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RESEARCH

GPR65 contributes to constructing immunosuppressive microenvironment in glioma

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Abstract
Glioma, especially glioblastoma patients, present highly heterogeneous and immunosuppressive microenvironment, leading to their poor response to treatment and survival. Targeting the tumor microenvironment is considered a promising therapeutic strategy. M2 macrophages are highly infiltrated in glioma tissue, even up to 50% of the total number of bulk tissue cells. Here, we identified GPR65 as the hub gene of the M2 macrophage-related module in glioma through WGCNA analysis. The expression and prognosis analysis suggested that GPR65 was positively correlated with the malignancy and poor prognosis of glioma, and the heterogeneity analysis found that GPR65 was highly expressed in the vascular proliferation area of glioma, which matched the spatial expression characteristics of M2 macrophages. We further verified that GPR65 was highly expressed in macrophages but not tumor cells in the glioma microenvironment by single-cell data analysis and immunofluorescence. Most importantly, we found that inhibition of GPR65 was sufficient to reduce macrophages' polarization response to glioma cell and break the malignant cooperation with glioma cells. Our study reports the expression characteristics and malignant behavior of GPR65 in the glioma microenvironment, which provides a new alternative target of treatment to glioma microenvironment.

Keywords Glioma · GPR65 · GPCRs · Macrophage

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Introduction

Glioma is the most common primary malignant tumor of the central nervous system and one of the most refractory tumors in humans. The World Health Organization (WHO) classifies glioma into four grades (1 to 4) based on pathological and molecular expression characteristics [1–3]. Among them, IDH-Wildtype glioblastoma (GBM) has the highest degree of malignancy and extremely poor prognosis, with extreme invasiveness and resistance to therapies including radiotherapy, chemotherapy, targeted therapy, immunotherapy and so on [4, 5]. Research progress in recent years has revealed that the complexity and diversity of the glioma microenvironment account for a lot of treatment failure [6–10]. The glioma microenvironment contains many non-tumor cells, mainly macrophages. The infiltration of macrophages is up to 50% of the total cells in GBM and positively correlated with the malignancy of gliomas [11, 12]. Recent studies have revealed some key factors of the crosstalk between glioma cells and macrophages. However, a better understanding of this complex cellular ecosystem is needed to improve the efficiency of targeting tumor microenvironment (TME) and benefit more glioma patients [13–15].

GPR65, also known as T Cell Death Associated Gene 8 (TDAG8), was first identified as a G protein-coupled receptor associated with activation-induced T-cell apoptosis in 1996 [16]. It was later identified as a proton sensor of extracellular pH, which can enhance the production of cAMP in response to the acidified extracellular environment [17–19]. GPR65 was reported to promote the adaptation of the non-small cell lung cancer cell line NCI-H8 to the acidic microenvironment to enhance its survival and proliferation in the acidic microenvironment, and promote tumor progression [20]. GPR65 has also been reported to be involved in the suppression of pro-inflammatory cytokine production in peritoneal macrophages induced by extracellular acidification [21]. BTB09089 has been identified as a specific activator of GPR65, and can regulate cytokines of T cells and macrophages, inhibit TNF-α and IL-6 production, and increase IL10 production to reduce immune-mediated inflammation [22]. In melanoma, acidosis induces G protein-coupled receptor-dependent expression of the transcriptional repressor ICER in tumor-associated macrophages, leading to their functional polarization towards a non-inflammatory phenotype and promotion of tumor growth [23]. Interestingly, another study has found that glioblastoma cells (H4, SW1088, A172) can produce more lactate than melanoma cells (SK-MEL3) in vitro [24]. Yang et al. identified 5 gene modules for GBM enriched in inflammatory response, of which GPR65 was recognized as the outstanding genes in survival analysis [25]. Wang et al. reported that GPR65 is overexpressed in glioblastoma and its high expression predicts unfavorable clinical outcome for patients [26]. However, there is a lack of a comprehensive analysis of the role of GPR65 in glioma, especially in the glioma microenvironment.

Weighted gene co-expression network analysis (WGCNA) aims to find co-expressed gene modules and explore the association between gene networks and phenotypes of interest, as well as the core genes in the network [27]. In this study, through WGCNA, we focused on the phenotype of M2 macrophages infiltration in glioma patients, and identified GPR65 as the hub gene related to the M2 macrophage module in glioma patients, and further data analysis and experimental verification proved that GPR65 mediates the M2 polarization of glioma-associated macrophages, and inhibiting GPR65 can break the malignant cooperation between glioma cells and macrophages.

Materials and methods

Data collection and analysis

The results shown here were in part based upon data generated by the TCGA Research Network(https://www.cancer.gov/tcga) and CGGA (http://www.cgga.org.cn) [28]. Download mutation, RNAseq, and clinical data of TCGA GBM and lower grade glioma (LGG) patients with the help of the R package "TCGAbiolinks" [29]. A total of 2159 glioma patients were included and specifically screened for downstream analyses. Immune cells infiltration data for TCGA samples were downloaded online from the TIMER (http://timer.cistrome.org/) [30]. The estimation result generated by CIBERSORT was used [31]. The Ivy Glioblastoma Atlas Project provided foundational resource for exploring the anatomic and genetic basis of glioblastoma at the cellular and molecular levels (https://glioblastoma.alleninstitute.org/) [32].

Tumor tissue microarrays were collected from patients with glioma who underwent surgery in Tianjin Medical University General Hospital, containing 12 Grade II, 12 Grade III, and 28 Grade IV gliomas and 2 non tumor tissues. Written informed consents were obtained from all donors or their relatives. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Tianjin Medical University General Hospital.

The R language (v.4.0.2) was used for statistical analysis and Visualization. Genes with significantly different expression between groups were estimated by t-test or ANOVA (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001). The KM survival analysis and visualization of GPR65 was completed by the survival R

package [33]. The correlation between GPR65 and gene mutation status was obtained by calculating the Pearson correlation coefficient with R language. GSEA enrichment analysis was done by the clusterProfiler R package [34].

Single-cell data analysis was based on data generated by GSE131928 and completed in Single Cell Portal (https://singlecell.broadinstitute.org/single_cell) [35].

Cell culture and co-culture

Human glioma cell line A172, human leukemia mononuclear cell line THP1 were purchased from Procell Life Science&Technology Co.,Ltd. And the Cell STR identifications were also provided by Procell. A172 was cultured in DMEM (Gibco, USA), THP1 was cultured in 1640 (Gibco, USA) medium containing 10% fetal bovine serum (FBS) in a 5% CO2, 37 °C humidified cell incubator. GPR65 knockdown cell lines were constructed, and GPR65 siRNA was purchased from GenePhama, China: si-GPR65-1 (sense: 5'-GCAUUAACUCUCCCUUUAUTT-3'; antisense: 5'-AUAAAGGGAGAGUUAAUGCTT-3') si-GPR65-2 (sense: 5'-GCGAACUUACCACAAUGUAUTT-3'; antisense: 5'-AUACAUUGUGUAAGUUCGCTT-3'), si-GPR65-3 (sense: 5'-CCUGAUCUGCAACCGGAAATT-3'; antisense: 5'-UUUCCGGUUGCAGAUCAGGTT-3').

For conditioned co-culture: use PMA to induce THP1/THP1 (siGPR65) for 48 h to adhere, then centrifuge and collect 6 ml A172 medium after 24-hour culture and treat adhered THP1 for 48 h with the collected medium. For co-culture: the lentiviruses containing red fluorescent gene (RF) and green fluorescent gene (GF) were from GENECHEM. Lentiviral transfection was performed in A172 (RF) and THP1 cells (siGPR65) (GF). In 6-well plate, induce 5×10^5 THP1/THP1 (siGPR65) for 48 h to adhere and add 2×10^5 glioma cell A172 for co-culture, after A172 completely adhered about 8 h, capture with the fluorescence microscope after 24, 48, 72 h. Fluorescent cell counting was performed using Imagej software, followed by graphing and statistical analysis using Graphpad.

Western blot

Western blot test was carried out following the protocol [36]. The protein expression of CD163 and GPR65 of THP1 was detected. WB primary antibody: GPR65 (Signalway Antibody, C92629Bio), CD163 (CST, 93498).

Immunohistochemistry (IHC) and immunofluorescence (IF)

Paraffin-embedded tissue sections were used for immunohistochemical staining and immunofluorescence double staining. Firstly, the slices were baked in an incubator and then dewaxed in xylene, graded ethanol debenzene, washed with distilled water and PBS, and then the antigen retrieval solution was prepared for antigen retrieval, and then incubated with 3% H_2O_2 at room temperature to remove endogenous peroxidase activity. Using goat serum blocking to reduce non-specific antigen staining, and finally the corresponding primary antibody was diluted and incubated overnight with the tissue. For the IHC, the goat anti-rabbit IgG two-step detection kit (PV-9000, ZSGB-Bio, China) was used to detect the markers, followed by counterstaining the slides with Mayer's hematoxylin solution (G1080, Solarbio, China) for nuclear staining, and finally the sections were scanned and photographed. For the IF, Alexa-Fluor 594-labeled Goat anti-mouse IgG (Invitrogen, USA) and Alexa-Fluor 488-labeled Goat anti-rabbit IgG (Invitrogen, USA) were used for double-color fluorescent staining. The nuclei were labeled with DAPI staining solution (Solarbio, China), and finally photographed using a confocal fluorescence microscope. IHC and IF primary antibody: CD163 (Abcam, ab156769), GPR65(Proteintech,20306-1-AP).

Results

GPR65 was identified as the hub gene of the M2 macrophage-associated module and associated with poor prognosis in glioma

Firstly, we clarified that higher enrichment of M2 macrophages was associated with poor prognosis of glioma patients through K-M survival analysis($p<0.0001$) (Fig. 1A), which is consistent with previous research results. And then, we further clustered the selected high variance genes (MAD top 5000) of glioma patients into 7 modules by WGCNA analysis. Among the modules, the turquoise module showed the highest positive correlation with M2 macrophages ($r=0.49$, $p<0.0001$), and at the same time had a significant negative correlation with follicular helper cells (r=-0.35, $p<0.0001$) (Fig. 1B). To obtain the hug genes, we further set the threshold, that is, those genes whose correlation with turquoise module is above 0.9, and the correlation with M2 macrophage trait is above 0.5. GPR65, FCGR2A, MSR1, IFI30 stood out. Among them, GPR65 had the highest correlation with the module ($r=0.92$) (Fig. 1C). More importantly, higher GPR65 expression patients performed a poorer prognosis based on TCGA and CGGA data sets. The median survival time was much lower than that of patients with low GPR65 expression (Fig. 1D-F).

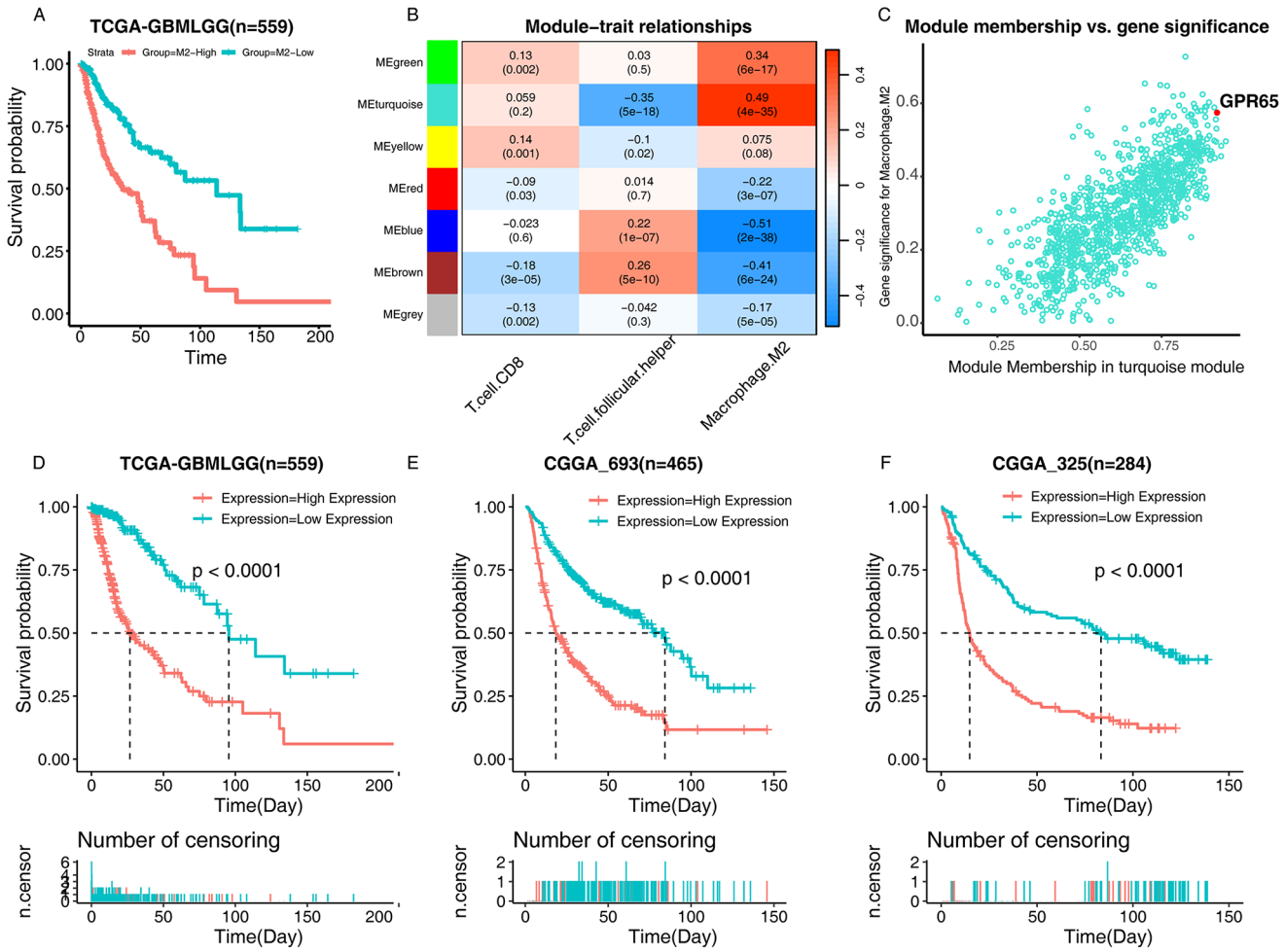

Fig. 1 Identify GPR65 as the hub gene of the M2 macrophage-associated module and K-M survival analysis of GPR65. **A**. K-M survival analysis of glioma patients with different M2 macrophage infiltration. **B**. Module-trait relationship heatmap. **C**. Turquoise module genes' module membership and gene significance. D-F. K-M survival analysis of glioma patients with different GPR65 expression in TCGA-GBMLGG, CGGA (693), CGGA (325) dataset

M2-like tumor-associated macrophage is regarded as an obstacle to glioma treatment, and a breakthrough is urgently needed [37–39]. Here, GPR65 was identified as a malignant gene highly correlated with glioma M2 macrophages, which has not been reported in glioma. Therefore, we would research on the role of GPR65 in glioma, especially the correlation with macrophages as following.

GPR65 is highly expressed in more malignant gliomas

First, we assessed the pan-cancer expression of GPR65 in the TCGA database and found that it was upregulated in 14 cancers including GBM and LGG, downregulated in 9 cancers, and had no significance in the remaining cancers (Fig. 2A). It is suggested that GPR65 may play different roles in different cancers, which is deserved further explanation but not in this study. Next, we stratified the expression of GPR65 in glioma samples in TCGA and CGGA databases based on WHO grade (Fig. 2B-D). We found that the mRNA expression level of GPR65 increased with tumor grade, and highest in WHO IV glioblastoma. These results indicated that the expression of GPR65 may contribute to the malignance of glioma.

To verify the expression of GPR65 at the tissue level of glioma, we determined the expression of GPR65 protein using immunohistochemistry in tissue microarrays containing 12 Grade II, 12 Grade III, and 28 Grade IV gliomas and 2 non tumor tissues (Fig. 2E-G). The results showed that GPR65 was abundantly expressed in WHO grade IV glioma tissue, but was hardly detected in WHO grade II glioma tissue, which also proved the correlation between GPR65 and tumor grade at the tissue level.

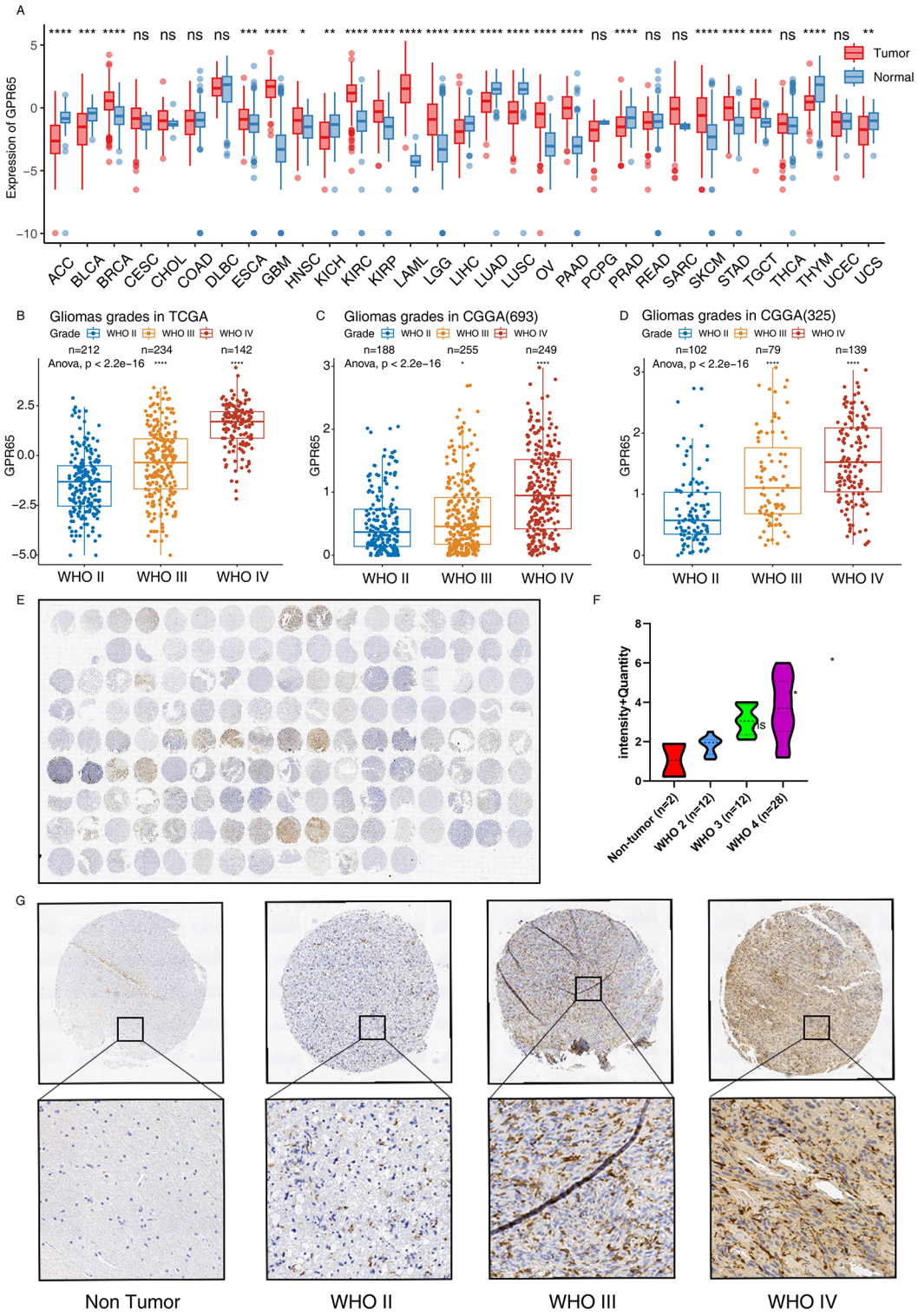

Fig. 2 GPR65 is highly expressed in more malignant gliomas. **A**. The expression profile of GPR65 in 31 kinds of cancers and their paired normal tissues from TCGA database. **B**-**D**. The relationship between GPR65 mRNA expression and WHO glioma grades in the TCGA and CGGA databases. **E**-**G**. Higher GPR65 level is observed with increasing tumor grade in glioma clinical samples (ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

GPR65 is highly expressed around angiogenesis niche and matches the spatial expression signature of M2 macrophages

Tumors have a high degree of intertumoral and intratumoral heterogeneity, making it hard to precisely target [40, 41]. Therefore, it is necessary to further analyze the expression heterogeneity of GPR65 in glioma. First, we analyzed the differences in GPR65 expression among patients with different hotspot mutations, age, and 7p10q status of glioma to evaluate the intertumoral expression heterogeneity of GPR65. We found that GPR65 was positively correlated with EGFR, PTEN mutations, older age, 7p gain and 10q loss, and negatively correlated with IDH1, CIC mutation and 1p/19q codeletion (Fig. 3A). This suggested that patients with IDH1 wild, CIC wild, EGFR mutation, PTEN mutation, older age, 7p gain and 10q loss may benefit more from GPR65 targeting. In addition, we analyzed the intratumoral expression heterogeneity of GPR65 in GBM (WHO grade 4 glioma) in the Ivy glioblastoma atlas, and we found that GPR65 was more expressed in the vascular proliferation area (Fig. 3B). Significantly, this region was also more highly enriched for M2 macrophages and immune checkpoint molecules (Fig. 3B). Further analysis found that among the immune checkpoint molecules we collected, the expressions of more than half (12/20) of the checkpoint molecules were significantly positively correlated with the expression of GPR65 (Fig. 3C). GSEA enrichment analysis of HALLMARK and Reactome pathways also suggested that the high expression of GPR65 enriched several immunosuppression-related pathways like IL-10 signaling (Fig. 3D&E).

In conclusion, here we revealed both intertumoral and intratumoral heterogeneity of GPR65 expression in glioma and found that it is highly correlated with the immunosuppressive microenvironment of glioma, and in particular, it closely matches the spatial expression signature of M2 macrophages.

GPR65 is highly expressed on macrophages but not tumor cells in the glioma microenvironment, especially on M2-like cells

Through analysis above, we found that GPR65 highly matched the spatial expression characteristics of M2 macrophages. Before further study, we were willing to clarify which cells mainly expressed GPR65 in glioma diverse ecosystem. First, we analyzed single-cell data set from GSE131928, and GPR65 was found highly expressed in macrophages which also expressed abundant M2 macrophage marker CD163 (Fig. 4A-C). Therefore, we concluded that GPR65 is highly expressed in tumor-associated macrophages, but not tumor cells, in the tumor microenvironment and appears to show an association with the M2 phenotype. To verify this conclusion, we performed GPR65 and CD163 immunofluorescence co-staining on LGG and GBM patient tissues, and the results verified our findings that GPR65 and CD163 have higher expression in GBM, and there was co-localization of their expression (Fig. 4D).

In conclusion, we directly demonstrated that GPR65 is highly expressed in M2-like macrophage populations by single-cell data analysis and immunofluorescence validation, which explains the correlation of GPR65 and macrophage enrichment we found above from bulk sequencing data. Next, the correlation between GPR65 and M2 polarization of macrophages needs to be further studied.

GPR65 inhibition can reduce the response of macrophages to glioma cell-induced polarization

We further investigated whether GPR65 plays driver's role in the cooperation between tumor macrophages with tumor cell or acts as a polarization marker. First, we collected the culture medium of A172 cells and used it to culture THP1 for 48 h, while the control group was cultured with ordinary culture medium. We found that compared with the control group, the expression of GPR65 in THP1 cells in the co-culture group was significantly up-regulated, and the M2 marker CD163 was also significantly up-regulated (Fig. 5A-B). Then, we knocked down the expression of GPR65 in THP1 before co-cultivation, and the control group was treated with transfection reagents. It was found that after GPR65 knockdown, the polarization responsiveness of THP1 to the medium of A172 cells was reduced (Fig. 5C-D). We also found the same result by immunofluorescence (Fig. 5E). This suggests that in the tumor microenvironment, GPR65 regulates the M2 polarization response of macrophages to the microenvironment. More importantly, when we directly co-cultured GPR65-knockdown THP1 with A172, we found that the growth rate of A172 was significantly slower than that of the control group (Fig. 5F&G). This result suggested that GPR65 can break the malignant cooperation between macrophages with glioma cells by down-regulating the M2 polarization of macrophages.

Discussion

GPR65 was identified as an extracellular pH sensor [19]. Our study suggests that GPR65 may have a huge impact on the tumor microenvironment. More analysis and

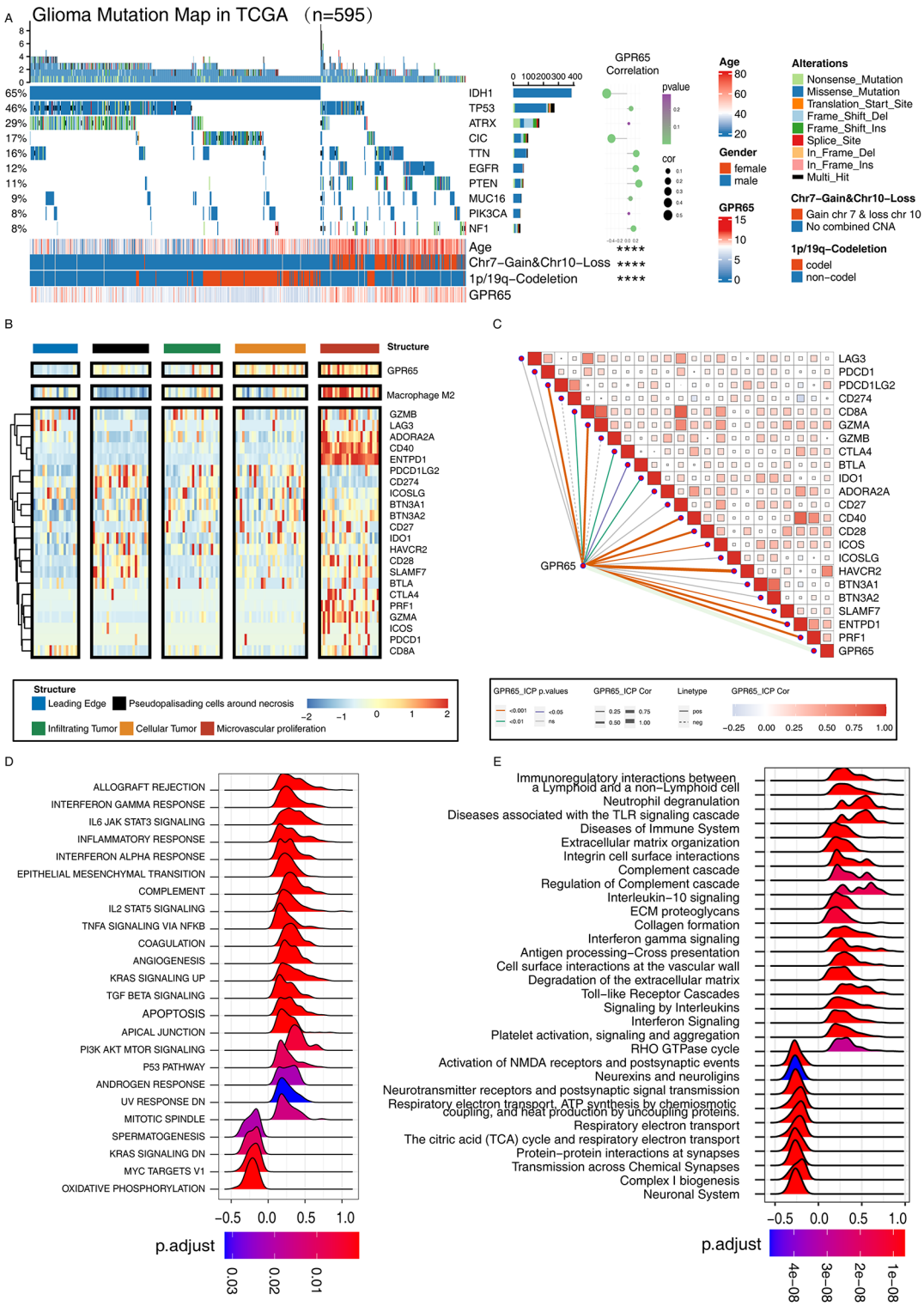

Fig. 3 GPR65 is highly expressed around angiogenesis niche and matches the spatial expression signature of M2 macrophages. **A**. Pearson correlation analysis between GPR65 and the top 10 mutation frequencies genes, age, chromosome 7 acquisition, chromosome 10 deletion and 1p / 19q combined deletion. **B**. Heatmap of the spatial expression pattern of GPR65, M2 macrophages and immune checkpoints based on Ivy glioblastoma atlas. **C**. GPR65 closely relates with immune checkpoint genes. **D** & **E**. The GSEA results of GPR65 related genes in HALLMARK and Reactome pathway datasets respectively

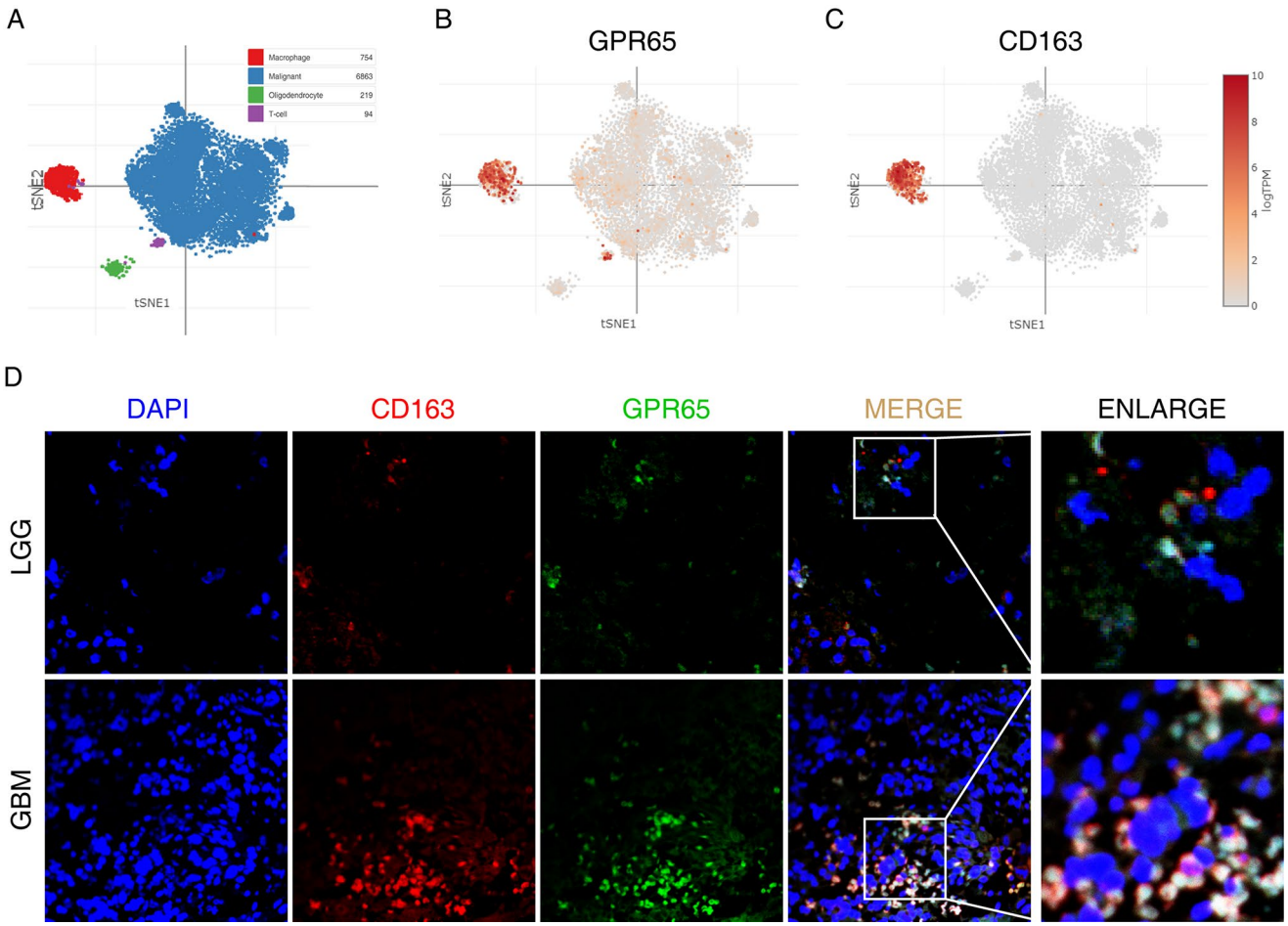

Fig. 4 GPR65 is highly expressed on macrophages but not tumor cells in the glioma microenvironment, especially on M2-like cells. **A**-**C**. Single cell RNA-Seq data analysis shows GPR65 is highly expressed on macrophages but not tumor cells, so as CD163. **D**. Double-colored fluorescent staining showed GPR65 co-expresses with CD163

experiment are needed to verify whether the acid production ability of glioma cells can regulate this impact. Radiomics analysis revealed that IDH-mutant gliomas exhibited lower acidity and lower hypoxia compared with IDH wild-type gliomas, and tumor acidity in glioma patients was significantly associated with both overall survival and progression-free survival, independent of patient age, IDH status, and treatment status [42]. Warburg proposed preferential anaerobic glycolysis of tumor cells, anaerobic glycolysis would generate lactic acid and acidify the tumor microenvironment to induce immunosuppression [43–47]. Studies have shown that different tumor cells have different ability to produce acid [24], but the molecular mechanism behind this has not yet been elucidated. Therefore, we suggest it is necessary to disentangle the mechanisms underlying the acid-producing ability of gliomas and to investigate whether these mechanisms further drive an immunosuppressive shift in the tumor microenvironment through GPR65.

More detailed studies on how GPR65 upregulation induces phenotypic changes in macrophages are still needed. Our enrichment analysis shows that GPR65 is related to various immune suppression-related pathways like IL-10 signaling. IL10 has been proven to be a key factor in the tumor immunosuppressive microenvironment [48–51]. It is worth further analyzing the relationship between GPR65 and IL-10 signaling pathways in the glioma microenvironment.

In conclusion, we report here that the extracellular pH sensor GPR65 induces M2 polarization of macrophages in the glioma microenvironment. Knockdown of the GPR65 expression of macrophage THP1 in the co-culture system inhibited the M2 polarization of macrophages and the proliferation rate of glioma cells in the co-culture system. Since studies on GPR65 in glioma are few and far between now, our study in vitro also cannot represent the real tumor environment in vivo, more studies are needed, especially more realistic in vivo studies, to further explore the effect of GPR65 interference on the glioma microenvironment, to

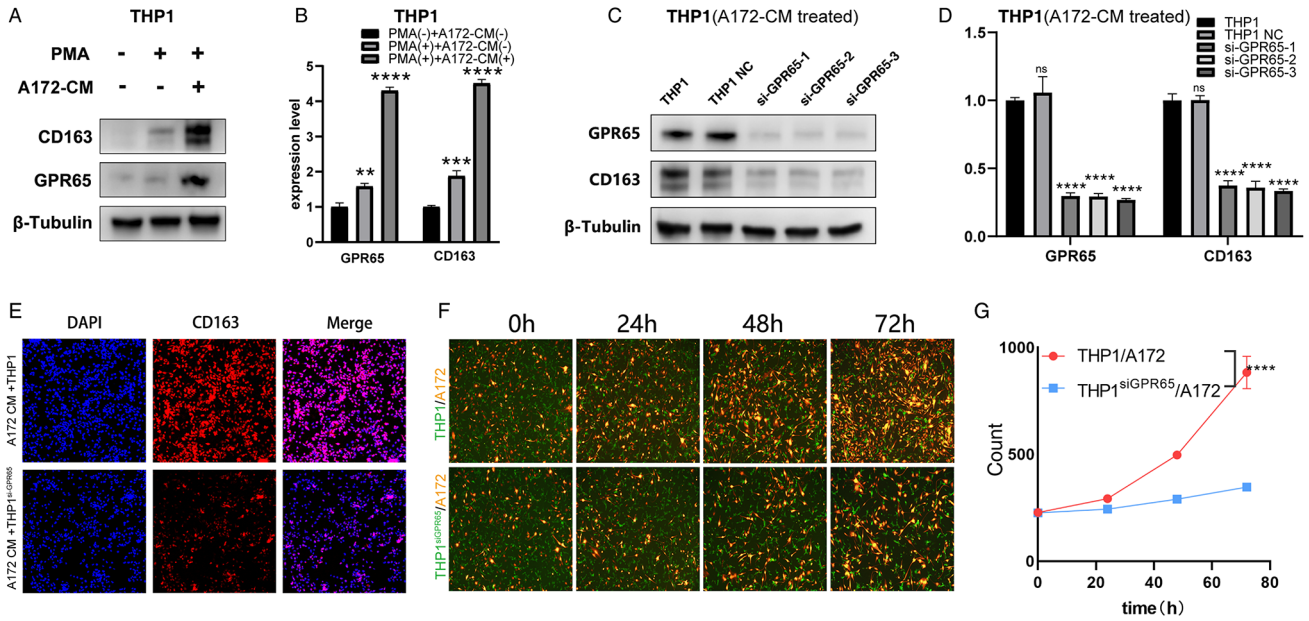

Fig. 5 GPR65 inhibition can reduce the response of macrophages to glioma cell-induced polarization. **A** & **B**. GPR65 and CD163 highly expressed in THP1 after A172 medium treated. **C**-**E**. Knockdown of GPR65 damaged the ability of THP1 to response to A172 medium. **F** & **G**. Co-culture of THP1(si-GPR65) with A172

evaluate whether it can release or even reverse the inhibitory effect of tumor-associated macrophages on T cells, so as to achieve the purpose of killing tumor cells.

Conclusion

GPR65 mediates the M2 polarization of glioma-associated macrophages, and inhibiting GPR65 can break the malignant cooperation between glioma cells and macrophages.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10143-024-02633-4.

Author contributions Jikang Fan, Jie Liu, Bin Zhang designed the experimental validation, performed experiments, analyzed the data, and drafted the manuscript. Xuya Wang, Xisen Wang, Jianshen Liang and Yiming Li carried out parts of the experiments. TaoLi, Chen Zhang and Shengping Yu provided constructive discussion about experiments. Xuejun Yang provided effective work direction. All authors read and approved the final manuscript.

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Data availability The public data can be online downloaded from TCGA, CGGA, Ivy Glioblastoma Altlas, GSE131928. The experimental data that support the findings of this study are available from Jikang Fan upon reasonable request.

Declarations

Ethical approval For human glioma samples, written informed consent was obtained from all donors and their relatives. The study was carried out in accordance with the principles of the Helsinki Declaration and approved by the ethical committee at Tianjin Medical University General Hospital (March, 2018).

Competing interests The authors declare no competing interests.

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