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ORIGINAL ARTICLE

MiR-181b modulates EGFR-dependent VCAM-1 expression and monocyte adhesion in glioblastoma

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Tumor-associated macrophages (TAMs) originate as circulating monocytes, and are recruited to gliomas, where they facilitate tumor growth and migration. Understanding the interaction between TAM and cancer cells may identify therapeutic targets for glioblastoma multiforme (GBM). Vascular cell adhesion molecule-1 (VCAM-1) is a cytokine-induced adhesion molecule expressed on the surface of cancer cells, which is involved in interactions with immune cells. Analysis of the glioma patient database and tissue immunohistochemistry showed that VCAM-1 expression correlated with the clinico-pathological grade of gliomas. Here, we found that VCAM-1 expression correlated positively with monocyte adhesion to GBM, and knockdown of VCAM-1 abolished the enhancement of monocyte adhesion. Importantly, upregulation of VCAM-1 is dependent on epidermal-growth-factor-receptor (EGFR) expression, and inhibition of EGFR effectively reduced VCAM-1 expression and monocyte adhesion activity. Moreover, GBM possessing higher EGFR levels (U251 cells) had higher VCAM-1 levels compared to GBMs with lower levels of EGFR (GL261 cells). Using two- and three-dimensional cultures, we found that monocyte adhesion to GBM occurs via integrin α4β1, which promotes tumor growth and invasion activity. Increased proliferation and tumor necrosis factor-α and IFN-γ levels were also observed in the adherent monocytes. Using a genetic modification approach, we demonstrated that VCAM-1 expression and monocyte adhesion were regulated by the miR-181 family, and lower levels of miR-181b correlated with high-grade glioma patients. Our results also demonstrated that miR-181b/protein phosphatase 2A-modulated SP-1 de-phosphorylation, which mediated the EGFR-dependent VCAM-1 expression and monocyte adhesion to GBM. We also found that the EGFR-dependent VCAM-1 expression is mediated by the p38/STAT3 signaling pathway. Our study suggested that VCAM-1 is a critical modulator of EGFR-dependent interaction of monocytes with GBM, which raises the possibility of developing effective and improved therapies for GBM.

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INTRODUCTION

Despite aggressive therapy, most patients with glioblastoma multiforme (GBM) die within 2 years after diagnosis.[1,2] Development of novel and effective strategies ensures improvement of the poor prognosis of patients with GBM. A recent study reported that tumor-associated macrophages (TAM) facilitate glioma proliferation and migration,[3] which represents an alternative therapeutic target of glioma.[4] Thus, understanding the mechanism of interaction between TAM and glioma may identify therapeutic strategies for glioma therapy. An inflammatory microenvironment promotes proliferation and survival of malignant cells,[5] and therefore, cytokines have a significant role in cancer diagnosis, prognosis and therapy. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are the primary cancer-related inflammatory cytokines with pro-tumor functions.[6] Moreover, TNF-α has also been reported to be a major cytokine in the tumor microenvironment,[7] whose expression correlates with GBM tumor grade.[8] TAMs can produce IL-1β, which in turn promotes inflammation, cell proliferation and tumor formation.[9]

Several studies reported that the brain-resident microglia and the infiltrating monocytes/macrophages of blood are the major glioma-associated inflammatory cells that constitute the tumor microenvironment.[10] Our group[11] and others[12] previously demonstrated that microglia promotes tumor growth. Importantly, a recent report[13] and a clinical study[14] revealed that monocyte/macrophage, but not microglia and lymphocytes, are the most predominant TAMs in GBM. Monocytes, cells of the myeloid lineage, are released during inflammation and differentiate into macrophages to maintain immune homeostasis.[15] A previous study suggested that circulating monocytes are cytotoxic to tumor cells;[16] however, when monocytes reach the tumor mass, the tumor molecular milieu induce differentiation to new cell types per tumor requirement.[17] A recent report indicated that reduction in the tumor-promoting effects of monocytes in GBM can be considered as an adjuvant treatment for glioma.[4] However, the fate of the GBM-adhered monocytes and their effect on GBM growth are still obscure.

Adhesion molecules are known to mediate cell–cell interactions, particularly between immune cells and target tissues. Vascular cell adhesion molecule-1 (VCAM-1) is an inducible adhesion molecule that facilitates tight attachment to the monocyte-associated integrin α4β1 (VLA4).[18] VCAM-1 expressed on the surface of

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tumors interacts with VLA4 on monocytes, which promotes tumor invasion, angiogenesis and metastasis.[19] VCAM-1 (also known as CD106) and ICAM-1 (also known as CD54) have been recognized as the major adhesion molecules of brain endothelial cells that facilitate attachment and penetration of leukocytes in the brain.[20] High expression of VCAM-1 correlates with abundant monocyte/macrophage infiltration in tumors,[21,22] and are associated with cancer recurrence, metastasis, and survival.[23,24] Interestingly, expression of VCAM-1 in tumors promote T-cell migration away from tumors, resulting in escape of tumor cells from immune surveillance.[25] Furthermore, higher levels of VCAM-1 in glioma correlate with lymphocytic infiltration.[26] However, the actual mechanism by which monocytes/macrophages affect GBM progression through VCAM-1 and ICAM-1 are still obscure.

Amplification and mutation of epidermal growth factor receptor (EGFR) are present in approximately 50% of GBMs,[27,28] which are associated with GBM proliferation[29] and invasion.[30] Amplification of EGFR in patients with primary GBM show significant correlation with poor patient outcome and survival rate.[31–33] In addition, macrophages modulate the tumor microenvironment by secreting

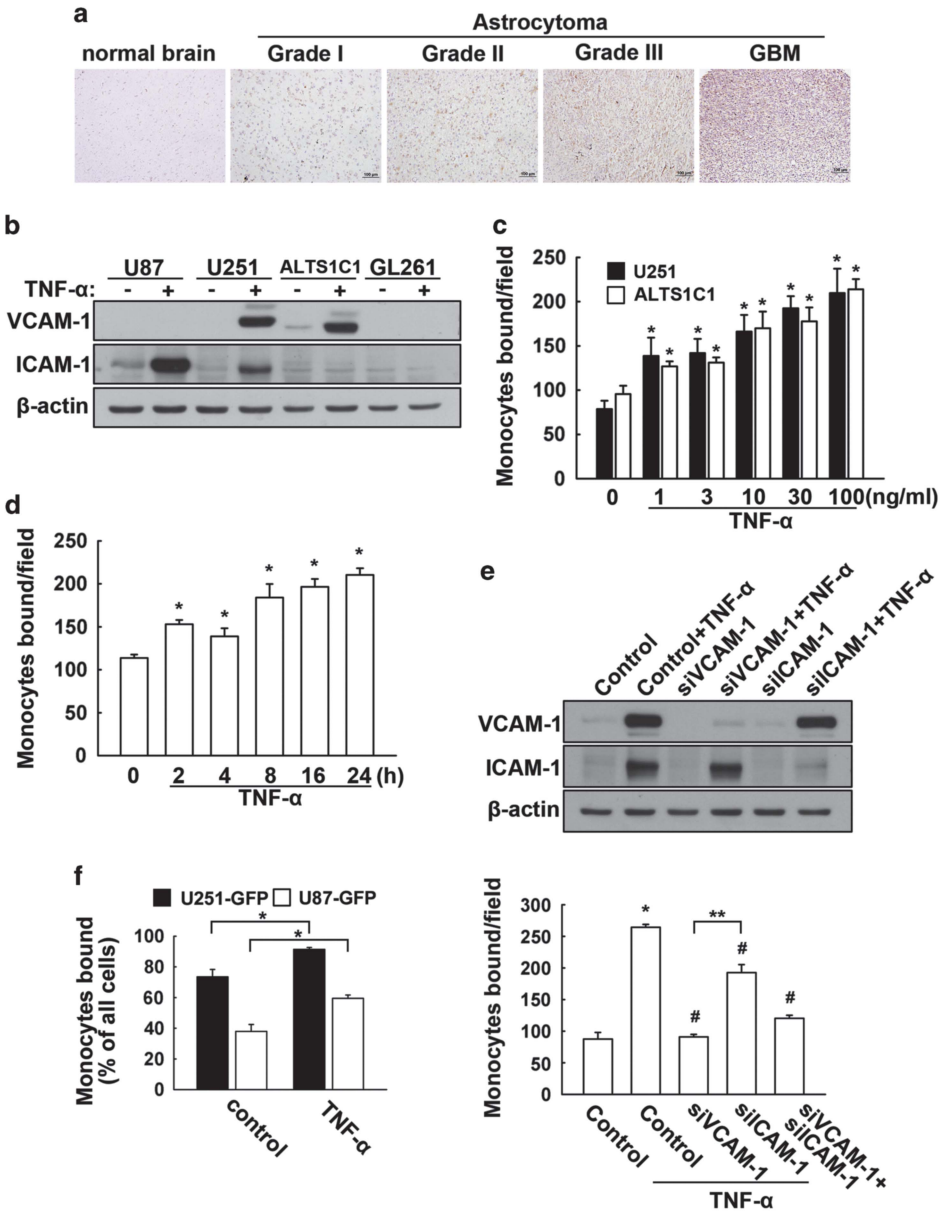


Figure 1. VCAM-1 involves in monocyte adhesion to GBM. (**a**) VCAM-1 expression in normal human brain and astrocytoma tissues. (**b**) U87, U251, ALTS1C1 or GL261 were treated with TNF-α (10 ng/ml) for 24 h. VCAM-1 and ICAM-1 expression were determined using western blot analysis. (**c**) U251 and ALTS1C1 were treated with various concentrations (1, 3, 10, 30 or 100 ng/ml) of TNF-α for 24 h. BCECF-AM-labeled-THP-1 were added to U251 and ALTS1C1 for 30 min, and then the adherence of THP-1 was measured by fluorescence microscopy. (**d**) U251 were treated with TNF-α (10 ng/ml) for indicated time periods (2, 4, 8, 16 or 24 h). The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy. (**e**) U251 were transfected with Control, VCAM-1, or ICAM-1 siRNA for 24 h and treated with TNF-α (10 ng/ml) for another 24 h. VCAM-1 and ICAM-1 expression, and monocyte adhesion ability were determined using western blot analysis (upper panel) and monocyte-binding assay (lower panel), respectively. (**f**) U251-GFP and U87-GFP cells were seeded at 3D culture for 24 h and treated with TNF-α (10 ng/ml) for another 24 h, followed by incubation with THP-1 for 24 h. The monocyte-binding ability was determined by using flow cytometry. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the TNF-α treatment group. $^{**}P < 0.05$ VCAM-1 siRNA compared with the ICAM-1 siRNA.

various growth factors such as EGF, which in turn promote cancer progression.[17,34] Furthermore, EGF triggers EGFR activation that promotes VCAM-1 expression in prostate cancer.[35] Recent studies have also documented that EGF-mediated EGFR activation promotes VCAM-1 expression in glioma cells.[36] Furthermore, there is a paracrine interaction between GBM and microglia, which involves EGF and EGFR-mediated GBM invasion.[37] In clinical trials, small molecule inhibitors targeting the EGFR tyrosine kinase such as gefitinib and erlotinib have provided encouraging results in patients with GBM.[38]

In this study, immunohistochemistry revealed that higher levels of VCAM-1 correlated with poor prognosis in glioma patients. Our results also show that EGFR-dependent VCAM-1 expression promotes human monocyte adhesion to GBM. The miR-181b/PP2A/p-SP-1 signaling pathways upregulate EGFR, which regulates VCAM-1 expression and monocyte adhesion through the p38/STAT3 pathway in GBM. The interaction of adherent monocytes and GBM increased the levels of TNF-α and IFN-γ, monocyte proliferation and GBM growth.

RESULTS

TNF-α induces VCAM-1 expression and increases monocyte adhesion in GBM

As shown in Figure 1a, higher levels of VCAM-1 correlated with higher clinico-pathological grades of human glioma as observed by immunohistochemistry (Figure 1a). Analysis of the GSE4290 dataset showed that VCAM-1 levels were higher in the GBM group than in the non-tumor group and grade II glioma (Supplementary Figure 1a). To examine the effect of cytokines on VCAM-1 expression in GBM, U251 were treated with various cytokines. As shown in Supplementary Figure 1b, IL-1β and TNF-α strongly induced VCAM-1 and ICAM-1 expression. TNF-α is a major cytokine in the tumor microenvironment[7] and its expression correlates with

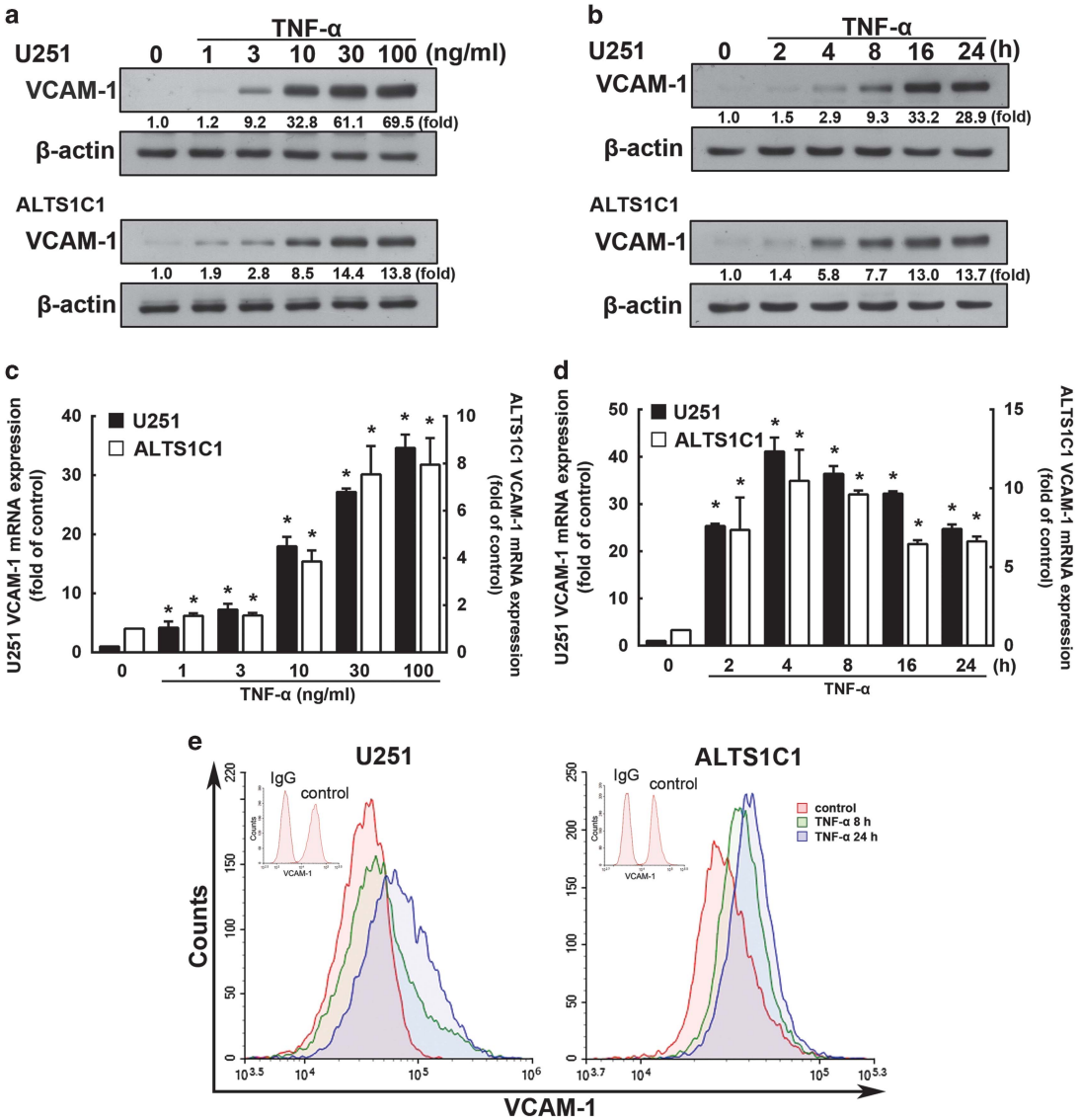


Figure 2. Effects of TNF-α induces VCAM-1 expression in U251 and ALTS1C1 GBM. Cells were treated with various concentrations (1, 3, 10, 30 or 100 ng/ml) of TNF-α for 24 h, and VCAM-1 expression was determined using western blot analysis (**a**) and real-time PCR (**c**), respectively. Cells were treated with TNF-α (10 ng/ml) for indicated time periods (2, 4, 8, 16 or 24 h), and VCAM-1 expression was determined by using western blot analysis (**b**) and real-time PCR (**d**), respectively. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). *$P < 0.05$ compared with the control group. (**e**) Cells were treated with TNF-α (10 ng/ml) for indicated time periods (8 or 24 h), and expression of VCAM-1 cell surface were determined using flow cytometry.

GBM tumor grade.[8] We further examined the effect of TNF-α on VCAM-1 expression in GBM. TNF-α induced high expression of VCAM-1 in U251 and ALTS1C1 (Figure 1b). The ability of monocytes to bind to GBM was further determined using the monocyte-binding assay. As shown in Figure 1c, treatment of GBMs with TNF-α increased THP-1 monocyte adhesion in a dose-dependent manner (green color; Supplementary Figures 1c and d). Similarly, treatment of GBM with TNF-α increased primary human monocyte adhesion in a dose-dependent manner (Supplementary Figure 1f). Furthermore, treatment of U251 with TNF-α increased monocyte adhesion in a time-dependent manner (Figure 1d and Supplementary Figure 1e). Transfection with siRNA against VCAM-1 or ICAM-1 significantly decreased the expression of VCAM-1 and ICAM-1 (Figure 1e, upper panel). The increase in

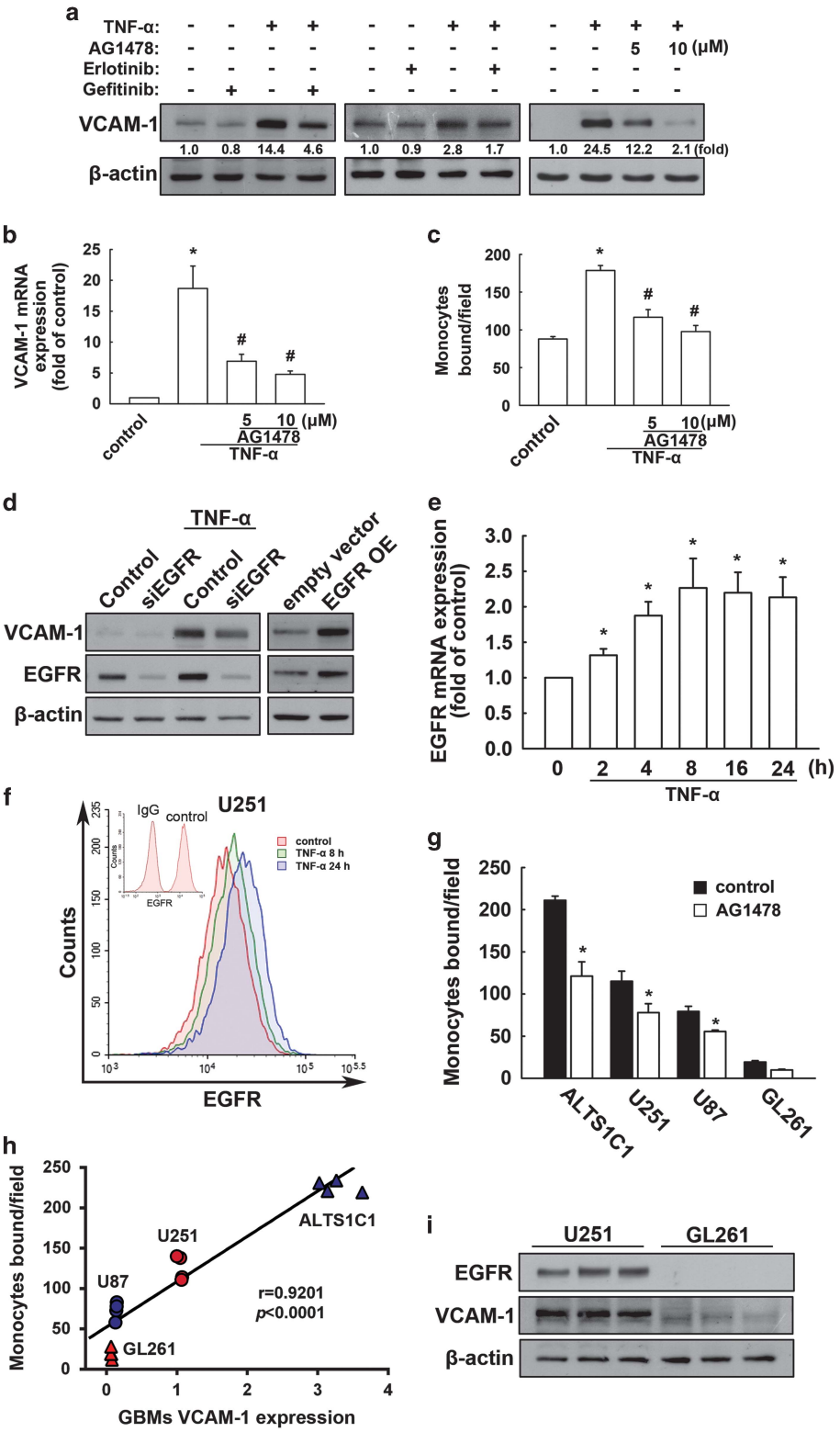


Figure 3. For caption see page 5010.

the adhesion of THP-1 monocytes to GBM was also reduced by VCAM-1 or ICAM-1 siRNAs, respectively (Figure 1e, lower panel). Similarly, the increase in the adhesion of primary human monocytes to GBM was reduced by VCAM-1 siRNA (Supplementary Figure 1g). Importantly, reduction of monocyte adhesion was stronger in transfection with the VCAM-1 siRNA than with the ICAM-1 siRNA (Figure 1e). The increase in monocyte adhesion to GBM was also observed in three-dimensional (3D) culture model (Figure 1f). The TNF-α-induced VCAM-1 protein and mRNA expression were observed in GBMs in a dose-dependent manner (Figures 2a and c). In addition, TNF-α treatment for different durations also induced VCAM-1 expression in a time-dependent manner (Figures 2b and d). Moreover, we found that the TNF-α-induced VCAM-1 was expressed on the GBM surface (Figure 2e). These results indicated that monocyte adhesion to GBM occurs via VCAM-1, and the levels of VCAM-1 correlated significantly with the clinico-pathological grade of glioma.

Involvement of EGFR in the TNF-α-induced VCAM-1 expression and monocyte adhesion

We further investigated whether EGFR is involved in VCAM-1 expression in GBM. Treatment with EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, reduced the TNF-α-induced VCAM-1 expression in U251 (Figure 3a) and ALTS1C1 (Supplementary Figure 2a). Similarly, AG1478, the pharmacological inhibitor of EGFR, also markedly reduced the levels of VCAM-1 mRNA and protein (Figures 3a and b, Supplementary Figure 2a). Moreover, AG1478 administration also reduced human monocyte adhesion (Figure 3c, Supplementary Figure 2b). Transfection with EGFR siRNA effectively reversed the TNF-α-induced VCAM-1 expression (Figure 3d, left panel). Interestingly, transfection with wild-type EGFR dramatically increased VCAM-1 expression (Figure 3d, right panel). We also found a significant time-dependent increase in EGFR mRNA levels and cell surface expression of the protein in U251 treated with TNF-α (Figures 3e and f, respectively). The monocyte-binding ability of GBM were all reduced by AG1478 treatment (Figure 3g). Moreover, Pearson's correlation analysis showed a positive correlation between GBM VCAM-1 levels and activity of THP-1 or primary monocyte attachment (Figure 3h and Supplementary Figure 2c, respectively). Furthermore, higher EGFR levels in U251, but not in GL261 (approximately 38.5-fold), paralleled the higher levels of VCAM-1 (~6.8-fold) (Figure 3i). These results suggest that EGFR is a critical modulator of VCAM-1 expression and monocyte adhesion to GBM.

Integrin α4 and β1 mediate human monocyte adhesion, and promote GBM progression

The TNF-α-induced adhesion of THP-1 and primary human monocytes was attenuated by the anti-integrin-α4 or -β1 neutralizing antibodies (Figure 4a and Supplementary Figure 3, respectively). Similar effects of the inhibitors were observed using the 3D culture model (Figure 4b), where the monocyte-attached GBM exhibited higher proliferation rates and invasion ability after TNF-α stimulation, compared to the rates observed in the mono-culture model (Figures 5a and b). In addition, the adherent monocytes also dramatically increased the levels of TNF-α and IFN-γ, but not those of ARG1, IL-10 and CD163 in the 3D co-culture model (Figure 6a). Moreover, GBM stimulated with conditioned medium collected from the GBM/monocyte co-culture increased monocytes adhesion, but not the conditioned medium from GBM or monocytes alone (Figure 6b). In addition, the effect of GBM/monocyte conditioned medium on monocyte adhesion was repressed by the administration of an anti-TNF-α neutralizing antibody (Figure 6b). Furthermore, adherent monocytes exhibited higher proliferation rates after TNF-α stimulation in 3D cultured GBM (Figure 6c). These results suggest that the VCAM-1-dependent monocyte adhesion to GBM might increase adhesion molecule expression in GBM with subsequent monocyte adhesion in a positive feedback loop, which leads to tumor progression.

Involvement of miR-181b/PP2A in TNF-α-induced VCAM-1 expression in GBM

A miRNA microarray profiling study was undertaken using total RNA from TNF-α challenged GBM. We selected miRNAs whose expression changed in the presence of TNF-α. We found that four miRNAs were upregulated and seven were downregulated upon stimulation with TNF-α (Figure 7a); of these, we chose two candidate miRNAs, namely, miR-181a and miR-181b. The sequences of miR-181a and miR-181b were highly similar, with only three differing nucleotides (Figure 7b). As shown in Figure 7c, the expression of miR-181a and miR-181b were significantly reduced by TNF-α treatment. Transfection of U251 with miR-181b or miR-181a mimics downregulated the protein and mRNA levels of VCAM-1, respectively (Figures 7d and e). As shown in Figure 7f, lower levels of miR-181b correlated significantly with high-grade glioma in patient specimens. The miR-181 family was further analyzed using a miRNA target database (DIANA Tools, TarBase v7.0) for identifying the miR-181a that might target protein phosphatase 2A (PP2A; data not shown). As shown in Figure 8a, PP2A activity decreased mildly after a short-term TNF-α stimulation (Figure 8a, left panel). Besides, TNF-α treatment enhanced PP2A activity in a time-dependent manner, and the PP2A activity increased by 20% at the 24 h time point (Figure 8a, right panel). Next, transfection with either miR-181a or miR-181b mimics significantly reduced PP2A activity (Figure 8b). A PP2A pharmacological inhibitor, okadaic acid (OA), effectively antagonized the TNF-α-induced VCAM-1 protein and mRNA expression (Figures 8c and d, respectively). In addition, the increase in adhesion of THP-1 and primary monocytes to GBM also decreased with OA treatment (Figure 8e and Supplementary Figure 4a). Furthermore, transfection with a siRNA against the alpha catalytic subunit of PP2A (PP2CA) markedly inhibited the TNF-α-induced VCAM-1 protein expression (Figure 8f). It has been reported that NF-κB, MAP kinase

Figure 3. EGFR involves in TNF-α-induced VCAM-1 expression and monocyte adhesion. (**a**) U251 were treated with gefitinib (50 nM), erlotinib (50 nM) and AG1478 (5 or 10 μM) for 30 min and treated with TNF-α (10 ng/ml) for another 24 h. VCAM-1 expression were determined using western blot analysis. U251 were treated with AG1478 (5 or 10 μM) for 30 min and treated with TNF-α (10 ng/ml) for another 6 (**b**) or 24 h (**c**). VCAM-1 expression and monocyte adhesion ability were determined using real-time PCR (**b**) and measured the adhesion activity of BCECF-AM-labeled-THP-1 (**c**), respectively. (**d**) U251 were transfected with Control or EGFR siRNA and treated with TNF-α (10 ng/ml) for another 24 h, or cells were transfected with empty vector or wild-type EGFR for 24 h. VCAM-1 and EGFR expression were determined using western blot analysis. (**e**) U251 were treated with TNF-α (10 ng/ml) for indicated time periods (2, 4, 8, 16 or 24 h), and EGFR expression were determined using real-time PCR. (**f**) U251 were treated with TNF-α (10 ng/ml) for indicated time periods (8 or 24 h), and expression of EGFR cell surface were determined using flow cytometry. (**g**) U87, U251, ALTS1C1 or GL261 were treated with AG1478 (10 μM) for 24 h. The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the TNF-α treatment group. (**h**) Pearson's correlation analysis between adherent monocyte ability and VCAM-1 expression in GBMs ($r = 0.9201$, $P < 0.0001$). (**i**) U251 and GL261 cell lysates were harvested from three deferent passages, and EGFR and VCAM-1 expression were determined using western blot analysis.

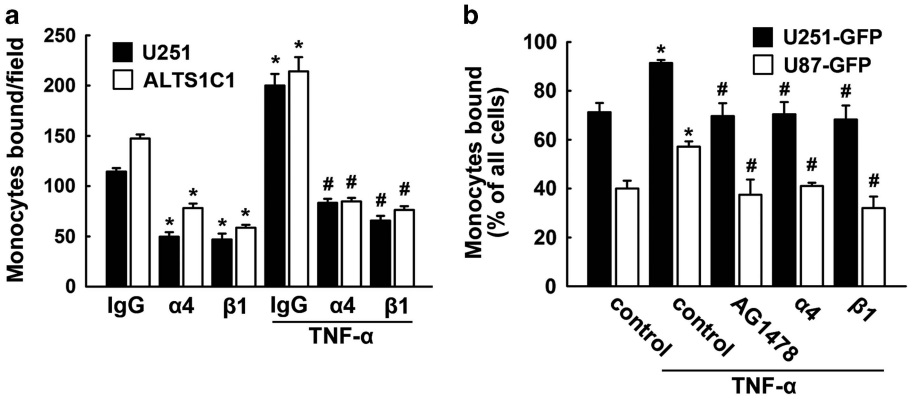


Figure 4. Integrin α4β1 mediates ability of monocyte adhesion to GBM. (**a**) U251 and ALTS1C1 were treated with TNF-α (10 ng/ml) for 24 h, followed by incubation with BCECF-AM-labeled-THP-1 in present anti-integrin-α4- or β1-neutralizing antibodies (2 μg/ml) for 30 min. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). *$P < 0.05$ compared with the IgG group. #$P < 0.05$ compared with the TNF-α treatment group. (**b**) U251-GFP and U87-GFP were seeded in 3D culture for 24 h, and treated with TNF-α (10 ng/ml) for another 24 h, followed by incubation with THP-1 in present anti-integrin-α4 or -β1 neutralizing antibodies (2 μg/ml) for 24 h. The monocyte-binding ability was determined by using flow cytometry. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). *$P < 0.05$ compared with the control group. #$P < 0.05$ compared with the TNF-α treatment group.

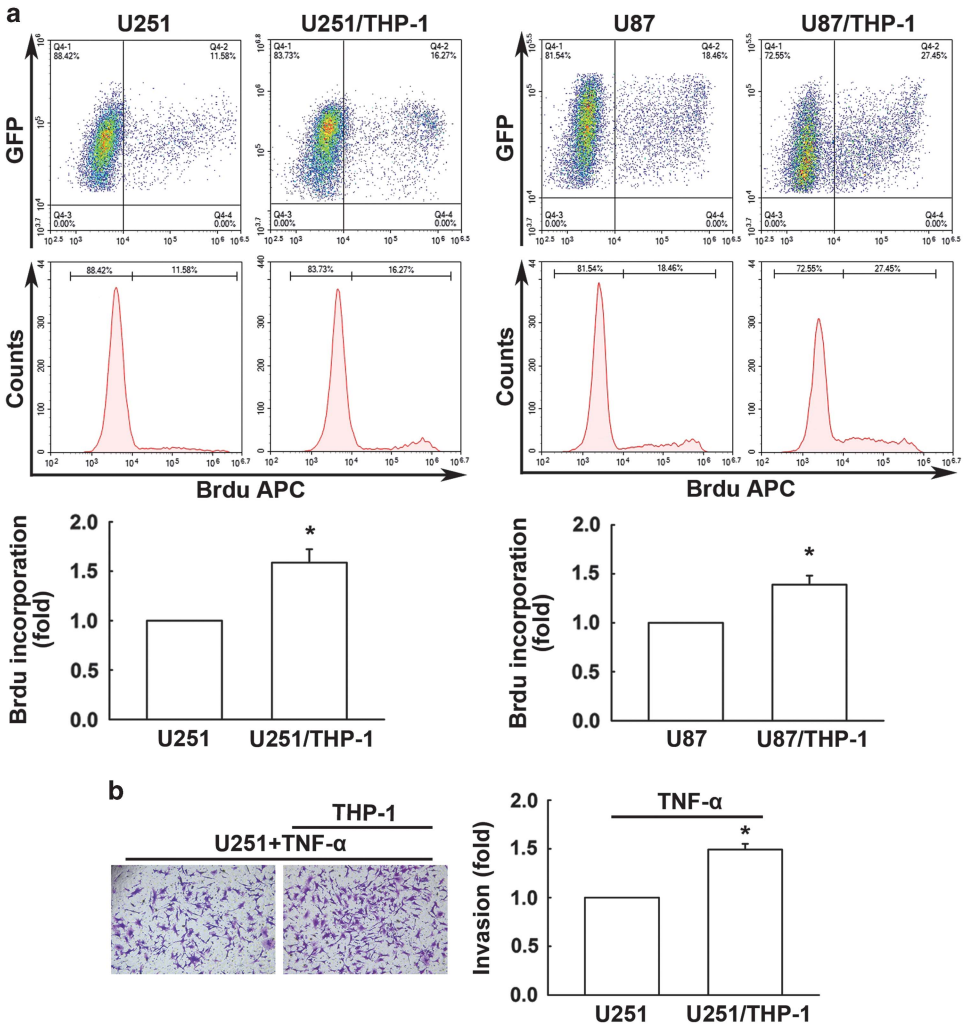
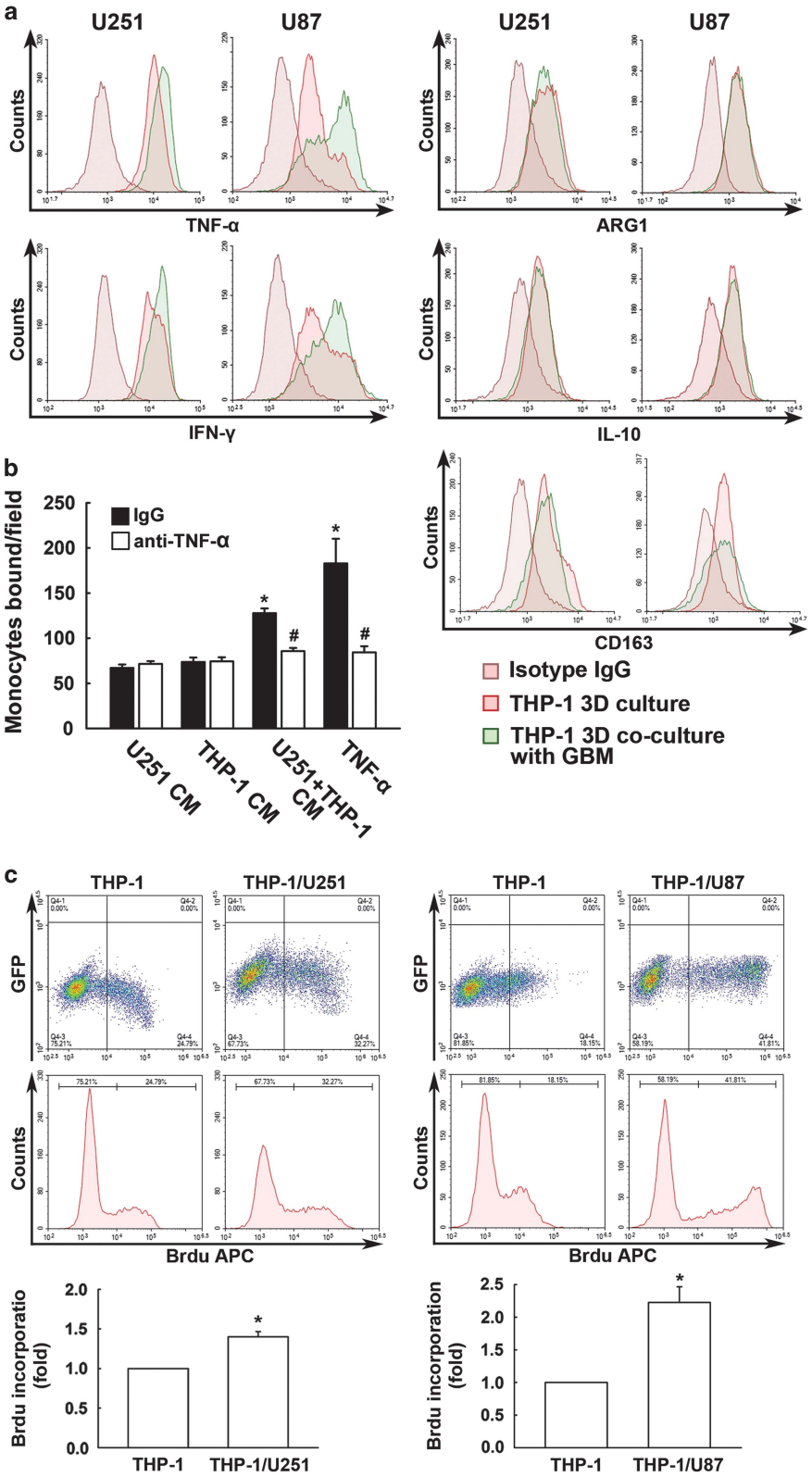


Figure 5. Monocyte adhesion increases GBM proliferation and invasion in 3D co-culture model. (**a**) U251-GFP or U87-GFP were treated with TNF-α for 24 h, and then co-cultured with THP-1 in 3D model for another 24 h. The 3D cultured cells were plotted on side scatter versus GFP, after incubation of Brdu for 4 h. The GFP-positive cells indicate GBM, whereas the GFP-negative cells indicate THP-1. The GFP-positive gated GBM were analyzed on dot plot of Brdu incorporation. (**b**) U251 were pre-treated with TNF-α for 24 h, next seeded in the matrigel-coated transwell inserts for 1 h. THP-1 were then loaded on top of U251 for further 24 h. The cell invasion was examined by transwell assay. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). *$P < 0.05$ compared with the GBM group.

pathway and STAT3 are downstream targets for PP2A.[39–41] Treatment of U251 cells with OA increased p65 and JNK phosphorylation in a time-dependent manner (Supplementary Figure 4b). Transfection with the PP2CA siRNA increased phosphorylation of JNK, but did not affect the TNF-α-induced JNK phosphorylation (Supplementary Figure 4c). Treatment with the JNK pharmacological inhibitor, SP600125, attenuated the TNF-α-induced VCAM-1 protein expression and p65 phosphorylation (Supplementary Figure 4d). Furthermore, transfection with the p65 siRNA effectively antagonized the TNF-α-induced VCAM-1 protein



and mRNA expression (Supplementary Figure 4e). Interestingly, transfection with p65 siRNA also decreased EGFR protein and mRNA levels (Supplementary Figure 4e). These results suggest that VCAM-1 expression and monocyte adhesion to GBM is mediated by EGFR activation through the miR-181/PP2A and JNK/p65 pathways.

Involvement of SP-1 in the TNF-α-induced EGFR/VCAM-1 expression in GBM

The transcription factor-specific protein-1 (SP-1) is a major target of the JNK pathway.[42,43] JNK and SP-1 were phosphorylated after treatment with PP2A inhibitor OA (Figure 9a and Supplementary Figure 5a). On the other hand, transfection with miR-181a or miR-181b mimics increased JNK and SP-1 phosphorylation, whereas transfection with miR-181a or miR-181b inhibitors decreased JNK and SP-1 phosphorylation (Figure 9b and Supplementary Figure 5b). JNK phosphorylation decreased in a dominant-negative mutant of JNK when GBM was treated with OA, without affecting SP-1 phosphorylation (Supplementary Figure 5c). As shown in Figure 9c, treatment with the SP-1 pharmacological inhibitor, WP631, effectively attenuated the TNF-α-induced VCAM-1 protein and mRNA expression. Importantly, WP631 also significantly decreased the TNF-α-induced EGFR mRNA expression as well (Figure 9c, right panel). In addition, treatment with WP631 also decreased THP-1 and primary human monocyte adhesion (Figure 9d and Supplementary Figure 5d). Furthermore, transfection with a SP-1 siRNA reduced the TNF-α-induced EGFR and VCAM-1 protein and mRNA expression (Figure 9e). Interestingly, transfection with wild-type SP-1 dramatically increased VCAM-1 and EGFR protein levels (Figure 9f, left panel). We also found that overexpression of SP-1 increased monocyte adhesion (Figure 9f, right panel). Furthermore, TNF-α-mediated binding of SP-1 to the SP-1-binding site on the EGFR promoter was repressed by WP631 treatment (Figure 9g). Next, inhibition of EGFR by AG1478 or EGFR siRNA effectively antagonized the TNF-α-induced phosphorylation of p38 and STAT3 but not that of p65 (Figure 9h and Supplementary Figure 5e). In contrast, stimulation of EGF increased p38 and STAT3 phosphorylation (Figure 9i). These results indicated that SP-1 is a downstream effector of miR-181b/PP2A for the TNF-α-mediated modulation of EGFR/VCAM-1 expression.

Involvement of p38/STAT3 in the TNF-α-induced EGFR/VCAM-1 in GBM

TNF-α-stimulation increased p38, STAT3, and p65 phosphorylation in a time-dependent manner (Figure 10a and Supplementary Figure 6a). In addition, transfection with p65 siRNA reduced the TNF-α-induced VCAM-1 expression (Supplementary Figure 6b). Similarly, transfection with a p38 siRNA or treatment with the p38 inhibitor SB203580 antagonized the TNF-α-induced VCAM-1 expression (Figures 10b and c). Moreover, transfection with a STAT3 shRNA or treatment with the STAT3 inhibitor S3I-201 effectively attenuated the TNF-α-induced VCAM-1 expression (Figures 10d and e). Next, we examined whether JAK1 and JAK2 were involved in the TNF-α-induced modulation of VCAM-1 expression. As shown in Supplementary Figure 10c, stimulation with TNF-α increased JAK1 and JAK2 phosphorylation. Both JAK1 and JAK2 inhibitors dramatically reduced the TNF-α-induced VCAM-1 protein and mRNA expression (Supplementary Figures 6d–f). In addition, both JAK1 and JAK2 inhibitors also markedly blocked monocyte adhesion (Supplementary Figure 6g). Interestingly, transfection with wild-type STAT3 increased VCAM-1 protein expression and monocyte adhesion (Figure 10f). Furthermore, transfection with a p38 siRNA or dominant-negative mutant of p38 inhibited the TNF-α-induced STAT3 phosphorylation (Figures 10g and h). In addition, TNF-α-mediated binding of STAT3 to the STAT3-binding site on the VCAM-1 promoter region was effectively repressed by AG1478 (Figure 10i). These results indicated that the VCAM-1-associated monocyte adhesion to GBM is mediated through the EGFR/p38/STAT3 pathway.

DISCUSSION

The present study demonstrated that VCAM-1 expression was induced in GBM treated with TNF-α, which subsequently promoted the interaction between monocytes and GBM. First, by DNA microarray (GEO DataSets) and immunohistochemical analysis, we found that higher expression of VCAM-1 in patients with glioma correlated with poor prognosis. Importantly, there was a positive correlation of VCAM-1 expression with the activities of adherent monocytes in GBM. Second, upregulation of VCAM-1 expression was dependent on EGFR expression, and gefitinib and erlotinib effectively reduced the enhancement of VCAM-1 expression. In addition, overexpression of EGFR upregulated VCAM-1, and inhibition of EGFR significantly antagonized the monocyte adhesion to GBM. Third, using two-dimensional and 3D cultures, we found that monocyte adhesion to GBM occurred via integrin α4β1, which promoted GBM invasion. Moreover, the 3D co-culture model showed enhancement of proliferation and increase in TNF-α and IFN-γ levels in adherent monocytes, along with increased proliferation of GBM. Fourth, using miRNA array, we found that miR-181b was involved in the TNF-α-induced VCAM-1 expression. Furthermore, lower levels of miR-181b correlated significantly with high-grade glioma patients. Finally, miR-181b/PP2A-modulated SP-1 de-phosphorylation, which mediated the EGFR-dependent VCAM-1 expression and monocyte adhesion to GBM. These EGFR-dependent phenomena are regulated by the p38/STAT3 signaling pathways (Figure 10j). Our study suggests that VCAM-1 expressed on the surface of GBM allows adhesion and interaction with integrin α4β1 expressed on monocytes, which is benefits the tumor microenvironment by increasing proliferation and invasion. Adherent monocytes on GBM further expressed TNF-α, which possibly amplified VCAM-1 expression in the monocyte-adhered GBM and consequently enhanced monocyte adhesion.

Glioma-associated microglia has been reported to secrete immunosuppressive factors such as IL-10, which contributes to immune surveillance.[44] The glioma-secreted factor IFN-γ facilitated TAM survival, whereas expression of immunosuppressive cell markers decreased in surviving TAMs, which is consistent with impaired tumor-promoting functions.[13] Our results corroborated the results of a previous study that showed that adherent

Figure 6. Alternation of monocyte behaviors in 3D co-culture model. (**a**) THP-1 monocytes were co-cultured with U251-GFP or U87-GFP in 3D model for 24 h. The 3D cultured cells were plotted on side scatter versus GFP. The monocytes (GFP-negative cells) were analyzed levels of TNF-α, IFN-γ, ARG1, IL-10 and CD163 by flow cytometry. (**b**) U251 were incubated with various conditioned media (U251-, THP-1- or U251/THP-1 co-culture-CM) in present anti-TNF-α neutralizing antibody (0.5 μg/ml) for 24 h. The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy. Note that TNF-α treatment group was as the positive control. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with U251 CM group. $^{\#}P < 0.05$ compared with IgG treatment group. (**c**) THP-1 were co-cultured with TNF-α-treated-U251-GFP or U87-GFP in 3D model for 24 h, and then incubation of Brdu for another 4 h. The 3D cultured cells were plotted on side scatter versus GFP. The monocytes (GFP-negative cells) were analyzed for Brdu incorporation. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the THP-1 group. CM, conditioned medium.

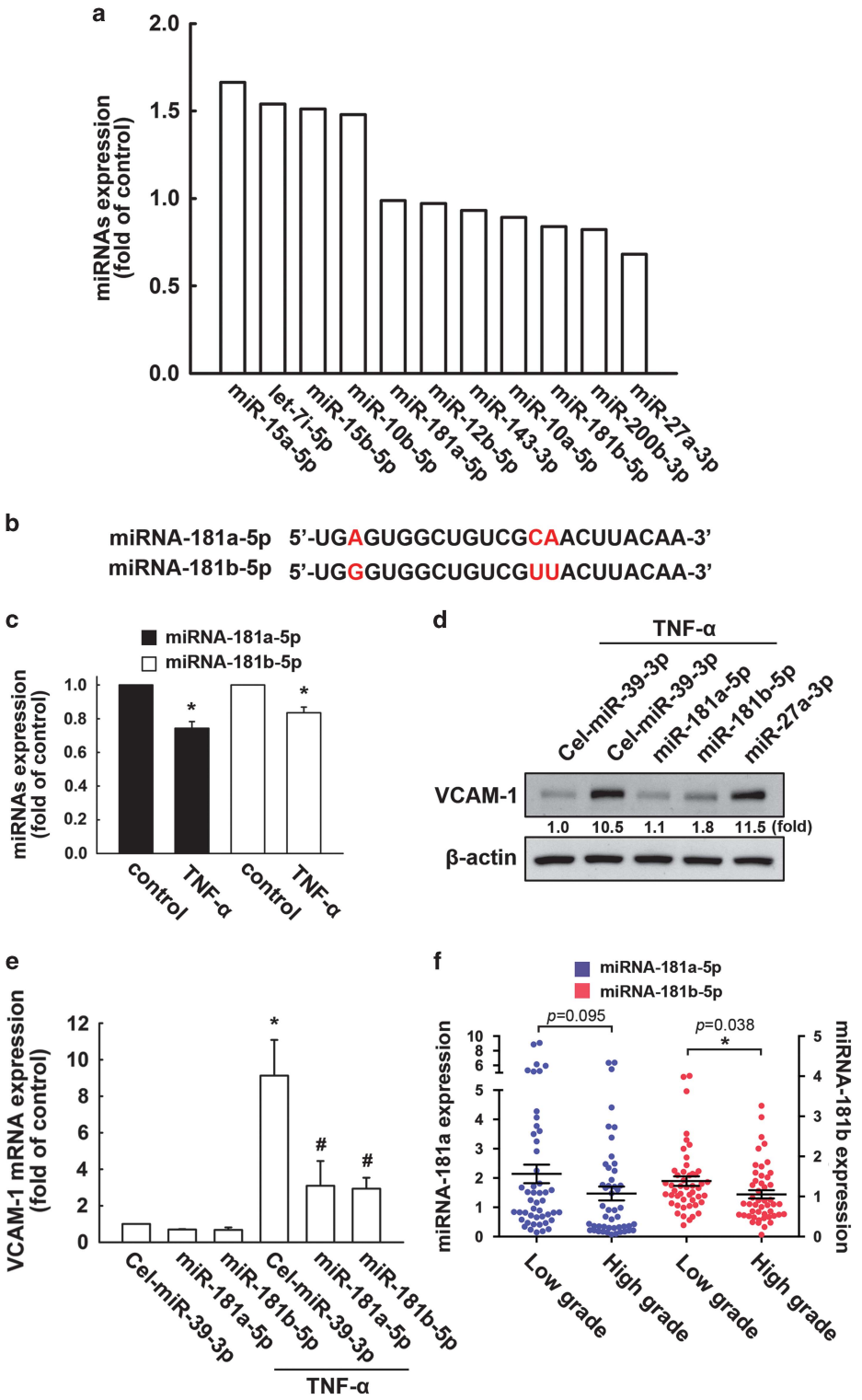


Figure 7. TNF-α induces VCAM-1 expression by downregulation of miR-181 family in GBM. (**a**) U251 were treated with TNF-α (10 ng/ml) for 6 h, and miRNAs expression were determined using real-time PCR for Cancer Focus microRNA PCR Panel. (**b**) Mature sequences of miR-181a-5p and miR-181b-5p. (**c**) U251 were treated with TNF-α (10 ng/ml) for 6 h, miR-181a-5p and miR-181b-5p expression were determined using real-time PCR. U251 were transfected with Cel-miR-39-3p (mimic control), miR-181a-5p, miR-181b-5p or miR-27a-3p mimics for 24 h and then treated with TNF-α (10 ng/ml) for another 24 (**d**) or 6 h (**e**). VCAM-1 expression was determined using western blot analysis (**d**) and real-time PCR (**e**), respectively. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the TNF-α treatment group. (**f**) MiR-181a-5p and miR-181b-5p expression in low-grade and high-grade glioma patients were determined real-time PCR. Both low-grade and high-grade have 48 specimens each. Quantitative data are presented as mean ± s.e.m., $^{*}P < 0.05$ compared with low-grade group.

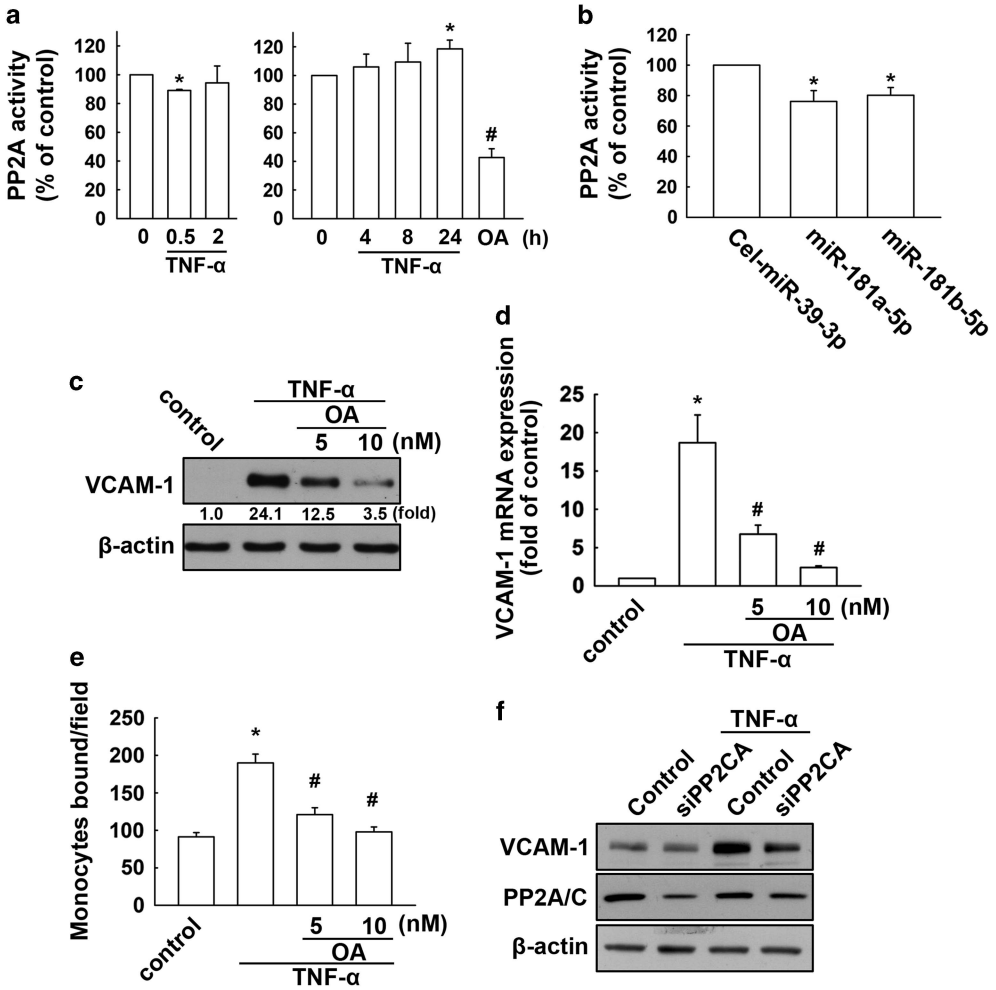


Figure 8. PP2A activation involves in TNF-α-mediated VCAM-1 expression and monocyte adhesion in U251 GBM. Cells were treated with TNF-α (10 ng/ml) for indicated time periods (**a**; 0.5, 2, 4, 8 or 24 h), or cells were transfected with Control, miR-181a-5p or miR-181b-5p mimics for 24 h (**b**). PP2A phosphatase activities were measured by using PP2A phosphatase kit. Cells were treated with OA (5 or 10 nM) for 30 min and treated with TNF-α (10 ng/ml) for another 24 (**c**) or 6 h (**d**). VCAM-1 expression was determined using western blot analysis (**c**) and real-time PCR (**d**), respectively. (**e**) Cells were treated with OA (5 or 10 nM) for 30 min and treated with TNF-α (10 ng/ml) for another 24 h. The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy. (**f**) Cells were transfected with Control or PP2CA siRNA for 24 h and treated with TNF-α (10 ng/ml) for another 24 h, and VCAM-1 and PP2A/C expression were determined using western blot analysis. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the TNF-α treatment group.

monocytes expressed the immune cytokines TNF-α and IFN-γ, promoted TAM proliferation and enhanced glioma cell growth, which has implications in the cytokines secreted from TAM modulates GBM microenvironment leading to gliomagenesis. However, reduction of CD14 was observed in glioma-infiltrating monocytes.[14] Surprisingly, no M2 markers, namely, ARG1, IL-10 and CD163 were expressed on the GBM-adhered monocytes in the 3D model (Figure 6a). Macrophages are classified as M1 or M2 based on their distinct characteristics, and have also been identified to possess anti-tumoral or pro-tumoral activities. In lower-grade glioma, the TAMs were strongly manifested with MHCII, an M1 marker.[45] Otherwise, TAMs in GBM tumors expressed strong M2 markers.[46] However, a recent report suggested that both M1 and M2 stimuli are required to engage monocytes toward pro-tumoral functions, which does not correlate with the M1 or M2 phenotypes.[47] The polarizations of TAM and the influence of its secreted cytokines on GBM need further investigation.

Previous reports indicate that the expression of VCAM-1 in tumor cells has been considered as a potential therapeutic target in immune evasion[48] and tumor metastasis.[21] In four different GBMs, the levels of VCAM-1 in the GBM correlated highly with the activities of monocyte binding to GBM (Figure 3h). Monocyte recruitment to GBM through the integrin α4β1-VCAM-1 interaction promoted GBM invasion and tumor growth (Figures 5a and b). We also found that GBM with higher EGFR levels (U251 cells) possessed higher levels of VCAM-1 than GBM with lower EGFR levels (GL261 cells) (Figure 3i). A recent study reported that EGF mediated the increase in expression of VCAM-1 in activated EGFR-promoted macrophage-glioma interaction and cell invasion.[36] Furthermore, blocking VCAM-1 expression significantly suppressed glioma growth and prolonged survival of glioma-bearing rats.[49] Importantly, anti-integrin-α4 inhibitors are used to treat diseases by macrophage and leukocyte infiltration, and have acquired the FDA approval in USA for clinical trials.[21] Our results provide evidence that TNF-α enhances VCAM-1 expression and monocyte adhesion, which is regulated by EGFR expression, treatment with gefitinib and erlotinib effectively reduced the TNF-α-induced VCAM-1 expression. It has been reported that JAK2/STAT3 pathway is essential for the EGFR-drive cell migration and invasion in GBM.[50,51] STAT3 is mainly activated via activators include JAK kinase and growth factor receptor (EGFR),[52,53] which

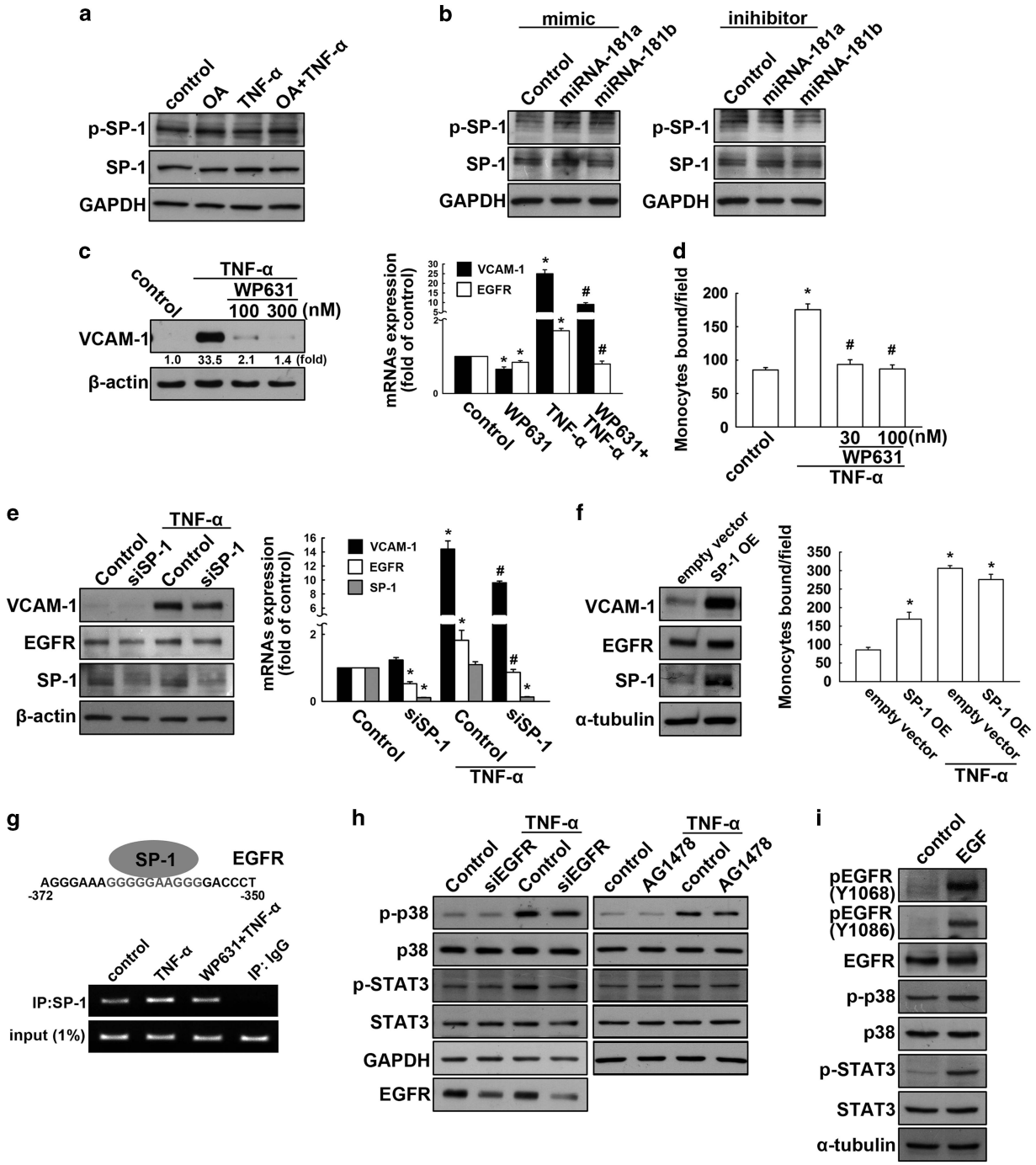


Figure 9. SP-1 involves in EGFR associated monocyte adhesion induced by TNF-α in U251 GBM. (**a**) Cells were treated with OA for 30 min and treated with TNF-α (10 ng/ml) for another 1 h, and p-SP-1 expression was determined using western blot analysis. (**b**) Cells were transfected with miR-181a and miR-181b mimics or miR-181a and miR-181b inhibitors for 24 h, and p-SP-1 expression was determined using western blot analysis. (**c**) Cells were treated with WP631 (100 or 300 nM) and treated TNF-α (10 ng/ml) for another 24 h (left) or 6 h (right). VCAM-1 and EGFR expression were determined using western blot analysis and real-time PCR, respectively. (**d**) Cells were treated with WP631 (30 or 100 nM) for 30 min and treated with TNF-α (10 ng/ml) for another 24 h. The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy. (**e**) Cells were transfected with Control or SP-1 siRNA for 24 h and TNF-α (10 ng/ml) for another 24 h (left) or 6 h (right). VCAM-1, EGFR and SP-1 expression were determined using western blot analysis and real-time PCR, respectively. (**f**) Cells were transfected with empty vector or wild-type SP-1 for 24 h, VCAM-1 and EGFR protein levels were determined by western blot analysis (left). The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy (right). (**g**) Cells were treated with WP631 (100 nM) for 30 min, followed by stimulation with TNF-α (10 ng/ml) for 2 h, and cells were then fixed and subjected to chromatin immunoprecipitation assay using antibodies to SP-1 or mouse normal IgG. Levels of immunoprecipitated chromatin fragments of EGFR promoter or input were examined by PCR. (**h**) Cells were transfected with Control or EGFR siRNA for 24 h (left), or treated with AG1478 (10 μM) for 30 min (right), and treated with TNF-α (10 ng/ml) for another 1 h. The expression of p-p38, p-STAT3 and total EGFR were determined using western blot analysis. (**i**) Cells were treated with EGF (100 ng/ml) for 1 h, and expression of p-EGFR, p-p38 and p-STAT3 were determined using western blot analysis. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^{*}P < 0.05$ compared with the control group. $^{\#}P < 0.05$ compared with the TNF-α treatment group.

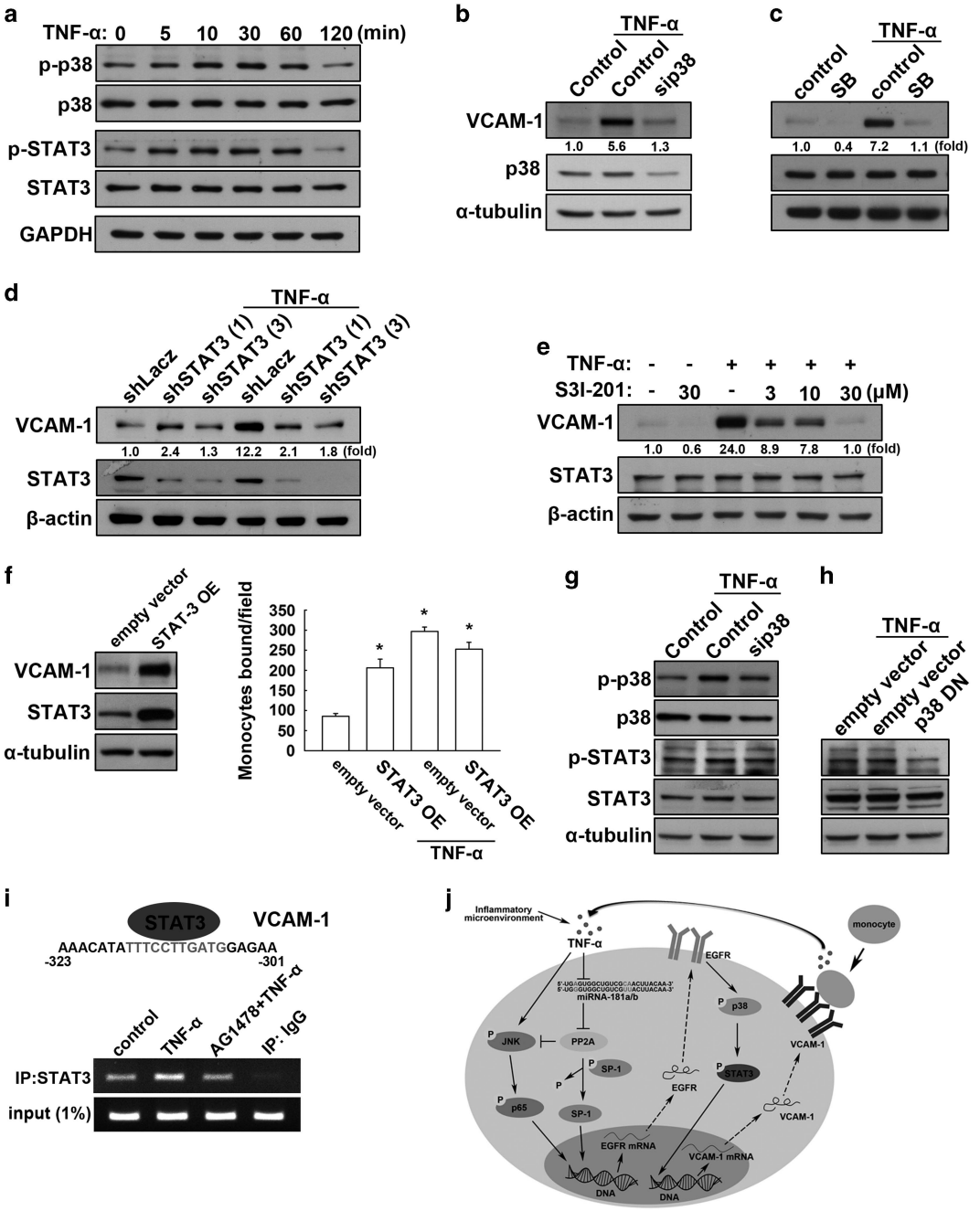


Figure 10. p38/STAT3 pathway involves in the EGFR associated VCAM-1 expression and monocyte adhesion induced by TNF-α in U251 GBM. (**a**) Cells were treated with TNF-α (30 ng/ml) for indicated time periods (5, 10, 30, 60 or 120 min), and p-p38, and p-STAT3 expression were determined using western blot analysis. (**b**) Cells were transfected with Control or p38 siRNA for 24 h and treated with TNF-α (10 ng/ml) for another 24 h. VCAM-1 and p38 expression were determined using western blot analysis. (**c**) Cells were treated with SB203580 (10 μM) for 30 min and treated with TNF-α (10 ng/ml) for another 24 h, and VCAM-1 and p38 expression were determined using western blot analysis. (**d**) Cells were transfected with Lacz (as a control) or STAT3 shRNA for 24 h and treated with TNF-α (10 ng/ml) for another 24 h. VCAM-1 and STAT3 expression were determined using western blot analysis. (**e**) Cells were treated with S3I-201 (3, 10 or 30 μM) for 30 min and treated with TNF-α for another 24 h, and VCAM-1 and STAT3 expression were determined using western blot analysis. (**f**) Cells were transfected with empty vector or wild-type STAT3 for 24 h. VCAM-1 and STAT3 expression were determined by western blot analysis (left). The ability of monocyte adhesion to GBM were measured the adhesion activity of BCECF-AM-labeled-THP-1 by fluorescence microscopy (right). Cells were transfected with p38 siRNA (left) (**g**), or DN mutant of p38 (right) (**h**) for 24 h and treated with TNF-α (10 ng/ml) for another 1 h. p-p38, p38, and p-STAT3 expression were determined using western blot analysis. (**i**) Cells were treated with AG1478 (10 μM) for 30 min, followed by stimulation with TNF-α (10 ng/ml) for 2 h. The cells were then fixed and subjected to chromatin immunoprecipitation assay using antibodies to STAT3 or mouse normal IgG. Levels of immunoprecipitated chromatin fragments of VCAM-1 promoter or input were examined by PCR. Quantitative data are presented as mean ± s.e.m. (representative of $n = 3$). $^*P < 0.05$ compared with the control group. (**j**) Schematic diagram of the signaling pathways in TNF-α-induced VCAM-1 expression in GBM. TNF-α could release from tumor surrounding inflammatory microenvironment, which TNF-α activates PP2A/SP-1 pathways through downregulation of miR-181b, and JNK/p65 pathways to enhance EGFR expression. TNF-α also induces EGFR upregulation through p38 and STAT3 pathways to modulate VCAM-1 expression and subsequently monocyte adhesion. Adherent of monocyte were further secreted more TNF-α to stimulate GBM to form a positive feedback loop. DN, dominant-negative.

results in activation of STAT3 target genes that promote tumor progression including cell proliferation, migration and invasion.[54] STAT3 activation is associated with the degree of immune infiltration and glioma patient poor prognosis.[55,56] Importantly, administration of a JAK/STAT3 inhibitor decreased the infiltration monocytes to glioma.[57] On the other hand, targeting JAK2/STAT3 sensitizes gliomas to anti-EGFR agent gefitinib and alkylating agents temozolomide and carmustine in human gliomas.[58] Our results, demonstrated that STAT3 translocate to nuclear binding with promoter region of VCAM-1 and VCAM-1-associated monocyte adhesion through EGFR/JAK/STAT3 pathways.

Previous studies showed that miR-181b reduces VCAM-1 expression in response to pro-inflammatory stimuli.[59] MiR-181a has been reported to be a target for enhancing the effect of radiation treatment on malignant glioma cells.[60] MiR-181b acts as a tumor suppressor that causes apoptosis and inhibition of invasion in glioma cells.[61,62] Importantly, miR-181b levels correlate negatively with glioma grade,[63] and lower expression of miR-181b is significantly associated with poor survival in patients with glioma.[64] Array-based miRNA profiling of human cancer cells revealed that miR-181b and miR-181a were the potential targets involved in EGFR-dependent monocyte adhesion in GBM. We also analyzed the miR-181b and miR-181a expression profile in specimens from patients with low-grade and high-grade glioma. Our results confirm the previous report that miR-181b expression negatively correlates with glioma grade and poor prognosis of patients with glioma.[63] Moreover, our results also support previous report that the tumor-suppressive effect of miR-181b in glioma cells is more apparent than the effect of miR-181a.[65]

PP2A is a major serine/threonine phosphatase, which has been shown to play a decisive role in cancer development.[66] Treatment with a PP2A inhibitor effectively inhibited PP2A activity and enhanced the radio-sensitivity of GBM cells *in vitro* and *in vivo*, respectively.[67] Another study showed that pharmacologic inhibition of PP2A combined with temozolomide therapy enhanced the sensitivity of GBM chemotherapy in a mouse model.[68] It has also been reported that PP2A activity is associated with poor outcome in patients with GBM and increased survival of GBM-derived stem-like cells.[69] Our results also show that PP2A is involved in the TNF-α-induced VCAM-1 expression, which promoted monocyte adhesion to GBM, and this phenomenon was abolished by the PP2A inhibitor. The miRNA target database (DIANA Tools, TarBase v7.0) also confirmed that miR-181a might target PP2A. Lacroix *et al.*[70] demonstrated an upregulation of SP-1 transcriptional activity by PP2A in dividing T cells. Vicart *et al.*[71] reported that the PP2A-mediated dephosphorylated form of SP-1 is more tightly associated with chromatin than phosphorylated SP-1. Furthermore, SP-1 upregulated the downstream gene expression, which correlated with the self-renewal capability of glioma stem cells[72] and cell proliferation, and promoted glioma tumorigenesis.[73] Importantly, treatment with an SP-1 inhibitor effectively induced cell death and reduced cell migration in GBM.[74] Moreover, upregulation of SP-1 in glioma cells promoted cell invasion and predicted poor clinical outcome.[75]

In conclusion, we provided evidence that VCAM-1 is a critical modulator of the EGFR-dependent interaction of monocytes and GBM, raising the possibility that anti-EGFR drugs, such as small molecule inhibitors targeting the EGFR tyrosine kinase, may lead to the development of effective therapies of GBM. These adherent monocytes further enhanced cell proliferation and TNF-α expression, at least in part, promoting VCAM-1 expression on GBM and consequently enhanced monocyte adhesion. Our results also indicate that the miR-181b/PP2A/SP-1 and STAT3 signaling pathways may be used as novel therapeutic targets for GBM therapy. Thus, our present work provides novel insights into the tumor microenvironment and might assist the identification of new targets for cancer diagnosis and therapy.

MATERIALS AND METHODS

Materials (reagents and antibodies)

IL-1β, TGF-β (transforming growth factor beta), TNF-α, IL-18 and EGF were purchased from PeproTech (Rocky Hill, NJ, USA). AG490, SP600125 and SB203580 were purchased from Sigma-Aldrich (St. Louis, MO, USA). S3I-201 was purchased from Selleckchem (Houston, TX, USA). JAK I and AG1478 were purchased from Calbiochem (San Diego, CA, USA). OA was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Primary antibodies specific for GAPDH (G8795) and α-tubulin (T5168) were purchased from Sigma-Aldrich. Primary antibodies specific for VCAM-1 (ab134047), SP-1 (ab133596), β-actin (ab6276) and p-SP-1 (Thr^{453}, ab59257) were purchased from Abcam (Cambridge, UK). Primary antibodies specific for ICAM-1 (sc-8439), p38 (sc-535), p65 (sc-7151), STAT3 (sc-482), ERK2 (sc-1647), JNK1/3 (sc-474), Akt1 (sc-5298), SP-1 (sc-59), p-STAT3 (Ser^{727}, sc-56747), p-ERK (Tyr^{204}, sc-7383) and p-Akt1/2/3 (Ser^{473}, sc-7985-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies specific for p-JNK (Thr^{183}/Tyr^{185}, 9251), p-JAK1 ($Tyr^{1022/1023}$, 3331), p-JAK2 ($Tyr^{1007/1008}$, 3776), p-p38 (Thr^{180}/Tyr^{182}, 9211), p-p65 (Ser^{536}, 3031), EGFR (4267), p-EGFR (Tyr^{1068}, 3777), p-EGFR (Tyr^{1086}, 2220) and PP2A/C (2038) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibody specific for VCAM-1 (11444-1-AP) was purchased from Proteintech (Rosemont, IL, USA). Neutralizing antibodies specific for human TNF-α (MAB610), integrin α4 (BBA37), integrin β1 (MAB17781) and mouse isotype IgG control (MAB002) were purchased from R&D Systems (Minneapolis, MN, USA). On-Target smart pool VCAM-1, ICAM-1, PP2CA, EGFR, p38, p65 and SP-1 small interfering (si)RNA and control non-targeting siRNA were purchased from Dharmacon (Lafayette, CO, USA). The miRNA mimics hsa-miR-181a-5p, has-miR-181b-5p, has-miR-27a-3p and cel-miR-39-3p (as a negative control), and miRNA inhibitors were purchased from EXIQON (Woburn, MA, USA). The short hairpin (sh)RNA against STAT3 and Lacz were purchased from National RNAi Core Facility (NRC) (Academia Sinica, Taipei, Taiwan). EGFR ORF clone was purchased from OriGene (Rockville, MD, USA). The overexpressing constructs containing the coding sequence (CDS) of SP-1 (2358 base pair, 98-2455) or STAT3 (2313 base pair, 241-2553) were amplified by PCR of cDNA obtained from U251 cells. PCR products were cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) between XhoI and HindIII restriction sites. All constructs were sequenced to verify that contained the CDS inserts.

Cell culture

U251 human GBM cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB No. IFO50288, Japan). U87 human GBM cells, ALTS1C1 mouse GBM cells, and THP-1 human monocytes were obtained from the Bioresource Collection and Research Center (BCRC No. 60360, 60582 and 60430; Taiwan). U251-GFP and U87-GFP cells were established and maintained in our laboratory. Briefly, the pAS2-EGFP.puro plasmids were transfected into either U251 or U87 cells. Stable clones were initially selected using 1.5–2.5 μg/ml of puromycin (Sigma) for 2 weeks and then maintained in 2 μg/ml of puromycin in culture medium. The U251-GFP or U87-GFP cells were seeded at 100 cells into 96 well plate and growing for 2 weeks. Multiple clones of U251-GFP and U87-GFP were established to stably express GFP, and the expression were verified by fluorescence microscope and western blotting (Supplementary Figure 7a). U251 and U87 were maintained with Minimum Essential Medium, ALTS1C1 and GL261 were maintained with Dulbecco's Modified Eagle Medium, and THP-1 was maintained with RPMI-1640. All cells were cultured in medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Conditioned medium from U251, THP-1 cultures or U251/THP-1 co-cultures grown in 6 ml of culture medium with 10% fetal bovine serum were collected and centrifuged at 2000 r.p.m. for 5 min. All cells used in this study were tested for mycoplasma.

Patients and specimen preparation

This study was performed following the guideline of the Institutional Review Board of Chang Gung Memorial Hospital (104-2656B), and all subjects provided informed written consent before their enrollment. Glioma tissue specimens were acquired from patients who had been diagnosed with glioma and had undergone surgical resection. The pathological grades of each glioma specimen were verified through histological examination by a pathologist according to the World Health

Organization criteria. The tissue samples for miRNA examination were sharply excised, placed in sterile tubes and frozen immediately in liquid nitrogen. The samples were stored at − 80 °C until analysis.

Primary human monocytes isolation

Human monocytes were prepared from healthy donor blood by performing Lymphoprep (Axis-shield PoC AS, Oslo, Norway) density gradient centrifugation followed by RoseteSep Human Monocyte Enrichment Cocktail CD14+ negative selection (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Briefly, collect whole blood into a tube containing anticoagulant, and added RosetteSep Enrichment Cocktail 50 µl/ml of whole blood. After incubation at room temperature for 20 min, dilute blood sample with an equal volume of phosphate-buffered saline containing 2% fetal bovine serum and then mix gently. Layer the diluted blood sample on top of Lymphoprep density gradient medium, and centrifuge at 1200 *g* for 20 min, with the break off. Remove the CD14+ monocytes enriched cells from the density gradient medium (plasma interface). After washing with phosphate-buffered saline, monocytes enriched cells are lysed with RBC lysis buffer to remove residual red blood cells, the purity of the monocytes enriched cells was 75–85%.

Immunohistochemistry

Human brain tumor tissue array (BS17017a) was purchased from Biomax (Odenton, MD, USA) in the form 5 µm sections of paraffin-embedded tissue on glass slides. After rehydration and incubation in 3% hydrogen peroxide (H_2O_2) to block endogenous peroxidase activity, slides than incubated with Triton X-100. After blocking with bovine serum albumin, the slides were incubated with primary antibody, monoclonal rabbit anti-human VCAM-1 (1:50) at 4 °C overnight. After several washes with TBST (0.1% tween 20 in tris buffered saline), binding was detected using a biotin-conjugated secondary antibody and an ABC reaction kit (Vector Laboratories, Burlington, CA, USA). Visualization was performed using 0.05% chromogen diaminobenzidine (Sigma-Aldrich), then counterstained with hematoxylin, dehydrated, incubated with xylene and mounted.

Monocyte-binding assay

THP-1 cells or primary human monocytes were labeled with 0.1 µg/ml BCECF/AM (Invitrogen, Green) at 37 °C for 1 h followed by washed twice with growth medium. GBM cells were treated with TNF-α for the indicated periods of time or dose. The medium was removed from the wells, and 2.5×10^5 BCECF/AM-labeled-THP-1 cells were added to a monolayer GBM cells. After incubation at 37 °C for 30 min, the wells were gently washed twice with warm growth medium to remove non-adherent cells. The cells were then photographed under a fluorescence microscope to calculate adherent cells. For 3D culture model-binding assay, the method was full described in 3D co-culture. The GBM-GFP+ and THP-1 cells were quantified by flow cytometry. The ratio of adherent THP-1 cells to GBM-GFP+ cells were calculated as the ratio of THP-1 cells to 5000 GBM-GFP+ cells.

3D co-culture

For 3D co-cultures, 150 µl phenol red free Matrigel (Corning, New York, NY, USA) was added to 24-well plates. After polymerization at 37 °C for 30 min, cultures of GBM cells including U251-GFP or U87-GFP were seeded at 2×10^5 cells per well and maintained in culture medium condition for 24 h. Then, treated with TNF-α for another 24 h followed by incubation with 2×10^5 THP-1 cells per well, and the wells were gently washed twice with PBS to remove non-adherent THP-1 cells. After 24 h, 3D co-cultured cells were extracted from matrigel using Cell Recovery Solution (Corning) according to the manufacturer's instructions. 3D co-cultured cells were isolated single cell using 1 mg/ml Collagenase/Dispase (Roche, Basel, Switzerland) in culture medium and stir slowly at 37 °C for 1 h in dark. After cell separation, cells were washed with PBS twice, and THP-1 binding assay and flow cytometry analysis were undertaken (Supplementary Figure 7b).

Flow cytometry analysis

GBM cells were incubated with antibodies against anti-VCAM-1-conjugated PE (eBioscience, 12-1069), EGFR-conjugated eFluor 660 (eBioscience, 50-9509) or isotype IgG control (eBioscience, San Diego, CA, USA) for 30 min at 4 °C. Expression of these surface receptors was determined using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA). Ten thousand events were recorded and GBM cells were examined the VCAM-1 and EGFR expression.

For 3D culture monocytes intracellular flow cytometric analysis based on a BD Cytofix/Cytoperm Plus fixation/permeabilization protocol (BD Biosciences, San Jose, CA, USA). The 3D co-cultured cells were blocked for nonspecific binding by using anti-CD16/CD32 antibody (eBioscience) for 20 min, and the 3D co-cultured cells were fixed and permeabilized with BD Cytofix/Cytoperm solution for 20 min at 4 °C. The 3D co-cultured cells were washed with BD Perm/Wash buffer, re-suspended in BD Perm/Wash buffer, and incubated with either anti-TNF-α-APC (eBioscience, 17-7349), anti-IFN-γ-PE (eBioscience, 12-7319), anti-CD163 (BioLegend, 326509), anti-ARG1-PE (BioLegend, San Diego, CA, USA, 369703), anti-IL-10-eFluor 660 (eBioscience, 50-7108) and isotype IgG control (eBioscience) for 30 min at 4 °C. The 3D co-cultured cells were then washed with Perm/Wash buffer and re-suspended in FACS buffer.

For proliferation assays of 3D co-culture based on modified BrdU Staining Kit for Flow Cytometry protocol (eBioscience). The 3D co-cultured cells were treated with BrdU (eBioscience, 20 µM) for 4 h, and then isolated from matrigel as described in method of 3D co-culture. After cell isolation, the cells were fixed and permeabilized with BD Cytofix/Cytoperm solution for 20 min at 4 °C. The cells were washed with BD Perm/Wash buffer, re-suspended in 10% DMSO/FBS, and the cells were frozen to -80 °C for least 12 h to permeabilize the nuclear membrane. After quick thawing of the cells at 37 °C the cells were washed with PBS. Cells were re-suspended in BD Cytofix/Cytoperm solution for 10 min at 4 °C. After washed with BD Perm/Wash buffer, the cells were incubated DNase I (eBioscience) at 37 °C for 1 h in the dark, and then incubated with anti-BrdU-conjugated APC for 30 min at room temperature in the dark.

Western blotting

The procedure of Western blotting was descripted previously. Briefly, protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% dry skim milk in TBST and probed overnight with a primary antibody at 4 °C and then, incubated with peroxidase-conjugates secondary antibodies (Santa Cruz Biotechnology). The blots were visualized using enhanced chemilumminescence and Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA). Quantitative data were obtained using an ImageJ software.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and was quantified using the BioDrop spectrophotometer (Cambridge, UK). The target mRNA levels were detected using quantitative real-time PCR. The reverse transcription (RT) reaction was performed using 2 µg of total RNA converted into cDNA using the Invitrogen RT Kit and amplified using the following oligonucleotide primers: VCAM-1: 5′-CAAAG GCAGA GTACG CAAAC AC-3′ and 5′-GGCTG ACCAA GACGG TTGTA TC-3′; ICAM-1: 5′-CCCCC CGGTA TGAGA TTGT-3′ and 5′-GCCTG CAGTG CCCAT TATG-3′; EGFR: 5′-ATGCT CTACA ACCCC ACCAC-3′ and 5′-GCCCT TCGCA CTTCT TACAC-3′; SP-1: 5′-GCCTC CAGAC CATTA ACCTC AG-3′ and 5′-TCATG TATTC CATCA CCACC AG-3′; β-actin: 5′-AGAGC TACGA GCTGC CTGAC-3′ and 5′-AGCAC TGTGT TGGCG TACAG-3′; GAPDH: 5′-AGGGC TGCTT TTAAC TCTGG T-3′ and 5′-CCCCA CTTGA TTTTG GAGGG A-3′. The protocol of qPCR was referred to our previous report.[76] The expression of miRNAs were detected using qPCR and Cancer Focus microRNA PCR Panel (EXIQON) following the manufacturer's protocols. For miRNA RT reaction, ten nanogram of total RNA from each samples was reverse-transcribed using the Universal cDNA Synthesis Kit II (EXIQON). PCR amplification were performed in final volumes of 10 µl using ExiLENT SYBR Green master mix (EXIQON). The expression of miR-103a-3p was used as an internal control to normalize expression levels of miRNAs. The threshold was set within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT).

Cell transfection

GBM cells were transiently transfected with shRNA against STAT3 or Lacz as a negative control, and dominant-negative mutant of JNK or p38 using Lipofectamine (LF)3000 (Invitrogen) for 24 h. Plasmid DNA and LF3000 were premixed in serum-free medium for 5 min and then applied to the cells. The LF3000-containing medium was replaced with fresh serum-free medium after 24 h. GBM cells were transiently transfected with either hsa-miR-181a-5p, has-miR-181b-5p, has-miR-27a-3p mimics and cel-miR-39-3p

as a negative control, or miRNA inhibitors (EXIQON), and smart pool siRNA (Dharmacon) against VCAM-1, ICAM-1, PP2CA, EGFR, p38, p65, SP-1 or control siRNA using DharmaFECT transfection reagents (Dharmacon). MiRNA mimics, miRNA inhibitors or negative control, and siRNAs or negative control were premixed with DharmaFECT transfection reagents (Dharmacon) in serum-free medium for 25 min and applied to the cells. After 24 h, the reagent-containing medium was replaced with fresh serum-free medium.

Chromatin immunoprecipitation (ChIP) assay

The protocol of ChIP was performed using EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, using isolated nuclei from the formaldehyde-cross-linked U251 cells. Immunoprecipitation was performed using primary antibody anti-SP-1 or anti-STAT3, and magnetic beads. Normal mouse IgG was used as negative control. 2 μg of antibody was used for each reaction. The diluted chromatin was then incubated on a rotator at 4 °C for overnight, then extracted and purified. Purified DNA was subjected to PCR. The amplified using the following oligonucleotide primers: The primers 5′-ATTAT CCGAC GCTGG CTCTA-3′ and 5′-GGGTG CCCTG AGGAG TTAAT-3′ were utilized to amplify across the EGFR promoter region. The primers 5′-CCTTG TTTTG GCAGC AATTT-3′ and 5′-TGCCA TGTGA ATTGA TTTTC T-3′ were utilized to amplify across the VCAM-1 promoter region. PCR products were resolved by 2% agarose gel electrophoresis and visualized by UV light.

Protein phosphatase 2A activity assay

PP2A activity was evaluated using a PP2A DuoSet IC kit (R&D Systems) according to the manufacturer's instructions. Briefly, whole-cell lysates containing 200 μg of protein were loaded into 96 well plates coated with a capture antibody-specific PP2A (R&D Systems) for immunocapture at 4 °C for 3 h. After washing, synthetic phosphopeptide substrates (200 μM) were added for the de-phosphorylation reaction catalyzed by PP2A. The levels of free phosphate were determined by a sensitive dye-binding assay using malachite green and molybdic acid according to the manufacturer's instructions followed by measurement of the absorbance at 620-650 nm.

Cell invasion assay

In vitro invasion assays were performed using Costar Transwell inserts (8-μm pore size; Costar, New York, NY, USA) in 24-well plates. The transwell inserts were coated with 100 μl of matrigel in cold serum-free medium at 37 °C for 1 h. GBM cells (5×10^4 in 100 μl of serum-free medium) were seeded in the upper chamber for 1 h. THP-1 cells (5×10^4 in 100 μl of serum-free medium) were then loaded on top of the U251 cells. After 24 h of incubation, GBM cells that invaded the matrigel and passed through the filters were fixed with 3.7% formaldehyde for 5 min, and then stained with 0.05% crystal violet for 30 min. The non-invading cells were wiped from the inside of the inserts by cotton-tipped swabs, and the GBM cells on the undersides of the filter were photographed using a digital camera mounted onto a microscope.

GEO glioma patients gene expression data

The dataset (GSE4290) of glioma patients from the publicly available GEO (Gene Expression Omnibus) databases was used as one of the validation set.[77] The study subjects in this cohort were collected from the Henry Ford Hospital. The cohort consists of 180 patients with histologically confirmed gliomas of different grades: 23 non-tumors, 45 grade II (seven astrocytomas, 38 oligodendrogliomas), 31 grade III (19 astrocytomas, 12 oligodendrogliomas), 81 grade IV astrocytomas (GBM). The VCAM-1 gene expression value obtained from GSE4290 dataset to evaluate correlation with grading of human glioma.

Statistical analysis

Statistical analysis was done using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) and SigmaPlot software (Systat Software Inc., San Jose, CA, USA). Data are presented as mean ± s.e.m. and all experiments were performed with three biologically independent replicates, unless stated otherwise. Sample sizes are calculated to allow significance to be reached. Student's *t*-test, as appropriate, was used to determine statistical significance ($P < 0.05$). No pre-test was used to choose sample size. No data points were excluded. The Pearson's correlation test was used to examine association between adherent monocyte ability and VCAM-1 expression in GBMs. The number of patient specimens used per experiment is stated in each figure legend. The *n* and *P*-value are indicated in the figure legends.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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