CSF-1R inhibition alters macrophage polarization and blocks glioma progression

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Glioblastoma multiforme (GBM) comprises several molecular subtypes, including proneural GBM. Most therapeutic approaches targeting glioma cells have failed. An alternative strategy is to target cells in the glioma microenvironment, such as tumor-associated macrophages and microglia (TAMs). Macrophages depend on colony stimulating factor-1 (CSF-1) for differentiation and survival. We used an inhibitor of the CSF-1 receptor (CSF-1R) to target TAMs in a mouse proneural GBM model, which significantly increased survival and regressed established tumors. CSF-1R blockade additionally slowed intracranial growth of patient-derived glioma xenografts. Surprisingly, TAMs were not depleted in treated mice. Instead, glioma-secreted factors, including granulocyte-macrophage CSF (GM-CSF) and interferon-γ (IFN-γ), facilitated TAM survival in the context of CSF-1R inhibition. Expression of alternatively activated M2 markers decreased in surviving TAMs, which is consistent with impaired tumor-promoting functions. These gene signatures were associated with enhanced survival in patients with proneural GBM. Our results identify TAMs as a promising therapeutic target for proneural gliomas and establish the translational potential of CSF-1R inhibition for GBM.

GBM, the most aggressive form of glioma, has an invariably terminal prognosis, as patients respond minimally to currently used therapies, including surgery, radiation and chemotherapy. One challenge in treating GBM is substantial tumor-cell and genetic heterogeneity, leading to aberrant activation of multiple signaling pathways. Underscoring this heterogeneity was the identification of several GBM molecular subtypes: proneural, neural, mesenchymal and classical. In contrast, noncancerous stromal cells in the tumor microenvironment are genetically stable therapeutic targets. TAMs in particular are associated with high tumor grade and poor prognosis in many cancers, including gliomas. A paracrine CSF-1–epidermal growth factor (EGF) signaling loop has also been implicated in breast cancer and glioma invasion.

Several approaches have been used to ablate TAMs or inhibit their tumor-promoting functions in mouse models of cancer. One strategy is CSF-1R inhibition, which depletes macrophages and reduces tumor volume in several xenograft models. Here we used a potent, selective CSF-1R inhibitor in multiple preclinical GBM models. These included RCAS-hPDGFB (the human platelet-derived growth factor B gene)/nestin–Tv-a; Cdkn2a−/− transgenic mice, referred to here as PDGF-B–driven glioma (PDG) mice, and intracranial xenografts of either proneural GBM cell lines or patient-derived tumor spheres. We found that CSF-1R inhibition blocks glioma growth and progression through a mechanism in which TAMs are not depleted but are instead ‘re-educated’ within the glioma microenvironment.

RESULTS

Macrophages are targets of CSF-1R inhibition in PDG GBMs

In PDG mice, nestin+ glial progenitors express the RCAS virus receptor, Tv-a, which enables specific delivery of the initiating oncogene, PDGFB. PDG tumorigenesis recapitulates pathological and molecular features of human proneural GBM. We first investigated whether macrophages accumulate in PDG GBMs as in human gliomas. Indeed, the numbers of CD11b+ myeloid cells and macrophages were markedly increased in PDG GBMs compared to normal brain, and the mRNA expression of Csf1, Csf1r and Csf2 was elevated in the PDGs (Supplementary Fig. 1a,b). Glioma cells and TAMs express Csf1, whereas Csf1r is only expressed in macrophages (Fig. 1a,b and Supplementary Fig. 1c–e).

BLZ945 is a brain-penetrant inhibitor of CSF-1R (Supplementary Fig. 2a and Supplementary Methods). The biochemical half-maximum inhibitory concentration (IC50) for CSF-1R is 1 nM, which is >3,200-fold higher than its affinity for other kinases (Supplementary Table 1). Treatment of wild-type (WT) bone marrow–derived macrophages (BMDMs) with BLZ945 inhibited CSF-1–dependent proliferation (half-maximum effective concentration (EC50) = 67 nM) and...
CSF-1R inhibition specifically targets macrophages, improves survival and decreases glioma malignancy in the transgenic PDG model. (a) Expression of Csf1 and Csf1r in different cell populations from GFP-expressing PDGs; a mixed population of live cells (DAPI-), purified glioma cells (GFP+) and macrophages (CD11b+Gr-1-). Csf1rb and Ty-a were used as cell type-specific control genes for macrophages and glioma cells, respectively. Expression is shown relative to that in the live cell fraction and was normalized to Ubc for each sample (n = 3 mice per group). (b) Representative immunofluorescence images of normal brain or GBM PDGs co-stained with CSF-1R and DAPI. Scale bars, 50 µm. (c) Graph showing that the CSF-1R inhibitor BLZ945 blocked BMDM survival, with an effect comparable to that of CSF-1 deprivation, assessed by MTT assays. n = 13 independent replicates. (d) Graph showing MTT assays for BLZ945 treatment of independent PDGF-driven glioma cell (PDGC) primary lines derived from PDG mice (Supplementary Figs. 3d and 7c). Concentrations up to 6,700 nM BLZ945 (100 times the dose required to effectively kill PDGF-driven glioma cell (PDGC) primary lines) had no effect on glioma cell survival or proliferation. n = 3 independent replicates. (e) Experimental design for the long-term survival trial: PDG mice were injected with RCAS-hPGDFB-hemagglutinin (HA) (5–6 weeks of age, n = 22) or BLZ945 (200 mg/kg body weight (mg kg-1), n = 14) treatment groups at 2.5 weeks after injection. Mice were dosed once daily until they developed symptoms or reached the trial endpoint. (f) Symptom-free survival curves for the mice described in e. (g) Histological grading of the vehicle (n = 14) and BLZ945 (n = 13) treatment groups. The data in a, c, and d are shown as the mean ± s.e.m. Statistical significance was calculated by unpaired two-tailed Student’s t-test (c,d), log-rank (Mantel-Cox) test (f) or Fisher’s exact test (g). *P < 0.05, ***P < 0.001.

CSF-1R inhibition blocks glioma progression and improves survival

We next assessed BLZ945 in preclinical trials using PDG mice. At 2.5 weeks after glioma initiation, 30% of the animals had small tumors (grade II or III) and 70% had tumors by histology or magnetic resonance imaging (MRI) (Supplementary Fig. 4a,b). We treated mice with BLZ945 or vehicle and evaluated them for symptom-free survival (Fig. 1e). The median survival time in the vehicle-treated cohort was 5.7 weeks. In contrast, BLZ945 treatment significantly improved long-term survival, with 64.3% of the treated mice surviving to the trial endpoint at 26 weeks (Fig. 1f). We chose this endpoint because Cdkn2a-/- mice develop spontaneous tumors, including lymphomas and sarcomas, beginning at ~30 weeks. BLZ945 was well tolerated over the long-term treatment with no visible side effects (Supplementary Fig. 4e), which is consistent with results from previous histopathological studies. Histological grading revealed high-grade, invasive gliomas in all vehicle-treated mice. In contrast, BLZ945-treated animals had tumors that were significantly less malignant, and there were no detectable lesions in 55.6% of the mice that were asymptomatic at the trial endpoint (Fig. 1g). We then monitored the effects of BLZ945 on established high-grade PDGs, which are histologically similar to human GBMs at time of diagnosis. We performed a 7-d trial incorporating MRI to assess initial tumor volume and subsequent growth (Fig. 2a). We randomized PDG mice with detectable tumors (4.5–40 mm3) into BLZ945 or vehicle treatment cohorts. In the vehicle treatment group, tumor growth increased substantially. In contrast, BLZ945 halted glioma growth, and the majority of tumors decreased in size in the mice treated with
BLZ945 (Fig. 2b,d,e and Supplementary Fig. 5a,b). We then studied a third cohort of mice with large gliomas (>40 mm³) that we treated with BLZ945, and the vast majority of these mice showed regression (Fig. 2c–e and Supplementary Fig. 5c). Comparison with a size-matched vehicle-treated cohort was not possible, as most of those mice would not have survived to the trial endpoint. We confirmed inhibition of CSF-1R phosphorylation in all BLZ945-treated tumors, and hPDGF-B production by glioma cells was unaffected by treatment with BLZ945 (Supplementary Fig. 6).

Because we used a PDGF-driven model for these initial trials, we wanted to exclude possible off-target effects of BLZ945 on glioma PDGF receptor (PDGFR) signaling. Glioma cells express PDGFR-α, and pericytes express PDGFR-β (Supplementary Fig. 7a,b). PDGFR inhibition reduces U-87 MG cell viability in a dose-dependent manner (Supplementary Fig. 7c). The affinity of BLZ945 for PDGFR-α is approximately 10,000-fold lower than for CSF-1R (Supplementary Table 1). Unlike PDGFR inhibitors, treatment with BLZ945 or an antibody to CSF-1R did not affect the viability of the U-87 MG or PDG cell lines (Fig. 1d and Supplementary Figs. 3d–e and 7c). Collectively these data establish that the therapeutic effects of BLZ945 in vivo are through CSF-1R inhibition and not off-target inhibition of PDGFR.

Multiple hallmarks of cancer are altered by CSF-1R blockade

To determine the mechanisms underlying the marked response to CSF-1R inhibition in established gliomas, we investigated multiple tumorigenic processes, including tumor grade, proliferation, angiogenesis and resistance to apoptosis. (Supplementary Table 2). We performed analyses on tissues from the 7-d BLZ945 trial, including tissues taken at the midpoint (3 d) and endpoint (7 d) to capture the dynamic effects of CSF-1R inhibition. Whereas all vehicle-treated mice had high-grade gliomas, BLZ945-treated animals exhibited a pronounced histological response that was characterized by tumor-cell depopulation (Fig. 3a and Supplementary Fig. 8a). Correspondingly, glioma cell numbers within the regions of the original tumors decreased markedly (Fig. 3b–d).
Proliferation analyses revealed a 67–98% reduction in glioma-cell proliferation after BLZ945 treatment (Fig. 3b,c). In addition, apoptosis increased by 9- to 17-fold after 3 d (Fig. 3b,f), and tumor vascularity decreased. This reduction was probably not due to direct effects of BLZ945 on endothelial cells, as their viability was unaffected in cultures (Supplementary Fig. 8b–f). Thus, CSF-1R inhibition effectively reduces the growth and malignancy of gliomas through the combined effects of blocking cell proliferation and increasing apoptosis.

**CSF-1R inhibition slows intracranial growth of proneural GBM xenografts**

We next asked whether CSF-1R inhibition is effective in experimental models of human proneural GBM, including primary tumor spheres (TS573 and TS1137; Supplementary Table 3) and established cell lines (U251 and LN229) (Supplementary Methods). The tumor spheres and cell lines were either negative for CSFIR expression or showed negligible CSFIR mRNA levels compared to the human macrophage line THP-1, and none of these tumor spheres or GBM cell lines was sensitive to CSF1R expression or showed negligible CSF1R mRNA levels compared to the human macrophage line THP-1, and none of these tumor spheres or GBM cell lines was sensitive to BLZ945 in monoculture (Fig. 4a,b and Supplementary Fig. 9a,b).

We intracranially injected the glioma tumor sphere or cell lines into NOD-SCID mice and treated established tumors with either vehicle or BLZ945. All four human lines responded to BLZ945 in vivo, showing a clear histological response and significantly reduced tumor growth and invasion (Fig. 4c–f and Supplementary Fig. 9c–e). Collectively these results demonstrate the preclinical efficacy of CSF-1R inhibition across multiple proneural GBM models, including genetically engineered mice and human-derived xenografts.

**Glioma-supplied survival factors protect TAMs from BLZ945-induced depletion**

Macrophage survival depends on CSF-1R signaling25, and we thus expected CSF-1R inhibition to deplete macrophage numbers. Indeed, BLZ945 treatment reduced the number of CD11b<sup>+</sup>Ly6G<sup>-</sup> cells in the blood of WT mice. Microglia numbers in normal brains were substantially decreased by BLZ945 treatment; surprisingly, however, CSF-1R inhibition did not affect TAM numbers in PDGs compared to vehicle treatment (Fig. 5a,b and Supplementary Figs. 10 and 11). Similarly, TAMs were not depleted in human tumor–sphere and cell-line xenografts (Supplementary Fig. 12a,b).

BLZ945-treated TAMs retained CSF-1R expression in vivo, and there were no significant differences in expression compared to vehicle-treated PDGs (Supplementary Fig. 13a). We examined whether the relative proportions of resident microglia and recruited BMDMs were altered in the PDG model. We performed BM transplantation experiments using GFP<sup>+</sup> BM and initiated gliomas after confirming BM reconstitution (Supplementary Fig. 13b,c). The proportion of GFP<sup>+</sup>CD68<sup>+</sup> macrophages in gliomas, determined by immunofluorescence analysis, was unchanged after BLZ945 treatment (Supplementary Fig. 13d), indicating no evident alteration in peripheral recruitment. We evaluated additional strategies to deplete macrophages in other tumor types or the brain and found that although the numbers of macrophages in peripheral tissues or normal brain decreased, there was no effect on TAM numbers in PDGs (Supplementary Fig. 14). We analyzed other immune cell types in gliomas after BLZ945 treatment and found no significant differences in cell numbers (Supplementary Fig. 13e). In addition, given varying reports of CSF-1R expression by neurons26,27, we examined neuron numbers in gliomas and normal brain and found no differences after treatment with BLZ945 (Supplementary Fig. 13f), which is consistent with our detection of CSF-1R in macrophages only (Supplementary Fig. 1c–e).

To explore the mechanisms by which glioma TAMs are specifically protected from BLZ945-induced death, we investigated whether glioma-supplied factors support macrophage survival in the...
Figure 4 BLZ945 inhibits orthotopic tumor growth of patient-derived proneural tumors and cell lines in vivo. (a) CSF1R mRNA expression in human proneural tumor sphere cells (TS573) and in the human proneural cell line U251 compared to the human macrophage line THP-1 (positive control). Expression is normalized to β2M (also called B2M) for each sample. n = 3 independent replicates. (b) MTT assays of BLZ945 treatment tested in TS573 and U251 human glioma cells demonstrating no effect of BLZ945 at concentrations up to 6,700 nM, n = 3 independent replicates. (c,d) Relative tumor growth determined by bioluminescence imaging (BLI) output in NOD-SCID mice injected intracranially with 5 × 10^4 TS573 cells (c) or 2.5 × 10^5 U251 cells (d). Treatment with BLZ945 was initiated when tumors were in a positive growth phase determined by BLI, which corresponded to day 14 for TS573 cells and day 7 for U251 cells. Mice were randomly assigned to vehicle (n = 12 for TS573, n = 16 for U251) or BLZ945 (n = 11 for TS573, n = 17 for U251) treatment groups. Tumor growth was evaluated every 5 d for 15 d, at which point vehicle-treated mice became symptomatic and both cohorts were euthanized for further analyses. (e,f) Representative images of the vehicle-treated and BLZ945-treated TS573 (e) and U251 (f) xenograft tumors described in c and d stained for GFP (tumor cells) and DAPI. Representative H&E images of both treatment groups for the U251 xenografts are shown in the bottom row in f. Scale bars (e,f), 50 μm. The data in a–d are shown as the mean ± s.e.m. Statistical significance was calculated by nonparametric two-tailed Mann-Whitney test. *P < 0.05, **P < 0.01, ***P < 0.001.

presence of CSF-1R inhibition. Indeed, glioma cell–conditioned medium (GCM) from PDGs or human cell lines protected BMDMs from death induced by BLZ945 (Fig. 5c and Supplementary Figs. 12c and 15a,b). This was analogous to the TAM maintenance that we observed for all of our in vivo models (Fig. 5a and Supplementary Figs. 11, 12a and 13d) and also indicated that the survival factors were secreted. We screened GCM from a panel of PDG cell lines and identified multiple protective lines and one nonprotective line (Supplementary Fig. 16a). By comparing the GCM from the protective lines to that from the nonprotective line using antibody arrays, we identified differentially secreted proteins (Supplementary Fig. 16b,c), which we tested in parallel with known macrophage survival factors36. From this screen, we identified three cytokines that promoted the survival, proliferation or both of BMDMs in the presence of BLZ945: GM-CSF (also called CSF-2), IFN-γ and chemokine (C-X-C motif) ligand 10 (CXCL10) (Fig. 5d,e and Supplementary Figs. 17 and 18a–d). Notably, when we added these factors in combination to GCM from the nonprotective line, it conferred protection from BLZ945-induced killing to the BMDMs (Supplementary Fig. 18e) in association with elevated Akt activation in macrophages, similarly to treatment with GCM from protective lines (Fig. 5f,g). Consistent with their protective effect, expression of these cytokines and their receptors was substantially elevated in PDGs compared to normal brain and were also produced by TAMs themselves (Supplementary Fig. 19). These results establish that glioma-supplied factors promote TAM survival in the presence of a CSF-1R inhibitor.

CSF-1R inhibition reduces M2 macrophage polarization in treated gliomas

We next asked how CSF-1R inhibition elicited such a potent antitumor response in vivo despite no evident TAM depletion. We hypothesized that TAMs from CSF-1R inhibitor–treated animals were functionally altered, and we used microarray expression profiling of TAMs isolated from BLZ945- and vehicle-treated glioma to identify 257 differentially expressed genes. 52 genes were significantly upregulated and 205 were downregulated; together this is referred to as the total gene signature (Fig. 6a, Supplementary Fig. 20a,b and Supplementary Table 4). CSF-1R inhibition in BLZ945-treated TAMs was corroborated by downregulation of targets of early growth receptor 2 (Egr2), a transcription factor that is located downstream of CSF-1R29 (Supplementary Fig. 20c). To determine the minimal set of genes that discriminated the treatment groups, we employed a lasso logistic regression model30 (Online Methods). This method identified a five-gene signature for BLZ945 treatment: Adm (adrenomedullin), Arg1 (arginase 1), F13a1 (encoding a clotting factor), Mrc1 (mannose receptor C type 1, also called Cd206), Serpinb2 (encoding a protease inhibitor and also called Pa12) (Fig. 6b); this is referred to as the minimal gene signature. Of these genes, four were downregulated after BLZ945 treatment. Quantitative PCR analyses on whole gliomas and sorted cell populations confirmed these findings (Supplementary Fig. 20d,e). Serpinb2, the only upregulated gene in the minimal signature, correlates positively with increased survival in breast cancer31. Interestingly, each of the downregulated genes has been associated previously with alternatively activated/M2 macrophage polarization32–35 (Supplementary Table 4).
Figure 5 CSF-1R inhibition depletes normal microglia but not TAMs in treated PDGs as a result of the production of glioma-supplied survival factors. (a) Representative images of PDGs (top) and adjacent normal brain from the contralateral hemisphere of tumor-bearing mice (bottom) from the 7 d BLZ945 trial stained for CD68 and DAPI. White arrows indicate CD68+ macrophages in adjacent normal brain from both the vehicle- and BLZ945-treated groups. Scale bars, 50 μm. (b) Quantification of the mean number of CD11b+ macrophages per 63× field of view (FOV) within each mouse tumor of the different treatment groups. (c) Graph showing MTT assays of BMDMs demonstrating that GCM induced BMDM proliferation and protected BMDMs from BLZ945-induced apoptosis. n = 19 independent replicates. For comparison, BMDMs were cultured in nonconditioned medium supplemented with CSF-1. (d,e) Results compiled from MTT assays demonstrating that GM-CSF and IFN-γ individually protected BMDMs against BLZ945-induced death (n = 9 independent replicates; d), whereas CXCL10 promoted proliferation (n = 6 independent replicates; e). These effects were not reproduced by other candidate factors, for example, CXCL1 and vascular endothelial growth factor A (VEGF-A), which are shown here and in Supplementary Figure 17. (f) Western blots showing activation of Akt (Ser473 phosphorylation site) in BMDMs. BMDMs were stimulated with freshly prepared GCM from protective (PDGC-23, PDGC-17 and PDGC-02) or nonprotective (PDGC-55) cell lines with or without BLZ945. In addition, GCM from nonprotective PDGC-55 cells was supplemented with survival factors (GM-CSF, IFN-γ and CXCL10) with or without BLZ945. (g) Quantification of pAkt normalized to total Akt demonstrating significant changes in pAkt levels between BMDMs stimulated with the protective compared to the nonprotective GCM with or without BLZ945, n = 4 independent replicates. RU, relative units. BLZ945 was used at 670 nM in all cell culture assays unless otherwise specified. The circles in b represent individual mice (n = 7 for vehicle, n = 5 for 3 d BLZ945, n = 5 for 7 d BLZ945, n = 5 for 3 d BLZ945 large, n = 5 for 7 d BLZ945 large), and horizontal lines represent the means. The data in c, d, e and g are shown as the mean ± s.e.m. Statistical significance was calculated by unpaired two-tailed Student’s t test. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001. There were no significant differences for any of the comparisons performed in b.

In many tumors, TAMs are M2 polarized, which is associated with protumorigenic functions. Further, macrophages in human gliomas exhibit an M2-like phenotype, which is correlated with higher tumor grade. Given the striking M2 gene enrichment in the minimal signature, we examined the total gene list to determine whether other M2-associated markers were similarly downregulated by BLZ945, which revealed five additional genes (Supplementary Fig. 20f and Supplementary Table 4). Apart from interleukin-1β (IL-1β), classically activated/M1 genes were not correspondingly upregulated (Supplementary Fig. 20g). Interestingly, CSF-1 favors M2 macrophage polarization in culture, which is consistent with our finding that BLZ945 reduces M2 gene expression in vivo.

To explore how glioma cells and CSF-1R inhibition regulate M2 macrophage polarization, we used cell-based assays in which we exposed BMDMs to GCM to model microenvironmental interactions. Expression of all nine differentially expressed M2 genes (Supplementary Fig. 20f) significantly increased, and these increases were reversed by BLZ945 (Fig. 6c). Analysis of a subset of these genes in two microglia cell lines, BV-2 and CRL-2467, showed similar results (Supplementary Fig. 21a,b). We next examined Mrc1 expression in cocultures and freshly isolated mouse glioma microenvironment cultures containing TAMs. We selected Mrc1 because it is a well-established M2 flow cytometry marker, which facilitated the determination of its macrophage-specific expression in mixed cultures. Mrc1 expression also decreased in response to BLZ945 (Fig. 6d and Supplementary Fig. 21c,d), paralleling its downregulation in vivo.

Interestingly, human GCM suppressed macrophage phagocytosis in vitro in conjunction with M2 polarization. To determine whether BLZ945 altered macrophage phagocytosis in vivo, we co-stained tissues for the macrophage cell-surface marker CD11b, the glioma cell marker Olig2 (ref. 42) and cleaved caspase 3 (CC3). Phagocytic capacity (number of CD11b+ macrophages that had engulfed Olig2+CC3+ glioma cells), increased 7.1-fold in the group of PDG mice with large gliomas treated with BLZ945 compared to mice treated with vehicle (Supplementary Fig. 22 and Supplementary Table 2). To control for enhanced glioma cell death, we analyzed phagocytic capacity after irradiation and found that it increased fourfold. Therefore, macrophages in BLZ945-treated gliomas undergo an increase in phagocytic function that is apparently not dependent solely on enhanced tumor apoptosis. Together these data suggest that after CSF-1R inhibition, glioma TAMs lose M2 polarization and show enhanced phagocytosis, providing a molecular corollary for their impaired tumor-promoting functions.
CSF-1R inhibition abrogates macrophage glioma–cell heterotypic signaling

As BLZ945 blocked PDG proliferation in vivo, we examined whether glioma-cell proliferation was modulated by exposure to macrophages in cell culture and whether it was reduced by CSF-1R inhibition. We stimulated WT BMDMs with GCM to mimic their ‘education’ in the glioma microenvironment (referred to hereafter as stimulated BMDMs) and subsequently added them to glioma cells in coculture (Supplementary Fig. 15a). Cell-cycle analysis showed that coculture of stimulated BMDMs with PDG cells markedly increased their proliferation. Notably, this induction was abrogated when we collected the CM from stimulated BMDMs in the presence of BLZ945 (Supplementary Fig. 23c), which implicates this pathway as an important driver of signaling in the GBM microenvironment. We also investigated whether macrophages promoted glioma-cell proliferation in vivo by orthotopically co-injecting BMDMs with PDG cells or injecting PDG cells alone. Notably, co-injection of macrophages with glioma cells enhanced tumor growth by 62% compared to injection of glioma cells alone, and this induction was blocked by treatment with BLZ945 (Supplementary Fig. 23d; \( P < 0.05 \)). Collectively these data demonstrate that macrophages and glioma cells have reciprocal effects on the survival, proliferation and/or polarization of each other to promote tumorigenesis, and this heterotypic signaling can be blocked by CSF-1R inhibition.
Gene signatures of CSF-1R inhibition predict survival advantage in proneural GBM

We next examined whether BLZ945 gene signatures generated from mouse TAMs might have translational value through differential survival associations in individuals with GBM. We used the total gene signature to classify subjects in The Cancer Genome Atlas (TCGA) and a second combined series into ‘BLZ945-like’ and ‘vehicle-like’ classes. Notably, there was a proneural-specific increase in median survival for subjects in the BLZ945-like class that ranged from 7.5 to 31.5 months (Supplementary Fig. 24a,b). We also used the minimal gene signature and observed that this markedly smaller gene set recapitulated the BLZ945-like survival advantage in subjects with proneural GBM (Fig. 6g,h, Supplementary Fig. 24a–d and Supplementary Tables 5 and 6). Notably, this survival increase was not evident in other GBM subtypes and was independent of glioma CpG island methylator phenotype (G-CIMP) status (Supplementary Fig. 24e and Supplementary Table 7).

To determine whether the minimal gene signature predicted survival independently of macrophage number, we used expression of macrophage-specific genes (AIF1, CD68 and ITGAM) as surrogates for macrophage number in a Cox proportional hazards model (Supplementary Tables 8 and 9). None of the macrophage-specific genes correlated with worse prognosis, indicating that the minimal gene signature of BLZ945 treatment is a subtype-specific, prognostic gene signature of BLZ945 treatment is a subtype-specific, prognostic predictor of the survival of patients with GBM that is independent of total macrophage number. This also suggests that the TAM phenotype within a tumor may predict overall survival better than TAM number per se. The subtype specificity for this survival difference is consistent with the minimal and total gene signatures having been generated from the PDG model, which most closely represents proneural GBM.

DISCUSSION

We show here that a new CSF-1R inhibitor blocks malignant progression, represses established gliomas and markedly enhances survival in a transgenic mouse model of proneural GBM. Moreover, multiple proneural GBM human xenografts responded similarly to CSF-1R inhibition, underscoring the therapeutic relevance of these findings. Surprisingly, we found that TAMs in all models were specifically protected from CSF-1R inhibitor–induced death. This contrasted with the observed depletion of microglia in the normal brain and the depletion of macrophages in other tissues, which is consistent with previous reports. These results suggest that glioma-supplied factors facilitate TAM survival in the presence of BLZ945, whereas neighboring microglia outside of the tumor mass are not exposed to these protective signals and are thus depleted. Modeling these microenvironment-mediated effects in culture allowed us to screen and identify GM-CSF and IFN-γ as glioma-supplied factors that facilitate macrophage survival in the context of CSF-1R inhibition. Notably, expression of these factors (or their receptors) was enriched in gliomas compared to normal brain, which is consistent with their prosurvival functions. This led us to ask whether TAM functions were impaired after CSF-1R inhibition, given that their numbers were not altered. Indeed, expression analyses of surviving TAMs revealed a decrease in alternatively activated M2 macrophage markers, which is consistent with blunted tumor-promoting functions. We also modeled this phenomenon in culture and demonstrated that induction of M2 polarization by glioma-secreted factors and its reversal by CSF-1R inhibition are both direct effects on macrophages. Together these data indicate that the presence of survival factors in the glioma microenvironment protects TAMs from CSF-1R inhibitor-mediated killing, enabling their re-education and an associated marked antitumor response.

There are several potential clinical implications of these findings. First, macrophage accumulation correlates with malignancy in human gliomas, which supports therapeutic targeting of TAMs. Second, we found that alteration of TAM tumor-promoting functions can significantly abrogate malignancy and that depletion is not strictly necessary for effective macrophage-targeted therapy. Third, in light of our preclinical data in proneural human xenografts and mouse models and the prognostic advantage associated with BLZ945-like signatures in subjects with proneural GBM, it is possible that proneural GBMs are particularly dependent on the tumor-promoting functions of TAMs. As such, it will be interesting to determine in future studies whether other GBM subtypes respond similarly to CSF-1R inhibition. Fourth, myeloid cells, including macrophages, blunt the chemotherapy response in breast cancer models and promote revascularization after irradiation in GBM xenografts. Thus, it may be instructive to consider CSF-1R inhibitors in combination with glioma cell–directed therapies, opening the possibility for synergistic effects.

In sum, we uncover a new therapeutic strategy for targeting cells in the glioma microenvironment. Rather than depleting stromal cells, as has been the goal with many microenvironment-targeted therapies thus far, re-educating these cells has the potential to not only abolish their protumorigenic functions but also actively enlist them as suppressors of tumorigenesis.

METHODS

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

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AUTHOR CONTRIBUTIONS

S.M.P., L.A., A.J.S., L.S., D.F.Q. and O.C.O. performed and analyzed experiments. R.L.B., M.S. and C.S.L. performed computational analyses. M.L.Q., V.T., Y.O., A.P. and J.Z. provided technical assistance or derived patient tumor sphere lines. J.T.H. and J.A.J. conceived, designed and supervised the study and wrote the manuscript. All authors edited or commented on the manuscript.

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The authors declare no competing financial interests.
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ONLINE METHODS

Mice. All animal studies were approved by the Institutional Animal Care and Use Committee of MSKCC under protocol 04-08-022. The nestin–Tv-a; Cdkn2a( Ink4a/Arf)-/- mouse model11,12 (mixed strain background), transgenic CCR2- diphtheria toxin receptor (DTR)-CFP mouse (C57BL/6)13 (referred to here as CCR2-DTR) and transgenic CD1lb-DTR mice (FVB/N)14–16 have been described previously. WT C57BL/6 and NOD-SCID mice were purchased from Charles River Laboratories, β-actin–GFP (C57BL/6)17 mice2 were from Jackson Laboratories, and athymic nude mice were from the National Cancer Institute Frederick. All lines were bred within the MSKCC animal facility.

Culture of established cell lines. The U-87 MG (HTB-14)18 human glioma and CRL-2467 (ref. 59) mouse microglia cell lines were purchased from ATCC, and human umbilical vein endothelial cells (HUVECs) and human brain microvascular endothelial cells (HBMECs) were from ScienCell. The BV-2 (ref. 60) mouse microglia cell line and the U251 and LN229 human glioma cell lines were kind gifts from E.C.H. All cells were grown at 37 °C with 5% CO2, passaged with trypsin and maintained in DMEM, 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) unless otherwise noted. The CRL-2467 cell line was cultured in DMEM and 10% FBS with 30 ng/ml recombinant mouse CSF-1 (R&D Systems). HUVECs and HBMECs were cultured on gelatin-coated cell culture dishes in endothelial cell medium (ScienCell) and 10% FBS supplemented with endothelial cell growth factors.

Culture and characterization of human primary glioma tumor spheres. Human glioma–derived tumor sphere lines (TS1137 and TS573) were derived from consenting patients under Institutional Review Board (IRB)-approved protocols for the banking of excess tumor tissue during routine surgical resection (MSKCC IRB# 99-125A(2) and 06-107), as previously described61. Tumor spheres were maintained in the human NeuroCult NS-A Proliferation Kit (Stem Cell Technologies) and were dissociated with Accutase cell detachment solution (Millipore). Characterization and molecular subtyping were performed as described in the Supplementary Methods.

Thymidine kinase–GFP-luciferase cell labeling. The indicated cell lines (TS573, TS1137, U251, LN229 and PDGC-23) were labeled with a triple-imaging vector (thymidine kinase (TK)-GFP-luciferase (Luc) (TGL))62 to allow for noninvasive in vivo imaging of tumor growth. A standard protocol for retroviral transduction was used. Briefly, GP2-293T cells were transfected with the TGL construct and pCl-Ampo at a 1:1 ratio using Fugene (Promega) and OptiMEM (Gibco). 12 h later, the medium was replaced with complete antibiotic-free DMEM and was collected for 3 d for the transduction of target cells.

Intracranial injection for tumor initiation in the mouse PDG and human glioma models. Adult mice were fully anesthetized with ketamine and xylazine and the local anesthetic bupivacaine at the surgical site before intracranial injection to the right frontal cortex (approximate coordinates: 1.5 mm lateral, 1 mm caudal from bregma, depth of 2–3 mm) using a fixed stereotactic apparatus (Stoelting) as previously described16–24. Additional details are available in the Supplementary Methods. TGL-labeled human cell lines were resuspended in antibiotic-free serum-free medium, and 5 × 105 (TS1137, TS573 and LN229) or 2.5 × 105 (U251) cells in a volume of 2 μl were injected into 6-week-old female NOD-SCID mice. Athymic nude mice (6-week-old females) were injected with 2 μl of either 5 × 105 TGL-labeled PDGC-23 cells alone or PDGC-23 cells mixed with 30% RFP+ BMDMs (1.66 × 105) in antibiotic-free serum-free medium.

BLZ945 treatment in vitro and in vivo. The CSF-1R inhibitor BLZ945 (supplied by Novartis) was synthesized as described in the Supplementary Methods. For cell culture studies, a 10 mM stock of BLZ945 was formulated in DMSO; DMSO was used as the vehicle control. For administration to mice, BLZ945 was formulated in 20% Captisol at a concentration of 12.5 mg ml-1. For in vivo dosing, mice received 200 mg per kg body weight BLZ945 or vehicle (20% Captisol) by oral gavage once daily. For long-term survival studies in PDG mice, dosing was begun at 17 d after injection of RCAS-hPDGFB-HA. For studies at a fixed time point, PDG mice underwent MRI scans at 4–5 weeks after injection of RCAS-hPDGFB-HA, as previously described15. Additional information can be found in the Supplementary Methods.

Immunodeficient (NOD-SCID or athymic nude) animals were orthotopically transplanted with TGL-labeled PDGC-23 cells (in athymic nude mice), TS573, TS1137, U251 or LN229 cells (in NOD-SCID mice). Mice were randomly assigned to BLZ945- or vehicle-treatment cohorts starting at the indicated time points after intracranial injection. Mice were dosed daily for 15 d, and tumor growth was monitored every 5 d by bioluminescence imaging using a Xenogen IVIS-200 Optical In-vivo Imaging System.

Radiation treatment. Tumor-bearing PDG mice were sedated with ketamine and xylazine before a single radiation dose of 10 Gy (115 cGy min-1) using the X-RAD 320 from Precision X-Ray. Mice were euthanized 24 h after irradiation, and tissues were collected for histological analysis of macrophage phagocytic capacity.

Bone marrow transplant (BMT). For BMT experiments, nestin–Tv-a; Cdkn2a( Ink4a/Arf)-/- mice were bred to WT C57BL/6 mice to generate nestin–Tv-a; Cdkn2a( Ink4a/Arf)-/- transplant recipients. Nestin–Tv-a; Cdkn2a( Ink4a/Arf)-/- mice were also bred to C57BL/6 β-actin–GFP transgenic mice to generate Cdkn2a( Ink4a/Arf)-/- bone marrow donors that ubiquitously expressed GFP. For BMT, recipient mice were lethally irradiated (10 Gy) at 4–4.5 weeks of age. The next day, donor-mouse bone marrow was flushed from femurs and tibiae with X-Vivo 20 Media (Cambrex) and resuspended in sterile PBS at a concentration of 106 cells/ml. Recipients received 1 × 106 donor BM nucleated cells in 100 μl of PBS by intravenous tail vein injection. After 4 weeks, successful engraftment was assessed by the percent-age of GFP+ cells in the blood of BMT recipients by flow cytometry. If engraftment was less than 60%, mice were excluded from all subsequent experiments.

DT/DTR and clodronate liposome-mediated macrophage depletion. A detailed description of the experimental procedures can be found in the Supplementary Methods.

Mouse euthanasia and tissue harvest. Mice were euthanized at the time points described in the figure legends or when they became symptomatic from their tumors, which included signs of poor grooming, lethargy, weight loss, hunching, macrocephaly or seizures. To isolate tissues for snap freezing in liquid nitrogen or BMDM isolation, mice were euthanized 1 h after the final treatment dose by CO2 asphyxiation or fully anesthetized with Avertin (2,2,2-tribromoethanol; Sigma) and cervically dissected. For flow cytometry, mice were fully anesthetized with Avertin and transcervically perfused with 20 ml of PBS. The brain was then isolated, and the tumor was microdissected from the surrounding normal tissue. For proliferation analysis, mice were injected intraperitoneally with 100 mg per g body weight of BrdU (Sigma) 2 h before euthanasia. To isolate tissues for frozen histology, mice were fully anesthetized with Avertin, transcervically perfused with 10 ml of PBS and 10 ml of 4% paraformaldehyde in PBS (PFA). The brain was post-fixed in PFA overnight at 4 °C, and other tissues were cryopreserved in 30% sucrose at 4 °C. After post-fixation, the brain was transferred to 30% sucrose at 4 °C for at least 2 d. All tissues were then embedded in optimal cutting temperature compound (OCT) (Tissue-Tek), and 10-μm cryostat tissue sections were used for all analyses.

Preparation of glioma single-cell suspensions. For the derivation of primary glioma cell cultures or investigation of brain macrophage populations by flow cytometry, microdissected PDGs were digested to a single-cell suspension by 8–12 min of incubation at 37 °C with 5 ml of papain digestion solution (0.94 mg ml-1 papain (Worthington), 0.48 mM EDTA, 0.18 mg ml-1 N-acetyl-l-cysteine (Sigma) and 0.06 mg ml-1 DNase I (Sigma) diluted in Earle’s Balanced Salt Solution (EBSS) and allowed to activate at room temperature for at least 30 min). After digestion, the enzyme was inactivated by the addition of 2 ml of 0.71 mg ml-1 ovomucoid (Worthington). The cell suspension was passed through a 40-μm mesh filter to remove undigested tissue and washed with Neural Stem Cell (NSC) Basal Medium (Stem Cell Technologies) for subsequent culture or FACS buffer (1% IgG-free BSA in PBS (Jackson ImmunoResearch)) for flow cytometry and centrifuged at a low speed, 750 r.p.m. (Sorvall Legend RT), to remove debris and obtain the cell pellet.

As many immune-cell epitopes are papain sensitive, for investigation of immune-cell infiltrates by flow cytometric analysis, tumors were digested to a...
single-cell suspension by incubation for 10 min at 37 °C with 5 ml of 1.5 mg ml−1 collagenase III ( Worthington) and 0.06 mg ml−1 Dnase I in 1× Hank’s Balanced Salt Solution (HBSS) with calcium and magnesium. The cell suspension was then washed with PBS and passed through a 40-µm mesh filter to remove undigested tissue. To remove myelin debris, the cell pellet was resuspended in 15 ml of 25% Percoll (room temperature) prepared from stock isotonic Percoll (90% Percoll (Sigma) and 10% 10× HBSS) and then spun for 15 min at 1,500 r.p.m. (Sorvall Legend RT) with the accelerator and brake set to 1. The cell pellet was then washed with 1× HBSS before being resuspended in FACS buffer.

**Derivation of mouse primary glioma cultures, tumor spheres and glioma cell lines.** PDG single-cell suspensions were resuspended in DMEM containing 10% FBS to derive mouse primary glioma cultures, which were used at early passage (2–3) and contained a mixture of different cell types, including tumor cells, macrophages and astrocytes (Supplementary Fig. 21c). For immunostaining, primary glioma cultures were grown for 24 h on poly-l-lysine–coated coverslips (BD Biocoat), fixed with PFA overnight at 4 °C, permeabilized with 0.1% Triton-X for 5 min and blocked with 0.5% PNB (phosphate-NaCl buffer) for at least 1 h before antibody staining for CD11b macrophages (1:200), nestin+ glioma cells (1:500) and GFAP+ astrocytes (1:1,000) (Supplementary Table 10).

For tumor sphere cultures, the PDG cell pellet was resuspended in mouse NSC Basal Medium with NSC proliferation supplement, 1 mg ml−1 heparin ( Stem Cell Technologies), 10 ng ml−1 recombinant human EGF (Invitrogen) and 20 ng ml−1 recombinant human basic fibroblast growth factor (FGF) (Sigma). Fresh medium was added every 72 h for 2 weeks. Primary tumor spheres were collected, mechanically disaggregated to a single-cell suspension and propagated by serial passaging. For the secondary tumor sphere–formation assay, 5 × 103 cells were plated in a six-well plate in neurosphere medium in the presence of BLZ945 or DMSO as a vehicle. The medium was changed every 48 h, and the number of tumor spheres present after 2 weeks was counted.

To generate PDGC lines, secondary tumor spheres were dissociated to a single-cell suspension and cultivated in DMEM and 10% FBS as a monolayer63. Multiple glioma cell lines were derived from independent mice, denoted as PDGC lines. The PDGC-23 line was infected with a pBabe-H2B-mCherry construct as described previously64.

**Isolation of BMDMs.** For BM isolation followed by macrophage derivation, femurs and tibiae were harvested under sterile conditions and flushed. For all experiments, unless otherwise specified, BM was isolated from WT C57BL/6 mice. The marrow was passed through a 40-µm strainer and cultured in 30-ml Teflon bags (PermaLife PL-30) in DMEM and 10% FBS supplemented with 10 ng ml−1 recombinant mouse CSF-1 (R&D Systems). BM cells were cultured in Teflon bags for 7 d with fresh CSF-1–containing medium changes every other day to induce macrophage differentiation, after which they were referred to as BMDMs.

**Histology, immunohistochemistry, flow cytometry and staining analysis.** A detailed description of the experimental procedures can be found in the Supplementary Methods and in Supplementary Tables 10 and 11.

**Preparation of glioma-cell and BMDM CM and heterotypic glioma cell and BMDM stimulation experiments.** CM from PDGC lines or human glioma cell lines was generated by incubating cells in serum-free DMEM or NeuroCult medium (for human tumor spheres) for 24 h (Supplementary Fig. 15a). Collected CM was passed through 0.22-µm filters to remove cellular debris and is referred to here as GCM. Undiluted GCM was used to stimulate differentiated C57BL/6 WT or β-actin–GFP+ BMDMs without further addition of CSF-1 or other media. Control macrophages were fresh DMEM, 10% FBS and 10 ng ml−1 recombinant mouse CSF-1. When indicated, differentiated BMDMs were cultivated in GCM containing either DMSO as vehicle, 75 ng of total RNA was reverse transcribed and labeled using the Genechip 3’ IVT Express Kit (Affymetrix). The resulting cRNA was hybridized to Affymetrix MOE 430A 2.0 chips. Raw expression data were analyzed using GCOS 1.4 (Affymetrix). Data were normalized to a target intensity of 500 to account for differences in global chip intensity. The microarray data are deposited in NCBI Gene Expression Omnibus (GEO) under the accession number GSE37475.

**Microarrays and gene expression profiling.** All samples were prepared and processed by the genomics core facility at MSKCC. RNA was isolated using TRizol, and the quality was assessed by running on an Agilent Bioanalyzer. 75 ng of total RNA was reverse transcribed and labeled using the Genechip 3’ IVT Express Kit (Affymetrix). The resulting cRNA was hybridized to Affymetrix MOE 430A 2.0 chips. Raw expression data were analyzed using GCOS 1.4 (Affymetrix). Data were normalized to a target intensity of 500 to account for differences in global chip intensity. The microarray data are deposited in NCBI Gene Expression Omnibus (GEO) under the accession number GSE37475.

**Microarray analysis.** All bioinformatics analyses were completed in R using the Bioconductor suite of packages. Expression values were computed using the robust multiarray average (RMA) method and then quantile normalized in the ‘affy’ package66,67. The ‘limma’ package68 was used to identify differentially expressed genes between the vehicle- and BLZ945-treated samples. Differential expression was considered significant at a fold change of ±2 with a false discovery rate of 10%. Gene set enrichment analysis (GSEA) was used as described previously69. For subsequent analyses and comparison to human data sets, mouse expression values were mean centered across all samples.

**Lasso regression method for gene signature identification.** Mouse expression data were normalized and mean centered as described above. Differentially expressed genes were used for further analysis. We trained a logistic regression model with lasso constraints to differentiate between vehicle- and BLZ945-treated samples using the ‘glmnet’ package80 by setting the ‘family’ parameter to ‘binomial’ in the glmnet function. The regularization parameter for lasso regression was chosen by fourfold cross validation.
Patient glioma data sets, subtyping of non-TCGA patients, human glioma classification and stratification of patients by G-CIMP status. A detailed description of the analyses can be found in the Supplementary Methods.

Survival analyses. Survival analyses were completed using the ‘survival’ package in R70. Hazard ratios were determined using the ‘coxph’ function from the ‘survival’ package. Patients were stratified on the basis of the probability of the lasso logistic regression classification, G-CIMP status or both, as indicated. P values were generated using Wald’s test.

Plots for patient analyses. All Kaplan-Meier survival curves, heat maps and volcano plots were generated in R v 2.14.1 using the ‘gplots’ and ‘ggplot2’ packages. Patient glioma data sets, subtyping of non-TCGA patients, human glioma classification and stratification of patients by G-CIMP status.

Data presentation and statistical analyses. Data are presented as the means with their respective standard errors (s.e.m.) or as statistical scatter plots generated using GraphPad Prism Pro5. Within each experiment, independent biological replicates represent a minimum of three technical replicates. Numerical data were analyzed by unpaired two-tailed Student’s t test unless otherwise noted. For survival curves, P values were obtained using the log-rank (Mantel-Cox) test or the two-tailed Mann-Whitney test when indicated, and Fisher’s exact test was used for histological tumor grading. P < 0.05 was considered statistically significant.

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