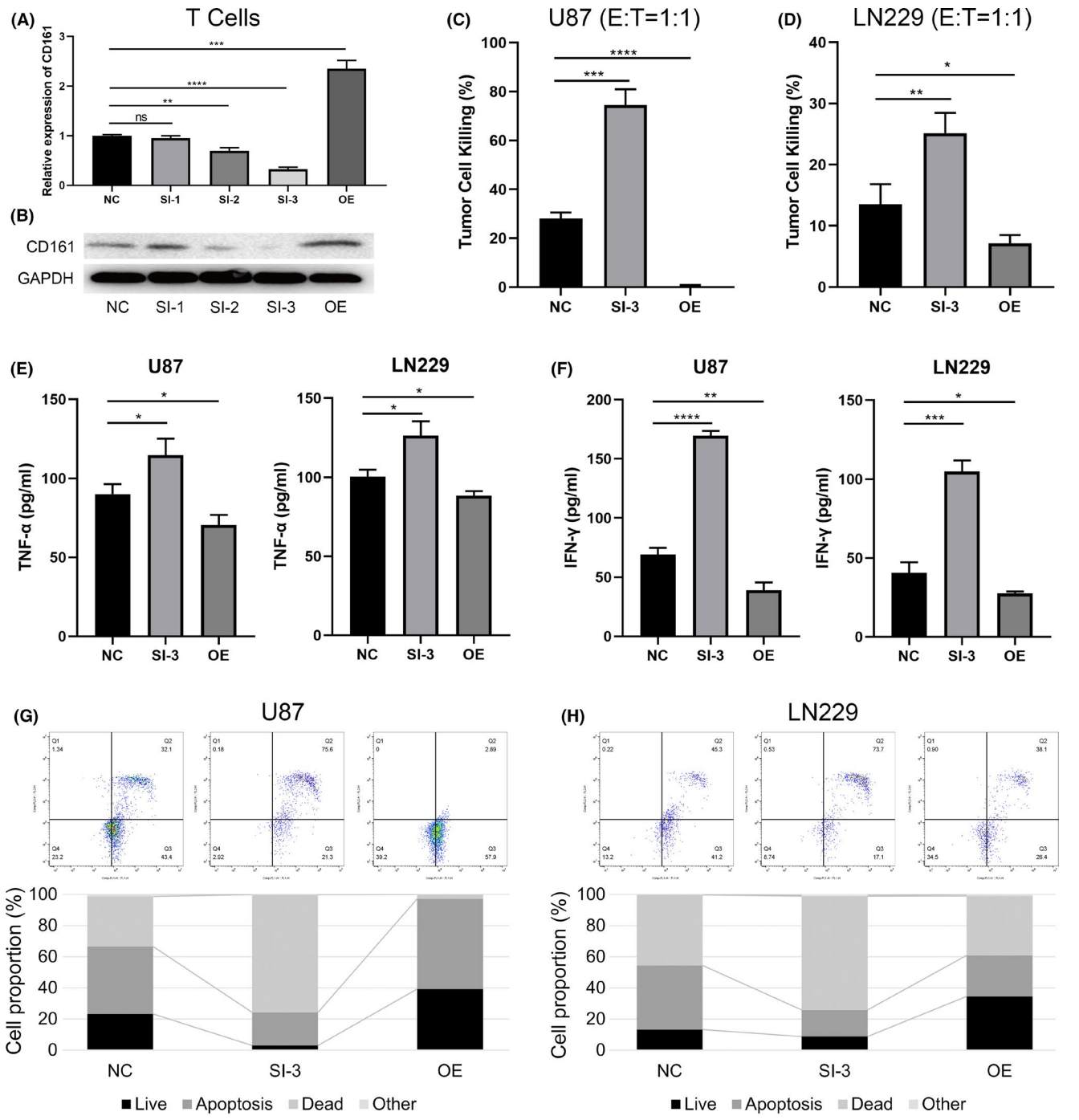

FIGURE 10 Negative correlation between CD161 expression level and cytotoxicity of T cells. A, qPCR analysis of KLRB1 (CD161) expression in T cells after transfecting siRNA and plasmid. B, SDS-PAGE analysis of KLRB1(CD161) expression in T cells after transfecting siRNA and plasmid. C and D, Lactate dehydrogenase (LDH) assay in U87 and LN229 cell lines cocultured with transfected T cells. E:T, effective cells to tumor cells ratio. E and F, Tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) in the supernatant of the coculture model were detected with ELISA. G and H, Apoptosis analysis using Annexin V/PI. Q4: viable cells (Annexin V−/PI−), Q3: early apoptosis (Annexin V+/PI−), Q2: late apoptosis (Annexin V+/PI+), and Q1: necrosis (Annexin V−/PI+). Data given as means ± standard deviations (SDs) (*means under *t* test, $P < .05$, **means under *t* test, $P < .01$, ***means under *t* test, $P < .001$)

and glioma cell lines. Overexpression of CD161 in T cells leads to the opposite results (Figure 10E,F). In addition, the cell viability of tumor cells in the coculture model was also tested. The proportion of death tumor cells was significantly increased in the coculture models of CD161–knocked down T cells and glioma cell lines (Figure 10G,H).

The above results indicated that CD161 expression level significantly affected cytotoxicity and secretion level of T cells and glioblastoma cell apoptosis induced by cytotoxic T cells in vitro. Collectively, CD161 affects the survival of patients by regulating the cytotoxicity of T cells on tumor cells.

4 | DISCUSSION

Glioma is the most common and deadly cancer of the central nervous system.[21] Conventional therapies, typically including surgical resection, radiotherapy, and temozolomide chemotherapy, have not shown satisfactory outcomes in patients with glioblastomas[22] overall. Although many patients with various cancers have benefited from immunotherapy, the clinical effects of immunotherapy in glioblastoma patients are still disappointing. It is postulated that the main contributor to this barrier is the unique inhibitory immune microenvironment in glioblastoma,[23] also known as immunological cold disease. Thus, illustrating the molecular mechanisms of tumor-induced immunosuppression and developing new therapeutics that exploit those are imperative for clinical treatment and success.

Recently, CD161, an NK cell receptor encoded by the KLRB1 gene, was identified as the prominent receptor expressed on glioma-infiltrating T cells leading to their exhaustion/inhibition in the tumor cell microenvironment.[9,10,24] This incremental study demonstrated that the inactivation of CD161 on T cells enhanced the antitumor activity of tumor-infiltrating T cells in vitro and in vivo, at least in part due to the increased secretion of inflammatory cytokines.[9] The data presented in this study validate the accumulating evidence that CD161 is indeed a new potential immunotherapeutic target in the context of neuro-oncology, especially glioblastoma. Our research contributes to the knowledge of brain tumor cell–expressed CD161 using large-scale clinical samples derived from different ethnic backgrounds and state-of-the-art molecular pathology technologies.

This study analyzed the expression levels of CD161 in distinct pathological types of gliomas based on mRNA-seq data of 916 glioma patients from the CGGA and TCGA databases. Our results elucidated CD161 enrichment in more malignant subtypes of gliomas, especially in the mesenchymal phenotype. As expected, CD161 mainly functioned as a T cell receptor. Consistent with previous research, it was negatively associated with the T cell–mediated immune response to tumor cells based on GO, functional enrichment, and GSVA. Moreover, we found that CD161 had the same expression pattern and inflammatory response effect as inhibitory immune checkpoints like PD-1, CTLA-4, and TIM-3.

Furthermore, data from functional studies of glioma stem cells cocultured with healthy donor–derived immune cells revealed that CD161 is enriched in cytotoxic CD4+ and CD8+ T cells interacting with the glioma cells. Meanwhile, in patient-matched tissue specimens from glioma resections, we found CD161 was only enriched in cytotoxic CD8+ T cells in matched glioma specimens, and the expression level increased during gliomagenesis. These results indicated that CD161 persistently existed in cytotoxic CD8+ T cells. Thus, CD161 seems to regulate the biological functions of CD8+ T cells through its dynamic expression during the longitudinal course of gliomagenesis. In addition, to validate these computational data, we constructed a coculture model with patient-derived T cells and glioblastoma cell lines. Our experimental data indicated that CD161 expression level was closely related to the cytotoxicity of T cells and apoptosis of glioblastoma cells induced by T cells.

In addition to supporting the concept that CD161 serves as a marker for reduced tumor cell immunity and inhibited tumor immune microenvironment in glioma, CD161 seems to possess different roles in different tumor types. Kunduri et al were able to improve the potential of CAR T cell therapy against pancreatic cancer using high CD161 expression on CD8+ T cells as a positive selection marker, as opposed to using similarly engineered bulk-PBMC or CD161-negative CD8+ T cells as therapies (https://pubmed.ncbi.nlm.nih.gov/33952672/). CD161 activation on immune cells in lung cancer models indicated increased immune cell activation and tumor defense. Moreover, total KLRB1 expression in lung cancer tissue specimens predicts patient survival, independent of the extent of tumor cell invasion (https://pubmed.ncbi.nlm.nih.gov/29721382/). In contrast, a recent high-profile study on recurrent hepatocellular tumors revealed that elevated CD161 expression in the tumor immune ecosystem was hypothesized to be responsible for mediating cancer cell immune evasion (https://pubmed.ncbi.nlm.nih.gov/33357445/). Our present work reveals CD161 as a tumor antigen associated with the molecular properties of the disease, and its expression aligns with the clinical findings from glioma patients. We confirm its role as a prominent receptor in the glioma immune ecosystem and indicate its dynamic regulation during gliomagenesis.

However, there are still some limitations in this study. We did not develop a new inhibitor of CD161. Therefore, it remains a question how to treat patients with gliomas targeting the CLEC2D-CD161 pathway effectively. Also, the dynamic evolution of CD161 in glioma-infiltrating lymphocytes was based on the comparison between cell coculture models and patent samples. We hope to validate these results in a new animal model with continuity, which could undoubtedly improve the precision of this study.

Furthermore, future studies could investigate whether standard therapies cause glioma cells—or the corresponding invading immune cells—to respond with altered CD161 activation. This may indicate unrecognized secondary effects of the current clinical management of these patients. For example, a recent study on immune checkpoint–treated melanoma patients revealed that ipilimumab therapy causes severe alterations in the expression of CD161 on innate lymphocytes (https://pubmed.ncbi.nlm.nih.gov/33810032/).

Traditionally, glioblastoma was identified with a highly immunosuppressive tumor microenvironment. However, immune checkpoint inhibitors targeting PD-1 and CTLA 4 did not show perspective potentials in clinical trials. CD161, a newly found inhibitory immune checkpoint in glioblastoma, highly expressed in many subtypes of tumor-infiltrating lymphocytes, may contribute to a new immunotherapy strategy in the future.

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DISCLOSURE

No potential conflict of interest was disclosed.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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