

The prognostic value of tumor-associated macrophages in glioma patients

Yang Shen

Department of cerebrovascular disease, Huizhou First People's Hospital

Dingke Zheng

Department of cerebrovascular disease, Huizhou First People's Hospital

Dong Hu

Department of cerebrovascular disease, Huizhou First People's Hospital

Baoxin Ma

Department of cerebrovascular disease, Huizhou First People's Hospital

Chunsheng Cai

Department of cerebrovascular disease, Huizhou First People's Hospital

Wei Chen

Department of cerebrovascular disease, Huizhou First People's Hospital

Jiahao Zeng

Department of cerebrovascular disease, Huizhou First People's Hospital

Junran Luo

Department of cerebrovascular disease, Huizhou First People's Hospital

Dan Xiao

Department of cerebrovascular disease, Huizhou First People's Hospital

Yao Zhao

Department of cerebrovascular disease, Huizhou First People's Hospital

Zhiyan Wu

Department of cerebrovascular disease, Huizhou First People's Hospital

Guojie Jing

Department of cerebrovascular disease, Huizhou First People's Hospital

yituan xie (✉ xyt39410@163.com)

Department of cerebrovascular disease, Huizhou First People's Hospital

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Abstract

Glioma is a complex tumor composed of both neoplastic and non-neoplastic cells, including tumor-infiltrating leukocytes (TILs), and each cell type contributes to tumor formation and malignant progression. Among TILs, tumor-associated macrophages (TAMs) are of great importance and play a key role in the immune response to cancer. In this study, 22 types of adaptive and innate TILs were evaluated in gliomas. TAMs, which account for 38.7% of all these cells, are the most abundant immune infiltrates in the tumor microenvironment (TME). In addition, we observed different immune cell patterns in low-grade glioma (LGG) and glioblastoma (GBM). Our research indicated that there was a connection between TILs, and 13 of 22 TILs were significantly associated with patient outcomes. Finally, the prognosis and diagnostic value of TAMs were revealed using Kaplan-Meier analysis. We identified the optimal cut-off point of TAMs at an infiltrating level of 0.47 to predict patient prognosis, with a median overall survival (OS) of 448 days in patients with higher TAM infiltration levels and 2660 days in patients with lower TAM infiltration levels. These findings provide a new idea for glioma to regulate tumor-specific immunity, clarify the potential effects of TAMs on disease pathology, and provide a theoretical basis for immune intervention treatment of gliomas.

1. Introduction

Gliomas, including glioblastoma and low-grade gliomas, are the most common intracranial malignant brain tumors ^[1]. Although there is a high chance of recurrence and unsatisfactory life expectancy, neurosurgical resection, adjuvant chemotherapy, and radiation therapy have significant potential to reduce discomfort and extend survival time. It is noteworthy that practically all glioma patients survive less than one year, and only approximately 5% of patients do so after undergoing the best course of treatment ^[2]. The therapeutic effects on glioma have not improved significantly despite years of study and investigation ^[3]. Consequently, there is a significant need to develop more focused and effective therapeutic strategies.

In recent years, the TME has gradually become a focus of tumor research. Likely many solid tumors, gliomas are composed of highly heterogeneous cell populations ^[4] interacting in complex networks, in which the heterogeneity of tumor cells is considered as a key challenge for therapeutic effectiveness. Moreover, the interplay between tumor and immune cells in the TME is crucial for tumorigenesis. Furthermore, cancer cells must overcome the immune surveillance barrier to achieve their final escape. While TAMs are a vital component of the TME, increasing evidence suggests that TAMs play a critical role in fueling cancer progression by inducing tumor cell proliferation and invasion ^[5-8]. Nevertheless, despite their being an essential constituent element of the immune system, the functions of TAMs in tumor immune evasion are largely unknown. With the emergence of immunotherapy, it is increasingly important to further understand the mechanism of action of TAMs in tumors and use the immune system to fight cancer ^[9]. A current primary approach is immune checkpoint blockade (ICB), which has exhibited significant clinical success. Despite promising outcomes, their curative effect was apparent in only a

subset of patients^[10, 11], not including glioma patients. However, immunotherapy is limited mainly by the dysfunction of tumor-induced immune cells and the collapse of immune T cells^[12]. Intriguingly, recent research has revealed that TAMs play a critical role in the immune checkpoint axis. Functionally, macrophages can remove the anti PD1 antibody from the surface of T cells and weaken the anti-tumor effect of T cells^[13]. In addition, macrophages can express PD1 protein on their cell surface, which weakens their phagocytic function^[14]. Thus, TAM inhibition is very important in cancer treatment, improving the success rate of ICB treatment. Moreover, numerous previous studies have shown that increased TAMs densities correlate with shorter overall survival in many human cancer types, including breast cancer, lung cancer, and Hodgkin's lymphoma^[15-17]. However, the exact relationship between TAM infiltration, glioma grade, tumor progression, and patient outcome has yet to be established and merits further investigation.

For the past few years in the field of glioma research, advances in tumor related molecular research benefit from the availability and reliability of the public dataset of The Cancer Genome Atlas (TCGA)^[18-23], which contains molecular features, including gene expression and DNA methylation related to prognosis^[24-26]. For example, mutations in isocitrate dehydrogenase genes 1 and 2 (IDH1/IDH2) can distinguish between different subsets of the hypermethylation phenotype (G-CIMP) and glioblastoma (GBM) with good prognosis^[24, 27]. In contrast, deletion of the IDH mutation in low-grade glioma (LGG) indicates poor prognosis^[19, 23]. This example fully illustrates TCGA's crucial role in translating scientific payoffs from bench to bedside and implies a better application potential.

In this study, we assembled the TCGA dataset, comprising 529 LGG patients and 169 GBM patients, and analyzed the infiltration of 22 types of immune cells in gliomas using the CIBERSORT method. First, we investigated TAM infiltration levels in different glioma patients. Subsequently, the association between TAM density and glioma grade was evaluated, and the correlation between different inflammatory immune cellular constituents in the glioma microenvironment was constructed to identify their interrelationships. Survival and prognostic analyses were performed to elucidate the prognostic value of TAMs in patients with gliomas. Herein, we provide valuable information that may assist in the investigation of potential candidate cell populations for prognosis and drug targets for gliomas.

2. Materials And Methods

2.1. Data mining of TCGA cohort

The analyzed TCGA data for LGG, GBM gene expression profiles, and corresponding clinical features were downloaded from the Broad Institute TCGA Genome Data Analysis Center (<https://cancergenome.nih.gov>). A total of 698 samples, including 169 GBM and 529 LGG tumor samples, were collected.

2.2. Acquisition of tumor-infiltrating immune cells

The CIBERSORT method was used to calculate the relative abundance of various immune cells^[47]. Gene expression-based methodology was used to examine 22 immune cell phenotypes. These cell types included macrophages, B cells, T cells, dendritic cells, plasma cells, natural killer cells, and mast cells. RNA sequencing data from TCGA were used as the input for the deconvolution technique, which was then applied using the CIBERSORT program. The *P*-value cut-off was set to 0.05. The predicted immune cell-type fractions for each sample totaled 1.

2.3. Correlation analysis of macrophage markers (CD68) with immune molecules

We used the Gene Expression Analysis Interactive Analysis (GEPIA)^[48] database to analyze the correlation and prognosis between the expression of CD68 and other immune molecules. GEPIA provides a powerful capacity for analysis based on the sequencing data in TCGA and GTEx databases^[49], including correlation analysis, differential expression analysis, and patient survival analysis.

2.4. Statistical analysis

We used the Kaplan-Meier curves and log-rank test to analyze and evaluate associations between immune cell infiltration and the corresponding clinical follow-ups. The best critical value and a plot of the different Youden indices were constructed using ROC curve analysis. All statistical analyses were performed using R (version 3.5.2), SPSS 16.0, statistical software (SPSS, Chicago, IL, USA), and GraphPad Prism (GraphPad Software Inc., San Diego, CA). All experimental procedures were approved by the Department of Cerebrovascular Disease of Huizhou First People's Hospital (Huizhou, China).

3. Results

3.1. The degree of infiltration of different immune cells in glioma

We identified the degree of infiltration of several immune cell populations in the TME of glioma using data from 22 immune cell types by CIBERSORT analysis. A few important cell types associated with adaptive immunity are represented in this population, including plasma cells, activated memory CD4 T cells, T follicular helper cells (Tfh), memory B cells, naive B cells, resting memory CD4 T cells, naive CD4 T cells, regulatory T cells, CD8 T cells and gamma delta T (Tgd) cells. In addition, innate immunity-related cell types, including monocytes, neutrophils, resting natural killer (NK) cells, resting mast cells, eosinophils, activated mast cells, activated NK cells, and macrophages (M0-M2) were also included (Fig. 1A).

Tumor-associated macrophages (TAMs, M0-M2, 38.7%) were the most prevalent immune infiltrates in glioma, followed by monocytes (22.4%), resting memory CD4 T cells (13.0%), activated mast cells (6.8%), and activated NK cells (2.3%), according to the results of cellular characterization of the tumor-infiltrating immune cells (Fig. 1B). Furthermore, we found a correlation between glioma grade and immune cell

invasion levels. Our data suggest that specific types of immune cells may function as crucial regulators in the glioma immune microenvironment, since glioma, particularly GBM, is one of the most immunotherapy-resistant tumor types. We also examined inflammatory cell infiltration in LGG and GBM. The findings revealed that TAMs cells, monocytes, and resting memory CD4 T cells constituted majority of the invading inflammatory cells in LGG and GBM (Fig. 1C-D). Therefore, we focused on the pathophysiology of inflammatory cells in gliomas in TAMs in subsequent analyses and research. Thus, we investigated whether there were any differences in the degree of immune cell infiltration between LGG and GBM.

3.2. LGG and GBM specific immune cells infiltration patterns

The heatmap shows the immune cell infiltration status in LGG and GBM patients in TCGA dataset (Fig. 2A). We observed a significant difference in infiltration levels of different cell types between the two groups. The infiltration levels in gliomas showed in Fig. 2B, such as TAMs ($P < 0.001$), monocytes ($P < 0.001$), activated NK cells ($P < 0.001$), CD8 T cells ($P < 0.001$), eosinophils ($P < 0.001$), and neutrophils ($P < 0.001$) (Fig. 2B). In GBM, the proportion of TAMs was significantly higher than that in LGG. More specifically, TAMs were related to the degree of malignancy of glioma and drive the malignant progression of tumors. As a non-immuno-privileged organ, the immunologic features of the brain and subtypes of tumor-infiltrating lymphocytes have been investigated over the past decade. For example, studies have found that CD8+, CD4+ T cells and regulatory T cells play an important role in suppressing the tumor invasion and TAMs also play crucial roles in supporting tumor growth in GBM [9, 13]. However, the specific relationships between different TILs in gliomas are poorly understood and further investigation is needed.

A correlation analysis among different subtypes of tumor-infiltrating lymphocytes was applied to illustrate the nature of tumor-infiltrating immune cell interactions and offer some clues to elucidate the mechanisms of the co-evolution of tumor cells and their microenvironment. The results demonstrated that there was a strong negative correlation ($|r| > 0.5$) between M0 macrophages and monocytes, and the coefficient of association was -0.73 . Similarly, resting NK cells and activated NK cells, M2 macrophages, and activated mast cells also showed strong negative correlations, and their separate co-efficiencies of association ranged from -0.6 to -0.5 . Tregs and resting NK cells had a strong positive correlation, with a coefficient of association of 0.57 . These data suggest that the four pairs of cells may have the potential to influence each other in a specific manner. Furthermore, we found moderate correlations ($0.3 < |r| < 0.5$) between eosinophils and activated mast cells ($r = 0.49$), activated mast cells and activated NK cells ($r = 0.41$), resting mast cells and activated mast cells ($r = 0.41$), activated memory CD4⁺ cells and plasma cells ($r = 0.4$) and other paired cells subsets, implying there exists some regulatory mechanisms underlying these correlations (Fig. 2C). Furthermore, we characterized the prognostic landscapes of all 22 tumor-infiltrating immune cells in patients with glioma to identify cell types that are of great importance in tumor progression from a macroscopic perspective.

3.3. The prognostic significance of tumor-infiltrating immune cells in glioma

In gliomas, 13 of the 22 tumor-infiltrating immune cells were significantly associated with patient outcome in the Kaplan-Meier analysis (Fig. 3A-M). In the groups of eosinophils, macrophages (M0), activated mast cells, monocytes, activated NK cells, CD8⁺ T cells, and gamma delta T cells, the log-rank *P*-value was ≤ 0.001 . In the groups of plasma cells and activated memory CD4⁺ cells, $0.001 < \text{log-rank } P\text{-value} \leq 0.01$. In the groups of activated dendritic cells, macrophages (M1), macrophages (M2), and resting NK cells, $0.01 < \text{log-rank } P\text{-value} \leq 0.05$. We observed that high tumor-infiltrating levels displayed markedly longer survival in the groups of activated dendritic cells, eosinophils, activated mast cells, monocytes, activated NK cells, and plasma cells (Fig. 3A-F).

Low tumor-infiltrating levels displayed markedly longer survival in the groups of macrophages (M0), macrophages (M1), macrophages (M2), resting NK cells, activated memory CD4⁺ cells, CD8⁺ T cells, and gamma delta T cells (Fig. 3G-M). Intriguingly, the positive infiltrates of all three types of macrophages, M0, M1, and M2, could provide independent predictive factors for glioma. Generally, these macrophages represent different phenotypes: M0 (unstimulated phenotype), M1 (pro-inflammatory phenotype), and M2 (alternative phenotype). However, the current literature has reported that the current M0, M1, and M2 classification schemes are not absolute, but only constitute relative definitions when studying TAMs *in vivo*. Consequently, we investigated whether TAMs, which contain various phenotypes of macrophages and microglia, could serve as a better prognostic predictor of clinical outcomes in glioma.

3.4. Optimal cut-off points of TAM infiltrating levels in relation to patient outcome

To characterize the best critical value, we built a receiver operating characteristic (ROC) curve, area under the curve (AUC), and a plot of different Youden indices of the different levels of TAM infiltration. The maximum Youden index of 0.4722 was obtained for TAMs infiltration levels (Fig. 4B). The sensitivity, specificity, and AUC of this cut-off were 0.56, 0.8, 0.67, respectively (Fig. 4A). Therefore, the critical value was set to 0.47. The median OS was 448 days in patients with high TAM infiltration levels (> 0.47) and 2660 days in patients with low TAM infiltration levels (< 0.47). The difference between the two groups was statistically significant ($P < 0.0001$) (Fig. 4C).

Thus, TAMs proportion in TME is an independent prognostic factor in patients with glioma. Infiltration levels exceeding 0.47 of TAMs in the glioma microenvironment are related to a significant increase in poor outcome risk.

3.5. Correlation analysis of macrophage marker (CD68) with immune molecules

Immune regulatory molecules play an important role in tumor immunity. In particular, CD274, IDO1, LAG3, and PDCD1 proteins play important regulatory roles in glioma prognosis. Therefore, we further analyzed the correlations between macrophage-specific expression of CD68 and CD274, IDO1, LAG3, and PDCD1 genes. We found that the expression of CD68 was positively correlated with these immune molecules (Fig. 5A-D). Moreover, survival curve analysis demonstrated that patients with high expression of CD274, IDO1, LAG3, and PDCD1 genes had poor prognosis (Fig. 5E-H). Therefore, we further determined that TAM infiltration predicted poor prognosis in patients.

4. Discussion

The application of immunotherapy in clinical oncology has greatly improved the prognosis of many patients with malignant tumors. Immuno-targeted therapy using antibodies that block immune checkpoints, including cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD-1), is a proven effective treatment in a variety of solid tumors. Thus, immunotherapy has become the standard treatment for non-small cell lung cancer (NSCLC) and blood malignancies, including other solid tumors with advanced malignancies, regardless of their pathology [28]. Previous research has shown that the combination of traditional therapy and immunotherapy can further improve the remission rate of patients. However, the efficacy of this combination therapy remains limited, and more than 50% of tumor patients fail to achieve clinical remission, which may be due to the presence of highly heterogeneous and non-responsive lesions in these tumors [29]. For instance, because of the high heterogeneity and adaptive drug resistance of glioblastoma, less than 10% of patients responded to immunotherapy [30, 31]. At present, preclinical model data show that immunotherapy is a feasible method for GBM, but this has not been confirmed in clinical trials of GBM patients [32]. With the development of some clinical studies, the efficacy of immune checkpoint inhibitors in glioma has been preliminarily proven in clinical application [33–37]. However, a clinical trial found that nivolumab (a monoclonal antibody to PD-1) could not improve the overall survival rate of patients with GBM (clinical trial NCT02017717). Therefore, the third phase clinical trial in which nivolumab and bevacizumab (a monoclonal antibody to the growth factor VEGF-A), were used as recurrent GBM therapy, was also forced to stop. Therefore, there is an urgent need to determine the mechanism of resistance against the antitumor immune response in GBM. By analyzing the TCGA dataset, we determined the composition of the immune microenvironment of glioma, which 22 immune cell types and their infiltration levels were analyzed in different glioma patients. Furthermore, we revealed that TAMs are the most abundant immune infiltrates in gliomas.

TAMs in GBM are derived from either microglia or peripheral macrophages, which are myeloid cells that have recently become the focus of intense research [38]. In adults, the uninflamed CNS comprises almost exclusively tissue-resident microglia, whereas in the inflammatory setting, increased permeability of the blood-brain barrier, combined with upregulation of inflammatory chemokines in intracranial tumors, promotes the entry of monocytes to the brain from peripheral blood [38]. In return, M2 type TAMs promote tumorigenesis and progression via several mechanisms, including cancer stem cell support, genetic

instability sustaining, adaptive immunity inhibition, and the promotion of epithelial-to-mesenchymal transition [39].

In our study, we demonstrated broad connections between tumor-infiltrating immune cells in glioma, in which M0 macrophages and monocytes, M2 macrophages, and activated mast cells showed strong correlations, highlighting that a direct interaction between TAMs and other immune cells play an important role in glioma immune monitoring and tumor recurrence. While an increasing number of studies have found that the presence of TILs can serve as a prognostic indicator of clinical outcome [40, 41], including the prognostic accuracy of an 'immuno-score' based on intratumoral density of CD3⁺ and CD8⁺ T cells in colorectal cancer, the prognostic value of TILs in glioma is not consistent [42-46]. This may be attributed to the scarcity of lymphocytes in glioma [38]. However, according to our findings, TAMs constitute the most abundant immune infiltrate in gliomas. Considering the association of TAMs with clinical outcomes, we assumed that TAMs can predict clinical behavior. As expected, we found that the level of TAMs infiltration could be an independent predictive factor in glioma, and we further demonstrated that 0.47 could be chosen as the optimal cut-off point of the level of TAMs infiltration in relation to patient outcome with high sensitivity and specificity.

In conclusion, our study sheds new light on the importance of TAMs in evaluating glioma progression and provides clues for studying the mechanisms of immune resistance of the antitumor response in glioma. Moreover, our findings are valuable for guiding clinical practice in terms of individualized therapy for patients.

5. Conclusion

This study revealed 22 immune cell types in the glioma microenvironment; LGG and GBM had specific immune cell infiltration patterns. Additionally, different tumor-infiltrating immune cells have inextricable connections. In addition, 13 of all 22 tumor-infiltrating immune cells were significantly associated with patients' clinical outcomes, and TAMs proportion measurement in TME can serve as a valuable prognostic factor in patients with glioma using the optimal cut-off point of TAMs infiltration level of 0.47.

Declarations

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

AUTHOR CONTRIBUTIONS

Conception and study supervision: Y. X. Development of methodology and analyses: Y. S. Assisted analysis of data: D. Z, D. H, B. M, C. C, W. C. Prepared the manuscript and revision: Y. S, J. Z, J. L, D. X, Y. Z, Z. W, G. J and Y. X. All authors read and approved the final manuscript.

DATA ACCESSIBILITY

The data sets used and/or analyzed during the present study are available from the TCGA dataset.

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Figures

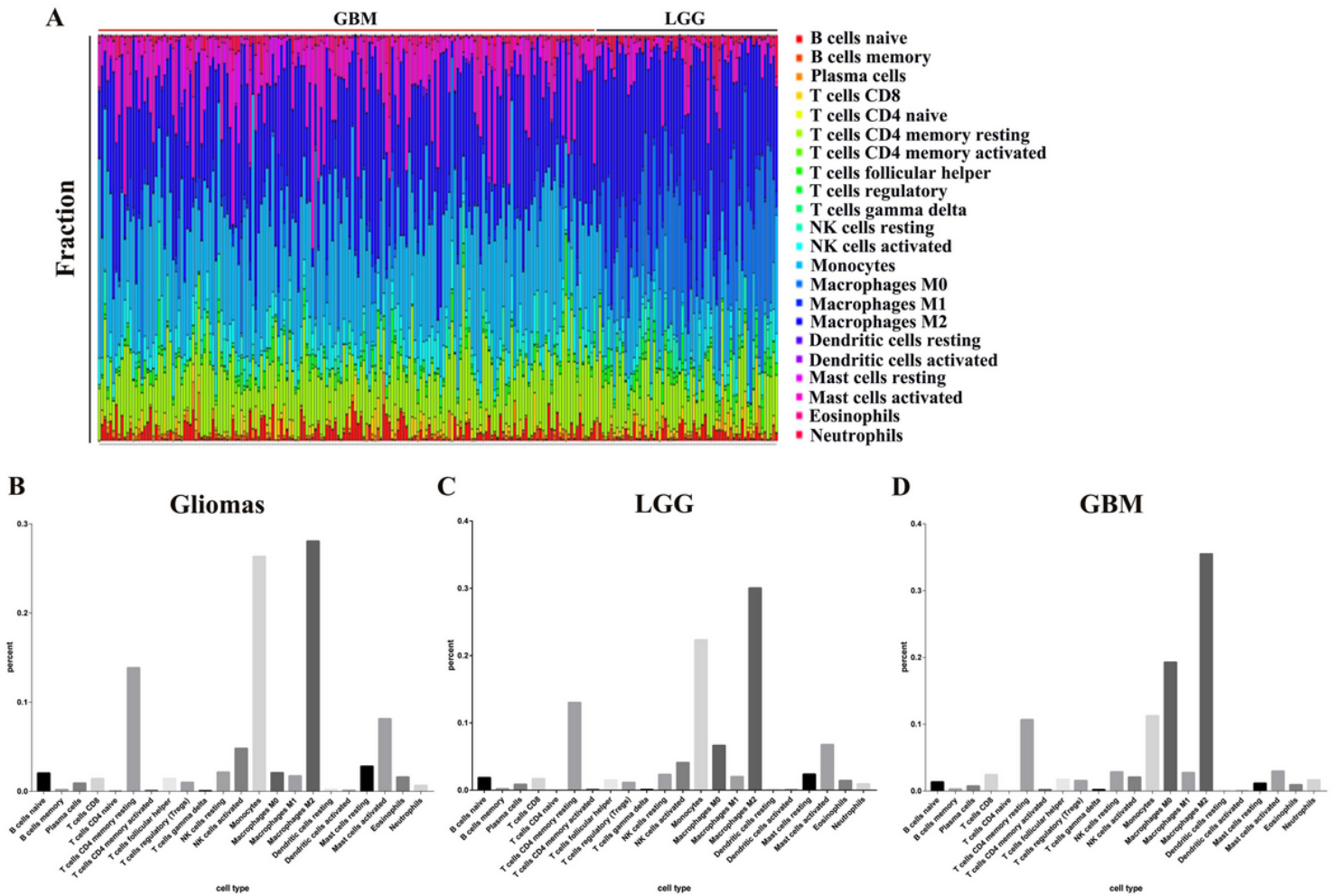
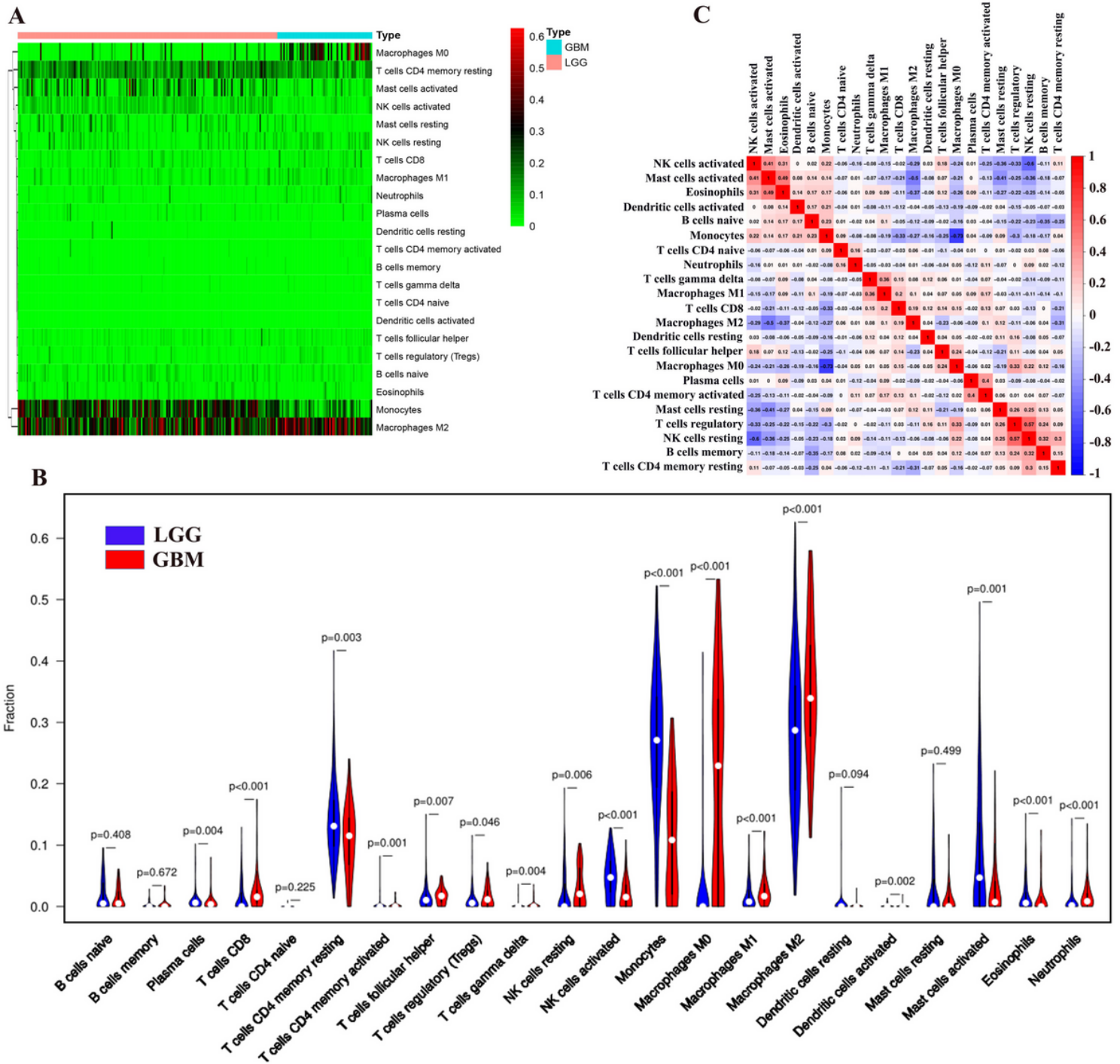


Figure 1

The proportion of tumor-infiltrating immune cells in glioma.

(A) The percentage of 22 types of adaptive and innate immune cells in glioma at the individual level; (B-D) The percentage of 22 types of adaptive and innate immune cells in glioma at the population level, representing glioma patients (B); LGG patients (C); and GBM patients (D); respectively.



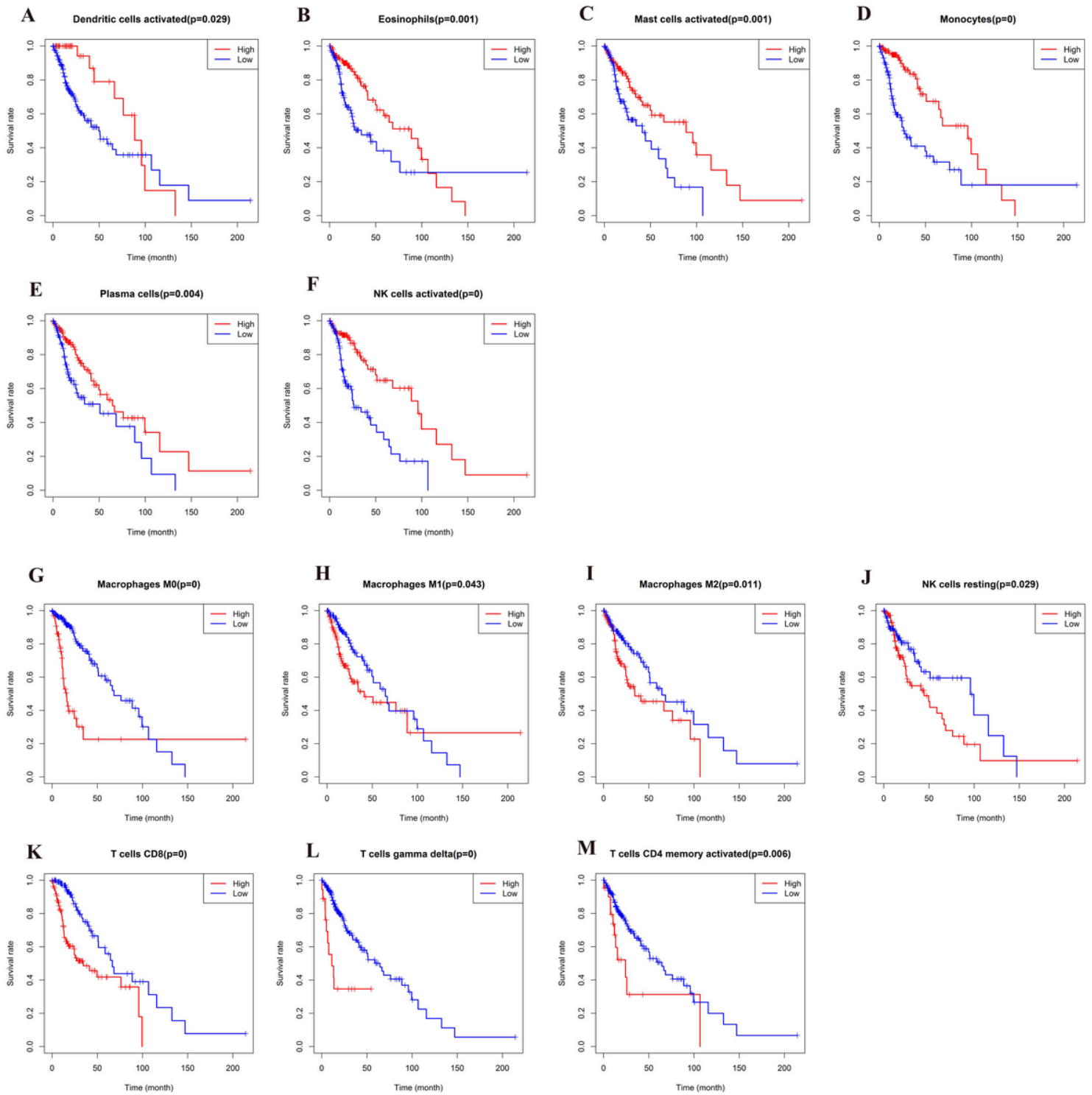


Figure 3

The Kaplan-Meier survival curve of tumor-infiltrating immune cells in glioma.

(A-F) Patients with high infiltration levels of immune cells have a longer survival period; (G-M) patients with low infiltration level of immune cells have a longer survival period.

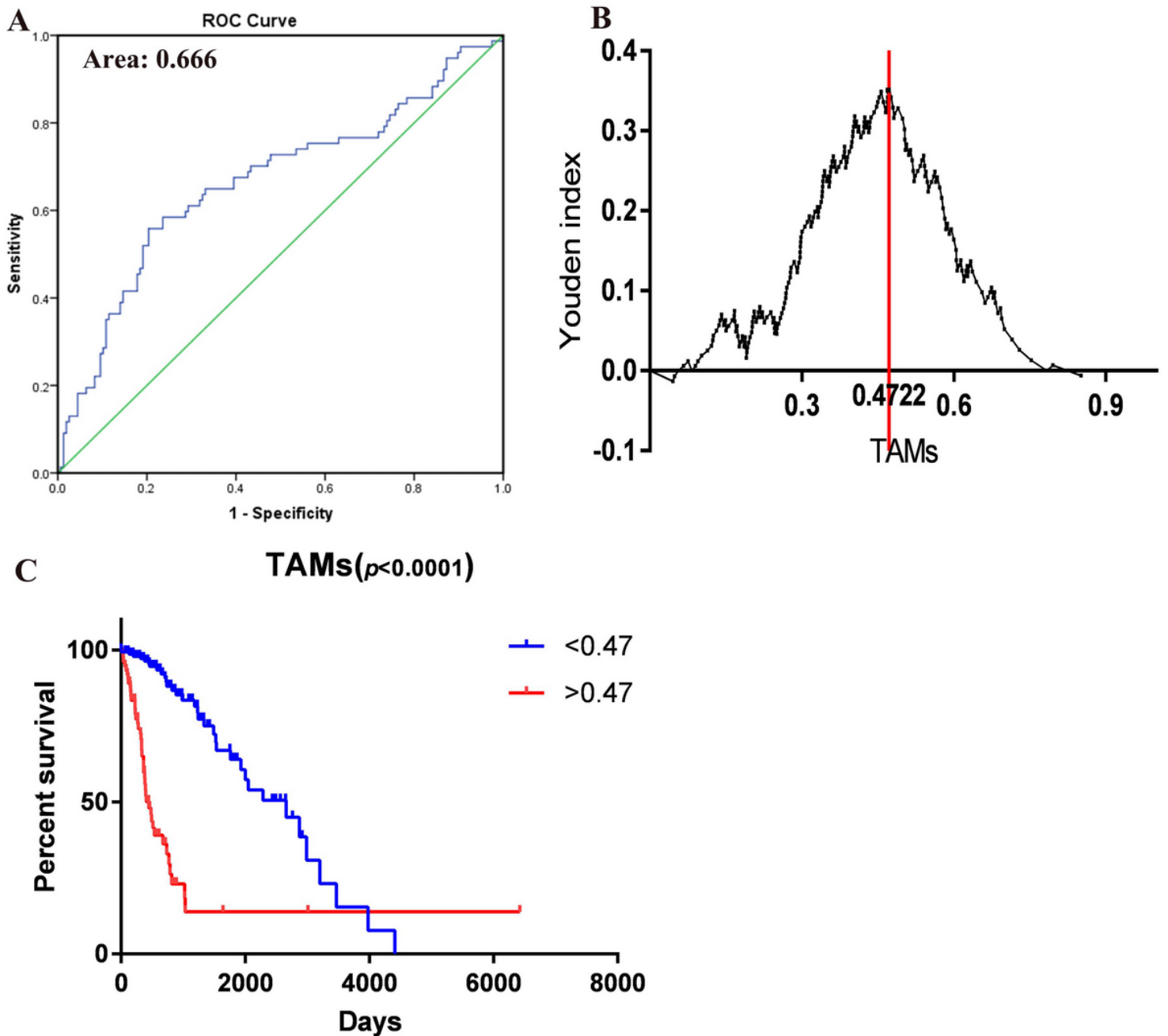


Figure 4

The prognostic value of TAMs in patients with glioma.

(A) ROC curves showed the predictive efficiency of the levels of TAMs; (B) Youden index for each possible cut-off point for the level; (C) Based on the expression level of TAM, patients were divided into high-risk and low-risk groups, and the overall survival curve of patients was calculated using the Kaplan–Meier method.

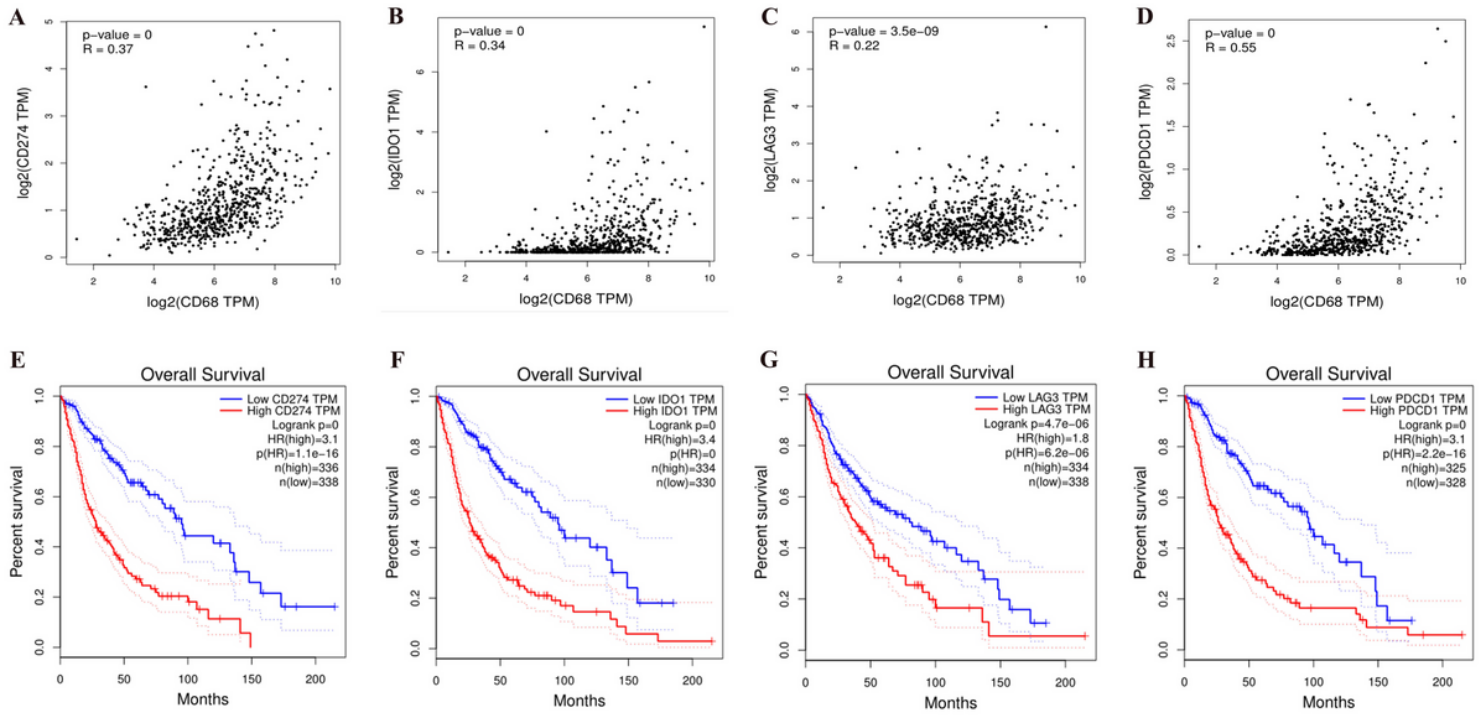


Figure 5

Correlation analysis of macrophage markers (CD68) with immune molecules.

(A-D) Correlation analysis between CD68 with CD274, IDO1, LAG3, and PDCD1; (E-H) survival curve analysis of CD274, IDO1, LAG3, and PDCD1 in glioma.