Malignant Astrocytic Tumor Progression Potentiated by JAK-mediated Recruitment of Myeloid Cells

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Abstract

Purpose: While the tumor microenvironment has been known to play an integral role in tumor progression, the function of nonresident bone marrow–derived cells (BMDC) remains to be determined in neurologic tumors. Here we identified the contribution of BMDC recruitment in mediating malignant transformation from low- to high-grade gliomas.

Experimental Design: We analyzed human blood and tumor samples from patients with low- and high-grade gliomas. A spontaneous platelet-derived growth factor (PDGF) murine glioma model (RCAS) was utilized to recapitulate human disease progression. Levels of CD11b+/GR1− BMDCs were analyzed at discrete stages of tumor progression. Using bone marrow transplantation, we determined the unique influence of BMDCs in the transition from low- to high-grade glioma. The functional role of these BMDCs was then examined using a JAK 1/2 inhibitor (AZD1480).

Results: CD11b+ myeloid cells were significantly increased during tumor progression in peripheral blood and tumors of glioma patients. Increases in CD11b+/GR1+ cells were observed in murine peripheral blood, bone marrow, and tumors during low-grade to high-grade transformation. Transient blockade of CD11b+ cell expansion using a JAK 1/2 Inhibitor (AZD1480) impaired mobilization of these cells and was associated with a reduction in tumor volume, maintenance of a low-grade tumor phenotype, and prolongation in survival.

Conclusions: We demonstrate that impaired recruitment of CD11b+ myeloid cells with a JAK1/2 inhibitor inhibits glioma progression in vivo and prolongs survival in a murine glioma model.

Introduction

Glioblastoma multiforme carries a dismal prognosis with a median survival of approximately 17 months (1). Glioblastoma multiforme broadly exists as two overlapping entities. In de novo glioblastoma multiforme, the tumor is discovered with a malignant phenotype and has pathologic characteristics suggestive of a high-grade tumor (1). Secondary glioblastoma multiforme may develop as a process of inexorable malignant transformation from a low-grade phenotype over the course of many years through mechanisms still being elucidated. Previous studies have suggested this process is heavily reliant on the interaction of the tumor with several nontumor cells recruited from nonmalignant sites. Relevant components of the tumor microenvironment include endothelial cells, fibroblasts, microglia, and bone marrow–derived myeloid cells (BMDC), which synergistically potentiate tumor progression and tumor-associated neangiogenesis (2–6). The angiogenic switch, which is defined as a state of rapid tumor growth supported by exponential neovascularization during which the malignant phenotype is initiated, is an important mechanism within low-grade glioma transformation. The distribution of CD11b+ cells within high-grade tumors supports an important role for a myeloid-derived cell population during this process (2–4). Tumor neovascularization provides nutrients and blood supply to the tumor core and is characterized by a metabolic profile exceeding that of neighboring brain parenchyma. BMDCs are key mediators of this angiogenic switch and initiate, support, and perpetuate malignant transformation (2–4).

BMDCs such as macrophages, dendritic cells, neutrophils, eosinophils, mast cells, and myeloid-derived suppressor cells...
Translational Relevance

Ineffective CNS drug penetration through the blood–brain barrier for brain tumors has stalled the development of efficacious treatment paradigms. The majority of current first-line therapies for low-grade and high-grade gliomas rely on inhibiting tumor cells: our work is innovative in that it directly targets bone marrow-derived cells (BMDC) within the tumor extrinsic microenvironment. Without having a direct effect on the CNS, we modulate the tumor microenvironment and delay progression of disease, which has major clinical relevance. Roughly 40% of gliomas present as lower grade lesions that progress to more invasive, malignant phenotypes. These slow growing, indolent tumors have the capacity to transform into highly malignant and aggressive high-grade gliomas. Despite standard therapeutic regimens, transformation to high-grade glioma is common. These data suggest a potential opportunity to consider treatment with JAK inhibitors in patient cohorts that show an upregulation of BMDCs in their peripheral blood prior to transformation.

Materials and Methods

Cell lines

A Ntu-a RCAS high-grade cell line was generated through the homogenization of high-grade tumors derived from explants harvested from 10-week-old Ntu-a tumor bearing mice. Tumor tissues were minced and subsequently incubated at 37°C with PAPAIN (0.1 mg/mL) in PIPEs buffer with DNAase. Tissue suspensions were then gently shaken for 20 minutes. After quenching the digestion with FBS, the suspension was again disrupted with a pipette and passed through a 100-μm cell strainer (BD Biosciences). Cells were collected by centrifugation (1,300 rpm × 5 minutes) and seeded onto poly-i-lysine–coated petri dishes with complete medium containing 10% FBS in DMEM overnight. Cell lines were subsequently grown and passaged in complete medium. The cell line described above is not authenticated. Adherent glioblastoma multiforme–derived cell lines (U87 and U251) and oligodendroglioma-derived neurospheres have been described previously (20, 21).

Drug preparation and administration

AZD1480 was purchased from ChemieTek. For in vitro experiments, AZD1480 was dissolved in 1% DMSO. For in vivo experiments, AZD1480 was dissolved in water, 0.5% hypermethylcellulose (HPMC), and 0.1% Tween 80. At 3 weeks of age, animals began treatment with AZD1480, which was administered via oral gavage once a day, 5 days a week, over 3 consecutive weeks. The treatment course was stopped at 6 weeks of age for the short-term groups, whereas it was continued indefinitely in the long-term cohorts. For transplant cohorts, treatment initiation was started at 7 weeks of age and was administered for 3 weeks until animals reached 10 weeks of age. The drug was administered through an oral gavage needle at a dose of 60 mg/kg. Control animals received identical volumes of vehicle without AZD1480 by oral gavage.

Tumor-generating mice

The Ntu-a model system was utilized as described previously. To derive glioma-bearing mice, Ntu-a (WT) mice were mated with Niu-a Ink4aArf+/− LPTEN mice to produce offspring (22–26). RCAS-HPDGF-B-HA and RCAS-Cre vectors were transfected into DEF-1 chicken cells (ATCC) using the FuGENE 6 Transfection Kit (Roche). DEF-1 cells transfected with RCAS-PSG were cultured in 10% FBS-DMEM under standard conditions. For tumor induction, transfected DEF-1 cells were trypsinized, centrifuged, and injected into the brain parenchyma of Ntu-a mice with a heterozygous Ink4aArf+/−, PTEN+/− background at postnatal day 0–2 (24, 25, 27).

Magnetic resonance imaging

All animals were imaged with a Bruker Mouse Brain Surface Coil (Bruker Biospin MRI, Inc) while anesthetized under 1.5%–2% isoflurane (Baxter). Images were acquired on a Bruker BioSpec 70/30 USR 7T (Bruker Biospin MRI, Inc) utilizing gadolinium contrast at a 9.1 saline to Magnevist (Bayer Healthcare Pharmaceuticals Inc) ratio. The mouse brain was imaged in the axial orientation using RARE T1-weighted and TurboRARE T2-weighted fast spin-echo rapid acquisition. T1 sequence repetition time was 1,500 milliseconds with echo time = 17.4 milliseconds and an echo train length of 6. T2 repetition time was 2,014 milliseconds with echo time = 48.4 milliseconds and echo train length of 10. For both T1 and T2, the field of view was 2,014 milliseconds with echo time = 48.4 milliseconds and echo train length of 10. For both T1 and T2, the field of view...
was $20 \times 20$ mm with slice thickness of 1.0 mm and spatial resolution of $0.078 \times 0.078$ mm/pixel. Tumor progression was characterized between 2 and 12 weeks.

**Tumor volumetric analysis**

T1- or T2-weighted MRI images representing axial sections of subject brains were uploaded into Imagel software. The slice halfway through each animal’s dataset, typically image 7 of 14, was determined to show the most representative image of tumor development and size. These slices were subsequently analyzed using the Free Hand Tracing and Measure tools to outline the two-dimensional area of brain tissue present. Traces were initiated slightly posterior to the ventral aspect of the skull and followed dorsally, forming a closed loop with the initial trace point. The area formed by this outline was then calculated in square millimeters using the Measure function. Each pixel represents 1 mm of depth; thus, overall brain volume was obtained by multiplying measured area by 1 mm. Animal brain volumes for controls and experimental conditions were averaged per group and presented graphically. In an attempt to minimize baseline differences in brain size and development, comparative volumetric analysis was only performed between animals treated under the same experimental conditions and from the same litter.

**Brain tissue processing**

Animals used for histologic examination were sacrificed using carbon dioxide (CO$_2$) and brains were removed and fixed in 4% paraformaldehyde for 24 hours followed by dehydration in 70% ethanol. The fixed, paraffin-embedded brain tissues were serially sectioned (10 μm), slide-mounted, and then deparaffinized in Histoclear (Allan Scientific) and soaked in serial alcohol gradients.

**Flow cytometry analysis**

Flow cytometry analysis was performed as described previously (19). Peripheral blood drawn from human subjects with histologically confirmed grade II ($n=10$) and IV ($n=10$) gliomas and healthy controls was centrifuged at 500 g for 10 minutes at 4°C. Isolation of white blood cells was performed using a Ficoll-Paque Gradient (GE Healthcare) and stained with CD11b (BD Biosciences 1:200) and GR1 antibodies (BD Biosciences). Blood and bone marrow from mice were harvested and stained with CD11b (BD Biosciences 1:200) and high-grade gliomas were stained with pSTAT3 (Tyr705; Cell Signaling Technology).

**Adoptive bone marrow transplantation**

Transplants were performed with GFP$^+$ bone marrow from age-matched donor Black 6 (BL6) mice. Bone marrow was harvested by flushing the femurs of BL6/GFP$^+$ donor mice. A total of $5 \times 10^6$ bone marrow cells from BL6/GFP$^+$ donor mice were transplanted into lethally irradiated (9.5 Gy) 2-week-old Nfu-a heterozygous knockout mice by retro-orbital injection. During the irradiation procedure, mice heads were shielded with a custom-designed lead helmet apparatus. After 4 weeks, GFP$^+$ bone marrow–reconstituted animals (day 49) were started on a 3-week treatment regimen as previously described. Animals were sacrificed at week 11 (day 77). Brain tissues were dissected and fixed in 4% paraformaldehyde or in a mix of 2% paraformaldehyde and 20% sucrose solution overnight and embedded in Tissue-Tek optimal cutting temperature medium (OCT; Electron Microscopy Sciences). Once embedding medium was in place, the OCT blocks were frozen over dry ice. These blocks were sectioned at 10-μm thickness using a cryotome (Leica) and analyzed for GFP$^+$ fluorescence and subsequent immunostaining (CD11b, pSTAT3).

**Cell proliferation and Western blot assays**

An RCAS high-grade cell line was cultured at a density of $10^5$ cells per well in 96-well plates (Corning). Using the RCAS high-grade cell line, AZD1480 was introduced at 1.0 μM/L concentration and cells were counted after coinubcation for both 48 and 72 hours. U87 and U251 cells were cultured in complete medium, and TS603 cells were cultured in Neurocult basal medium (StemCell Technologies) containing Neurocult supplement, EGF, FGF, and Heparin. To measure cell growth, 3–5 × 10$^5$ cells were seeded per well with either AZD1480 (0.5 μM/L and 1 μM/L) or DMSO. Cells were then counted at 24-, 48-, and 72-hour time intervals. Each experiment was performed in triplicate and cell counting was performed using a hemocytometer.

Western blot analysis was performed as described previously (19). All of the glioma cell lines were treated with AZD1480 for 5 hours before cell lysis for the analysis of pSTAT3 (Y705) and total STAT3 (Cell Signaling Technology).

**Immunohistochemistry**

IHC was performed on a Leica Bond system using the standard protocol. For IDH1 (R132H) staining, tissue sections were subjected to antigen retrieval using a Tris-EDTA buffer for 20 minutes and incubated with DIA-H09 (1:50) for 15 minutes at room temperature. For CD11b staining, the sections were subjected to antigen retrieval using a sodium citrate buffer for 30 minutes and incubated with anti-CD11b (ab52478 1:100) for 25 minutes at room temperature. Signals were detected using an HRP-conjugated compact polymer system and visualized using DAB as the chromogen. Each tumor was scored as positive if at least one tumor core showed >10 CD11b$^+$ cells with circumferential staining.

**Immunofluorescence**

To assess the degree of pSTAT3 staining in human gliomas, two separate tissue microarrays comprising formalin-fixed, paraffin embedded (FFPE) tissue cores of human low- and high-grade gliomas were stained with pSTAT3 (Tyr705; 0.2 μg/mL; Cell Signaling Technology) and counterstained with DAPI. Immunofluorescence experiments were performed using Discovery XT processor (Ventana Medical Systems) at the Molecular Cytology Core Facility (MSKCC, New York, NY). The slides were scanned at high resolution and analyzed using Panoramic Viewer Software. Up to 3 tissue cores per case were scanned at low power for an area of maximal pSTAT3 staining. A representative 400× field from this area was selected for quantification. Distinct nuclear staining above background was considered positive. In each image, 100 to 500 cells were manually counted using ImageJ software’s cell counter and grid macros. A two-tailed Student $t$ test was used to assess for a significant difference in positive pSTAT3 staining between
high-grade glioma (n = 41) and low-grade glioma (n = 38) samples (total n = 79). Multiplex immunofluorescent staining for pSTAT3/GFP/CD11b was performed as described previously (28).

Statistical analysis and quantification
Data is represented as mean ± SD. N values indicate the number of animals or slides used for interpretation of data for a particular experimental animal cohort. Tumor histology was analyzed in a blinded fashion for experimental cohorts and quantified for hallmark characteristics of gliomas by a neuropathologist. Significant differences between experimental groups were measured by a Student t test with P values indicated less than 0.05 (*) or 0.01 (**).

Results
CD11b⁺ myeloid-derived cells are increased in high- versus low-grade gliomas
We hypothesized that CD11b⁺ BMDCs contribute to the transition of low- to high-grade gliomagenesis. To test this hypothesis, we collected peripheral blood from patients with low-grade gliomas, high-grade gliomas, and non-tumor controls. Flow cytometry was used to measure CD11b⁺ cells, demonstrating an increased number of circulating CD11b⁺ cells in patients with high-grade gliomas (66%; grade IV) as compared with low-grade gliomas (42%; grade II; Fig. 1A). Next, we examined whether these findings could be recapitulated in a PDGF-driven murine model of low- to high-grade glioma progression (Fig. 1B). This animal model, termed Replication-Competent shows various images and data analyses.
ASLV long terminal repeat (RCAS), allows for a murine retrovirus (MoMuLV) to deliver the PDGF-B oncogene into glial progenitor cells of mice deficient for the tumor suppressor PTEN, and heterozygous for Ink4a/Arf (Nestin-tva, P16(INK4a)+/−; P14(ARF)+/−; PTEN(fl/fl); Fig. 1B; Supplementary Fig. S1A; refs. 24, 25, 27). Following tumor induction, mice first developed low-grade gliomas (3–4 weeks), which transformed into high-grade gliomas (7–10 weeks; Fig. 1B). We examined the number of CD11b+/GR1− cells in the peripheral blood (Fig. 1C) and bone marrow (Fig. 1D) of non-tumor bearing mice, low-grade gliomas, and high-grade gliomas by flow cytometry. We observed an increase of CD11b+/GR1− cells in the blood and bone marrow of mice bearing high-grade gliomas as compared with low-grade and non-tumor controls.

We then hypothesized that these circulating myeloid cells could infiltrate the tumor microenvironment during the low- to high-grade transformation. Accordingly, we examined CD11b+ cells in human and murine low- and high-grade gliomas by immunohistochemical (IHC) and immunofluorescent (IF) analyses. Increased CD11b+ staining was observed in high-grade gliomas as compared with low-grade gliomas (Supplementary Fig. S1B).

In addition, consistent with the literature, increased vascularity was observed by CD31+ staining in the high-grade tumors (ref. 29; Supplementary Fig. S1B).

These data demonstrate that the process of glioma transitioning from a low- to high-grade state is associated with an increased number of CD11b+ cells in the peripheral circulation and within the tumor of high-grade gliomas.

**BMDCs are recruited to the tumor microenvironment**

The above data led us to hypothesize that as low-grade gliomas transition to a high-grade state, CD11b+ cells are mobilized from the bone marrow into the peripheral circulation and subsequently are recruited to the tumor microenvironment. To test this hypothesis and differentiate between recruited and resident CD11b+ cells, we transplanted GFP+ bone marrow into the PDGF murine glioma model (GFPnegative; Supplementary Fig. S2). We compared the number of GFP+/BMDCs+ in the low- and high-grade gliomas by IF and flow cytometry. A (30-fold) increase in the number of GFP+/BMDCs+ was observed in high- (week 8) versus low-grade gliomas (week 4; Fig. 2A and B). In addition, the GFP+/BMDCs+ were found in a perivascular distribution (Fig. 2A).

![Figure 2](image-url)

**Figure 2.**

Bone marrow–derived cells (BMDC) are recruited to the tumor microenvironment. A, Representative photomicrographs of tumor cross sections from GFP+ bone marrow–transplanted mice counterstained with CD31+ at low (n = 4) and high-grade stages (n = 4; 4 and 8 weeks, respectively). Scale bar, 50 μm. B, Representative flow cytometric analysis of brain tumor tissue from RCAS murine animals gated for GFP+ cells in low- (n = 4) and high-grade tumors (n = 4). P < 0.05. C, Confocal microscopic analysis of high-grade tumor sections (n = 3 animals, >6 sections/tumor) stained for DAPI and CD11b from GFP+ bone marrow–transplanted RCAS mice. Scale bar, 50 μm. D, Quantification of cells labeled with GFP+, CD11b−, and costained (GFP+|CD11b+). Percentage of CD11b+/GFP+ cells.
To determine what proportion of these GFP+/BMDCs were myeloid-derived, we performed a GFP+/CD11b+ costain of tumor sections. Of the GFP+/BMDCs recruited population, approximately 33% of these cells were CD11b+ (Fig. 2C and D). These data demonstrate that a third of recruited tumor-infiltrating myeloid cells (CD11b+) are derived from the bone marrow.

**JAK 1/2 inhibition following bone marrow transplantation mitigates GFP+/BMDC recruitment and tumor burden**

The IL6/JAK/STAT3 signaling pathway has been shown to promote tumorigenesis in part through the expansion and activity of CD11b+ myeloid cells in models of renal and breast cancer (19, 30). We hypothesized that preventing the expansion and mobilization of these cells with an inhibitor of this pathway could abrogate the transition from low- to high-grade glioma. To test this hypothesis, we utilized the transplanted GFP+/bone marrow in the PDGF murine glioma model (Fig. 3A; Supplementary Fig. S2). Upon radiographic evidence of low-grade tumors, animals were treated with a JAK 1/2 inhibitor (AZD1480) for 3 weeks (Fig. 3A). By MRI, vehicle control–treated animals had increased tumor burden as compared with AZD1480-treated animals (Fig. 3B). To determine whether tumors from AZD1480-treated mice harbored fewer BMDCs as compared with vehicle control–treated mice, animals were sacrificed and tumors were analyzed for GFP+ cell recruitment within brain tumors. Vehicle control–treated animals demonstrated a (10-fold) increase of GFP+ cells within the tumor as compared with AZD1480-treated animals (Fig. 3C). These results demonstrate that AZD1480 blocks the recruitment of BMDCs to the tumor, which correlates with a reduction in tumor burden. Also, to further examine the effect of AZD1480 on BMDCs and the resident microglial population, we performed a costain with GFP+/CD11b+ on these tumors. Vehicle control–treated animals demonstrated a (10-fold) increase in GFP+/CD11b+ cells within the tumor as compared with AZD1480-treated animals (Fig. 3D). In addition, vehicle control animals demonstrated a (5-fold) increase of resident microglia (CD11b+/GFP+) when compared with AZD1480–treated animals (Fig. 3D). Coincident with a reduction in infiltrating myeloid cells (CD11b+/GFP+), we also observed a (5-fold) reduction in CD11b+/pSTAT3 cells in AZD1480–treated animals when compared with vehicle control animals (Supplementary Fig. S3C and S3D). These data suggest that AZD1480 blocks the recruitment of CD11b+ cells to the tumor, and decreases the number of resident microglia.

**JAK 1/2 inhibition prevents low- to high-grade tumor progression**

These data led us to hypothesize that AZD1480 impairs the transformation from low- to high-grade glioma in part through a blockade of BMDC recruitment. Mice with radiographically confirmed low-grade gliomas were treated with AZD1480. In contrast to the cohort described above, nontransplanted mice were used, as radiation (required for bone marrow transplant) delays tumor formation (31). Mice with radiographically confirmed low-grade tumors were treated for 3 weeks with either AZD1480 or vehicle control (Fig. 4A). We assessed tumor burden in these animals by MRI and performed hematoxylin and eosin (H&E) staining of the brain sections. MRI revealed reduced tumor burden in AZD1480–treated animals when compared with vehicle control–treated animals (Fig. 4B). Morphologic examination of tumors from vehicle control animals by H&E confirmed features consistent with glioblastoma multiforme including increased cellularity, pseudopalisading cytarchitecture, nuclear pleomorphism, hemorrhage, and necrosis, along with microvascular proliferation and mitotic figures (Fig. 4B, Supplementary Fig. S3). In contrast, the AZD1480–treated animal brain tumors had decreased microvascular proliferation and no mitotic figures present and appeared less aggressive morphologically (Fig. 4B; Supplementary Fig. S3A) To examine the effect of AZD1480 on the production and mobilization of CD11b+/GR1+ cells, flow cytometry was performed on the bone marrow and blood of animals treated with vehicle control and AZD1480. After 3 weeks of treatment with AZD1480, animals showed a 7-fold reduction in CD11b+/GR1+ cells in the peripheral blood while a 2.5-fold reduction of these cells was observed in the bone marrow (Fig. 4C). These data demonstrate that AZD1480 impairs tumor progression and inhibits the mobilization of CD11b+/GR1+ cells. Given these observations, we then asked whether transient or short-term treatment regimen would have an impact on survival while minimizing potential systemic toxicity. The transient or short-term treatment approach included a 3-week regimen with AZD1480 until 6 weeks of age. Once low-grade tumor formation was confirmed radiographically around 3 weeks of age, animals were treated with AZD1480 or vehicle control for a duration of 3 weeks. Animals were observed for clinical symptoms of progression (macrocephaly, weight loss, hunched back, poor feeding, and/or immobility; ref. 27). The survival of AZD1480–treated animals when compared with vehicle control–treated animals was increased by approximately 2-fold (Fig. 4D). A long-term or continuous treatment approach was also utilized to assess overall survival where animals were treated with AZD1480 or vehicle control starting at 3 weeks indefinitely until death. Similarly, the survival of AZD1480–treated animals when compared with vehicle control–treated animals was increased by (2-fold) with an indefinite treatment regimen (Fig. 4E). In addition to increased vascularity and increased recruitment of CD11b+ cells in high-grade gliomas, we also examined pSTAT3 levels (a JAK 1/2 target) by IHC in human high- and low-grade gliomas. As expected, human high-grade gliomas had increased pSTAT3 staining when compared with low-grade gliomas (ref. 32; Supplementary Fig. S4A). Given the paucity of pSTAT3 in low-grade tumors, our data suggest that AZD1480 prevents low- to high-grade transformation by impairing bone marrow–derived cell recruitment. While the presence of pSTAT3 in tumor cells does not necessarily predict a dependence on pSTAT3 for growth, our data suggest that AZD1480 had an effect on the low-grade tumor microenvironment by impairing the mobilization of BMDCs. Similar to human low- and high-grade gliomas, we performed pSTAT3 IHC on vehicle control and AZD1480–treated animal brain sections which demonstrated increased pSTAT3 staining in vehicle control–treated animals when compared with AZD1480–treated animals (Supplementary Fig. S4B). pSTAT3 has been shown in previous studies to be highly expressed in tumor cells along with microenvironment cells including BMDCs (15, 19, 33). We treated a small cohort (n = 5) of animals with radiographically confirmed high-grade gliomas for 1 week with AZD1480 and no impact on survival was observed (the mice all died within one week). In addition to AZD1480’s direct effect on targeting pSTAT3 in BMDCs, we
Figure 3.
JAK 1/2 inhibition following bone marrow transplantation mitigates CD11b⁺/GR1⁺-mediated low-grade glioma transformation. A, Schematic of RCAS low-grade murine glioma bearing animals postirradiation (2 weeks) and GFP⁺ bone marrow transplantation, treated with AZD1480. B, Representative image of T2-weighted 7T MRI of vehicle control (n = 7) and AZD1480-treated (n = 7) animals at 10 weeks. Animals were imaged around 7 weeks. C, Representative photomicrographs of tumor cross sections from mice treated with vehicle control (n = 5) and AZD1480 (n = 5). Quantification of average number of GFP cells per high power field in bar graph format, (n = 10). Scale bar, 50 μm, **P = 0.004. D, Representative photomicrographs of tumor cross sections from mice treated with vehicle control (n = 5) and AZD1480 (n = 5) costained with GFP⁺ and CD11b⁺. Quantification of average number of cells per high power field in bar graph format labeled with GFP⁺, CD11b⁺ and double labeled with GFP⁺/CD11b⁺, (n = 10). Scale bar, 50 μm, *P < 0.05.
examined its effects on high-grade tumor cell proliferation in vitro (Supplementary Fig. S4C). AZD1480 at 1.0 μmol/L (which effectively blocked pSTAT3 expression) had no effect on cellular proliferation when compared with vehicle control–treated cells (Supplementary Fig. S4C and S4D). In addition to AZD1480’s direct effect on targeting pSTAT3 in BMDCs, we examined its effects on murine high-grade and human-derived glioma cell proliferation (TS603, U87, U251; Supplementary Fig. S4C and S4D).

Figure 4.
JAK 1/2 inhibition prevents low- to high-grade tumor progression. A, Animal schematic depicting experimental design to examine effect of AZD1480 (treatment for 3 weeks) on tumor progression. Treatment with AZD1480 was stopped at 6 weeks. B, Representative images of T2-weighted 7T MRI of vehicle control (n = 9) and AZD1480 (n = 9) treated animals at 6 weeks. Representative images of H&E staining of tumor sections from control (n = 8) and AZD1480 (n = 8) treated animals at 6 weeks. Scale bar, 100 μm. C, Representative flow cytometry graphs of CD11b+/GR1+ from blood (n = 3/group) and bone marrow (n = 7/group) of vehicle control and AZD1480-treated tumor bearing mice at 6 weeks. *P = 0.0386 blood; P = 0.0556 bone marrow (BM). D, Kaplan-Meier symptom-free survival curve for RCAS mice treated for 3 weeks only with vehicle control (n = 9) and AZD1480 (n = 9), P < 0.0001. E, Kaplan-Meier symptom-free survival curve for RCAS mice treated indefinitely with vehicle control (n = 6) and AZD1480 (n = 6), P < 0.0001.
Discussion

The role of BMDCs in promoting angiogenesis and invasion during tumor progression has been previously described in cancer (7, 8). However, the multiple molecular mechanisms converging within the microenvironment to promote tumor progression in low-grade gliomas remain under investigation. Ultimately, the role of BMDCs within the microenvironment during low- to high-grade glioma transformation in this patient population is poorly understood. There exists a delicate balance that is achieved between the tumor and stroma; we suggest that one of the earliest demarcations of malignant progression in gliomas is the expansion of the tumor-associated stroma including a large infiltration of BMDCs.

Recent studies have described the importance of CD11b\(^+\)/Gr1\(^+\) cell recruitment and infiltration in solid tumors (9, 10, 14, 29, 34). These studies demonstrate that myeloid cells support the tumor endothelium by producing proangiogenic elements such as matrix metalloproteinase 9 (MMP-9; refs. 15, 18), highlighting a role for myeloid cell–mediated angiogenesis within the microenvironment. The STAT3 signaling pathway has been shown to be critical for myeloid-cell dependent tumor angiogenesis within multiple murine models including B16 melanoma, 4T1, and polyoma middle t murine breast cancer models (19, 33). We explored a similarly predicted role for CD11b\(^+\)/Gr1\(^+\) cells mediating tumor progression within the glioma microenvironment and via JAK/STAT signaling with our PDGF-driven RSU1286 murine glioma model. This model lacks IDH alterations often seen in a majority of diffuse astrocytomas (35, 36); however, given that we are not studying the molecular pathways of gliomagenesis, rather the tumor microenvironment–immune system modulation, we believe this is a useful model given its wide utilization and our internal controls using other nonspontaneous mouse models (20).

We have studied the modulation of the glioma microenvironment through BMDC recruitment during transformative glioma transformation and the influence of JAK/STAT signaling. Previous groups have focused on impairing the JAK/STAT3 pathway within tumor cells using in vitro and immunocompromised xenograft models with the U87 cell line (37). In contrast, our work utilizes an immunocompetent transgenic murine glioma model that recapitulates low- to high-grade glioma transformation. Targeting glioma tumor cells remains the current standard of oncologic care for patients with malignant glioma; tumor escape mechanisms harbored within the stromal infrastructure remain an under-targeted aspect of complex malignant glioma biology. Neuro-oncologists lack a wide treatment armamentarium based upon tumor-intrinsic therapies beyond early-generation anti-VEGF antibodies. Our in vitro studies with AZD1480 showed no effect on cellular proliferation (Supplementary Fig. S4C and S4D), suggesting a tumor-intrinsic effect of BMDC targeting.

Achieving adequate CNS penetration of agents through the blood brain barrier is also a significant problem for tumor-intrinsic therapies (38, 39). Therefore, new agents that target specific BMDCs warrant consideration and discussion. Ultimately, a more comprehensive understanding of the many facets, both intrinsic and extrinsic, that comprise the molecular complexity of tumor progression may help us to stratify patients into targeted regimens.

Our data demonstrates the peripheral upregulation of CD11b\(^+\)/Gr1\(^+\) cells within the bone marrow and increased mobilization into the peripheral blood en route to the tumor site. Similar to peripheral blood data from our murine glioma model, human patients with high-grade gliomas had a higher level of CD11b\(^+\) cells found in the peripheral blood when compared with those with low-grade gliomas (Fig. 1A). We suggest cross-talk occurs between the tumor and microenvironment via tumor cell–secreted factors that lead to tumor progression. More studies are necessary to define these factors. Understanding the cross-talk between the tumor and microenvironment (BMDCs), particularly with respect to how AZD1480 influences the recruitment of BMDCs during the low- to high-grade transition is paramount. Using our RSU1286 murine glioma model with GFP\(^+\) bone marrow transplant, we also attempted to sort GFP\(^+\)/BMDCs\(^{\text{GFPpositive}}\) to allow us to run a cytokine array comparing cells from AZD1480-treated and vehicle control animals. However, despite several attempts, the BMDC cellular yield from this brain-derived population was too low to permit this type of analysis. It is important to identify the cytokines, growth factors, and chemokines that may play a significant role in the recruitment process of BMDCs and subsequent studies should address this important point. The role of non-CD11b\(^+\) cells and resident microglia (BMDC\(^{\text{GFPpositive/GFPnegative}}\)) should also be further studied as infiltrating CD11b\(^+\) myeloid cells are only 33% of the recruited GFP\(^+\) population in our animal model.

To dissect the contribution of BMDCs during low- to high-grade transformation, bone marrow transplant studies were performed demonstrating the recruitment of GFP\(^+\) BMDCs, which correlated with high tumor burden. We observed a higher quantity of CD11b\(^+\) cells in both the peripheral (blood and bone marrow) and at the tumor site in high-grade tumor-bearing animals. We also observed a perivascular distribution of CD11b\(^+\) cells suggesting a role in neovascularization. These results support previous extensive reports describing myeloid-associated tumor angiogenesis in cancer (11, 40–43).

After observing BMDC mobilization in our animal model, we attempted to impair recruitment before low-grade glioma transformation to mitigate the effects of the angiogenic switch. The JAK/STAT pathway has been actively studied with respect to its crucial role regulating myeloid cell development and extravasation. Previous studies in other solid tumors have shown the effectiveness of AZD1480 in targeting the JAK/STAT3 pathway, including in CD11b\(^+\)/Gr1\(^+\) cells (15, 30). We examined the effects of AZD1480 on CD11b\(^+\) cell recruitment in the low- to high-grade murine glioma model. We observed reduced CD11b\(^+\) cell levels in the bone marrow, peripheral blood, and at the regional tumor site in AZD1480-treated animals. We also demonstrated significant impairments in tumor progression by histologic analyses, radiographic findings, and overall survival in animals treated with AZD1480 compared with vehicle control mice. While previous findings in other solid tumor model systems have demonstrated myeloid cell infiltration, to our knowledge, we are the first to report that this process holds true in early-stage gliomagenesis. Reports have also demonstrated BMDCs (using a CSFR-1 inhibitor) as an important factor that promote angiogenesis and invasion in high-grade gliomas (44). AZD1480-treated animals demonstrated decreased microvascular proliferation when compared with vehicle control animals. These data suggest AZD1480 may have an antiangiogenic effect in our murine glioma model. Limitations of antiangiogenic therapies have been described in the glioma literature including a lack of definitive data confirming antitumor effect with these agents (45).
Furthermore, inhibiting angiogenesis in high-grade gliomas may antagonize the efficacy of chemotherapeutic agents by normalizing the blood brain barrier. The earliest stages of gliomagenesis represent a unique stage of glioma biology during which the microenvironment may be more actively evolving than the tumor cells and thus may represent a novel approach to cancer therapy. Currently, glioma progression is monitored largely by MRI. More precise tumor surveillance may both reduce unnecessary treatments for patients who are not likely to progress and yet simultaneously prompt earlier and more appropriate interventions for patients with low-grade gliomas at the earliest stages of malignant transformation. Our data demonstrate one important factor in glioma progression: the BMDC recruitment process from the bone marrow. These cells can be easily monitored during critical stages of glioma progression and may serve as a biomarker of low-grade transformation with a simple peripheral blood assay. Utilizing this data along with more traditional markers of progression such as MRI may more accurately predict early glioma progression. Studies aimed at collection of peripheral blood samples of patients with low-grade (grade II) lesions may help quantify and characterize mobilized BMDCs. RNA-sequencing assays of this crucial cellular population may help identify the factors regulating their recruitment and their interactions within the tumor microenvironment. Appreciating differences in these BMDC populations during stages of glioma progression may serve as a platform to stratify which patients may benefit from early-stage anti-CD11b⁺ therapy and which patients would be better served by observation. Further studies in select glioma patient cohorts utilizing JAK 1/2 inhibitors that are currently used in clinical practice for other indications warrant further consideration in this unique patient population.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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