Cancer cell intrinsic TIM-3 induces glioblastoma progression
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SUMMARY
Glioblastoma (GBM) is identified to share common signal pathways between glioma and immune cells. Here, we find that T cell immunoglobulin domain and mucin domain protein 3 (TIM-3) is one of the most common co-inhibitory immune checkpoints in GBM shared by tumor and non-tumor cells. Glioma cell-intrinsic TIM-3 is involved in not only regulating malignant behaviors of glioma cells but also inducing macrophage migration and transition to anti-inflammatory/pro-tumorigenic phenotype by a TIM-3/interleukin 6 (IL6) signal. In mechanism, as one of the major regulators of IL6, TIM-3 regulates its expression through activating NF-kB. Blocking this feedback loop by Tocilizumab, an IL6R inhibitor, inhibited the above effects and repressed the tumorigenicity of GBM in vivo. Our work identifies glioma cell-intrinsic functions of TIM-3/IL6 signal mediating the crosstalk feedback loop between glioma cells and tumor-associated macrophages (TAMs). Blocking this feedback loop may provide a novel therapeutic strategy for GBM.

INTRODUCTION
As the most fatal primary CNS malignancy in adults, glioblastoma (GBM) is characterized by its resistance to routine treatments, including maximal surgical resection, chemotherapy, and radiotherapy. The median survival of GBM remains only 15 months (Molinaro et al., 2019). One important reason for this dilemma is immunosuppressive microenvironment shaped by glioma and its non-tumor compartments. As a hallmark of cancers, sustained immune suppression facilitates immune evasion of tumor cells (Hanahan and Weinberg, 2011; Sadahiro et al., 2018). Immune checkpoints contribute to immune suppression in the tumor microenvironment (TME) (Sadahiro et al., 2018). Multiple immune checkpoint inhibitors (ICIs) strategies designed to target solid tumors are currently under investigation, and differential expression of immune checkpoints has been employed as a biomarker for predicting the efficiency of ICI monotherapy (Garg et al., 2017). Solid tumors such as melanomas and lung cancers, which have a positive response to ICIs against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), express these two immune checkpoints at a significantly higher level than GBM (Garg et al., 2017). ICIs strategies, including the highest expressed immune checkpoint involved in GBM immune suppression, may achieve a better therapeutic efficacy. Therefore, it is crucial to investigate which immune checkpoints have elevated expression in glioma and play a decisive role in TME (Kleeffl et al., 2015).

Here, we first examined the expression of immune checkpoint in glioma, and found that T cell immunoglobulin domain and mucin domain protein 3 (TIM-3) was one of the most upregulated co-inhibitory immune checkpoints in glioma, and closely related with the unfavorable prognosis of GBM (Serensen et al., 2021). We further found that there was a tumor-intrinsic TIM-3 signaling which enhanced the tumorigenicity of glioma cells. This signaling activates NF-kB signaling to promote the transcription of interleukin 6 (IL6), which increases IL6 secretion and leads to the migration and anti-inflammatory/pro-tumorigenic transition of macrophages. Moreover, alternative activation of macrophages further triggered feedback signaling on TIM-3 expression of glioma cells. Blocking this TIM-3 feedback loop by an IL6 receptor antagonist, Tocilizumab, had a good therapeutic effect and effectively inhibited tumor growth in vitro and in vivo. Collectively, we demonstrate a cancer cell-intrinsic TIM-3/IL6 signal promoting glioma progression and involving constituting a positive feedback loop between GBM cells and anti-inflammatory/pro-tumorigenic tumor-associated macrophages (TAMs). These findings revealed a TIM-3-dependent crosstalk mechanism for...
Figure 1. TIM-3 is one of the most highly elevated immune checkpoints in GBM and is expressed by both non-tumor and tumor cells

(A) The expression analyses of immune checkpoints in GBM, in comparison with non-tumor tissue and LGG, identified 7 overlapping elevated immune checkpoints, including 2 co-inhibitory immune checkpoints (TIM-3 and LAIR1) and 5 co-stimulatory immune checkpoints (SLAMF8, CD300A, TYPOBP, CD58, and BTN3A2) (TCGA-seq, GBM, n = 155; LGG, n = 515; non-tumor, n = 4; GSE16011, GBM, n = 155; LGG, n = 116; non-tumor, n = 8). (B) RT-qPCR analyses of CTLA4, TIM3, LAIR1, PD1, PDL1, PDL2 and IDO 1 in clinical GBM samples (n = 10, means ± SEM, one-way ANOVA). (C) Immunohistochemical staining (Left, scale bar, 50 µm) and analyses (Right, non-tumor, n = 13; Grade II, n = 6; Grade III, n = 17; Grade IV, n = 77; means ± SEM, one-way ANOVA) of TIM-3 in clinical samples. (D) The Kaplan-Meier analyses of clinical glioma samples reveal the correlation of TIM-3 with poor prognosis in GBM (TIM-3 high vs. low, p = 0.0003, log rank test). (E) CIBERSORT analysis of non-tumor cell populations associated with high TIM-3 expression in TCGA RNA-seq GBM dataset (Pearson correlation analysis). (F) The tSNE plot of TIM-3 expression profile in GBM and immune cells with single-cell RNA-seq data (GSE131928). (G) Representative immunofluorescence images of TIM-3 and GFAP staining in clinical GBM samples (scale bar, 50 µm). (H) FACS analysis that clinical GBM samples contained low CD45, and high TIM-3 cell populations (n = 7). (I) qPCR analysis of TIM-3 mRNA expression in NHA, indicated glioma cell lines (U87, U251, and LN229), primary glioma cells (PGC1228, PGC21, PGC24, and PGC40), and PBMC from patients with glioma (G-PBMC1 and G-PBMC2) and healthy donor (PBMC1 and PBMC2) (n = 3, means ± SEM, one-way ANOVA). (J) Western blot analyses of TIM-3 in NHA, indicated glioma cell lines, and primary glioma adherent and neurosphere cells (n = 3, means ± SEM, one-way ANOVA). (K) The schematic diagram describing the co-culture system of THP-1-derived anti-inflammatory/pro-tumorigenic (M2) macrophages and glioma cells (left panel), and representative western blot images of TIM-3 in indicated glioma cell cocultured with THP-1-derived anti-inflammatory/pro-tumorigenic TAMs (right panel): (ns p ≥ 0.05, *p < 0.05; **p < 0.01; ****p < 0.0001).

tumor progression and macrophage activation in GBM, which may contribute to developing novel therapeutic strategies for GBM.

RESULTS
Integrated profiling identifies TIM-3 are an immune checkpoint with highly elevated expression and indicates poor survival in glioblastoma

To identify the critical immune checkpoints in glioma, we summarize a list of 121 immune checkpoints (Table S1) (Schildberg et al., 2016; Yshii et al., 2017). We firstly analyzed their expression in GBM relative to normal tissue in The Cancer Genome Atlas (TCGA) and GSE16011 glioma datasets. Then we compared their expression in GBM and LGG in TCGA and GSE16011 glioma datasets. We selected overlapping genes with log FC > 0.3 and p < 0.002 in these two datasets. The analyses identified seven immune checkpoints with elevated expression in GBM compared with non-tumor and LGG tissues, including 2 co-inhibitory immune checkpoints (TIM-3 and LAIR1), and 5 co-stimulatory immune checkpoints (SLAMF8, CD300A, TYPOBP, CD58, and BTN3A2) (Figure 1A) (Schildberg et al., 2016). Owing to the importance of co-inhibitory immune checkpoints in driving tumor progression and their ability to act on the GBM microenvironment, we prioritized TIM-3 and LAIR1 for further investigation. RT-qPCR analysis in clinical GBM samples also confirmed that TIM-3 expression was higher than LAIR1 and the well-known immune checkpoints, including CTLA4, PD1, PDL1, PDL2, and IDO 1 (He and Xu, 2020) (Figure 1B). Therefore, we focus on examining the functions of TIM-3. Further analysis showed that TIM-3 expression in GBM was significantly higher than that of grades II and III (Figures S1A and S1B). This was verified with clinical samples by western blotting and immunohistochemical (IHC) staining (Figures 1C and 51C). In consistent with a previous report (Li et al., 2017), Survival analysis with our in-house specimens revealed that high TIM-3 expression indicated a short survival in GBM (Figure 1D). This is consistent with previous reports (Baize et al., 2017; Zhang et al., 2020). Together, these data showed that TIM-3 was not only a co-inhibitory immune checkpoint with elevated expression in glioma but also a clinically relevant molecule associated with unfavorable prognosis in GBM.

TIM-3 is expressed by both non-tumor and tumor cells in glioblastoma tumor microenvironment

We next sought to characterize TIM-3 expression in different components of GBM TME. Since TIM-3 was originally identified as a Th1-specific cell surface receptor (Monney et al., 2002), we first employed CIBERSORT to analyze the correlation between TIM-3 expression and non-tumor cell populations of GBM. The result showed that anti-inflammatory/pro-tumorigenic (M2-like) macrophages were the
Figure 2. Cancer cell intrinsic TIM-3 signaling enhances the malignant behaviors of GBM cells

(A) Western blot analyses of TIM-3 in indicated glioma cells transduced with lentiviral TIM-3 overexpression or control vector or transfected with siTIM-3 or negative control. (B and C) In vitro proliferation assays showed that the growth of indicated glioma cells was enhanced by TIM-3 overexpression and inhibited by TIM-3 knockdown (n = 3; NC vs. TIM-3 OE, t-test; siNC vs. siTIM3, means ± SEM, one-way ANOVA) (scale bar, 50 µm). (D and E) The clonogenicity (D, 500 cells/well; n = 3, means ± SEM, t-test) and neurosphere formation capability (E, left and middle: n = 10, extreme limiting dilution assay; right: stem cell frequency, n = 3; NC vs. TIM-3 OE, means ± SEM, t-test; siNC vs. siTIM3, means ± SEM, one-way ANOVA) of indicated glioma cells were increased by TIM-3 overexpression and decreased by TIM-3 knockdown, respectively. (F and G) Transwell assays showed that TIM-3 regulated the migration and invasion capabilities of glioma cells (n = 3; F, t-test; scale bar, 100 µm). G, means ± SEM, one-way ANOVA; scale bar, 50 µm. (H) The migration of GSC40 was significantly enhanced or inhibited by TIM-3 overexpression (n = 3, t-test) or knockdown (n = 3, means ± SEM, one-way ANOVA; scale bar, 50 µm). (I) Representative bioluminescence (at day 40) images of mouse brains transplanted with indicated GSC40 cells (n = 6, means ± SEM, t-test). (J) Representative H&E staining images of the brain sections (J) from mice intracranially transplanted with GSC40 cells transduced with indicated vectors (n = 6, t-test). (K) Survival plot of NOD mice intracranially transplanted with GSC40 cells transduced with indicated vectors (n = 6, log rank test). (L) Representative H&E staining images of CS7BL/6N mice intracranially transplanted with mouse spontaneous glioma cells using sleeping beauty (SB) transposon in vivo (Day 14, SB-Vector: SB cells transduced with control vector; SB-Tim3 OE: SB cells transduced with Tim-3 overexpression vector) (n = 5, t-test). (M) Survival plot of CS7BL/6N mice intracranially transplanted with SB neurosphere cells transduced with control or Tim3 overexpression vector (n = 6, log rank test). (N and O) The brain images (N) and analysis (O, n = 5) of BALB/C-Null nude mice in vivo limiting dilution assay orthotopic transplanted with indicated number of mouse SB glioma cells transduced with control (NC) or Tim-3 overexpression (Tim3 OE) vectors. (*p < 0.05, **p < 0.01, ***p < 0.001; ****p < 0.0001).

non-tumor cell population most positively correlated with TIM-3 expression in GBM (Figure 1E). IHC staining in clinical GBM samples also confirmed that GBM samples with high TIM-3 expression preferred to have high IBA1 expression, a marker of macrophages and microglia (Figure S1D). Consistent with these results, MCP-counter analyses with TCGA and GSE16011 datasets confirmed that monocytic lineage was the non-tumor cell population most positively associated with high TIM-3 expression in GBM (Figures S1E and S1F).

Previously, IHC staining of TIM-3 in murine glioma showed not only a positive expression on perivascular cells or tumor-infiltrating lymphocytes but also a staining pattern consistent with positive tumor cells (Kim et al., 2017). This was in line with a previously defined TIM-3 expression on astrocytes under hypoxia (Koh et al., 2015). These reports raise a question whether there are TIM-3-expressing cancer cells in GBM. Since cancer-derived immune checkpoints may play a crucial role in cancer progression, we next examine TIM-3 expression in cancer subpopulations of GBM. The analysis with single-cell RNA-seq data (scRNA-seq, GSE131928) (Neftel et al., 2019) showed that there were TIM-3-expressing tumor cells besides immune cells (Figure 1F). This was further supported by immunofluorescence (IF) in primary glioma tissue cell suspensions from clinical samples, which showed that there were cells expressing both TIM-3 and glial fibrillary acidic protein (GFAP), a glioma cell marker (Figure 1G). FACS further revealed that there were TIM-3+ GFAP+ CD45+ cells in clinical GBM samples (Figure 1H). Then, we evaluated TIM-3 expression in primary and established glioma cell lines. The result demonstrated that TIM-3 expression in glioma cells was significantly higher than that in human normal astrocytes (NHA) (Figures 1I, 1J, and S1G). Finally, we employed a co-culture system (0.4 µm) to examine the effect of anti-inflammatory/pro-tumorigenic TAMs on TIM-3 expression of glioma cells. The results demonstrated that anti-inflammatory/pro-tumorigenic TAMs increased TIM-3 expression in cocultured glioma cells (Figures 1K and S1H). Collectively, these data supported a cancer cell-intrinsic TIM-3 expression in GBM.

Glioma cell intrinsic TIM-3 signaling regulates the proliferation, and invasion capabilities of glioma cells in vitro

Next, based on the above observations, we sought to investigate the functions of glioma cell intrinsic TIM-3. Previously, TIM-3 in glioma cells was shown to associate with TMZ resistance (Zhang et al., 2019). However, the functions of glioma cell TIM-3 remain poorly understood. We employed two primary glioma cell lines (one glioma sphere: GSC40 from GBM, and one primary adherent cell line: PGC1228 from grade III glioma) and one routine glioma cell line, U87, to perform experiments. The data revealed that TIM-3 elevation induced with a lentiviral TIM-3 overexpression vector promoted glioma cells’ proliferation, and TIM-3 knock-down decreased their growth (Figures 2A–2C, S2A, and S2B). Furthermore, TIM-3 was required for the clonogenicity and self-renewal of glioma cells. No matter if TIM-3 was overexpressed or inhibited, a
previously defined an eight-gene signature associated with GBM TME regulation, including IL6 (Cheng et al., 2020). Blocking the interaction between TIM-3 and its ligands with TIM-3 in GBM. According to TCGA and GSE16011 datasets, Gal-9 was the ligand most positively associated with TIM-3 expression in GBM cells (Figures S3A and S3B). These data supported the role of Gal-9 in feedforwarding glioma intrinsic TIM-3 signaling. Then, we investigated whether TIM-3 could regulate Gal-9 expression through a feedback mechanism in intercellular communication among GBM cells. However, TIM-3 overexpression and knock-down didn’t significantly affect the concentration of Gal-9 in CM from GBM cells (Figures 2N and 2O). qPCR assay in SB glioma cells and GSC40 sample also revealed an elevated expression of markers associated with glioma stemness regulation including CD44, SOX4, and Nanog (Figures S2K and S2L). This may imply the possibility of TIM-3 involved in glioma stemness regulation. Together, these data highlighted the potential role of TIM-3 in promoting in vivo tumorigenicity and self-renewal capabilities of glioma cells.

**TIM-3 functions as a downstream molecule of Gal-9 and activates IL6 signaling in glioblastoma cells**

TIM-3 has three known ligands, Gal-9, HMGB1, and CEACAM1 (Das et al., 2017). To investigate the main functional receptor feedforward TIM-3 signaling, we first analyzed the expression tendency of these three ligands with TIM-3 in GBM. According to TCGA and GSE16011 datasets, Gal-9 was the ligand most positively associated with TIM-3 expression in GBM (Figures S3A and S3B). Blocking the interaction between Gal-9 and TIM-3 with a Gal-9 antibody efficiently attenuated the enhanced growth and self-renewal of GBM cells (Figures S3C–S3E). The migration and invasion of GBM cells induced by TIM-3 were significantly inhibited by the supplement of Gal-9 antibody (Figures S3F–S3H). These data supported the role of Gal-9 in feedforwarding glioma intrinsic TIM-3 signaling. Then, we investigated whether TIM-3 could regulate Gal-9 expression through a feedback mechanism in intercellular communication among GBM cells. However, TIM-3 overexpression and knock-down didn’t significantly affect the concentration of Gal-9 in CM from GBM cells (Figures 2N and 2O). Since the intercellular crosstalk via inflammatory cytokines feedback signaling in GBM TME is crucial for its progression, we screened the cytokines related to TIM-3 signal in glioma cells using ProteomeProfiler Human Cytokine Array. The data confirmed an elevation of CCL2, CXCL1, GCSF, GMCSF, IL1RN, IL6, and IL8 secretion in GSC40 with TIM-3 overexpression, in comparison with detection of MIF, SERPINE1, GCSF, GMCSF, IL6, and IL8 in the control group (Figure 3B). We previously defined an eight-gene signature associated with GBM TME regulation, including IL6 (Cheng et al., 2020).
et al., 2016b). According to TCGA, TIM-3 had a positive correlation with this eight-gene immune-related gene signature (Figure S4A). We performed TIM-3 overexpression to determine whether TIM-3 affected these eight gene expressions in glioma cells. The data demonstrated that IL6 was the only gene in this signature with significant mRNA elevation after TIM-3 overexpression (Figures S4B and S4C). Thus, we further focused on IL6 to examine the downstream mechanism of TIM-3 in GBM. Gene Set Enrichment Analysis (GSEA) in TCGA RNA-seq dataset revealed a significant enrichment of IL6 production and secretion, and its reported upstream regulator, NF-κB, signaling, in GBM with high TIM-3 expression (Figure S4D). Additionally, correlation analysis demonstrated a significant correlation between TIM-3 and IL6 production, indicating TIM-3 as a main regulator of IL6 in GBM (r = 0.860, p < 0.0001, Figure S4E). A previous report shows a mechanism of TIM-3 regulating IL6 at a transcriptional level through NF-κB (Zhang et al., 2018). However, when we analyzed IL6 expression in glioma cells with the scRNA-seq dataset (GSE131928), we found that IL6 was only expressed in a few immune and tumor cells in GBM (Figure S4F). This may explain why TIM-3 is not correlated with IL6 expression in GBM scRNA-seq data (GSE131928). As reported, the low transcript abundance of IL6 may limit its detection by the present scRNA-seq technique (Sankowski et al., 2019). Thus, we supposed that TIM-3 may affect the degradation of IL6 protein in addition to regulating IL6 at the transcriptional level, which has been demonstrated in other tumors (Zhang et al., 2018). Moreover, western blotting showed that TIM-3 overexpression increased the expression of IL6, MMP2, and phosphorylated p65, instead of Gal-9 (Figures 3F and S4G). In contrast, knock-down TIM-3 led to the decrease of IL6, MMP2, and phosphorylated p65 and no change of Gal-9 (Figure S4H). Based on the above observations, we concluded that TIM-3 may function through NF-κB/IL6 signal at the transcription level. Blocking of Gal-9 inhibited the elevation of IL6, and phosphor-p65 induced by TIM-3 (Figures 3F, S3G, and S3H). And IL6 concentration in CM from glioma cells significantly increased after TIM-3 overexpression (Figures 3G and S4I). IL6 significantly increased TIM-3 expression in glioma cells (Figures 3H and S4J), and rescued the decreased proliferation, migration, and neurosphere formation abilities of GSC40 cells induced by TIM-3 knock-down (Figures 3I–3K). Additionally, western blotting analysis revealed that TIM-3 overexpression didn’t significantly influence IL6R expression in glioma cells (Figure S4K). Altogether, these data suggested Gal-9 as a partner of TIM-3 and there was no feedback from TIM-3 to Gal-9. TIM-3 functioned through NF-κB by activating IL6 production, indicating TIM-3 as a main regulator of IL6 in GBM (r = 0.860, p < 0.0001, Figure S4E).

Glioma cell TIM-3 promotes monocyte migration and induces their phenotype transition to anti-inflammatory/pro-tumorigenic macrophages

To further analyze TIM-3 function, we summarized the overlapping genes positively associated with high TIM-3 expression in GBM from TCGA and GSE16011 datasets (Figure 4A and Table S2) and performed Gene Ontology (GO) analysis. The results showed an enrichment of myeloid leukocyte-related terms (Figure 4B). Gene Set Variation Analysis (GSVA) further confirmed that ten immune-related GO terms were associated with high TIM-3 expression (Figures 4C and S5A). Gene set enrichment analysis further disclosed a significant enrichment in gene sets of macrophage chemotaxis and differentiation with high TIM-3 expression (Figure 4D). Cancer cell-expressed immune checkpoints could mediate the crosstalk among tumor and non-tumor cells in TME (Kleeffel et al., 2015). Owing to the correlation of TIM-3 with the functions...
of macrophage populations in GBM, we further sought to examine this glioma cell-TIM-3 signal on the behaviors of macrophages in TME of GBM. First, we investigated whether the glioma cell-TIM-3/IL6 signal promoted macrophage migration in GBM. The data showed that CM from TIM-3 knockdown glioma cells significantly decreased macrophage migration compared with CM from the control group. This could be rescued by IL6 supplementation (Figures 4E and S5B). Interestingly, the incubation of CM from TIM-3 overexpressed glioma cells strongly increased macrophage migration, which could be blocked by the anti-Gal-9 antibody (Figures S5B and SSC). We further explored whether this glioma cell-TIM-3/IL6 signal could induce the phenotype transition of macrophages in GBM. As expected, CM from TIM-3 overexpressed glioma cells significantly increased anti-inflammatory/pro-tumorigenic marker expression and decreased pro-inflammatory/anti-tumorigenic marker expression (Yeini et al., 2021) (Figures 4F–4I, SSD, and SSE). TIM-3 knockdown induced an opposite effect (Figures 4G, 4I, SSD, and SSE). This pro-inflammatory/anti-tumorigenic and anti-inflammatory/pro-tumorigenic marker expression shift could be inhibited by the Gal-9 blocking antibody (Figures 4F, SSD, and SSE). With GL261 and the primary mice glioma sphere cells from sleeping beauty (SB) mice glioma model (Wiesner et al., 2009), we found that in the Tim3 OE group, more TAMs infiltrated into tumor tissue, while less CD8+ T cells and similar CD4+ T cells infiltrated into tumor tissue (Figures 4J and S7A). qPCR assay in bone marrow-derived macrophages (BMDM) cocultured with GL261 cells also confirmed decreased pro-inflammatory/anti-tumorigenic marker expression (Ifih1, Nos2, Il1b, and Tnfa; Figures S6A–S6D) and the elevated anti-inflammatory/pro-tumorigenic marker expression (Tgfbo, Cd206, and Trem2; Figures S6E–S6G). In vitro migration assay also confirmed the increased migration of these cells cocultured with Tim3 overexpression GL261 cells (Figure S6H), which was consistent with the qPCR and migration results of RAW264.7 cocultured with SB cells (Figures S6I–S6P). Moreover, in vivo, FACS analyses of GL261 glioma cells revealed a reduction of MHC II and an elevation of CD206+ CD11b+ cells after Tim-3 overexpression (Figures S7B and S7C). Pd1 positive cell ratio in CD45+ CD3+ or CD45+ CD3+ CD8+ cells were increased after TIM-3 overexpression (Figures S7D and S7E). The ratio of Ifng positive cells was decreased in the Cd45+ Cd3+ Cd8+ cell population after TIM-3 overexpression (Figure S7F), and Foxp3 positive cell ratio showed no significant change with TIM-3 overexpression (Figure S7G). The results of SB glioma cells were consistent with GL261 glioma cells in vivo (Figures S7H–S7M). This indicates that TIM-3 promotes the activation of anti-inflammatory/pro-tumorigenic macrophages and promotes T cell exhaustion to form the immune suppressive TME of GBM. We also found a decrease in pro-inflammatory/anti-tumorigenic markers (Tnfa, Ifih1, and Cd86) and an increase in anti-inflammatory/pro-tumorigenic macrophage marker expression (Cd206 and Stat3) in GL261 and SB glioma cells with Tim-3 overexpression by qPCR (Figures S8A–S8J). In addition, qPCR analyses in GL261 and mice SB glioma tumor tissue disclosed an elevation of Ccr2/Cx3cr1 ratio with TIM-3 overexpression (Figures S8K–S8P). This supports the macrophages infiltrating glioma tissue induced by TIM-3 overexpression are dominated by peripheral blood-derived macrophages. IHC staining of IL6, IBA1, CCR2, and CX3CR1 in GSC40 tumor tissue also confirmed this result (Figure S8Q). Collectively, these results showed that glioma cell-TIM-3 signaling promoted macrophage migration and induced activation to anti-inflammatory/pro-tumorigenic macrophages, which may involve in regulating glioma immune microenvironment. And the macrophages infiltrating glioma tissue are dominated by peripheral blood-derived macrophages.

Activation of NF-kB is required for glioma-intrinsic TIM-3/IL6 signaling

Since TIM-3 promoted the p65 phosphorylation, we further tested BAY 11-7082, an NF-κB inhibitor, on glioma TIM-3/IL6 signaling. The data showed that BAY 11-7082 significantly inhibited phospho-p65, not total p65, and IL6 in glioma cells (Figures S9A and S9B), and restrained their elevation induced by TIM-3 (Figures S9C and S9D). The increased proliferation, self-renewal, and migration abilities of glioma cells induced by TIM-3 were also attenuated by BAY 11-7082 (Figures S9E–S9H). Interestingly, the incubation of CM from TIM-3 overexpressed glioma cells strongly increased macrophage migration, which could be blocked by BAY 11-7082 (Figure S10A). Together, these results showed that the activation of NF-κB was required for glioma cell TIM-3/IL6 signaling.

Blockade of IL6 signaling attenuates glioma cell-TIM-3 induced proliferation, migration, and invasion of glioma cells, and the crosstalk between glioblastoma cells and anti-inflammatory/pro-tumorigenic tumor-associated macrophages

Next, we sought to evaluate the clinical potential strategy against GBM by blocking the tumor intrinsic TIM-3 signal. We employed two antibodies: an anti-TIM-3 antibody, and Tocilizumab, an anti-IL6 receptor antibody, previously recommended for the treatment of immune diseases (Jones and Jenkins, 2018; Ma et al., 2009). Consistent with results obtained from TIM-3 knock-down glioma cells, the blockade of
TIM-3 or its downstream effector, IL6 signal, significantly inhibited the proliferation and neurosphere formation capabilities of GSC40 cells (Figures S5A and S11A). We confirmed the effects of the anti-Tim3 antibody and anti-Il6r antibody in vivo experiments and found that anti-Tim3 antibody and anti-Il6r antibody significantly inhibited GL261 tumor growth (Figure S5B) as in vitro Figure 5A, while there was no significant difference between anti-Tim3 antibody and anti-Il6r antibody between tumor size and survival. Owing to the clinical availability of Tocilizumab, we then focus on it and investigated whether it could inhibit the enhanced malignant behaviors of glioma cells induced by TIM-3. The result showed that Tocilizumab (500 ng/mL) efficiently attenuated the malignant behaviors including growth, migration, and invasion of glioma cells (Figures S5C–S5H and S10A–S10D). Besides, IL6 incubation increased anti-inflammatory/pro-tumorigenic and decreased pro-inflammatory/anti-tumorigenic marker expression in PBMC-derived macrophages. This could also be partially blocked by Tocilizumab (Figure S5I). These data indicated a potential implication of Tocilizumab in attenuating TIM-3-related glioma malignant behaviors in vitro.

To further explore how glioma TIM-3/IL6 signals regulate the intercellular communication in GBM, we used CM from glioma cells to treat THP-1-derived macrophages. We observed the elevation of IL6, TIM-3, and phospho-p65 in these cells incubated with CM from glioma cells with TIM-3 overexpression, which could be blocked by Tocilizumab (Figures 5J and S11F). By contrast, the incubation of THP-1-derived macrophages with CM from TIM-3 knockdown glioma cells decreased their IL6 expression (Figure S11G). ELISA showed that IL6 concentration in CM of anti-inflammatory/pro-tumorigenic TAMs was higher than that of M0 (Figure S11H). Anti-inflammatory/pro-tumorigenic TAMs and macrophages stimulated with IL6 had higher TIM-3 and IL6 expressions than M0 or pro-inflammatory/anti-tumorigenic TAMs (Figure S11I). The incubation of CM from glioma cells with TIM-3 overexpression increased IL6 secretion of THP-1-derived macrophages, which could also be efficiently blocked by Tocilizumab (Figure S11J). Additionally, TIM-3 overexpression in M0 and pro-inflammatory/anti-tumorigenic macrophages led to IL6, TIM-3, and phospho-p65 elevation in these cells, which also be significantly inhibited by Tocilizumab (Figure S5K). In vivo, we also found an increase in pro-inflammatory/anti-tumorigenic markers (Cd68, Infγ, Irf7, Mhc II, and Tnfα) and a decrease in anti-inflammatory/pro-tumorigenic macrophage marker expression (Cd206 and Stat3) in GL261 glioma cells treated with anti-Tim3 antibody and anti-Il6r antibody by qPCR (Figures S12A–S12I). In addition, IHC staining of Ki67, IBA1, Cd68, Cd206, Cd3, Cd4, and Cd8 in GL261 tumor tissue also confirmed this result (Figure S12J). Collectively, combined with the data that anti-inflammatory/pro-tumorigenic TAMs increased TIM-3 expression in cocultured glioma cells (Figures 1K and S1H), these results indicated a potential regulatory loop contributing to GBM progression via TIM-3/IL6 signaling between glioma cells and anti-inflammatory/pro-tumorigenic TAMs, and supported IL6 as the core molecule in the crosstalk via TIM-3 signal between GBM cells and anti-inflammatory/pro-tumorigenic TAMs. TIM-3 suppression by Tocilizumab implied the feasibility of this clinical IL6 inhibitor to block TIM-3 signaling.
Blocking IL6 signaling inhibits the enhanced tumorigenicity of glioblastoma cells induced by TIM-3 in vivo

Finally, we employed a mouse orthotopic model to investigate whether TIM-3 overexpression alone or in combination with macrophages can promote tumors progression and whether Tocilizumab efficiently...
TIM-3 is mainly expressed in immune cells and regulates both innate immunity and adaptive immunity (Zhang et al., 2018). Unexpectedly, we found that TIM-3 was one of the most highly expressed immune checkpoints in GBM, instead of PD-1 and CTLA-4. This may be one reason why ICIs of PD-1 and CTLA-4 did not work well in GBM. TIM-3 has been shown to be expressed by liver cancer cells with a potential tumor-intrinsic mechanism (Zhang et al., 2018). In glioma, TIM-3 is upregulated in circulating NK cells and monocytes from patients with glioma and acts as a mesenchymal GBM marker and an indicator of increased their tumorigenicity in vivo. which indicated the regulatory role of TIM-3 in the malignant behaviors of glioma cells. TIM-3 has been shown to drive the self-renewal of human myeloid leukemia stem cells and promoted leukemia progression (Kikushige et al., 2015). Interestingly, we obtained similar data in GBM. Manipulating TIM-3 expression in glioma sphere cells significantly affected their self-renewal capabilities and the expression of markers associated with glioma stemness regulation like SOX4, CD44, and NANOG. This supported the crucial role of tumor-intrinsic TIM-3 in malignant glioma progression. To our knowledge, this function of TIM-3 has not been reported in gliomas.

The aggregation of anti-inflammatory/pro-tumorigenic macrophages is a characteristic of GBM, especially in the mesenchymal subtype. Thus, we investigated whether TIM-3 mediated the interaction between GBM cells and TAMs. We found an elevation of migration capabilities and anti-inflammatory/pro-tumorigenic markers, and a decreased expression of pro-inflammatory/anti-tumorigenic markers on macrophages after 24 h stimulation with CM from TIM-3 overexpressed glioma cells. These data provided a new insight into the role of TIM-3 in the phenotypic transition of macrophages in GBM. A positive feedback loop is central to high cytokine expression in TME (Nguyen et al., 2017). Previous reports showed that Gal-9 could bind with and activate TIM-3 by increasing phosphorylation at its tyrosine residues (Lee et al., 2011). AML cells could secret Gal-9 and constitute an autocrine loop with TIM-3 (Kikushige et al., 2015). We found that Gal-9 was the ligand most highly correlated with TIM-3 in GBM. Blocking of Gal-9 inhibited the effect induced by TIM-3, but TIM-3 overexpression did not lead to Gal-9 elevation in GBM cells. This suggested Gal-9 activated TIM-3...
through a feedforward mechanism and no feedback from TIM-3 to Gal-9 in GBM cells. Our previous study defined an eight-gene immune-related gene signature in GBM, including IL6 (Cheng et al., 2016b). Cytokine screening demonstrated elevated IL6 secretion induced by TIM-3 in glioma cells. This enhanced IL6 signal may enhance the crosstalk between GBM cells and macrophages, and triggered anti-inflammatory/pro-tumor-igenic activation of macrophages, and increased TIM-3 expression in these cells. This may illustrate the recruitment mechanism of TAMs in GBM, especially the mesenchymal subtype, which has the most abundant TIM-3 expression and TAMs infiltration. In mechanism, we got similar data to a previous report (Zhang et al., 2018). Gal-9/TIM-3 interaction among cancer cells increased IL6 secretion by activating NF-kB in GBM. However, owing to the low transcript abundance of IL6 (Sankowski et al., 2019), we explored the potential post-transcription regulatory mechanism associated with the accumulation of IL6 protein in GBM TME. The mass spectrometry analysis of ubiquitination modification showed the inhibition of ubiquitin modification on IL-6 protein after TIM-3 overexpression (Data not shown). This indicates the possible TIM-3-mediated mechanism on IL-6 protein ubiquitination. Further study is needed to clarify the detailed mechanism of this.

Finally, our work has potential clinical translational significance. We provided preclinical evidence of a clinical IL6 receptor antibody, Tocilizumab, effectively inhibited TIM-3-induced GBM growth, similar to previous reports (Wang et al., 2009). Clinical trial (NCT02017717) shows that GBM responding to PD-1 antibodies are rare. The current blockade strategy toward this TIM-3/IL6 feedback loop may be an alternative option to improve the GBM outcome. As suggested by our data, this strategy may not only directly target TIM-3/IL6 expression immune cells, but also aim at tumor counterpart. The current in vivo experiments did not directly use TIM-3 antibody, because IL6 is central to the TIM-3 positive feedback signal in GBM, and the effect of its blockade in vitro was similar to anti-TIM-3 antibody (Figures 5A and 5B). Another reason for choosing Tocilizumab is its clinical availability. The safety and efficacy of Tocilizumab for IL6 receptor have been established (Jones and Jenkins, 2018). This could make it easier to clinically translate our research. In the future, we will compare the efficacy of Tocilizumab with strategies directly toward other immunomodulatory molecules and explore combination therapies. Our data also suggested that tumor cells with TIM-3 elevation could proliferate rapidly and attract macrophages migrating to them. This may lead to a reduced purity and a more complicated TME and indicate an important role of TIM-3 signal in the progression of GBM. Additionally, among GBM subtypes, the prognosis of the mesenchymal subtype is the worst. An improvement in mesenchymal GBM outcomes may effectively increase the overall survival of patients with GBM. Since mesenchymal GBM expresses higher TIM-3 and IL6 than other subtypes, TIM-3 signaling blockade may be more effective in it. Further study is needed to test this hypothesis.

In conclusion, our study identified a TIM-3-mediated mechanism in the progression of GBM cells. As one of the major regulators of IL6 in GBM, TIM-3 increases its transcription through activating the NF-kB signal. This TIM-3/IL6 signal further induced anti-inflammatory/pro-tumorigenic activation and migration of monocytes in TME and mediated the crosstalk between glioma cells and TAMs. Antibody blockade of this loop prolonged the survival of tumor-bearing mice. Thus, targeting TIM-3 may provide a novel therapeutical opportunity for GBM.

Limitations of the study
Recently, increasing evidence confirms the cancer-intrinsic immune check-point mechanism in the immune evasion of tumor cells. According to the present study, we identify a TIM-3/NF-kB mediating IL6 regulatory mechanism in glioma cells at the transcriptional level, which promotes their tumorigenicity and regulates their crosstalk with TAMs. However, as a well-known immune-regulatory cytokine, IL6 is a cytokine with significant expression and low transcript abundance in GBM TME (Sankowski et al., 2019). The post-transcriptional regulatory mechanism associated with IL6 need to be further investigated to fully illustrate its accumulation in the TME of this devastating tumor. Additionally, the intervention time of IL6 blocking therapy in GBM is also worth discussing.

STAR METHODS
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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105329.

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AUTHOR CONTRIBUTIONS
Q. G., P. C., and A. H. W. conceived and designed the study. Q. G., S. S., G. F. G., C. Y. Z., J. Y. C., W. C., C. Z., and J. H. Y. performed experiments and collected data. Q. G. and W. C. performed the bioinformatics analysis. P. C., Q. G., G. L. W., X. Y. X., Z. G. L., L. C., and A. H. W. contributed to draft the article. A. H. W., P. C., and G. L. W. obtained funding. All authors read and approved the final article.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CTLA4 (F8)          | Santa Cruz Biotechnology | sc-376016, RRID: AB_10988256 |
| PDL1 (E1L3N)        | Cell Signaling Technology | 13684P, RRID: AB_2687655 |
| PDL2(D7U8C)         | Cell Signaling Technology | 82723T |
| IDO1                | Proteintech | 13268 |
| PD-1                | R and D Systems | AF1086, RRID: AB_354588 |
| MMP2                | Abcam | ab37150, RRID: AB_881512 |
| IL6                 | Abcam | ab6672, RRID: AB_2127460 |
| IL6                 | R & D Systems | AF206, RRID: AB_354392 |
| Flag                | Sigma-Aldrich | F1804, RRID: AB_262044 |
| Galectin-9          | Abcam | ab9630, RRID: AB_1268942 |
| TIM-3               | Proteintech | 60355, RRID: AB_2881464 |
| TIM-3               | Abcam | ab47997, RRID: AB_883007 |
| TIM-3 (DSDSR)       | Cell Signaling Technology | 45208, RRID: AB_2716862 |
| NF-κB p65(S536) (93H1) | Cell Signaling Technology | 30335, RRID: AB_331284 |
| NF-κB p65(D14E12)  | Cell Signaling Technology | 82425, RRID: AB_10859369 |
| GAPDH               | Proteintech | 60004-1, RRID: AB_2107436 |
| β-actin             | Sigma-Aldrich | A1978, RRID: AB_476692 |
| HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) | Proteintech | SA00001-1, RRID: AB_2722565 |
| HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) | Proteintech | SA00001-2, RRID: AB_2722564 |
| PE Mouse Anti-Human TIM-3 | BD Biosciences | 563422, RRID: AB_2716866 |
| Alexa Fluor® 647 Mouse anti-GFAP | BD Biosciences | 561470, RRID: AB_10646037 |
| FITC Mouse Anti-Human CD45 | BD Biosciences | 555482, RRID: AB_395674 |
| PE Mouse IgG1, κ Isotype Control | BD Biosciences | 554680, RRID: AB_395506 |
| PE Mouse Anti-Human CD11b/Mac-1 | BD Biosciences | 555388, RRID: AB_395789 |
| BV421 Mouse Anti-Human CD206 | BD Biosciences | 564062, RRID: AB_2738570 |
| APC Mouse Anti-Human CD11b/Mac-1 | BD Biosciences | 569906 |
| HRP-conjugated Affinipure Donkey Anti-Goat IgG(H+L) | Proteintech | SA00001-3 |
| Mouse IgG1 Isotype Control | R and D Systems | MAB002, RRID: AB_357344 |
| Purified anti-human Galectin-9 Antibody | BioLegend | 348902, RRID: AB_10612753 |
| Tocilizumab         | Roche | ACTEMRA |
| nVivoMAb human IgG1 isotype control | Bio X Cell | BE0297, RRID: AB_2687817 |
| Goat Anti-Mouse IgG(H+L), FITC conjugate | Proteintech | SA00003-1 |
| Goat Anti-Rabbit IgG(H+L), FITC conjugate | Proteintech | SA00003-2 |
| Goat Anti-Mouse IgG(H+L), Rhodamine conjugate | Proteintech | SA00007-1 |
| Goat Anti-Rabbit IgG(H+L), Rhodamine conjugate | Proteintech | SA00007-2 |
| BD Horizon^®BV510 Rat Anti-Mouse CD45 | BD Biosciences | 563891 |
| BD Pharmingen^®PerCP-Cy™5.5 Hamster Anti-Mouse CD3e | BD Biosciences | 551163 |
| BD Pharmingen^®APC-Cy™7 Rat Anti-Mouse CD4 | BD Biosciences | 552051 |
| BD Pharmingen^®FITC Rat Anti-Mouse CD8a | BD Biosciences | 553031 |
| BD Pharmingen^®PE Rat Anti-Mouse IFN-γ | BD Biosciences | 554412 |

(Continued on next page)
Continued

**REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER**
--- | --- | ---
BD Pharmingen™ APC Hamster Anti-Mouse CD279 | BD Biosciences | 562671
BD Pharmingen™ FITC Rat Anti-CD11b | BD Biosciences | 553310
BD Pharmingen™ PE Rat Anti-Mouse I-A/I-E | BD Biosciences | 557000
BD Pharmingen™ Alexa Fluor® 647 Rat Anti-Mouse CD206 | BD Biosciences | 565250
BD Pharmingen™ PE Rat anti-Mouse Foxp3 | BD Biosciences | 563101
Anti-CD3 Rabbit pAb | Servicebio | GB111337
Anti-CD4 Rabbit pAb | Servicebio | GB11064
Anti-CD8 alpha Rabbit pAb | Servicebio | GB11068
Ki67 | Servicebio | GB111141
IBA1 | Servicebio | GB113502
Anti-CX3CR1 Rabbit pAb | Servicebio | GB11861
TIM-3 (D3M9R) XP® Rabbit mAb (Mouse Specific) | Cell Signaling Technology | 83882; RRID: AB_2800033
CCR2 | Servicebio | GB11326
IL6R | Proteintech | 23457-1-AP

**Biological samples**

| Biological samples | Source | N/A |
| --- | --- | --- |
| Glioma samples, see Table S3 | First Hospital of China Medical University | N/A |
| Non-tumor samples, see Table S3 | First Hospital of China Medical University | N/A |

**Chemicals, peptides, and recombinant proteins**

| Chemicals, peptides, and recombinant proteins | Source | N/A |
| --- | --- | --- |
| Dulbecco’s Modified Eagle Medium (DMEM), high glucose (+GlutaMAX) | Gibco | 10566024 |
| FBS | Gibco | 16140071 |
| Penicillin-Streptomycin-Glutamine (100X) | Gibco | 10378016 |
| Roswell Park Memorial Institute 1640 Medium (RPMI-1640) (+GlutaMAX) | Gibco | 61870036 |
| Dulbecco’s Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12) | Gibco | 11320033 |
| B-27™ Supplement, minus insulin | Gibco | A1895601 |
| Animal-Free Recombinant Human EGF | Peprotech | AF-100-15 |
| Animal-Free Recombinant Human FGF-basic (154 a.a.) | Peprotech | AF-100-18B |
| Accutase(R) solution sterile-filtered, suitable for cell culture | Sigma-Aldrich | A6964 |
| Ficoll-Paque PLUS | GE Healthcare | 17544203 |
| Phorbolester 12-myristate 13-acetate (PMA) | Sigma-Aldrich | P1585 |
| Lipopolysaccharides (LPS) | Sigma-Aldrich | L2630 |
| Recombinant Human IFN-gamma Protein | R and D Systems | 285-IF |
| Recombinant Human IL-4 Protein | R and D Systems | 204-IL |
| RIPA Lysis Buffer | Beyotime Biotechnology | P0013B |
| Immobilon PVDF membranes, 0.45 um | Millipore | IPVH00010 |
| ECL reagent | Tanon | 180-5001 |
| DAB kit | ZSGB BIO | ZLI-9017 |
| 2-(4-Aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI) | Beyotime Biotechnology | C1002 |
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| TRIzol reagent | Invitrogen | 15596018 |
| Prime-Script™ RT Master Mix | TaKaRa | RR036Q |
| SYBR® Premix Ex Taq™ II | TaKaRa | RR820A |
| Polybrene | GeneChem | REVG0001 |
| Lipofectamine™ 3000 Transfection Reagent | Invitrogen | L3000008 |
| Difco™ Skim Milk | BD Biosciences | 232100 |
| Cell lysis buffer for Western and IP | Beyotime Biotechnology | P0013 |
| Phenylmethanesulfonyl fluoride (PMSF) | Beyotime Biotechnology | STS05 |
| Dynabeads™ Protein A for Immunoprecipitation | Invitrogen | 10002D |
| 4% paraformaldehyde | Solarbio Science & Technology | P1110 |
| Triton X-100 | Solarbio Science & Technology | T8200 |
| Crystal violet solution, 1% | Solarbio Science & Technology | G1059 |
| Ionomycin | Sigma-Aldrich | I3909 |
| BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Solution Kit with BD GolgiPlug™ | BD Biosciences | 555028 |
| BD Pharmingen™ Transcription Factor Buffer Set | BD Biosciences | 562574 |

Critical commercial assays

| Critical commercial assays | SOURCE | IDENTIFIER |
|---------------------------|--------|------------|
| Proteome profiler human cytokine array | R and D Systems | ARY005B |
| Human IL6 DuoSet ELISA | R and D Systems | DY206 |
| CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) | Promega | G5421 |
| kFluor488 Click-iT EdU imaging detection kit | KeyGEN BioTECH | KGA331 |

Experimental models: Cell lines

| Experimental models: Cell lines | SOURCE | IDENTIFIER |
|-------------------------------|--------|------------|
| Human: Primary patient-derived glioma (neuro)spheres: GSC4, GSC24, GSC28, and GSC40 | First Hospital of China Medical University | N/A |
| Human: Primary adherent cells (PGC1228, PGC21, PGC24, PGC28, and PGC40) | First Hospital of China Medical University | N/A |
| Human: U87 cells | GeneChem | RRID: CVCL_0022 |
| Human: U251 cells | GeneChem | RRID: CVCL_0021 |
| Human: LN229 cells | BIOWING | RRID: CVCL_0393 |
| Human: normal human astrocytes (NHA) | Provided by Professor Xin Meng | N/A |
| Human: THP-1 cells | First Hospital of China Medical University | N/A |
| Human: PBMCs | First Hospital of China Medical University | N/A |
| Mouse: Spontaneous glioma (neuro) spheres: SB | First Hospital of China Medical University | N/A |
| Mouse: GL261 | iCell | RRID: CVCL_Y003 |
| Mouse: RAW 264.7 | Fenghui Biological Technology | RRID: CVCL_0493 |

Experimental models: Organisms/strains

| Experimental models: Organisms/strains | SOURCE | IDENTIFIER |
|----------------------------------------|--------|------------|
| BALB/C-Null nude mice (six-week-old, male or female) | Charles River | 401 |
| C57BL/6N mice (six-week-old, male) | Charles River | 213 |
| NOD SCID mice (six-week-old, female) | Charles River | 406 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Anhua Wu (ahwu@cmu.edu.cn).

**Material availability**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Oligonucleotides** | This paper for human glioma cells, including U87, PGC1228 and GSC40. | N/A |
| siTIM3-1 sequence: Sense GGUCCUCAG AAGUGGAUATT, Antisense UAUUCCA CUUCUGAGGA CCTT | This paper | N/A |
| siTIM3-2 sequence: Sense GAGCCUCCCUGAUAAUAATT, Antisense AUUUAUAUCAGGGAGGCCU | This paper | N/A |
| Primers for Real-time PCR, see Table S6 | This paper | N/A |
| **Recombinant DNA** | GeneChem | GV146 |
| Lentiviral TIM-OE vector: Ubi-MCS-SV40-firefly_Luciferase-IRESPuromycin | GeneChem | GV308 |
| Lentiviral TIM-OE vector: TetIIP-MCS-3FLAG-Ubi-TetR-IRESPuromycin | GeneChem | 20207 |
| PT2/C-Luc/PGK-SB13 | Addgene | 20207 |
| pT/Nestin-SV40-LgT | This paper | N/A |
| pT/Nestin-NRASV12 | This paper | N/A |
| **Software and algorithms** | BD Biosciences | Version 10.4 |
| FlowJo | NIH | https://imagej.nih.gov/ij/ |
| Image J software | Microsoft | 2013 |
| Excel | GraphPad Software | Version 7 |
| PRISM | LHR (Huang et al., 2009) | https://david.ncifcrf.gov/tools.jsp |
| DAVID 6.8 | PMID: 23323831 (Henzelmann et al., 2013) | http://www.bioconductor.org |
| Gene Set Variation Analysis (GSVA) | Broad Institute(Mootha et al., 2003; Subramanian et al., 2005) | http://www.broadinstitute.org/gsea/index.jsp |
| Gene Set Enrichment Analysis (GSEA) | Standford (Newman et al., 2015) | https://cibersort.stanford.edu/ |
| CIBERSORT | PMID: 27765066 (Becht et al., 2016) | N/A |
| Microenvironment cell populations (MCP)-counter | R Foundation | version 3.4 |
| R | PMID: 19567251 (Huang et al., 2009) | http://bioinf.wehi.edu.au/software/elda/ |
| **Other** | Roche | 480II |
| PCR LightCycler 480 | Perkin Elmer | VICTOR Nivo |
| EnVision Plate Reader | Olympus | IX71 |
| Fluorescence Microscope | Leica | DM8 |
| Microscope | Leica | DM2500 LED |
| Microscope |
Data and code availability
All the detailed data in this paper are available upon request. This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics and human specimens (patient cohort)
This study was approved by the Ethics Committee of the First Hospital of China Medical University. All animal experiments were approved and performed under the supervision of the Institutional Animal Care and Use Committee in China Medical University. The patient samples used in this study were collected at the First Hospital of China Medical University (CMU1h samples, Table S3). The histological diagnoses were confirmed by two neuropathologists according to the 2016 WHO classification guidelines. The samples were de-identified before being processed. Informed consent was obtained from each patient.

Cell lines and cell culture
Primary patient-derived glioma (neuro)spheres (GSC4, GSC24, GSC28, and GSC40) and adherent cells (PGC1228, PGC21, PGC24, PGC28, and PGC40) were derived from fresh gliomas immediately after surgery at the First Hospital of China Medical University (Table S4) (Mao et al., 2013; Wu et al., 2019). The identities of PGC1228 and GSC40 have been authenticated by short tandem repeat (STR) analysis at Baihao Biotechnology (Kim et al., 2016) (Benxi, Liaoning, China, http://www.baihaobio.com/, Table S5). THP-1 cells were provided by Professor Xin Meng (Department of Biochemistry, China Medical University, China). Primary adherent cells were cultured in RPMI-1640 medium, containing 10% FBS and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO2. Glioma spheres were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12, supplemented with 2% B27 supplement, 20 ng/mL EGF, 20 ng/mL basic-FGF, and 1% penicillin/streptomycin (Cheng et al., 2016a). Human normal astrocytes (NHA) were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. PBMCs were isolated by centrifugation of human blood with Ficoll-Paque PLUS (Zhou et al., 2015). Monocytes were collected by attachment to plastic after 4 h culture in RPMI-1640 medium containing 10% FBS. THP-1 cells were maintained in RPMI-1640 medium containing 10% FBS, and RAW 264.7 cells were cultured in DMEM containing 10% FBS. GL261 cells were cultured in DMEM containing 10% FBS.

METHOD DETAILS

Isolation of bone marrow-derived macrophages and treatment conditions
Bone marrow-derived myeloid progenitor cells were isolated by harvesting marrow from the femurs of 5–6-week-old C57BL/6 mice. Non-adherent cells were collected by centrifugation and red blood cells lysed. Cells were then cultured in α-MEM with 20 ng/mL macrophage colony stimulating factor (M-CSF) (R & D Systems) for 3 days to generate bone marrow-derived, M-CSF dependent macrophages (BMDMs). GL261 cells transfected with control or Tim3 overexpression vector were pretreated with 5 μg/mL doxycycline for 24 hours and then co-cultured with BMDMs in 0.4 um co-culture chambers.

Immunohistochemistry (IHC) and IHC scoring
For IHC staining, tissue sections were fixed with 4% paraformaldehyde, embedded in paraffin, and then cut into 4 μm sections. A streptavidin-biotin immunostaining method was employed (Guo et al., 2019), and staining intensity of each tissue section was evaluated at 400x magnification using five randomly selected fields of view. TIM-3 expression was scored according to the German immunohistochemical score (GIS) method (Berns et al., 2007). Each tissue section was viewed at a 400x magnification using five randomly selected fields of view. The levels of TIM-3 expression were scored based on the staining intensity and distribution using the immunoreactive score (IRS) as follows: IRS = staining intensity (SI) × percentage of positive cells (PP) (Berns et al., 2007). The SI was determined as follows: Absent, 0; weak, 1; moderate, 2; and strong, 3. The PP were scored as follows: 0%, 0; 0%–25%, 1; 25%–50%, 2; 50%–75%, 3; and 75%–100%, 4 (Berns et al., 2007). The information of CMU1h patient samples for IHC staining were listed in Table S3 (TIM-3 expression analysis in glioma: P1-P13, G201-G206, G301-G307, G401-477; TIM-3 expression and survival analysis in GBM: G425-477; TIM-3 and IBA1 expression correlation analysis in GBM: G425-G453).
**Immunocytochemistry**

For immunocytochemistry, the cells were seeded into a dish at a density of $1 \times 10^3$ per well and incubated at 37°C with 5% CO$_2$ for 24 h. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 20 min. After 5% BSA incubation for 1 h, primary antibody was added and incubated overnight at 4°C. Following incubation with fluorescein or rhodamine conjugated secondary antibody and 4',6-diamidino-2-phenylindole (Booster, Tan Zhou, China), the samples were detected using a fluorescence microscope (BX53; Olympus, Tokyo, Japan).

**Preparation of conditioned medium (CM)**

CM was obtained from $2 \times 10^5$ glioma cells in 2 mL medium with 0.1% bovine serum albumin for 24 h. The medium was collected after 5 minutes of 1500 rpm centrifugation and stored at −80°C (Wang et al., 2018).

**Cell treatment**

For IL6 stimulation and the blocking of IL6, Gal-9, and TIM-3, the cells were incubated with recombinant human IL6 (40 ng/mL, 24 h), Tocilizumab (500 ng/mL, 24 h), an anti-Gal-9 blocking antibody (10 µg/mL, 12 h), and anti-TIM-3 blocking antibody (10 µg/mL, 24 h) (Markwick et al., 2015), respectively.

**Proteome profiler human cytokine array**

GSC40 cells transduced with TIM-3 overexpression or control lentiviral vectors were seeded into 6-well plates at a density of $1 \times 10^6$ cells in 1.5 mL F12 medium without B27 supplement, EGF, basic-FGF, penicillin/streptomycin (Gibco) per well. After culture at 37°C with 5% CO$_2$ for 24 hours, the medium was collected by centrifugation and then measured with proteome profiler human cytokine array method (ARY005B, R&D), following manufactures’ instructions. Protein expression was quantified using Image J software.

**Lentivirus mediated TIM-3 over-expression**

Lentiviral TIM-3 overexpression or control vectors were obtained from GeneChem. The lentivirus transduction was performed as manufacturer’s plan described in the presence of polybrene (Cheng et al., 2016a).

**Small interfering RNA (siRNA) and cell transfection**

Specific siRNA targeting TIM-3 and a negative control were synthesized by Sangon Biotech. siRNAs were transfected with Lipofectamine 3000 reagent (Life Technologies) according to the manufacturer’s instructions.

**RNA isolation and reverse-transcription quantitative PCR (RT-qPCR)**

Total RNA was isolated from cell and clinical tissue samples using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Total RNA was reversely transcribed into cDNA with Prime-Script RT Master Mix (TaKaRa). qPCR was performed in a thermal cycler (Roche) with SYBR Green Master Mix (TaKaRa). The following conditions were used: 1 cycle of 95°C for 30s, followed by 40 cycles of a two-step cycling program (95°C for 5s; 60°C for 30s). The mRNA expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH or 18S mRNA expression (Schmittgen and Livak, 2008). The PCR primer sequences were listed in Table S6.

**In vitro cell proliferation assays**

After transduction for 60 h, glioma cells (PGC1228 and U87) were seeded into 96-well plates at a density of $1 \times 10^3$ cells in 100 µL medium per well and incubated at 37°C with 5% CO$_2$ for 5 days. The cell growth was measured at day 0, 1, 2, 3, 4, and 5 by adding 20 µL MTS solution (Promega) into the wells and incubated for 3 h at 37°C. Optical density (OD) values of each well were determined with a microplate reader (Molecular Devices, SpectraMax M5) at the absorbance of 490 nm. After seeded into the plate, the proliferation rate of GSC40 was analyzed according to the size of neurospheres at day 10 (Cosset et al., 2017).

**EdU incorporation assay**

After dissociated into single cell solutions, glioma cells were seeded into 24-well chamber slides coated with (GSC 40) or without (PGC1228, U87) laminin (sigma) at the density of 20,000 cells per well. Then, 10 µM EdU were added into each well. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS.
and stained with the Click-iT EdU kit (KeyGEN BioTECH). Proliferation index was then determined by quantifying percentage of EdU labelled cells using confocal microscopy at 400× magnification (Venkatesh et al., 2017).

**Colony formation assay**

The cells were collected and resuspended as mentioned above. Then the cells were seeded into a six-well plate at a density of 500 cells each well, and incubated at 37°C with 5% CO₂ for 15 days. The cells were stained with 1% crystal violet solution. The number of colonies (the diameter larger than 20 μm) was counted under a microscope (Leica).

**Extreme limiting dilution analysis (ELDA)**

Neurosphere-forming capability of GSC40 was determined using the ELDA web-based tool (Hu and Smyth, 2009). In brief, the cells were dissociated with accutase and seeded into 96-well plates at 1, 10, 25, 50 cells/wells. After 14 days, the numbers of spheres with diameters larger than 60 μm in each well were counted.

**Wound healing assay**

The cells were seeded into six-well plates at a density of 5×10⁵ per well. After 24 h, the cells reached 100% confluence and a 200 μL pipette tip was used to scratch a neat and straight line in each well. Then the wells were washed with PBS twice to remove cell debris, and fresh serum-free medium added. Five fields of each wound were monitored at 0 and 24 h to evaluate the migration of cells. The wound healing rate was calculated with Image J.

**Cell migration and invasion assay**

Transwell inserts with a pore size of 8 μm (Corning, 3422) were used for cell migration and invasion assay *in vitro*. For migration assays, cells were resuspended as single cells in serum-free medium and seeded into the upper chambers at a density of 4×10⁴ per 200 μL (Zhou et al., 2015). Then 700 μL of conditioned medium was added into the lower chamber. For invasion assay, glioma cells were resuspended as single cells in DMEM containing 0.2% FBS and seeded into the upper chamber which was pre-coated with 500 ng/mL Matrigel solution (BD) at a density of 4×10⁴ per 200 μL. Then, 700 μL of DMEM containing 20% FBS was added into the lower chamber. After 24 h incubation, the cells on the upper side of the membrane were physically removed with a cotton swab. After fixation with methanol, the cells migrating or invading into the lower side of the membrane were stained with 1% crystal violet solution. The number of cells were counted under five different random high-power fields in each well.

**Protein extraction and western blotting**

Total proteins from tissues and cells were extracted by whole cell lysis buffer and quantitated using BCA test kit (Beyotime Biotechnology, Beijing, China) (Guo et al., 2019). Thirty micrograms of protein from each sample were loaded onto a lane and electrophoresed using 10% SDS-PAGE followed by transfer to a polyvinylidene difluoride (PVDF) membrane (0.45 μm; Millipore, Burlington, MA, USA). After being blocked with 5% skimmed milk, the PVDF membranes were incubated with the primary antibody overnight at 4°C. Then, the PVDF membranes were incubated with the secondary antibodies at room temperature for 1 h. Protein expression was visualized with a chemiluminescence ECL reagent (Tanon, Woburn, MA, USA), and quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

**Immunoprecipitation (IP)**

After cells were lysed in IP lysis buffer with protease inhibitors, TIM-3 antibody (1:100; 45208, CST) was added to the lysates and incubated overnight at 4°C, with rabbit IgG (2.5 μg) as control. Then, Dynabeads Protein A/G (10002D/10003D, Invitrogen) was added and incubated for 1 h at room temperature (Chen et al., 2019).

**Enzyme linked immunosorbent assay (ELISA)**

The concentration of IL6 in the conditioned medium (CM) were measured with ELISA method, according to manufactures’ instructions.
The induction of macrophage-like cells

Macrophage-like cells were induced as the following scheme (Shi et al., 2017; van den Bossche et al., 2018). Briefly, after polarized into macrophages using 10 nM Phorbol-12-myristate-13-acetate, peripheral blood mononuclear (PBMC) and THP-1 cells were induced into pro-inflammatory/anti-tumorigenic macrophages with lipopolysaccharides (1 μg/mL) and IFN-γ (20 ng/mL) or anti-inflammatory/pro-tumorigenic macrophages with 20 ng/mL IL-4 for 24 h (Osman et al., 2017). Then, the cells were collected and seeded into 6-well plate at the density of 2 x 10^6 cells/well. The cells were then incubated with CM from glioma cells for 24 h, followed by FACS and qPCR analyses to distinguish the expression of pro-inflammatory/anti-tumorigenic markers (MHCII and CD11c) and anti-inflammatory/pro-tumorigenic markers (ARG1 and CD163). Simultaneously, the migration of PBMC and THP-1 derived macrophages was examined with Transwell inserts accordingly (Osman et al., 2017; Qin, 2012).

Flow cytometry (FACS) and immunophenotyping

For FACS analyses, single cell suspensions were prepared from fresh tumor tissue and stained for indicated markers including CD45, TIM-3, and glial fibrillary acidic protein (GFAP) (Kleffel et al., 2015). To analyze the phenotype transition of macrophages, PBMC and THP-1 derived macrophages were stained for CD11b, MHC II, and CD206. Cells were acquired on the LSR II flow cytometer, and the data were analyzed using FlowJo software.

Intracranial and subcutaneous tumor xenograft transplantation

Six-week-old female NOD and nude mice were purchased from Beijing Vital River Laboratory Animal Technology. Mice were raised in laminar flow cabinets under specific pathogen-free conditions in the Laboratory Animal Center of China Medical University. For orthotopic transplantations, 3 μL glioma cell suspension (GSC40: 2 x 10^5, NOD mice; U87: 5 x 10^5, nude mice) were injected into the mice brains at a depth of 3.0 mm using stereotactic devices. The mice were euthanized when exhibiting neurological signs, or more than 20% of body weight loss.

Subcutaneous tumor xenograft transplantations: Six-week-old female BALB/C-Null nude mice were purchased from Beijing Vital River Laboratory Animal Technology, Beijing, China. Mice were raised in laminar flow cabinets under specific pathogen-free conditions in the Laboratory Animal Center of China Medical University. For subcutaneous xenograft transplantations, U87 cells (transduced with lentiviral TIM-3 overexpression or control vector) were injected into the right back flanks of nude mice at a density of 2 x 10^6 cells per 200 μL. The mice were fed ad libitum for 4 weeks and the tumor sizes were measured every 4 days with a vernier caliper. The following formula was used to calculate the tumor volume: V = (length x width^2)/2 (Naito et al., 1986). The mice were sacrificed by cervical dislocation at day 28 after implantation, and the tumors were weighed and photographed.

In vivo inhibitor experiments

For in vivo inhibitor experiments, GSC40 were transduced with lentiviral TIM-3 overexpression or control lentiviral firefly luciferase vector. Then, a 3 μL cell suspension (2 x 10^5 GSC40 cells with or without 1 x 10^4 THP-1 derived macrophages) were intracranially implanted into NOD mice (Zhou et al., 2015). After 1 week, tumor size was evaluated with an IVIS imaging system (Spectrum CT). Then the mice were randomly divided into control and Tocilizumab group. Tocilizumab or control IgG was intraperitoneally injected three time a week for 5 weeks at a dose of 10 μg/g body weight (Ham et al., 2019).

Mouse spontaneous intracranial glioma constructed with sleeping beauty (SB) transposon

Mouse spontaneous intracranial glioma was induced with SB transposon method (Wiesner et al., 2009). Mouse glioma sphere cells were derived from fresh tumor in the mouse brain with primary culture, and then transduced with tet-on lentiviral Tim-3 overexpression (SB-Tim3 OE cells) or control vector (SB-NC cells), respectively. Then, the mouse glioma sphere cells of Tim3 OE and NC were simultaneously treated with Doxycycline hyclate (DOX, 5 μg/mL). Transduction efficiency was determined by qPCR assay.

FACS and qPCR analyses of GL261 and SB mouse in vivo samples

SB-NC or SB-Tim3 OE cells (2 x 10^5 cells), and GL261-NC or GL261-Tim3 OE cells (2 x 10^5 cells) were intracranially injected into 6 weeks old male C57BL/6N mice. Doxycycline (2 mg/mL) were added into drinking water to induce Tim-3 overexpression at day 7 after injection. Tumor tissues were harvested at day 14 and
followed by FACS and qPCR analyses. The antibody panel and qPCR primer sequences were listed in key resources table. For FACS, single cell suspension was prepared and first stained with cell membrane fluorescein-conjugated antibodies. Before intracellular fluorescein-conjugated antibody staining, cells were treated with BD Cytofix/Cytoperm™ PlusFixation/Permeabilization Solution Kit. And BD Pharmin-gen™Transcription Factor Buffer Set was applied for the pretreatment before intranuclear fluorescein-conjugated antibody staining. For IFN-γ detection, cells were treated with BD GolgiPlug, ionomycin and PMA for 4 hours. BD LSRFortessa flow cytometer was employed for data acquiring. FlowJo was used for FACS data analysis. For the detection of TAMs source and polarization status in SB mouse samples, RT-qPCR was performed to detect the marker expression of microglia, peripheral monocyte, pro-inflammatory/anti-tumorigenic macrophage and anti-inflammatory/pro-tumorigenic macrophage polarization markers.

**In vivo limiting-dilution assays**

For in vivo limiting-dilution assay, SB-Tim3 OE or SB-NC cells (cell numbers: 10, 100, 1000 and 10000 cells) were intracranially injected into 6 weeks old male BALB/c nude mice (n = 5). Seven days after transplantation, doxycycline (2 mg/mL) were added into drinking water to induce TIM-3 overexpression. Then mouse brains were harvested at day 14 (for 10000 cells), at day 17 (for 1000 cells), at day 20 (for 100 cells), at day 23 (for 10 cells) and cross sections were used to determine whether tumors formed or not. Statistic was performed with ELDA (http://bioinf.wehi.edu.au/software/elda/).

**Dataset preparation for TIM-3 expression and survival analyses**

TIM-3 expression and the patient survival data of TCGA RNA Sequencing (RNA-seq) and GSE16011 GBM datasets were extracted from GlioVis portal (http://recur.bioinfo.cnio.es/) (Bowman et al., 2017). TCGA RNA-seq dataset included 629 samples (Nontumor: 4 cases; WHO grade II: 226 cases; grade III: 244 cases; 45 cases had no accurate grade in LGG; GBM: 155 samples). GSE16011 dataset included 269 samples (Nontumor: 8 cases; Grade I: 7 cases; Grade II: 24 cases; Grade III: 85 cases; GBM: 155 cases). A Kaplan-Meier curve was used to estimate the survival distribution, and followed by a log-rank test evaluating the difference between stratified groups. The median value was employed as the cutoff.

Single cell RNA-seq data were extracted from GSE131928. The t-distributed stochastic neighbor embedding (tSNE) plot was performed using the Seurat package with R, version 3.6.0. The cell identities of each cluster were assigned with R (Neftel et al., 2019).

**Gene ontology (GO) analysis, gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA)**

Microsoft Excel 2013 was employed to obtain correlation genes of TIM-3 with Pearson r > 0.6, and p Value <0.05. Then, the common upregulated genes associated with high TIM-3 expression were summarized from the indicated datasets. The relevant signaling pathways of TIM-3 were analyzed by DAVID 6.8 (https://david.ncifcrf.gov/tools.jsp) (Huang et al., 2009). The patients were stratified into 2 groups with the median value of TIM-3 mRNA expression. Gene Set Enrichment Analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) was performed to explore whether the identified sets of genes showed statistical differences between 2 groups stratified as described above. Normalized enrichment score (NES) and false discovery rate (FDR) were used to determine the statistical significance (Subramanian et al., 2005). Gene Set Variation Analysis (GSVA, http://www.bioconductor.org) was performed to further verify whether there were significant differences between these 2 groups, according to the gene sets of defined signaling pathways.

**CIBERSORT and microenvironment cell populations (MCP)-counter method**

Cell composition of complex tissues were characterized from their gene expression profiles using https://cibersort.stanford.edu/, which contained 22 types of immune cells (Newman et al., 2015). MCP counter method was applied to quantify the abundance of two non-immune stromal (endothelial cells and fibroblasts) and eight immune cell populations (T cells, CD8+ T cells, NK cells, cytotoxic lymphocytes, B cell lineage, monocytic lineage cells, myeloid dendritic cells, and neutrophils) infiltrating glioma tissue from transcriptomic data (Becht et al., 2016).
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted with Microsoft Excel 2013 and Prism 7, unless otherwise stated. Data was presented as mean ± standard deviation. Two tailed t-test or one-way analysis of variance (ANOVA) followed by Turkey’s post-test) were used to determine significant differences between and among groups. The heat maps and correlation analyses were performed with R, version 3.4.2, unless mentioned elsewhere. A value of p < 0.05 was considered statistically significant in all experiments.