Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth

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Tumour-associated macrophages (TAMs) are enriched in glioblastoma multiformes (GBMs) that contain glioma stem cells (GSCs) at the apex of their cellular hierarchy. The correlation between TAM density and glioma grade suggests a supportive role for TAMs in tumour progression. Here we interrogated the molecular link between GSCs and TAM recruitment in GBMs and demonstrated that GSCs secrete periostin (POSTN) to recruit TAMs. TAM density correlates with POSTN levels in human GBMs. Silencing POSTN in GSCs markedly reduced TAM density, inhibited tumour growth, and increased survival of mice bearing GSC-derived xenografts. We found that TAMs in GBMs are not brain-resident microglia, but mainly monocyte-derived macrophages from peripheral blood. Disrupting POSTN specifically attenuated the tumour-supportive M2 type of TAMs in xenografts. POSTN recruits TAMs through the integrin αvβ3, as blocking this signalling by an RGD peptide inhibited TAM recruitment. Our findings highlight the possibility of improving GBM treatment by targeting POSTN-mediated TAM recruitment.

GBM is the most common and lethal type of primary brain tumour¹. The median survival of GBM patients is less than 16 months despite optimal treatment². Abundant macrophage infiltration is a common feature of GBMs, but these TAMs in GBMs lack apparent phagocytic activity³. In addition, an inverse correlation between TAM infiltration and GBM prognosis has been reported⁴. Recent studies suggested that TAMs may promote GBM tumour progression in multiple aspects⁵–⁸. The TAM-secreted cytokines, including IL-6 and IL-10, have been shown to promote cancer cell proliferation in GBMs (ref. 8). TAMs could also facilitate GBM tumour growth by promoting neo-vascularization⁹,¹⁰. Moreover, TAMs can interfere with the anti-tumour functions of other immune cells, although TAMs do not exhibit traditional immunocyte properties. Thus, TAMs mainly play a tumour-supportive role in GBM progression.

As pioneering immunotherapies have shown initial promise for GBM treatment, interrogating immunologic regulation in GBMs may inform therapy⁹–¹¹. In addition, as TAMs in GBM may originate from either monocytes from peripheral blood or resident microglia in brain, identifying the source of TAMs in GBMs will provide critical information to determine the appropriate therapeutic approaches. Therefore, determining the cellular and molecular mechanisms underlying TAM recruitment may facilitate the development of therapeutics to effectively improve GBM treatment.

Macrophages can be categorized into M1 and M2 subtypes based on their polarization status¹². In tumours, the M1 or M2 subtype TAMs represent tumour-suppressive or tumour-supportive macrophages, respectively¹³. Cell surface markers including Iba1 and CD11b are commonly used to label total TAMs (refs 12,14). Several surface markers such as CD163, Fizz1 and Arg1 have been used to mark M2 subtype TAMs, whereas other surface markers including MHCII, CD11c and iNOS have been suggested for M1 subtype TAMs (refs 15–19). Although TAMs in lower grade astrocytomas were strongly stained with the M1 marker MHCII (ref. 20), TAMs in GBM tumours manifested strong M2 marker staining¹⁵–¹⁷. Such correlation between tumour grades and the M2 TAM abundance further suggests that the M2 type TAMs might play a critical role in GBM progression. However, TAMs in GBMs may be derived from circulating monocytes or resident microglia¹³,¹⁶. Despite the fact that these two populations may share some surface markers in brain¹⁷,¹⁸, studies using CX3CR1(+/GFP)/CCR2(+/RFP) knock-in

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Figure 1 POSTN is preferentially secreted by GSCs and its levels correlate with TAM density in GBMs. (a) Immunofluorescent staining of POSTN (green) and the GSC marker OLIG2 or SOX2 (red) in human primary GBMs. Frozen sections of GBMs (CCF2445 and CW672) were immunostained with antibodies against POSTN and SOX2 or OLIG2, and counterstained with DAPI to show nuclei (blue). POSTN was preferentially expressed in GSCs and distributed in the area near GSCs. Scale bars, 20 μm. (b) Graphical analysis of showing the fraction of POSTN+ cells in SOX2+ cells (GSCs) in human primary GBMs. More than 70% of SOX2+ GSCs showed POSTN staining (n = 5 GBMs; mean ± s.e.m.). (c,d) Immunoblot analysis of POSTN expression in cell lysates (c) and conditioned media (d) from GSCs (+) and matched NSTCs (−). Conditioned media were obtained by culturing equal numbers of GSCs and NSTCs in neurobasal media without supplements for 24 h and concentrating media by vacuum centrifugation. Endogenous tubulin amounts in the corresponding cells were used for control. (e) Immunofluorescent analysis of POSTN (red) and the TAM marker Iba1 (green) in GBM tissue microarray (US Biomax). Two sets of representative staining are presented to show the enrichment of TAMs in POSTN-abundant regions in GBMs. Areas indicated with squares are enlarged and shown on the right side of each picture. Scale bars, 80 μm. (f) Immunohistochemical staining of POSTN and the TAM marker Iba1 in two consecutive tissue microarray slides, respectively. Representative staining images show that the GBM (GL803a C6) with higher POSTN (POSTNH) levels contained more Iba1+ (Iba1H) cells (TAMs) and the GBM (GL803a E7) with lower POSTN (POSTNL) levels has fewer Iba1+ (Iba1L) cells (TAMs). Scale bars, 40 μm. (g) Graphical analysis of POSTN and Iba1 staining in the tissue microarray slides. 52.5% of GBM cases showed POSTNH and Iba1H staining, and 28.75% of GBM cases showed POSTNL and Iba1L staining. Only 12.5% of GBM cases showed POSTNH but Iba1L staining, and 6.25% of GBM cases showed POSTNL but Iba1H staining. Most (81.25%) GBM cases showed that POSTN levels positively correlate with TAM density. (Data from 80 tumours.) Uncropped images of blots are shown in Supplementary Fig. 9.
GBM exhibits a remarkable cellular heterogeneity and differentiation hierarchy. GSCs at the apex of this hierarchy are able to self-renew, differentiate and repopulate the whole tumour bulk, but the monocyte-derived macrophages are CCR2\(^+\)/CX3CR1\(^-\) (ref. 22), indicating that microglia and monocyte-derived macrophages in brains are different populations with distinct and specific surface antigens.

To investigate the potential molecular link between GSCs and TAM recruitment, we screened for candidate chemoattractants of TAMs from GSC-secreted proteins. We identified POSTN as a potent TAM attractant that is preferentially expressed in GSCs. POSTN (originally called OSF-2) is a member of the Fasciclin family and a disulphide-linked cell adhesion protein\(^{36}\). POSTN is involved in various aspects of tumorigenic processes through signalling pathways such as Akt/Pi3K, integrin and Wnt-1 (refs 37,38).

Recently, POSTN was shown to be preferentially upregulated in stromal fibroblasts of breast cancer and help the maintenance of breast cancer stem cells by increasing Wnt signalling; thus, POSTN plays a supportive role in cancer stem cell metastatic colonization and breast cancer metastasis\(^{38}\). In addition, POSTN plays crucial roles to promote tumour progression including growth, invasion and metastasis in several malignant cancers\(^{36,38,39}\). In this study, we found that TAM density positively correlates with POSTN protein levels in human GBMs. Disrupting POSTN markedly reduced TAM density, inhibited tumour growth and increased the survival of mice bearing the GSC-derived xenografts. We determined that TAMs in GBMs are mainly monocyte-derived macrophages (CCR2\(^+\)/CX3CR1\(^-\)) from peripheral blood but not resident microglia (CX3CR1\(^+\)/CCR2\(^-\)) from brain. Silencing POSTN specifically attenuated the tumour-supportive M2 type (CD163\(^+\)/Fizzl\(^+\)) of TAMs in GSC-derived tumours. We further demonstrated that POSTN mediates through the integrin \(\alpha_\text{v}\beta_3\) to recruit TAMs in vivo. Thus, POSTN secreted by GSCs recruits M2 type TAMs to support GBM growth.

RESULTS

POSTN is preferentially secreted by GSCs and correlates with TAM density in primary GBMs

To address whether GSC-secreted POSTN plays a role in TAM recruitment and GBM tumour growth, we examined the effect of POSTN knockdown on TAM density and tumour growth in GSC-derived GBM xenografts. Lentiviral-mediated short hairpin RNA (shRNA) silencing reduced POSTN expression in GSCs by 85–90% (Fig. 2a and Supplementary Fig. 2a). GSCs expressing POSTN shRNAs or non-targeting shRNA and firefly luciferase were transplanted into immunocompromised mice through intracranial injection. Bioluminescent imaging of orthotopic tumours in vivo showed a marked inhibition of tumour growth in the xenografts derived from GSCs expressing POSTN shRNA relative to those expressing non-targeting shRNA (Fig. 2b). Consistently, silencing POSTN also significantly extended the survival of mice bearing the GSC-derived tumours (Fig. 2c and Supplementary Fig. 2b,c). These data demonstrate a supportive role for POSTN in GBM tumour growth. Given that POSTN protein levels showed a positive correlation with TAM density in primary GBMs, we next examined the impact of POSTN downregulation on TAM density in GSC-derived xenografts with immunofluorescence staining of two well-defined macrophage...
Disrupting POSTN by shRNA markedly inhibited tumour growth and reduced TAM density in GSC-derived xenografts. (a) Immunoblot analysis of POSTN in GSCs expressing non-targeting (NT) shRNA or POSTN shRNA. Targeting POSTN by two distinct shRNA clones O56 and O57 through lentiviral infection reduced POSTN expression by 85–90% in GSCs (T387). (b) In vivo bioluminescent imaging analysis of tumour growth in mice bearing GBM xenografts derived from GSCs expressing non-targeting shRNA or POSTN shRNA. GSCs (T387) were transduced with firefly luciferase and POSTN shRNA or non-targeting shRNA and then engrafted intracranially into athymic nude mice. Representative images on day 15 post transplantation are shown (data from 5 mice). Silencing POSTN by both O56 and O57 shRNA clones markedly delayed tumour growth. (c) Kaplan–Meier survival curves of mice implanted with GSCs expressing POSTN shRNA or non-targeting shRNA (control). GSCs were transduced with POSTN shRNA or non-targeting shRNA (control). GSCs were transduced with POSTN shRNA (O56 or O57) or non-targeting shRNA through lentiviral infection and then were transplanted intracranially into athymic nude mice (20,000 cells per mouse). Survival curves were analysed by two-tailed log-rank test. Mice bearing GSC-derived tumours expressing POSTN shRNA showed a significant survival extension relative to the control group. ***, P < 0.001 (n = 6 mice for non-targeting shRNA and O57, and n = 8 mice for O56). (d) Immunofluorescent analysis of POSTN (red) and the TAM marker Iba1 (green) in GBM tumours derived from GSCs expressing POSTN shRNA or non-targeting shRNA. Frozen sections of GBM tumours derived from GSCs with POSTN shRNA (O56 and O57) or non-targeting shRNA were immunostained with antibodies against POSTN and Iba1 and counterstained with DAPI (blue). Marked reductions of POSTN and Iba1 (TAMs) signals were detected in the GSC-derived tumours expressing POSTN shRNA. Scale bars, 80 μm. (e, f) Graphical analyses of d showing a decrease of POSTN signal intensity by ~80% and a significant reduction of TAM density by ~60% in the GSC-derived tumours expressing POSTN shRNA. POSTN intensity and TAM density were analysed with ImageJ. **, P < 0.05; ***, P < 0.001 (n = 5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). Uncropped images of blots are shown in Supplementary Fig. 9.
Figure 3 TAMs in human primary GBMs and xenografts are monocyte-derived macrophages from peripheral blood. (a) Immunofluorescent staining of CCR2 (the marker for monocyte-derived macrophages) and CX3CR1 (the microglia marker) in GBM xenograft and normal brain tissue. Frozen sections of GSC-derived tumour (T387) and the adjacent normal mouse brain were immunostained with antibodies against CCR2 (green) and CX3CR1 (red) and counterstained with DAPI (blue). CCR2\(^+\) cells were detected only in tumour tissue, whereas CX3CR1\(^+\) cells were detected only in normal brain. Scale bars, 40\(\mu\)m. (b) Immunohistochemical staining of CCR2 and CX3CR1 in human primary GBMs and normal brain tissue. Two consecutive tissue microarray slides (US Biomax) containing GBMs and normal brain tissues were immunostained with antibody against CCR2 or CX3CR1 (brown) and counterstained with haematoxylin. GBM tumours showed abundant CCR2\(^+\) cells (the monocyte-derived TAMs) but not CX3CR1\(^+\) cells (microglia), whereas normal brain tissues contained CX3CR1\(^+\) cells but not CCR2\(^+\) cells. Scale bars, 40\(\mu\)m. (c) Graphical analysis of b in tissue microarrays showed that CCR2\(^+\) cells (monocyte-derived TAMs) were detected in most (82.6%) GBM cases (data from 23 tumours) and a minority (20%) of normal brains (data from 5 normal samples). In contrast, CX3CR1\(^+\) cells (microglia) were detected in all normal brains but only 4.3% of GBM tumour cases. (d) Immunofluorescent staining of Iba1 and CD31 in human primary GBMs. Frozen sections of a primary GBM (CCF2445) were immunostained with antibodies against Iba1 to label TAMs (green) and CD31 to mark vessels (red) and counterstained with DAPI (blue). Abundant TAMs are enriched in perivascular niches. Scale bars, 80\(\mu\)m. (e) Immunofluorescent staining of the TAM marker Iba1 and the endothelial marker Glut1 in GSC-derived xenografts. Frozen sections of GBM xenografts derived from GSCs (T387) were immunostained with antibodies against Iba1 (green) and Glut1 (red) and counterstained with DAPI (blue). TAMs (green) were localized near vessels but not in areas lacking blood vessels. Scale bars, 80\(\mu\)m.

TAMs in GBM intracranial xenografts (Supplementary Fig. 3d) and the isolated TAMs from the subcutaneous xenografts (Supplementary Fig. 3e). Collectively, these data demonstrate that GSC-secreted POSTN plays critical roles in promoting TAM recruitment and GBM tumour growth.

TAMs in GBM are monocyte-derived macrophages from peripheral blood

As the origin of TAMs in GBM has not been well defined, we next sought to address whether TAMs recruited by POSTN in GBM tumours are monocyte-derived macrophages from peripheral
Figure 4 POSTN-recruited TAMs are maintained as the M2 subtype in GSC-derived xenografts. (a) Immunofluorescent staining of the M2 TAM marker Fizz1 (green) and the pan macrophage marker Iba1 (red) in GSC-derived tumours expressing POSTN shRNA (056 and 057) or non-targeting shRNA (control). Nuclei were counterstained with DAPI (blue). Scale bars, 40 μm. (b,c) Graphical analyses of a showing a significant reduction of Fizz1+ M2 TAM density (b) and ratio (c) in POSTN-shRNA-expressing xenografts relative to xenografts expressing non-targeting shRNA. Relative M2 TAM density was normalized to total TAMs in xenografts expressing non-targeting shRNA. The M2 TAM fraction (ratio) was determined by the percentage of M2 TAMs (%). (d) Graphical analyses of d showing a significant reduction of CD163+ M2 TAM density (e) and ratio (f) in POSTN-shRNA-expressing xenografts relative to xenografts expressing non-targeting shRNA. "∗∗∗, P < 0.001 (n=5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). (g) Immunofluorescent staining of the M2 TAM marker Arg1 (green) and the pan macrophage marker CD11b (red) in GSC-derived tumours expressing POSTN shRNA or non-targeting shRNA. Scale bars, 40 μm. (h,i) Graphical analyses of g showing a significant reduction of Arg1+ M2 TAM density (h) and ratio (i) in POSTN-shRNA-expressing xenografts relative to xenografts expressing non-targeting shRNA. "∗, P < 0.05 (n=5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). (j) In vivo bioluminescent imaging showing the accelerated tumour growth of intracranial xenografts from POSTN-overexpressing (POSTN-OE) xenografts. Representative images on day 16 post transplantation are shown (data from 5 mice). (k) Graphical analysis of j showing an increase in relative M2 TAM density (k). CD163+ and Fizz1+ cell numbers were normalized to vessel density. "∗∗∗, P < 0.001 (n=5 tumours; mean ± s.e.m.; two-tailed unpaired t-test).
Figure 5 Silencing POSTN in GSCs increased the relative fraction of M1 TAMs in GSC-derived xenografts. (a) Immunofluorescent staining of the M1 TAM marker MHCI (green) and the pan macrophage marker Iba1 (red) in GSC-derived tumours expressing POSTN shRNA or non-targeting shRNA (control). Nuclei were counterstained with DAPI (blue). Scale bars, 40 μm. (b,c) Graphical analyses of a showing the reduced TAM density (b) but the increased MHCI⁺ M1 fraction (c) in POSTN-shRNA-expressing tumours relative to tumours expressing non-targeting shRNA. Relative TAM density was normalized to total TAMs in xenografts expressing non-targeting shRNA. The M1 TAM fraction was determined by the percentage of M1 TAMs within TAMs expressing non-targeting shRNA or POSTN shRNA xenografts, respectively. ***, P < 0.001; **, P < 0.01 (n = 5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). (d) Immunofluorescent staining of the M1 TAM marker CD11c (green) and the pan macrophage marker Iba1 (red) in GSC-derived tumours expressing POSTN shRNA or non-targeting shRNA. Scale bars, 40 μm. (e,f) Graphical analyses of d showing the reduced TAM density (e) but the increased CD11c⁺ M1 fraction (f) in POSTN-shRNA-expressing tumours relative to tumours expressing non-targeting shRNA. ***, P < 0.001 (n = 5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). (g) Immunofluorescent staining of the mature TAM marker F4/80 (green) and the pan macrophage marker Iba1 (red) in GSC-derived tumours expressing POSTN shRNA or non-targeting shRNA. Scale bars, 40 μm. (h,i) Graphical analyses of g showing the reduced TAM density (h) but the increased F4/80⁺ mature TAM fraction (i) in POSTN-shRNA-expressing tumours relative to tumours expressing non-targeting shRNA. ***, P < 0.001 (n = 5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). (j) qPCR analysis of multiple M1 and M2 macrophage markers STAB1 and Lyve1 were markedly upregulated. ***, P < 0.001; **, P < 0.01; NS, P > 0.05 (mean ± s.e.m.; n = 3 biologically independent samples per group, one representative experiment shown, and the experiment was repeated 3 times).
blood or resident microglia from brain. Previous reports have shown that microglia within normal brain are CX3CR1+/CCR2− whereas the monocyte-derived macrophages are CCR2+/CX3CR1− (refs 22, 42), so we applied these markers to analyse the TAMs in GSC-derived xenografts and primary GBMs. Consistently, most macrophages were CX3CR1+/CCR2− within the normal mouse brain (Fig. 3a, bottom panels), confirming they were resident microglia. However, only CCR2+/CX3CR1− macrophages were found in the GSC-derived GBM xenografts (Fig. 3a, top panels), demonstrating that POSTN-recruited TAMs in GBMs were monocyte-derived macrophages from peripheral blood. We further validated that TAMs in most human primary GBMs were also CCR2+/CX3CR1−, the monocyte-derived macrophages (Fig. 3b,c and Supplementary Fig. 9). Moreover, we found that TAMs were enriched around blood vessels in primary GBMs and the GSC-derived xenografts (Fig. 3d,e). Figure 4a). Disrupting POSTN in GSCs specifically reduces M2 tumour-supportive TAMs in vivo.

Recent studies indicated that there are at least two subtypes (M1 and M2) of TAMs in tumours43–45. The M1 subtype has an inhibitory effect on tumour growth whereas the M2 subtype plays a supportive role to promote tumour progression by secreting cell growth cytokines and survival factors46–48. To address which subtype of TAMs was recruited or maintained by POSTN in GBM tumours, we applied
M2-specific markers (Fizz-1, CD163 and Arg1)\cite{15,49,50} and M1-specific markers (MHCII and CD11c)\cite{14,51} to distinguish TAMs in GSC-derived tumours. More than 50% of TAMs in control tumours derived from GSCs expressing non-targeting shRNA were M2 subtype TAMs as they were positive for Fizz-1, CD163 and Arg1 (Fig. 4a–i), which mimics the situation in primary GBMs (Supplementary Fig. 4b–g). However, the M2 TAMs (Fizz-1\*, CD163\* or Arg1\*) were markedly reduced in the GBM tumours derived from GSCs expressing POSTN shRNA (Fig. 4a–i), indicating that disrupting POSTN in GSCs attenuated the M2 TAMs in the GSC-derived tumours. Consistently, overexpression of POSTN in GSCs accelerated xenograft growth and increased M2 TAM recruitment (Fig. 4j,k). In addition, a fraction (~50%) of TAMs was positive for the M1 subtype markers MHCII and CD11c in tumours derived from GSCs transduced with non-targeting shRNA (Fig. 5a–f). In contrast, most TAMs in the GBM tumours derived from GSCs expressing POSTN shRNA showed positive staining for the M1 markers MHCII and CD11c (Fig. 5a–f), although total TAMs were markedly reduced in the POSTN-silencing tumours relative to POSTN-expressing tumours. In addition, F4/80, a marker for macrophage maturation\cite{52}, was detected in most TAMs (76%~88%) within POSTN-silencing tumours (Fig. 5g–i). However, only a small proportion (10–15%) of TAMs in the tumours derived from GSCs transduced with non-targeting shRNA were F4/80 positive (Fig. 5g–i), suggesting an inhibition of macrophage maturation by POSTN in GBM tumours. Collectively, these data indicate that GSC-secreted POSTN also plays a critical role in maintaining the M2 subtype of TAMs in GBM tumours.

To further validate the role of POSTN in regulating the M1/M2 subtype of TAMs, we applied qPCR to determine the expression of multiple M2 and M1 markers in TAMs. CD11b\+ cells in GBM xenografts were isolated by magnetic-assisted cell sorting. When we examined M1/M2 marker expression in the CD11b\+ populations from xenografts derived from GSCs expressing non-targeting shRNA or POSTN-shRNA-expressing GSCs, we detected a significant downregulation of the M2 markers STAB1 and Lyve1 but a substantial upregulation of the M1-specific markers CCL17 and IL1b (ref. 43) in the CD11b\+ TAMs isolated from the xenografts derived from POSTN-silencing GSCs (Fig. 5j). These data further confirmed that POSTN not only recruits TAMs but also maintains the M2 subtype of TAMs in GBMs.

**POSTN exhibits a potent capacity to recruit macrophages/monocytes**

To validate whether POSTN functions as a potent GSC-secreted chemoattractant, we performed a series of migration and invasion assays to examine the capacity of POSTN to attract macrophages/monocytes. Conditioned media from GSCs attracted significantly more PMA-primed macrophage-like U937 cells than the media from matched NStCs (Fig. 6a,b). Pre-incubation of GSC-conditioned media with an anti-POSTN antibody attenuated the promoting effect of macrophage migration (Fig. 6c,d). Furthermore, silencing POSTN expression in GSCs by two specific shRNAs also significantly reduced this chemoattractant effect (Fig. 6e,f). When exogenous POSTN was overexpressed in NStCs (Fig. 6g), the resultant conditioned media exhibited a markedly increased ability to attract the U937-derived macrophage-like cells (Fig. 6h,i). The capacity of POSTN to attract monocyte/macrophages was further demonstrated with the isolated human primary monocytes in migration and invasion assays (Fig. 6j–m). To further address whether the POSTN-mediated attractant effect is dose dependent, we examined the impact of different concentrations of recombinant POSTN (rPOSTN) protein on macrophage migration. The migration of U937 macrophages towards POSTN was significantly enhanced with an increase of rPOSTN protein (Fig. 6n,o), indicating that POSTN attracts macrophages in a dose-dependent manner. Collectively, these data demonstrate that POSTN preferentially secreted by GSCs exhibits a potent capacity to attract macrophages.

**POSTN recruits TAMs through integrin α\_β3 signalling**

Next, we turned attention to the molecular mechanisms underlying the POSTN-mediated recruitment of TAMs. Previous reports suggested that the integrin α\_β3 is the key receptor for POSTN in mediating cell migration\cite{3,54}. Immunofluorescence staining showed that TAMs in GBM xenografts and primary GBM tumours express the integrin α\_β3 (Fig. 7a,b), suggesting that the POSTN–integrin α\_β3 axis may regulate TAM recruitment. To elucidate the signalling mediated by POSTN for TAM recruitment, we examined whether GSC-secreted POSTN can activate the integrin α\_β3 on monocyte-derived macrophages in vitro. We applied the U937 line that can be primed from monocytes to macrophage-like cells\cite{53,56} for the study, as most of the PMA-primed U937 cells are integrin α\_β3 positive (Fig. 7c). Consistently, POSTN induced activation of Akt in the primed U937 cells, which can be blocked by addition of anti-integrin α\_β3 antibody (Fig. 7d), indicating that POSTN can activate the integrin α\_β3 pathway in macrophages. We then examined the impact of an integrin inhibitor RGD peptide (Arg-Gly-Asp-d-Phe-Lys) related to a clinically developed anti-tumour agent, Cilengitide\cite{57}, on POSTN-mediated recruitment of U937 macrophages. Disruption of the integrin signalling in U937 cells by the RGD inhibitory peptide significantly attenuated the recruitment of the macrophages by rPOSTN protein (Fig. 7e,f), suggesting that the integrin α\_β3 on the macrophage surface is involved in the response to POSTN. To confirm whether the POSTN–integrin α\_β3 axis plays a critical role in TAM recruitment in vivo, we further investigated the inhibitory effect on TAM recruitment by the RGD peptide in GSC-derived GBM xenografts. Surprisingly, the GSC-derived tumours in the group of mice treated with the RGD peptide for 5 days showed an ~70% reduction in TAM density relative to the control tumours from mice treated with a control peptide (Fig. 7g,h). Taken together, these data indicate that POSTN recruits TAMs through integrin α\_β3 signalling in GBM tumours.

**Intracranial co-transplantation of M2 subtype TAMs with GSCs accelerates tumour growth of GSC-derived xenografts**

As silencing POSTN specifically reduces M2 type TAMs and potently inhibited tumour growth in GSC-derived xenografts, we next investigated the role of M2 TAMs in GBM tumour growth. Statistical analysis of the TCGA database showed that the M2 macrophage marker CD163 negatively correlated to GBM patient survival (Supplementary Fig. 4h), but the M1 macrophage marker MHCII positively correlated to prognosis (Supplementary Fig. 4i). In addition, immunofluorescent and immunohistochemical analyses
Figure 7 POSTN recruits macrophages through αVβ3 integrin signalling. (a) Immunofluorescent staining of the integrin αVβ3 (green) and the TAM marker Iba1 (red) in GSC-derived xenografts. Nuclei were counterstained with DAPI (blue). TAMs (Iba1+) stained positive for the integrin αVβ3 in the tumours. Scale bar, 40 μm. (b) Immunofluorescent staining of the integrin αVβ3 (green) and Iba1 (red) in human primary GBMs. A fraction of TAMs (Iba1+) stained positive for the integrin αVβ3 in GBM tumours (CCF2774 and CCF2467). Scale bars, 40 μm. (c) Immunofluorescent staining of the integrin αVβ3 (green) in PMA-primed U937 cells. U937 cells were stained positive with anti-integrin αVβ3 antibody. Scale bar, 40 μm. (d) Immunoblot analysis of POSTN-induced Akt activation that was attenuated by the antibody against the integrin αVβ3 in PMA-primed U937 cells. After serum starvation overnight, cells were incubated with 0.1 mg ml⁻¹ anti-integrin αVβ3 antibody or control IgG for 1 h followed by stimulation with 0.5 mg ml⁻¹ rPOSTN for 1 h. Pre-incubation with anti-integrin αVβ3 antibody but not the control IgG markedly inhibited POSTN-induced phosphorylation of Akt. (e) Representative images of U937 cells that migrated towards rPOSTN in the presence of inhibitory RGD peptide in Transwell assays. PMA-primed U937 cells pre-incubated with or without RGD peptide (1 mg ml⁻¹) were used for assessing cell migration towards rPOSTN protein (0.2 μg ml⁻¹) or 0.1% BSA. Scale bar, 40 μm. (f) Graphical analysis of e showing that pre-incubation of U937 cells with the RGD inhibitory peptide significantly reduced U937 cell migration towards rPOSTN. **, P < 0.01; NS, P > 0.05 (n = 5 fields; mean ± s.e.m.; the experiment was repeated 3 times and analysed by two-tailed unpaired t-test). (g) Immunofluorescent analysis of the TAM marker Iba1 (green) and the vessel marker Glut1 (red) in GBM xenografts treated with RGD inhibitory peptide or control peptide. Scale bars, 80 μm. (h) Graphical analysis of g showing a significant reduction of TAM density by ~70% in the GBM tumours treated with the RGD inhibitory peptide. ***, P < 0.001 (n = 5 tumours; mean ± s.e.m.; two-tailed unpaired t-test). Uncropped images of blots are shown in Supplementary Fig. 9.

demonstrated a positive correlation between POSTN expression and M2 subtype macrophage density in human GBM specimens (Supplementary Fig. 5a–c). POSTN treatment enhanced expression of M2 markers but reduced expression of M1 markers in mouse intraperitoneal macrophages and U937-derived macrophage-like cells (Supplementary Fig. 5d,e), although disrupting POSTN by shRNAs showed a mild impact on GSC proliferation in vitro (Supplementary Fig. 6a–c). These data suggest that the M2 subtype TAMs recruited and maintained by GSC-secreted POSTN may play a critical role in GSC-derived xenograft growth. To further explore the impact of M2 subtype TAMs on GBM tumour growth, we isolated CD11b+ TAMs that are enriched with M2 subtype TAMs from GSC-derived orthotopic xenografts, and then transplanted these TAMs with luciferase-expressing GSCs into mouse brains. Bioluminescent imaging demonstrated that intracranial co-transplantation of M2 TAMs with GSCs markedly accelerated GSC tumour growth relative to the transplantation of GSCs alone (Fig. 8a). Moreover, co-transplantation of M2 TAMs with GSCs also significantly shortened the survival of animals relative to the GSC transplantation alone (Fig. 8b). In addition, co-transplantation of M2 TAMs with GSCs expressing POSTN shRNA partially rescued the inhibition of GSC tumour growth caused by POSTN disruption (Fig. 8c), indicating an essential role for TAMs in mediating the POSTN-promoted tumour growth. The failure to fully rescue the inhibiting effect of POSTN silencing in GSC tumour growth by the co-transplanted M2 TAMs may be ascribed to the loss of M2 properties, impacted viability, or migration away of these TAMs from the tumour due to the absence of POSTN. Collectively, these data demonstrate that the M2 subtype TAMs have a potent capacity to promote GBM growth in vivo.

DISCUSSION

GBM often contains a large number of TAMs that constitute the dominant immune cell population within the tumour. Accumulating evidence suggests that TAMs rarely phagocytize cancer cells or behave like antigen-presenting cells45,58. M2 subtype TAMs are now widely regarded as immunosuppressive cells with a tumour-supportive...
role\textsuperscript{15,43}, although a role for M1 TAMs in anti-tumour immune responses has been reported\textsuperscript{39}. Tumours may recruit TAMs through secretion of CC chemokine ligand 2 (CCL2) and soluble colony-stimulating factor 1 (sCSF1) in other cancers\textsuperscript{40-42}. In this study, we demonstrated that TAMs in GBMs are mainly recruited by POSTN, a protein preferentially expressed by GSCs but rarely expressed in peripheral macrophages by GSC-secreted POSTN, as CCR2 is a potent chemoattractant to recruit monocyte-derived macrophages that secrete tumour-supportive factors to promote GBM growth and progression.

Our results demonstrated that disrupting POSTN in vivo significantly reduced recruitment of the tumour-supportive TAMs (M2 type) and inhibited GBM tumour growth. To determine other biological effects of POSTN downregulation in GBM tumours, we also examined the impact of targeting POSTN on several aspects of tumour biology such as vessel density, GSC population, cell proliferation and survival. POSTN knockdown had no obvious effects on vascular density in GSC-derived xenografts (Supplementary Fig. 7a-c). Similarly, blocking POSTN–integrin $\alpha_v\beta_3$ signalling by RGD treatment did not have a significant impact on vessel density (Supplementary Fig. 7d,e). However, the vessel morphology changed to some degree, which may be associated with the reduced TAM density. In addition, POSTN knockdown had no significant impact on GSC populations in tumour bulk as demonstrated by SOX2 immunofluorescence (Supplementary Fig. 8a,b). Furthermore, there was no significant difference in cancer cell proliferation between the GSC-derived tumours expressing POSTN shRNA and non-targeting shRNA as indicated by Ki-67 staining (Supplementary Fig. 8c,d). In contrast, targeting POSTN induced massive apoptosis in GSC-derived xenografts as demonstrated by TUNEL assay (Supplementary Fig. 8e,f). Moreover, qPCR analysis demonstrated a marked upregulation of M2 markers STAB1 and Lyve1 in CD11b$^+$ chemoattractant to recruit monocyte-derived macrophages that secrete tumour-supportive factors to promote GBM growth and progression.

Figure 8 M2 TAMs accelerated GSC tumour growth in mouse brains. (a) In vivo bioluminescent imaging analysis of tumour growth in mice bearing GBM xenografts derived from GSCs plus CD11b$^+$ cells (enriched with M2 TAMs) or GSCs alone. Fifty thousand CD11b$^+$ cells were isolated from GSC-derived xenografts and then co-transplanted with 20,000 GSCs intracranially into athymic nude mice. Representative images on day 18 post transplantation are shown (data from 5 mice). Co-transplantation of GSCs with CD11b$^+$ TAMs markedly promoted GSC tumour growth. (b) Kaplan–Meier survival curves of mice implanted with GSCs plus CD11b$^+$ cells or GSCs alone. Mice implanted with T387 GSCs plus CD11b$^+$ cells showed a significantly reduced survival relative to the mice implanted with POSTN-shRNA-expressing GSCs or POSTN-shRNA-expressing GSCs alone. Mice implanted with POSTN-shRNA-expressing GSCs plus CD11b$^+$ cells showed a significantly reduced survival relative to the mice implanted with POSTN-shRNA-expressing GSCs alone. $P = 0.039 \,(n = 5$ mice for each group; two-tailed log-rank test). (d) A schematic representation of the POSTN-mediated recruitment of monocyte-derived TAMs from peripheral blood during GBM development. POSTN preferentially secreted by GSCs attracts monocytes from peripheral blood to enter GBM tissues. The POSTN-recruited, monocyte-derived TAMs are co-localized in perivascular niches with GSCs and maintained as M2 macrophages that secrete tumour-supportive factors to promote GBM growth and progression.
cells (TAMs) from GBM xenographs relative to resident microglia from normal mouse brains (Supplementary Fig. 8g). These data suggest that the reduced M2 TAMs caused by POSTN disruption might be closely associated with the increased apoptosis and inhibited tumour growth in the GBM xenographs expressing POSTN shRNA. Thus, POSTN-mediated recruitment and maintenance of M2 TAMs largely contributes to the accelerated tumour growth by promoting cancer cell survival in GBMs, although POSTN itself may also partially play a direct role in promoting tumour cell survival in vivo. It will be important to determine how POSTN-recruited M2 TAMs augment cancer cell survival in GBMs in future studies.

Our studies uncovered the cellular and molecular mechanisms underlying TAM recruitment in GBMs. We also identified the origin of TAMs and their subtype in this lethal type of brain tumour. GSCs preferentially secrete POSTN to recruit TAMs into GBM tumours, although other factors may also affect immune cells in GBMs. As POSTN-recruited TAMs are mainly monocyte-derived macrophages from blood, the contribution of microglia from brain to TAMs in GBMs is not as critical as we previously thought. Thus, targeting monocyte-derived TAMs should be considered as the main focus of GBM immunotherapy. Furthermore, we demonstrated that POSTN-recruited TAMs are maintained as the pro-tumorigenic M2 subtype of macrophages, indicating that the recruited TAMs may produce important factors to augment GBM tumour growth. Consistently, silencing POSTN expression markedly reduced TAMs and inhibited GBM tumour growth by reducing cell survival. As previous studies in other tumours such as colon cancers have shown that POSTN promotes cancer cell survival through AKT activation, it is likely that POSTN may affect GBM tumour growth through multiple mechanisms besides recruitment and maintenance of M2 TAMs. Thus, targeting POSTN signalling, blocking POSTN-mediated TAM recruitment and eliminating the POSTN producer GSCs are likely to be attractive therapeutic approaches. As a reciprocal supportive interplay between cancer cells and TAMs may be critical to promote tumour progression, disrupting the interrelationship between TAMs and cancer cells, especially GSCs, may effectively inhibit GBM tumour growth. Recently, targeting TAMs by trabcetin has been shown to exhibit a significant anti-tumour activity in lung carcinoma, ovarian growth. Recently, targeting TAMs by trabectedin has been shown to interplay between cancer cells and TAMs may be critical to promote recruitment and eliminating the POSTN producer GSCs are likely. Thus, targeting POSTN signalling, blocking POSTN-mediated TAM mechanisms besides recruitment and maintenance of M2 TAMs. This, targeting POSTN signalling, blocking POSTN-mediated TAM recruitment and eliminating the POSTN producer GSCs are likely to be attractive therapeutic approaches. As a reciprocal supportive interplay between cancer cells and TAMs may be critical to promote tumour progression, disrupting the interrelationship between TAMs and cancer cells, especially GSCs, may effectively inhibit GBM tumour growth. Recently, targeting TAMs by trabectedin has been shown to exhibit a significant anti-tumour activity in lung carcinoma, ovarian cancer and fibrosarcoma. Our finding of molecular mechanisms underlying TAM recruitment by GSC-secreted POSTN may shed light on the development of therapeutics to improve GBM treatment. In addition, therapeutic targeting of POSTN-mediated TAM recruitment may synergize with current immunotherapies to effectively increase the survival of patients with GBMs and possibly other lethal cancers.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
W.Z. and S.B. designed the experiments, analysed data and prepared the manuscript. W.Z., S.Q.K., Z.H., X.F., I.J.P and L.W. performed the experiments. W.E. performed database analysis. A.E.S. and R.E.M. provided GBM surgical specimens. R.E.M. performed pathological analyses. J.N.R. and X.L. provided scientific input and helped to edit the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Human GBM specimens or xenografts through cell sorting and functionally characterized as previously described.16,27,56,65,66 Intracranial tumour formation in vivo was performed with an ApoTag Plus Peroxidase in situ apoptosis kit (Chemicon) according to the manufacturer's instructions.

Intracranial transplantation of GSCs or NSTCs. U937 monocyte Transwell assays. U937 cells (ATCC) were cultured in the suggested medium 24 h before priming. U937 monocytes were primed with 5 nM PMA (Sigma) for 4 h to become monocyte-derived macrophages as described previously.26. Transwell assays assessing cell migration or invasion potential were performed on 24-well plates with inserts (BD Biosciences) according to the manufacturer's instructions. Briefly, 5 × 10^4 primed U937 cells were cultured in the upper chamber and allowed to migrate or invade for 24–48 h before fixation.

Isolation of human monocytes from blood. Human monocytes were isolated by Ficoll-Paque PLUS (GE Healthcare) centrifugation of human blood following the collection of monocytes attached to the plastic surface after 4 h culture. Briefly, blood from donors was diluted in Hank's buffer to a 1:1 solution. The blood solution was placed over a half-volume of Ficoll Paque PLUS (GE Healthcare) and centrifuged at 1,600 rpm (515g) for 30 min. The middle layer of PBMCs was collected and cultured in RPMI/1640 medium supplemented with 10% FBS. Four hours after culture, the floating cells were washed away with PBS. The adherent monocytes were trypsinized and subjected to migration or invasion assays. All studies related to human subjects were approved by the Institutional Review Board of the Cleveland Clinic. Informed consent was obtained from all subjects. For Transwell assays, 5 × 10^4 human monocytes/macrophages were cultured in the upper chamber and reconstituted POSTN protein (0.5 μg ml^-1) in RPMI/1640 media with 0.1% BSA for 24 h and then used for the cell migration assay.

Isolation of CD11b^+ cells from GBM xenografts. Mouse brain tissues or GBM xenografts were digested with the Neural Tissue Dissociation Kit (Milteny Biotech) followed by magnetic-assisted cell sorting with PE-conjugated anti-CD11b antibody (Milteny Biotech, 130-091-240, 1:11) and anti-PE-conjugated microbeads (Milteny Biotech). Briefly, mouse brains or tumours were washed with PBS, minced and incubated with enzyme mixes. The dissociated brain tissue was passed through a 40 μm cell strainer. Tissue was then re-suspended in a 30% Percoll (Santa Cruz) PBS solution and centrifuged at 700 g for 10 min to remove myelin. Cells were re-suspended in 90 μl IMAG buffer (PBS without Ca^2+ and Mg^2+), supplemented with 0.3% BSA and 2 mM EDTA, pH 7.2. The IHC staining of POSTN was evaluated by two independent pathologists. Samples with a score of 0–1 were regarded as low expression (POSTN^-) and those with a score of 2–3 were regarded as high expression (POSTN^+). For Iba1 evaluation, 3 random areas from a single section were checked for Iba1^+ cells. If the average number of Iba1^+ cells was less than 100 Iba1^+ cells in 4.2 mm^2 (2.36 ± 1.78 mm^2), the sample was ascribed to Iba1 low (Iba1^b). Otherwise the sample was classified as Iba1 high (Iba1^h).

TUNEL assays. TUNEL assays detecting apoptosis in tumour sections were performed with an ApopTag Plus Peroxidase in situ apoptosis kit (Chemicon) according to the manufacturer's instructions.

U937 monocyte Transwell assays. U937 cells (ATCC) were cultured in the suggested medium 24 h before priming. U937 monocytes were primed with 5 nM PMA (Sigma) for 4 h to become monocyte-derived macrophages as described previously.26. Transwell assays assessing cell migration or invasion potential were performed on 24-well plates with inserts (BD Biosciences) according to the manufacturer's instructions. Briefly, 5 × 10^4 primed U937 cells were cultured in the upper chamber and allowed to migrate or invade for 24–48 h before fixation.
**METHODS**

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**shRNAs for knockdown.** Lentiviral-mediated shRNAs targeting POSTN (TRCN0000123055 for O55; TRCN0000123056 for O56, and TRCN0000123057 for O57) or control shRNA (SHC002) were purchased from Sigma-Aldrich.

**RT-qPCR of secreted proteins.** To identify potential chemoattractants differentially expressed by GSCs, we used RT-qPCR analysis to determine the expression of major secreted proteins, including most cytokines along with some secreted proteins relevant to tumorigenesis, in paired GSCs and NSTCs. qPCR primers were designed to span an intron of each target gene (Supplementary Table 1). Total mRNA was purified from paired GSCs and NSTCs with the RNeasy Mini Kit (74104, Qiagen). mRNA (500 ng) was reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (18080-044, Invitrogen). RT-qPCR assays were performed with SYBR Green PCR Master Mix (4310251, Applied Biosystems) on a 7900HT thermal cycler (Applied Biosystems). Three-step amplification was performed (95 °C 15 s, 60 °C 15 s, and 72 °C 30 s) for 45 cycles. For data analysis, GAPDH was used as the internal standard and fold changes of gene expression levels in GSCs relative to NSTCs were calculated. The target genes with a > 2-fold upregulation in GSCs were regarded as differentially expressed. RT-qPCR was performed with 3 pairs of matched GSCs and NSTCs and repeated three times. Average fold changes of target genes presented were from 3 representative experiments.

**Primers for other qPCR analyses.** mSTAB1-qF: 5'-ACGGGAACGTGCTTGAGTC-3'; mSTAB1-qR: 5'-ACTCAGCGTCATGTTGTCCA-3'; mLyve1-qF: 5'-CTGGCTTGTGCTACGGAATGGAAT-3'; mLyve1-qR: 5'-CATGAAAACCTGCTGTTG-3'; mCd17-qF: 5'-AGTGCAGCAGGTGATTCTCAAG-3'; mCd17-qR: 5'-CTGGACAGTCAGAAACACGG-3'; mIL12a-qF: 5'-GGAAGAACGCGAGGAATGGA-3'; mIL12a-qR: 5'-AACTTGAGGAGAAATGGTGAATGGA-3'; mIL1b-qF: 5'-GTGTGGATCCAAAGCAATAC-3'; mIL1b-qR: 5'-GTCTGCTCATCTGACAAG-3'; mGAPDH-qF: 5'-TGATGACATCAAGAAGGTGGTGAAG-3'; mGAPDH-qR: 5'-TCCTGGAGGCCATGTAGGCCAT-3'.

**Statistical analysis.** The level of significance was determined by a two-tailed unpaired Student’s t-test (bar graphs) or analysis of variance with $p = 0.05$ (survival curves) and analysed with GraphPad Prism 5 software. All quantitative data presented are the mean ± s.e.m. from at least three samples or experiments per data point.

**Repeatability of experiments.** Representative Transwell images and the corresponding statistical analyses (Figs 6b–d,f,i,m,o and 7f and Supplementary Fig. 1a,b) are shown from three experiments. Immunofluorescent analyses (Figs 2e,f, 4b,c,e,f,h,i,k, 5b,c,e,f,h,i and 7b and Supplementary Figs 2e, 3b,c, 7b,c,e and 8b,d,f) are shown from three independent experiment using cryosections from 5 different xenografts. qPCR analyses (Fig. 5) and Supplementary Figs 5d,e and 8g) are shown from three independent experiments. Immunofluorescent analyses of human primary GBMs (Fig. 1b and Supplementary Fig. 4eg) are shown from 5 patients. Immunofluorescent analyses of human primary GBMs (Supplementary Fig. 6e,g,i) are shown from 3 patients.

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**Supplementary Figure 1** Identification of POSTN as a dominant chemoattractant to macrophages, and the correlation between POSTN expression and GBM prognosis, GSC population, or TAM density. 

(a) and (b), Graphical analyses of migration (a) and invasion (b) assays showing that POSTN is a dominant chemoattractant in recruiting PMA-primed U937 macrophage-like cells. Migration was allowed for 24 hours and invasion was allowed for 36 hours. ***, p<0.001; *, p<0.05; ns, p>0.05. (n=5 fields; mean ± s.e.m.; two-tailed unpaired t-test; experiments were repeated 3 times).

(c) and (d), Bioinformatic analyses of the relationship between POSTN expression and overall survival (c) or progression-free survival (d) of GBM patients in TCGA dataset (The Cancer Genome Atlas) from Oncomine (www.oncomine.com). The threshold for high/low POSTN expression is determined by the average of POSTN expression levels in 525 GBM patients. The Kaplan-Meier survival curves demonstrate a significant inverse correlation between POSTN levels and overall survival (c) (n=525 patients; POSTN low, n=115 patients; POSTN high, n=400 patients; two-tailed log-rank test) or progression-free survival (d) (n=337 patients; POSTN low, n=164 patients; POSTN high, n=173 patients; two-tailed log-rank test) of GBM patients. 

(e), Immunofluorescent staining of POSTN (green) and the GSC marker SOX2 (red) in primary GBMs. POSTN was preferentially expressed in GSCs and distributed in areas near GSCs in GBMs. In CW690 GBM with less GSCs, POSTN showed little diffusion staining and strictly co-localized with SOX2+ cells. Scale bar, 40μm.

(f), Immunofluorescent analysis of POSTN (red) and the TAM marker Iba1 (green). Three sets of representative staining from 3 tumors (B7, C4 and D8) in a tissue microarray slide (GL803a, US Biomax) were presented to show enrichment of TAMs in POSTN abundant regions in GBMs. Areas indicated with squares were enlarged and shown on right side of each picture. Scale bar, 80μm.

(g), Graphical analysis of (f) showing the enrichment of TAMs in POSTN-high areas. Average levels of POSTN and Iba1 signals were determined by ImageJ. Iba1+ TAM densities from five random POSTN-high and POSTN-low areas in each GBM were normalized to total TAMs. **, p<0.01; *, p<0.05. (n=5 fields; mean ± s.e.m.; two tailed unpaired t-test).
Supplementary Figure 2 Silencing POSTN by shRNA reduced TAM density and extended survival of mice bearing the GSC-derived xenografts. 

a, Immunoblot analysis of POSTN in GSCs expressing non-targeting shRNA (shNT) or POSTN shRNA (shPOSTN, O55). Disrupting POSTN by the shRNA clone O55 through lentiviral infection reduced POSTN expression by >90% in GSCs. 

b and c, Kaplan-Meier survival curves of mice implanted with GSCs expressing shPOSTN or shNT (control). GSCs (T3691 or T4121) were transduced with shPOSTN (O55, O56, or O57) or shNT through lentiviral infection and then transplanted intracranially into athymic/nude mice (20,000 cells per mouse). Mice bearing GSC-derived tumors expressing shPOSTN showed a significant survival extension relative to the control group. (n=5 mice for each group; p=0.0477 for T3691-O55 vs. shNT; p=0.0026 for T4121-O56 vs. shNT; p=0.0027 for T4121-O57 vs. shNT; two-tailed log-rank test). 

d, Immunofluorescent analysis of the TAM marker Iba1 (green) in GBM tumors derived from GSCs expressing shPOSTN (O55) or shNT. Frozen sections of GBM tumors derived from GSCs (T3691) expressing shPOSTN (O55) or shNT (NT) were immunostained with antibodies against Iba1 and counterstained with DAPI (blue). A dramatic reduction of TAMs was detected in the GSC-derived tumors expressing shPOSTN. Scale bar, 40μm. 

e, Graphical analysis of (d) showing a significant reduction of TAM density by ~70% in the GSC-derived tumors expressing shPOSTN. POSTN intensity and TAM density were analyzed with ImageJ. **, p<0.01 (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test).
Supplementary Figure 3  TAMs in GSC-derived tumors were CD11b+ and Iba1+ macrophages that were reduced after POSTN disruption. a, Immunofluorescent analysis of POSTN (red) and the TAM marker CD11b (green) in GBM tumors derived from GSCs expressing POSTN shRNA (shPOSTN) or NT shRNA (shNT). Frozen sections of GBM tumors derived from GSCs with shPOSTN (O56 and O57) or shNT were immunostained with antibodies against POSTN and Iba1 and counterstained with DAPI (blue). Reductions of both POSTN and TAMs were detected in GSC-derived tumors expressing shPOSTN. Scale bar, 80μm. b, Graphical analysis of (a) showing a decrease of POSTN signal intensity by ~80% in the GSC-derived tumors expressing POSTN shRNA. **, p<0.01 (n=5 tumors; mean ± s.e.m; two tailed unpaired t-test). c, Graphical analysis of (a) showing a significant reduction of TAM density by ~60% in the GSC-derived tumors expressing shPOSTN (O56 or O57). POSTN intensity and TAM density were analyzed with ImageJ. ***, p<0.001 (n=5 tumors; mean ± s.e.m; two tailed unpaired t-test). d, Co-immunofluorescent staining of two TAM markers Iba1 (red) and CD11b (green) in sections of GSC-derived orthotopic xenografts. Frozen sections of GBM xenografts derived from GSCs (T387) expressing shNT or shPOSTN (O57) were immunostained with antibodies against Iba1 and CD11b and counterstained with DAPI (blue). Overlapping staining of Iba1 and CD11b was detected in the majority of TAMs in GSC-derived tumors. Scale bar, 80μm. e, Co-immunofluorescent staining of two TAM markers Iba1 (red) and CD11b (green) in TAMs isolated from subcutaneous xenografts. Subcutaneous tumor derived from GSCs (CW0322) were dissociated and cultured in complete Neurobasal media for 24 hours, and the attached, macrophage-like cells were trypsinized and transferred to RPMI/1640 media supplemented with 10% FBS. The attached cells were immunostained with the pan macrophage marker CD11b (green) along with Iba1 (red). All CD11b+ TAMs are also Iba1+, indicating that Iba1 is a universal TAM marker. Scale bar, 80μm.
**Supplementary Figure 4** TAMs in human GBMs are mainly monocyte-derived M2 macrophages that correlate with the tumor prognosis. 

**a,** Immunohistochemical (IHC) staining of monocyte-derived TAM marker (CCR2) or resident microglia marker (CX3CR1) in human primary GBM tumors. Two consecutive human GBM tissue microarray slides (GL806b, US Biomax) were immunostained with antibodies against CCR2 or CX3CR1, respectively. CCR2 or CX3CR1 staining in representative tumor cases (A4, B4, and B7) were presented. The majority of TAMs in GBMs were CCR2+/CX3CR1- (monocyte-derived macrophages). Representative image of the IHC staining in normal brain (H2) were presented to show CCR2-/CX3CR1+ microglia (indicated by arrays) in normal brain tissue. Scale bar, 40μm.

**b-d,** Immunofluorescent staining of the pan macrophage marker Iba1 (red) and M2 TAM markers CD163 (b) and Fizz1 (c), or M1 TAM marker HLA-DR (d) and in primary GBM tumors. Frozen sections of GBM tumors were immunostained with the indicated antibodies and counterstained with DAPI (blue). Scale bar, 40μm. 

**e-g,** Graphical analyses of (b), (c), (d) showing fractions of CD163+/Fizz1+ TAMs (M2 subtype) and HLA-DR+ TAMs (M1 subtype) in primary GBM tumors (n=5 human GBMs; mean ± s.e.m.). 

**h,** Bioinformatic analysis of the relationship between the M2 TAM marker CD163 expression and GBM patient survival in TCGA dataset (The Cancer Genome Atlas) from Oncomine (www.oncomine.com). The threshold for high/low CD163 expression is determined by the average of CD163 expression levels in 525 GBM patients. The Kaplan-Meier survival curves demonstrate a significant inverse correlation between CD163 levels and overall survival (n=525 patients; CD163 low, n = 244 patients; CD163 high, n=271 patients; two-tailed log-rank test; p=0.0013) of GBM patients.

**i,** Bioinformatic analysis of the relationship between the M1 TAM marker MHCII expression and GBM patient survival in TCGA dataset (The Cancer Genome Atlas) from Oncomine (www.oncomine.com). The threshold for high/low MHCII expression is determined by the average of MHCII expression levels in 525 GBM patients. The Kaplan-Meier survival curves demonstrate a significant positive correlation between MHCII levels and overall survival of GBM patients. (n=525 patients; MHCII low, n=280 patients; MHCII high, n=235 patients; two-tailed log-rank test; p=0.0007).
Supplementary Figure 5  The M2 subtype TAMs are enriched in POSTN abundant areas in GBMs and POSTN promotes M2 TAM marker expression.  

a, Immunofluorescent analysis of POSTN (red) and the M2 subtype TAM marker CD206 (green) in a GBM tissue microarray (GL806c, US Biomax). Two sets of representative staining were presented to show the enrichment of TAMs in POSTN abundant regions in GBMs. Scale bar, 80μm.

b, Immunohistochemical staining of POSTN and the M2 subtype TAM marker CD163 in two consecutive tissue microarray slides, respectively (GL806c, US Biomax). Representative staining images were presented to show that the GBM (GL806c-F1) with higher POSTN levels contains more CD163+ cells (M2 TAMs) and the GBM (GL806c-E3) with lower POSTN levels has less CD163+ (M2 TAMs). Scale bar, 40μm.

c, Graphical analysis of POSTN and CD163 staining in the tissue microarray slides. 63.2% of GBM cases showed POSTNHigh/CD163High staining, and 22.1% of GBM cases showed POSTNLow/CD163Low staining. Only 11.8% of GBM cases showed POSTNHigh/CD163Low staining, and 2.94% of GBM cases showed POSTNLow/CD163High staining. The majority (85.3%) of GBM cases showed that M2 TAM density correlates with POSTN protein levels (data from 68 tumors).

d, RT-qPCR analyses showing up-regulation of the M2 TAM markers induced by POSTN in the isolated mouse peritoneal macrophages. The peritoneal macrophages were stimulated with rPOSTN (0.5 µg/mL) in the RPMI media supplemented with 0.1% BSA for 24 hours. RT-qPCR analysis indicates upregulation of the M2 markers STAB1 and Lyve1, along with the downregulation of the M1 markers CCL17 and IL1b. **, p<0.01; ***, p<0.001. (n=3 biologically independent samples per group; mean ± s.e.m; two tailed unpaired t-test; experiment was repeated three times).

e, RT-qPCR analyses showing downregulation of M1 TAM markers induced by POSTN in PMA-primed macrophage-like U937 cells. PMA-primed U937 cells were treated with recombinant POSTN (0.5 µg/mL) in RPMI media supplemented with 0.1% BSA for 24 hours. RT-qPCR analysis indicated downregulation of the M1 TAM markers including CCL17, IL-12a, and iNOS. **, p<0.01; ***, p<0.001; ns, p>0.05. (n=3 biologically independent samples per group; mean ± s.e.m; two tailed unpaired t-test; experiment was repeated three times).
Supplementary Figure 6 POSTN disruption shows negligible impact on GSC growth in vitro and differentiated glioma cells rarely express POSTN in GBMs.  

**a**, Cell titer assay showing proliferation of GSCs expressing shPOSTN or shNT. GSCs were attached to human embryonic stem cell matrix and then transduced with shNT (NT) or shPOSTN (O56 or O57) through lentiviral infection. Cell proliferation was monitored with the luminescent cell viability assay kit at indicated time points. No significant difference was detected in cell proliferation between GSC populations expressing shPOSTN and shNT. (n=3 biologically independent samples per group; mean ± s.e.m.; one representative experiment shown, and the experiment was repeated 3 times).  

**b**, Immunoblot analysis showing knock-down efficiency of shPOSTN in GSCs through lentiviral infection on Day 2. An 85–90% decrease of endogenous POSTN was detected in GSCs transduced with shPOSTN.  

**c**, Representative images of attached GSCs expressing shNT or shPOSTN. Scale bar, 40μm. Only mild or negligible effect of POSTN disruption on GSC morphology was detected.  

**d** and **e**, Immunofluorescent staining of POSTN (green) and the neuron marker TUJ1 (red) in human GBM samples. Scale bar, 40μm. Statistical analysis (e) showing that less than 5% of TUJ1+ cells overlapped with POSTN staining (n=3 human GBMs; mean±s.e.m.).  

**f** and **g**, Immunofluorescent staining of POSTN (green) and the astrocyte marker GFAP (red) in human GBM samples. Scale bar, 40μm. Statistical analysis (g) showing that approximately 5% of GFAP+ cells are positive POSTN staining. (n=3 human GBMs; mean±s.e.m.).  

**h** and **i**, Immunofluorescent staining of POSTN (green) and the oligodendrocyte marker Galc (red) in human GBM samples. Scale bar, 40μm. Statistical analysis (i) showing that less than 6% of Galc+ cells overlapped with POSTN staining (n=3 human GBMs; mean±s.e.m.).
Disrupting POSTN or its signaling showed very mild effect on tumor vascularization. 
a, Immunofluorescent analysis of blood vessels marked by Glut1 (red) and TAMs labeled by Iba1 (green) in GBM xenografts derived from GSCs expressing shPOSTN or shNT. Frozen sections of GBM tumors derived from GSCs (T387) expressing shPOSTN (O56 and O57) or shNT were immunostained with antibodies against Glut1 and Iba1 and counterstained with DAPI (blue). A dramatic reduction of TAMs in the GSC-derived tumors expressing shPOSTN was confirmed, while the blood vessels were not affected by POSTN disruption. Scale bar, 100μm. 
b, Graphical analysis of (a) showing a significant reduction of TAM density by ~70% in the GSC-derived tumors expressing shPOSTN. ***，p<0.001 (n=5 tumors; mean ± s.e.m; two tailed unpaired t-test). 

c, Graphical analysis of (a) showing no significant difference in vessel intensity between control tumors (shNT) and the tumors expressing shPOSTN. ns, p>0.05. Vessel density and TAM density were analyzed with ImageJ. (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test). 
d, Immunofluorescent staining of the vessel marker Glut1 (red) in GBM xenografts treated with RGD inhibitory or control peptide. Mice bearing GBM xenografts were treated with the RGD inhibitory or control peptide at 25 mg/kg for 5 days through intraperitoneal delivery. Frozen tumor sections were immunostained with antibodies against Glut1 (red) and counterstained with DAPI (blue). Scale bar, 100μm. 
e, Graphical analysis of (d) showing no significant difference of vessel density between the GBM xenografts treated with the RGD inhibitory peptide and control peptide. ns, p>0.05 (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test).
Supplementary Figure 8 POSTN disruption did not affect GSC population and cell proliferation but induced apoptosis in GSC-derived xenografts. 

a, Immunofluorescent staining of SOX2 in GBM xenografts derived from GSCs expressing shPOSTN or shNT to determine the effect of POSTN knockdown on GSC populations. Frozen sections of GBM xenografts derived from GSCs (T387) expressing shNT or shPOSTN were immunostained with the antibody against SOX2 (red) and counterstained with DAPI (blue). Scale bar, 40μm.

b, Graphical analysis of (a) showing no significant difference in SOX2+ populations between tumors expressing shPOSTN and shNT (control). Scale bar, 40μm. p>0.05 (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test).

c, Immunohistochemical (IHC) staining of Ki67 (brown) in GBM xenografts derived from GSCs expressing shPOSTN (O56 or O57) or shNT to determine the effect of POSTN down-regulation on cell proliferation. Scale bar, 40μm.

d, Graphical analysis of (c) showing no significant difference in Ki67 staining between the tumors expressing shPOSTN and shNT. Scale bar, 40μm. p>0.05 (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test).

e, TUNEL assay detecting cell apoptosis in GSC-derived tumors expressing shPOSTN or shNT. More apoptotic cell deaths were detected in the tumors with POSTN down-regulation (shPOSTN, O56 or O57) than the control tumors (shNT). Scale bar, 20μm.

f, Graphical analysis of (e) showing a significant increase (250-300%) in TUNEL positive cells in tumors derived from GSCs expressing shPOSTN relative to the control tumors (shNT). ***, p<0.001 (n=5 tumors; mean ± s.e.m.; two tailed unpaired t-test).

g, qPCR analysis of multiple M1 and M2 macrophage markers in CD11b+ populations from normal mouse brains or GBM xenografts. M2 macrophage markers STAB1 and Lyve1 were markedly up-regulated in CD11b+ population isolated from GBM xenografts, whereas M1 marker IL1b was significantly down-regulated. ***, p<0.001; ns, p>0.05. (mean ± s.e.m.; n=3 biologically independent samples per group, one representative experiment shown, and the experiment was repeated 3 times).
Supplementary Figure 9: Scans of uncropped blots
| Fold Change | Genbank Accession | Description | Forward Primers | Reverse Primers |
|-------------|-------------------|-------------|-----------------|-----------------|
| 12.176      | NM_000475         | Periodin, stabilized specific factor | ACTCTAGATATGTTCCCTTT | GCTGGTAAAGTGAAGAA |
| 1.059       | NM_033736         | Vascular endothelial growth factor | AGCCGAGAAGCAGCAAGAT | GCGGCTGGATATGGAGCA |
| 4.694       | NM_000405         | Chemokine (C-C motif) ligand 3 | GCGGCAAGAAAGAAGAGC | CAGGACCTTTGCTGTTG |
| 4.112       | NM_000690         | Interleukin 6 (interleukin 2c) | ATTTCCTTTGATTTTTGTTT | GCTTGGTGAAGTGAAGAA |
| 2.9         | NM_004414         | Interleukin-1 inducing factor 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 5.145       | NM_017290         | Chemokine (C-C motif) ligand 2 | CTGCCCTTTGATTTTTGTTT | GCTTGGTGAAGTGAAGAA |
| 1.042       | NM_008100         | Interleukin-1 inducing factor 1 | GCTGCTGGATATGGAGCA | CAGGACCTTTGCTGTTG |
| 1.471       | NM_010217         | Interleukin 3 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.718       | NM_010468         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.712       | NM_010468         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.658       | NM_002399         | Lactate dehydrogenase, lactate 1 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.573       | NM_017171         | TGF beta | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.562       | NM_002969         | Chemokine (C-C motif) ligand 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.461       | NM_002969         | Chemokine (C-C motif) ligand 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.356       | NM_002969         | Chemokine (C-C motif) ligand 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.278       | NM_002969         | Chemokine (C-C motif) ligand 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.261       | NM_002969         | Interleukin 3 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.251       | NM_002969         | Interleukin 3 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.134       | NM_002969         | Interleukin 3 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 1.106       | NM_002969         | Interleukin-1 inducing factor 1 | GCTGCTGGATATGGAGCA | CAGGACCTTTGCTGTTG |
| 1.094       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.918       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.892       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.880       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.873       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |
| 0.857       | NM_002969         | Interleukin 2 | GTGAAGCTTGGTATGGTCTT | GCTGCTGGATATGGAGCA |

**Supplementary Table 1** A list of the secreted proteins differentially expressed by GSCs relative to matched non-stem tumor cells (NSTCs) from primary GBMs. The data were obtained through RT-qPCR analysis (qPCR array) of mRNA expression of a series of secreted proteins in matched GSCs and NSTCs (data from 3 biologically independent samples). Periostin (POSTN) was identified as a preferentially and highly expressed cytokine by GSCs in this small-scale expression profiling analysis of secreted proteins.

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