Malignant astrocytic tumor progression potentiated by JAK-mediated recruitment of myeloid cells

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Running Title

JAK inhibition impairs glioma progression

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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 (BMDCs) 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 who show an upregulation of BMDCs in their peripheral blood prior to transformation.

Abstract

Purpose: While the tumor microenvironment has been known to play an integral role in tumor progression, the function of non-resident bone marrow-derived cells (BMDCs) remains to be determined in neurological 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.

**Conclusion:** 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 (GBM) carries a dismal prognosis with a median survival of approximately 17 months (1). GBM broadly exists as two overlapping entities. In *de novo* GBM, the tumor is discovered with a malignant phenotype and has pathologic characteristics suggestive of a high-grade tumor (1). Secondary GBM 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 non-tumor cells recruited from non-malignant sites. Relevant components of the tumor microenvironment include endothelial cells, fibroblasts, microglia, and bone marrow-derived myeloid cells (BMDCs), which synergistically potentiate tumor progression and tumor associated neo-angiogenesis (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 (MDSCs) are often present in large numbers within the stroma of neoplasms (7-13). Myeloid cell surface markers include CD11b⁺, CD14, CD34, CD44, CD59, CD68, CD163, and F4/80. MDSCs, another subset of myeloid cells, consist of immature progenitor cells intended for neutrophil, monocyte, or dendritic cell fate. CD11b⁺/GR1⁺ cells are immature myeloid progenitor cells that could be classified as MDSCs. CD11b⁺ is a marker for myeloid cells from the macrophage lineage and GR1⁺ designates a granulocytic lineage of origin.
CD11b+/GR1+ cells have been shown in other solid tumors to secrete TGF-B, promote angiogenesis, tumor progression, and metastasis (14).

Both murine and human MDSCs exhibit a CD11b+CD14+/–MHCIIlowMHCI+ phenotype, however those found in mice are defined as CD11b+GR1+ and can be further divided into two subtypes: CD11b+GR1hi, which exude an immature neutrophil phenotype, and CD11b+GR1low, which resemble a monocytic phenotype (12). In our murine model, we selected to examine CD11b+/GR1+ cells since they are significantly increased (in bone marrow and blood) during low- to high-grade progression and we were unable to observe an increase in other myeloid cell markers. We have not functionally characterized these CD11b+/GR1+ cells as MDSCs in our study.

Myeloid cells have been the subject of rigorous investigation within the context of solid tumorigenesis and have been shown in certain models to depend on the JAK/STAT3 signaling pathway (15-17). We sought to examine the feasibility of regulating myeloid cell recruitment using a JAK 1/2 inhibitor (AZD1480) initially developed for the treatment of myeloproliferative disorders. AZD1480 was shown to restrict myeloid cell accumulation within the tumor microenvironment and impair tumor progression in murine models (18, 19). Here we demonstrated that the transition from low- to high-grade glioma was associated with increased BMDCs both within the circulation and tumor. Importantly, this process was potently blocked with AZD1480 treatment, leading to a survival advantage in murine models. These results suggest a novel therapeutic approach for the management of low-grade gliomas and inhibition of malignant progression.

**Materials and Methods**

**Cell Lines**

A Ntv-a RCAS high-grade cell line was generated through homogenization of high-grade tumors derived from explants harvested from 10 week old Ntv-a tumor bearing mice. Tumor tissues were minced and subsequently incubated at 37°C with PAPAIN (0.1mg/ml) in PIPES buffer with DNAase. Tissue suspensions were then gently shaken for 20 min. After quenching the digestion with Fetal Bovine Serum (FBS), the suspension was again disrupted with a pipette and passed through a 100 μm cell strainer (BD Biosciences). Cells were
collected by centrifugation (1300 rpm x 5 min) and seeded onto poly-L-lysine-coated petri-dishes with complete medium containing 10% FBS in Dulbecco’s Modified Eagle’s Medium (DMEM) overnight. Cell lines were subsequently grown and passed in complete medium. The cell line described above is not authenticated. Adherent GBM derived cell lines (U87 and U251) and Oligodendroglioma derived neurospheres have been previously described (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 Ntv-a model system was utilized as described previously. To derive glioma-bearing mice, Ntv-a (WT) mice were mated with Ntv-a Ink4aArf<sup>−/−</sup> LPTEN mice to produce offspring (22-26). RCAS-hPDGFB-HA and RCAS-Cre vectors were transfected into DF-1 chicken cells (ATCC, Manassas, VA) using the Fugene 6 transfection kit (Roche, Manheim, Germany). DF-1 cells transfected with RCAS-PSG were cultured in 10% FBS-DMEM media under standard conditions. For tumor induction, transfected DF-1 cells were trypsinized, centrifuged, and injected into the brain parenchyma of Ntv-a mice with a heterozygous Ink4a/Arf<sup>+/−</sup>, PTEN<sup>fl/fl</sup> 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, Billerica, MA) while anesthetized under 1-2% isoflurane (Baxter, Deerfield, Illinois). Images were acquired on a Bruker BioSpec
70/30 USR 7T (Bruker Biospin MRI, Inc, Billerica, MA) utilizing Gadolinium contrast at a 9:1 saline to Magnevist (Bayer Healthcare Pharmaceuticals Inc, Wayne, NJ) 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 1500 milliseconds with echo time = 17.4 milliseconds and an echo train length of 6. T2 repetition time was 2014 milliseconds with echo time = 48.4 milliseconds and echo train length of 10. For both T1 and T2, the field of view was 20 × 20 mm with slice thickness of 1.0 mm and spatial resolution of 0.078 x 0.078 mm/pixel. Tumor progression was characterized between 2 and 12 weeks or sacrifice.

Tumor Volumetric Analysis

T1 or T2 weighted MRI images representing axial sections of subject brains were uploaded into ImageJ software. The slice halfway through each animal’s data set, 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 mms 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 histological examination were sacrificed using carbon dioxide (CO₂) 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, Walldorf, Germany) and soaked in serial alcohol gradients.
Flow Cytometry Analysis

Flow cytometry analysis was performed as previously described (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 min at 4°C. Isolation of white blood cells was performed using a Ficoll-Paque Gradient (GE Healthcare) and stained with CD11b+ antibody (BD Biosciences 1:50). Blood collected from non-tumor controls was used solely for the purpose of comparison between low- and high-grade counterparts for circulating CD11b+ cells. Blood and bone marrow from mice were harvested and stained with CD11b (BD Biosciences 1:200) and GR1 antibodies (BD Biosciences 1:100).

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 x 10^6 bone marrow cells from BL6/GFP donor mice were transplanted into lethally irradiated (9.5 gy) 2 week old Ntv-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 O.C.T. (Electron Microscopy Sciences). Once embedding medium was in place, the O.C.T 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 Assay and Western Blot

RCAS high-grade cell line was cultured at a density of 10^5 cells per well in a 96 well plate (Corning, New York, NY). Using the RCAS high-grade cell line, AZD1480 was introduced at 1.0 μM concentration and cells were counted after co-incubation for both 48 and 72 h. U87 and U251 cells were cultured in complete medium, and the TS603 cells were cultured in Neurocult basel medium (Stemcell Technologies) containing neurocult
supplement, EGF, FGF and Heparin. To measure the cell growth, $3 \times 10^4$ to $5 \times 10^4$ cells were seeded per well with either AZD1480 (0.5 μM and 1 μM) or DMSO. Cells were then counted at 24 h, 48 h and 72 h time intervals. Each experiment was performed in triplicate and cell counting was performed using a hemacytometer.

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

**Immunohistochemistry**

Immunohistochemistry 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 min at RT. For CD11b staining, the sections were subjected to antigen retrieval using a Sodium Citrate buffer for 30 min and incubated with anti-CD11b (ab52478 1:100) for 25 min at RT. 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, Boston MA) and counterstained with DAPI. Immunofluorescence experiments were performed using Discovery XT processor (Ventana Medical Systems, Oro Valley, AZ) at the Molecular Cytology Core Facility (MSKCC). 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 400X 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's 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 previously described (28).

**Statistical Analysis and Quantification**

Data is represented as mean ± Standard Deviation (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’s 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⁺ BDMCs 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 to low-grade gliomas (42%) (Grade II) (Fig. 1A). Next, we examined if 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 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<sup>Ink4a+/−P14Arf+/−PTEN<sup>fl/fl</sup></sup>) (Fig. 1B, Supplemental Fig. 1A) (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 mice bearing no tumors, 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 to 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 to low-grade gliomas (Supplemental Fig 1B). Additionally, consistent with the literature, increased vascularity was observed by CD31+ staining in the high grade tumors (29) (Supplemental Fig. 1B).

These data demonstrate that the process of gliomas 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 recruited to the tumor microenvironment. In order to test this hypothesis and differentiate between recruited and resident CD11b+ cells, we transplanted GFP+ bone marrow into the PDGF murine glioma model (GFP negative) (Supplemental. Fig 2). 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, 2B). Additionally, the GFP+/BMDCs+ were found in a perivascular distribution (Fig. 2A).

In order to determine what proportion of these GFP+/BMDCs+ were myeloid-derived, we performed a GFP+/CD11b+ co-stain of tumor sections. Of the GFP+/BMDCs+ recruited population, approximately 33% of these cells were CD11b+ (Fig. 2C, Fig. 2D). 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 IL-6/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 expansion and mobilization of these cells with an inhibitor of this pathway could abrogate the transition from low- to high-grade gliomagenesis. In order to test this hypothesis, we utilized the transplanted GFP+ bone marrow in the PDGF murine glioma model (Fig. 3A, Supplemental. Fig 2). 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 to AZD1480-treated animals (Fig. 3B). To determine if tumors from AZD1480 treated mice harbored fewer BMDCs as compared to 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 to AZD1480 treated animals (Fig. 3C). These results demonstrate that AZD1480 blocks 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 co-stain with GFP+/CD11b+ on these tumors. Vehicle control treated animals demonstrated a (10-fold) increase in GFP+/CD11b+ cells within the tumor as compared to AZD1480 treated animals (Fig. 3D). Additionally, vehicle control animals demonstrated a (5-fold) increase of resident microglia (CD11b+/GFP+) when compared to 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 to vehicle control animals (Supplemental Fig. 3C,D). These data suggest that AZD1480 blocks recruitment of CD11b+ cells to the tumor, and decreases 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, non-transplanted 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 H&E staining of brain sections. MRI revealed reduced tumor burden in AZD1480-treated animals when compared to vehicle control treated animals (Fig 4B). Morphological examination of tumors from vehicle control animals by H&E confirmed features consistent with GBM including increased cellularity, pseudopalisading cytoarchitectecture, nuclear pleiomorphism, hemorrhage, and necrosis, along with microvascular proliferation and mitotic figures (Fig. 4B, Supplemental Fig. 3). In contrast, the AZD1480-treated animal brain tumors had decreased microvascular proliferation and no mitotic figures present and appeared less aggressive morphologically. (Fig. 4B, Supplemental Fig. 3A) In order to examine the effect of AZD1480 on 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 if transient or short-term treatment regimen would have an impact on survival while minimizing potential systemic toxicity. The transient or short-term treatment approach is 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) (27). The survival of AZD1480-treated animals when compared to 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 to 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 to low-grade gliomas (32) (Supplemental Fig. 4A). Given the paucity of pSTAT3 in low-grade tumors, our data suggests 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 suggests 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 to AZD1480-treated animals (Supplemental Fig. 4B).

PSTAT3 has been shown in previous studies to be highly expressed in tumor cells along with microenvironment cells including BDMCs (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 examined its effects on high-grade tumor cell proliferation in vitro (Supplemental Fig 4C). AZD1480 at 1.0 µM (which effectively blocked pSTAT3 expression) had no effect on cellular proliferation when compared to vehicle control-treated cells (Supplemental Fig. 4C,D). 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) (Supplemental Fig 4C,D). AZD1480 at 0.5 and 1.0 µM (which effectively blocked pSTAT3 expression) had no effect on cellular proliferation when compared to vehicle control-treated cells (Supplemental Fig. 4C,D).

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...
pro-angiogenic elements such as metallomatrix proteinase 9 (MMP-9) (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 RCAS 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 non-spontaneous mouse models (20).

We have studied the modulation of the glioma microenvironment through BMDC recruitment during transformative gliomagenesis 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-extrinsic therapies beyond early generation anti-VEGF antibodies. Our in vitro studies with AZD1480 showed no effect on cellular proliferation (Supplemental Figure 4 C-D), suggesting a tumor-extrinsic effect of targeting of BMDCs.

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 up-regulation 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 to those with low-grade gliomas (Fig 1A). We suggest cross-talk between the tumor and microenvironment via tumor cell secreted factors that leads to tumor progression. More studies are necessary to define these factors. Understanding the cross-talk between the tumor and microenvironment (BDMCs), particularly with respect to how AZD1480 influences the recruitment of BDMCs during the low- to high-grade transition is paramount. Using our RCAS murine glioma model with GFP+ bone marrow transplant, we also attempted to sort GFP+/BMDCs+ 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 BDMCs and subsequent studies should address this important point. The role of non-CD11b+ cells and resident microglia (BMDC+/GFPnegative) should also be further studied since infiltrating CD11b+ myeloid cells are only 33% of the recruited GFP+ population in our animal model.

In order to dissect the contribution of BDMCs during low- to high-grade transformation, bone marrow transplant studies were performed demonstrating the recruitment of GFP+ BDMCs, which correlated with high tumor burden. We observed a higher quantity of CD11b+ cells in both the periphery (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 histological analyses, radiographic findings, and overall survival in animals treated with AZD1480 compared to 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 promotes angiogenesis and invasion in high-grade gliomas (44). AZD1480 treated animals demonstrated decreased microvascular proliferation when compared to vehicle control animals. This data suggests AZD1480 may have an anti-angiogenic effect in our murine glioma model. Limitations of anti-angiogenic therapies have been described in the glioma literature including a lack of definitive data confirming anti-tumor 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 demonstrates 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 bone marrow-derived cell 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.

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**Author Contributions:**

P.R. conceived and developed the study with input from J.B. and J.G.

P.R., W.C, E.M., Y.H., J.Z performed experiments.


P.R., D.P. and J.G. collected and provided patient samples, T.C. and S.H. provided glioma cell line.

P.R., J.B. and J.G. wrote the paper.

J.B. and J.G oversaw the project.

All the authors discussed the results and commented on the manuscript.

**Competing Financial Interests:**

The authors declare no competing financial interests.
References


Figure Legends

Figure 1. Elevated CD11b+ expression in myeloid cells is associated with malignant human and murine gliomas. (A) Flow cytometric analysis of CD11b+ cells from the peripheral blood of patients with low (n=10) and high grade (n=10) glioma (WHO Grade II and IV) (n=20), ** p =0.01. (B) RCAS murine model depicting low to high-grade transition (3-12 weeks) and representative tumor histology at each stage. (C) Flow cytometric analysis of CD11b+/GR1+ cells in blood of RCAS mice bearing low (n=7) and high-grade tumors (n=9). Quantification of CD11b+/GR1+ cells at normal (n=4), low (n=7) and high grade tumor (n=9) stages, Student’s t-test, * P<0.05. (D) Flow cytometry analysis of CD11b+/GR1+ cells in bone marrow from RCAS mice bearing low (n=7) and high-grade tumors (n=9). Quantification of percentage of CD11b+/GR1+ cells at normal (n=4), low- (n=7) and high-grade (n=9) tumor stages is shown on the right. Student’s t-test, * p<0.05.

Figure 2. Bone marrow-derived cells (BMDCs) 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 50μm. (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 50μm (D) Quantification of cells labeled with GFP+, CD11b+, and co-stained (GFP+/CD11b+). Percentage of CD11b+/GFP+ cells.

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 post irradiation (2wks) 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 50μm, **p=0.004. (D) Representative photomicrographs of tumor cross sections from mice treated with vehicle control (n=5) and AZD1480 (n=5) co-stained 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 50μm, *p<0.05.

**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 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.
Figure 1

A

Human Peripheral Blood

Low Grade Glioma

High Grade Glioma

CD11b

FSC

**

% CD11b+ Cells

Low Grade

High Grade

B

Low to High Grade Glioma model (murine PDGF)

Injection (P3-P2) Low Grade High Grade

RCAS PDGF

C

Murine Peripheral Blood

No Tumor

Low Grade

High Grade

CD11b

Gr1

*

% CD11b+Gr1 (%)

D

Murine Bone Marrow

No Tumor

Low Grade

High Grade

CD11b

Gr1

*

% CD11b+Gr1 (%)

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Figure 3

A

B

C

D

CTRL (10 weeks) | AZD1480 (10 weeks)

T2

CTRL (10 weeks) | AZD1480 (10 weeks)

GFP DAPI

CTRL (10 weeks) | AZD1480 (10 weeks)

DAPI GFP CD11b

Flow/IF
Blood
Bone Marrow
Brain
for GFP+ BMDCs
Figure 4

A. Schematic representation of the experimental setup.

B. MRI images showing tumor volume changes over time.

C. Flow cytometry analysis of blood samples.

D. Survival analysis showing short-term treatment outcomes.

E. Survival analysis showing long-term treatment outcomes.
Malignant astrocytic tumor progression potentiated by JAK-mediated recruitment of myeloid cells


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