Tumors are capable of coopting hematopoietic cells to create a suitable microenvironment to support malignant growth. Here, we have demonstrated that upregulation of kinase insert domain receptor (KDR), also known as VEGFR2, in a myeloid cell sublineage is necessary for malignant progression of gliomas in transgenic murine models and is associated with high-grade tumors in patients. KDR expression increased in myeloid cells as myeloid-derived suppressor cells (MDSCs) accumulated, which was associated with the transformation and progression of low-grade fibrillary astrocytoma to high-grade anaplastic gliomas. KDR deficiency in murine BM-derived cells (BMDCs) suppressed the differentiation of myeloid lineages and reduced granulocytic/monocytic populations. The depletion of myeloid-derived KDR compromised its proangiogenic function, which inhibited the angiogenic switch necessary for malignant progression of low-grade to high-grade tumors. We also identified inhibitor of DNA binding protein 2 (ID2) as a key upstream regulator of KDR activation during myeloid differentiation. Deficiency of ID2 in BMDCs led to downregulation of KDR, suppression of proangiogenic myeloid cells, and prevention of low-grade to high-grade transition. Tumor-secreted TGF-β and granulocyte-macrophage CSF (GM-CSF) enhanced the KDR/ID2 signaling axis in BMDCs. Our results suggest that modulation of KDR/ID2 signaling may restrict tumor-associated myeloid cells and could potentially be a therapeutic strategy for preventing transformation of premalignant gliomas.
A proangiogenic signaling axis in myeloid cells promotes malignant progression of glioma

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Tumors are capable of coopting hematopoietic cells to create a suitable microenvironment to support malignant growth. Here, we have demonstrated that upregulation of kinase insert domain receptor (KDR), also known as VEGFR2, in a myeloid cell sublineage is necessary for malignant progression of gliomas in transgenic murine models and is associated with high-grade tumors in patients. KDR expression increased in myeloid cells as myeloid-derived suppressor cells (MDSCs) accumulated, which was associated with the transformation and progression of low-grade fibrillary astrocytoma to high-grade anaplastic gliomas. KDR deficiency in murine BM-derived cells (BMDCs) suppressed the differentiation of myeloid lineages and reduced granulocytic/macrophagic populations. The depletion of myeloid-derived KDR compromised its proangiogenic function, which inhibited the angiogenic switch necessary for malignant progression of low-grade to high-grade tumors. We also identified inhibitor of DNA binding protein 2 (ID2) as a key upstream regulator of KDR activation during myeloid differentiation. Deficiency of ID2 in BMDCs led to downregulation of KDR, suppression of proangiogenic myeloid cells, and prevention of low-grade to high-grade transition. Tumor-secreted TGF-β and granulocyte-macrophage CSF (GM-CSF) enhanced the KDR/ID2 signaling axis in BMDCs. Our results suggest that modulation of KDR/ID2 signaling may restrict tumor-associated myeloid cells and could potentially be a therapeutic strategy for preventing transformation of premalignant gliomas.

Introduction

The emerging role of BM-derived cells (BMDCs), including myeloid-derived suppressor cells (MDSCs), dendritic cells, neutrophils, and macrophages (1–6), has become a focus of recent research in malignant progression. Numerous studies have demonstrated that tumors directly influence hematopoietic cells toward protumoral phenotypes via complex molecular signaling pathways (4, 7). Interactions with stromal components or tumor cells allow BMDCs to promote angiogenesis, resistance, and invasion in various types of cancers, including brain tumors (5, 8).

Gliomas represent the most prevalent malignant brain tumors and are classified by WHO as grade I, II, III, or IV. WHO grades are uniquely defined by the presence of specific pathological features, including cell proliferation, necrosis, and the extent of angiogenesis (9). In comparison with their low-grade (grade II) counterparts, high-grade gliomas (grade III/IV) are characterized by microvascular proliferation and necrosis (9–11). While a percentage of high-grade gliomas develop de novo, these tumors may also result from malignant transformation of lower grade tumor variants (9, 10, 12). Patients with low-grade gliomas have an increased chance of cure using aggressive therapies, including total surgical resection and chemotherapy, and may live many progression-free years with residual tumor that cannot safely be removed through gross total resection (10, 12, 13). Once low-grade tumors undergo malignant transformation to a higher grade, however, prognosis often mirrors de novo diagnosis of high-grade gliomas and often leads to poor clinical outcomes (14, 15).

A more thorough understanding of the tumor microenvironment, particularly the role of BMDCs towards malignant glioma transformation, has substantial biological and clinical relevance insofar as arresting malignant progression has implications for long-term disease survival. In this study, we found that kinase insert domain receptor (KDR), also known as VEGFR2, is responsible for driving differentiation of hematopoietic progenitor cells (HPCs) into protumoral MDSCs. Through enhanced signaling of hematopoietic-derived inhibitor of DNA binding protein 2 (ID2), a member of the inhibitor of DNA binding proteins class, primary tumors direct myeloid progenitors to develop into tumor-associated BMDCs via upregulation of KDR and, in turn, stimulate the aggressive proangiogenic phenotype of gliomas.

Results

Intracellular KDR in glioma-associated myeloid cells. KDR has been identified in both endothelial and endothelial progenitor cells
within various models of tumor malignancy (2, 16–18). Recently, KDR was found to be expressed by BM-derived plasmacytoid dendritic cells (19), suggesting it may have a broader impact on tumor microenvironment than previously thought. To study the expression pattern of KDR in gliomas at different stages, we utilized \textbf{KdrGFP} mice, with \textbf{GFP} knocked into the \textbf{Kdr} locus, crossbred with \textbf{Nestin-tva Ink4a/Arf–/– Ptenfl/fl} mice. The replication-competent avian sarcoma-leukosis virus (ASLV) long terminal repeat (LTR) with a splice acceptor/tumor virus A (RCAS/TVA) system was used to induce PDGF/protein kinase B–driven (PDGF/Akt-driven) gliomas from neural progenitor cells in postnatal mice (20, 21). These mice develop low-grade gliomas that invariably progress to higher grades over a reproducible 12-week time course. Initially, at approximately week 5, the tumors mimic fibrillary astrocytomas, which are equivalent to the low-grade human grade II tumors but subsequently progress to anaplastic astrocytomas (grade III) and, by weeks 8–12, progress to malignant gliomas akin to human glioblastoma or WHO grade IV tumors. Use of the RCAS/TVA model allowed monitoring of KDR expression throughout both the formation and malignant progression of gliomas. During tumor progression from a low-grade stage (~week 5) to a high-grade stage (~week 8), the population of CD11b+KDRGFP+ cells within the peripheral blood increased 5-fold (Figure 1, A and B). During this transition, tumor-associated macrophages within tumor tissue were also increased 5-fold (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI86443DS1), mirroring the elevated populations of MDSCs (Supplemental Figure 1B). Within the CD11b+KDRGFP+ population in peripheral blood, the majority of cells phenotypically resemble MDSCs (Ly6G+ or Ly6C+). Approximately 60% of CD11b+KDRGFP+ cells had monocytic features versus 15% of the CD11b+KDRGFP– cells (Ly6C+; Figure 1, C and D). Among BM progenitor cells, more than 80% of Lin–KDRGFP+ cells were hematopoietic stem cells (HSCs) (Lin–/c-Kit+/Sca-1+) or HPCs (Lin–/c-Kit–/Sca-1–) (Supplemental Figure 1, C and D).
To further validate that KDR-expressing hematopoietic cells are restricted to the myeloid lineage, we isolated KDR<sup>GFP</sup>+/Lin– hematopoietic cells and performed a colony formation assay, which demonstrated that these cells were capable of forming myeloid-derived colonies. Compared with lineage-negative WT hematopoietic cells (Lin–WT), which demonstrated the potential to form all granulocyte-macrophage, granulocyte, and macrophage colonies, KDR<sup>GFP</sup>/Lin– cells were rarely found to potentiate granulocytic colonies (Figure 1E).

To investigate the expression pattern of KDR throughout the process of myeloid differentiation, Lin– hematopoietic cells were grown in tumor cell line–conditioned (GL261) medium for 1 week. Compared with values on day 0, the undifferentiated stem cell marker c-Kit was downregulated beginning on day 1 of differentiation, whereas differentiatied cell marker CD11b and late-stage differentiation marker CD68 were upregulated (Figure 1F). KDR expression was maximal on day 1 and day 2 in the early differentiation phase of myeloid lineage cells; however, expression began to drop thereafter, suggesting a role for KDR in the early programming of myeloid progenitors toward a protumoral lineage.

Myeloid KDR expression was also assessed in glioma patients. As part of this study, peripheral blood samples were prospectively collected for 3 years from a cohort of glioma patients diagnosed with different tumor grades. We detected significant amounts of KDR within permeabilized leukocytes of high-grade glioma patients. As shown in Figure 2A, there was a defined CD11b<sup>+</sup>KDR<sup>+</sup> population expressing KDR. Within each sample, the ratio of CD11b<sup>+</sup>KDR<sup>+</sup> versus CD11b<sup>+</sup> myeloid cells was quantified and plotted within each of the WHO-recognized pathological grades. The percentage of KDR<sup>+</sup> myeloid cells was significantly higher in blood samples derived from patients with high-grade gliomas as compared with samples from low-grade glioma patients (Figure 2, A and B). Specifically, the levels of CD11b<sup>+</sup>KDR<sup>+</sup> cells were higher in patients with grade III or IV tumors than they were in patients with grade I tumors and healthy controls (Figure 2B). Among all patients, 2 grade III patients and 4 grade IV patients had received chemotherapy, 7 grade IV patients had received steroids, and none had received radiotherapy or targeted therapy by the time of sample collection. Importantly, the elevated levels of CD11b<sup>+</sup>KDR<sup>+</sup> cells were not induced by treatments that patients had received (Supplemental Figure 2, A and B).

Perhaps the most striking of our findings was the apparent delineation of 2 distinct populations of patients, each with pathologically defined grade II astrocytoma. We divided patients with grade II astrocytoma into 2 subgroups, those with high versus those with low CD11b<sup>+</sup>KDR<sup>+</sup> values (cut-off of 1.0%). The clinical course and medical histories of the patients were closely followed over 12 months. Disease progression in patients with grade II glioma was assessed by MRI and subsequent histological diagnoses of grade III or IV glioma (Supplemental Figure 2C). The progression-free survival of our cohort of grade II patients is shown in Figure 2C. Patients with an elevated percentage of CD11b<sup>+</sup>KDR<sup>+</sup> (KDR<sup>Hi</sup>) myeloid cells presented with a significantly higher likelihood of tumor progression from fibrillary to anaplastic variants (Figure 2C). This recognition of a subclass within grade II astrocytomas represents what we believe is a novel finding as well as a prospective paradigm shift from observation to earlier active therapy in the treatment of patients with low-grade gliomas.

Complex interactions between the host immune system and malignant tumors are an essential component of how the tumor microenvironment is thought to modulate oncologic growth and transformation (1, 11). Our findings suggest that high-grade glio-
The distribution of KDR on endothelial cells was also shown on HUVECs and high-grade murine glioma tissue (Supplemental Figure 3, D and E). Immunoblotting of KDR on different cellular fractions of both myeloid and endothelial cells revealed that myeloid-derived KDR was intracellular and intact (Figure 2D).

**KDR controls differentiation of protumoral myeloid cells**. The observation that KDR expression was temporally associated with differentiation of tumor-associated myeloid cells prompted us to closely examine the role of BM-derived KDR in the immunomodulation of tumor microenvironments. Patients with more malignant phenotypes (Supplemental Figure 2, D and E). In contrast to the typical cell surface distribution of KDR in endothelial cells, our results show that KDR resides primarily within the cytoplasm of leukocytes (Supplemental Figure 3A and B). KDR expression was not detected at significant levels in nonpermeabilized leukocytes, indicating that KDR is mainly intracellular in its localization (Supplemental Figure 3C).
diethylenetriamine penta-acetic acid (gadolinium-DTPA) and gadolinium-albumin, which closely approximates blood flow (Supplemental Figure 6, A and B). Quantification of MRI on day 18 showed that brain tumors in the KDRfl/fl animals were 25% smaller and showed an 80% decrease in their contrast enhancement when compared with tumors in control animals (Supplemental Figure 6, C and D).

Endothelial and pericyte staining within tumors grown in KDRfl/fl mice demonstrated marked differences in blood vessel morphology, vasculature size, and pericyte coverage compared with those in the control group (Supplemental Figure 7, A and B). Wide-field tumor histology in tumors from KDRfl/fl mice demonstrated less hemorrhage at the tumor core and a less invasive tumor margin when compared with those in KDRfl/+ mice (Supplemental Figure 7C).

We noted that the microenvironment of xenograft brain tumor models may not always accurately reflect the complexity of invasive de novo brain tumors and thus approximated this intricacy more accurately by challenging findings from the GL261 allograft system using the spontaneous RCAS/TVA murine brain tumor model (20). In order to examine the role of KDR in BMDCs from RCAS/TVA glioma mice, we irradiated and transplanted RCAS/TVA mice at week 2 with Rosa26-CreERT2Kdrfl/fl or Rosa26-CreERT2Kdrfl/+ BMDCs from littermate donor mice. Tamoxifen was administered to induce targeted deletion of the KDR locus specifically within BMDCs before host animals first showed evidence of astrocytic tumors at approximately week 5. Beginning at week 6, recipient RCAS/TVA mice were monitored for tumor progression using weekly MRI scans and histological samples from representative sacrificed mice.
were analyzed to confirm anaplasia or malignant transformation when scans were suggestive of transformation (Figure 3A). Three cohorts of RCAS/TVA mice (i.e., those that received Rosa26-CreERT2Kdrfl/fl or control Rosa26-CreERT2Kdrfl/+ BM and those that did not undergo irradiation-transplantation) were analyzed based on their symptoms associated with late-stage glioma. These symptoms included hydrocephalus, lethargy, and weight loss. Kaplan-Meier symptom-free survival curves were utilized to demonstrate glioma progression in the 3 cohorts. Median symptom-free survival time (MSST) was 11 weeks for RCAS/TVA mice transplanted with control Rosa26-CreERT2Kdrfl/+ BM versus 32 weeks for RCAS/TVA mice transplanted with Rosa26-CreERT2Kdrfl/Kdr BM. For RCAS/TVA mice not treated with irradiation or a bone marrow transplant (BMT), MSST was 6 weeks (Figure 3B). We compared brain tumors in RCAS/TVA mice at weeks 6, 9, and 12 using MRI followed by histological examination (Figure 3C). Knocking out Kdr in BMDCs...
substantially delayed the malignant transformation of low-grade gliomas to higher grade states. By week 9, 60% of RCAS/TVA mice transplanted with control Rosa26-CreERT2Kdrfl/+ BM developed high-grade tumors that had typical malignant features, including robust gadolinium enhancement on MRI, pseudopalisading necrosis, and microvascular proliferation (Figure 3, D and E). In contrast, tumors in 70% of RCAS/TVA mice transplanted with Rosa26-CreERT2Kdrfl/fl BM remained in the low-grade stage without signs of malignant transformation at week 9 (Figure 3, C–E).

**Figure 6.** ID2 promotes proangiogenic phenotypes of tumor-associated myeloid cells. (A) Chimeric C57BL/6 mice transplanted with Id2+/+ BM cells (Id2+/+ BM cells as control) were implanted with luciferase-labeled GL261 tumors intracranially. Tamoxifen was applied at day 3 after implantation. The tumors were monitored by bioluminescence. Representative images were taken at day 14. The color bar on the right represents photon intensity. The experiments had 2 replicates. The tumor growth curve is based on bioluminescence. n = 10. **P < 0.01, 1-way ANOVA.** (B) Flow cytometry analysis of peripheral blood cells on CD11b, Ly6C, and Ly6G in Id2+/+ BMT and Id2–/– BMT groups. Quantification of CD11b+ cell frequency out of total white blood cells and of Ly6G+ cell frequency out of CD11b+ cells. **P < 0.01, 1-way ANOVA, for each group.** n = 7. (C) Tubule formations of HCMEC/D3 (GFP) cocultured with lineage-negative Id2+/+, Id2–/–, Id2–/– scrambled sequence (SC) control, or Id2–/– KDR overexpression (OE) HPCs pretreated with TGF-β1/GM-CSF. Quantifications of tubule lengths in the indicated group. ***P < 0.0001, 1-way ANOVA. n = 6. (D) Growth factor–reduced Matrigel plugs of lineage-negative Id2+/+, Id2–/–, Id2–/– SC CTL, or Id2–/– KDR overexpression HPCs pretreated with TGF-β1/GM-CSF. Blood vessels (red) were perfused with rhodamine-dextran. Lower panels show magnified views to highlight vascular permeability. Quantifications of based blood vessels density (BVD) in each group. **P < 0.01, 1-way ANOVA. n = 6. Data are represented as mean ± SD. Scale bars: 50 μm (D, upper panels); 20 μm (C, D, lower panels).

ID2 mediates KDR expression and granulocytic/monocytic cell differentiation. Our data demonstrate that KDR contributes to the differentiation of proangiogenic myeloid cells and, more importantly, suggest that these cells play a major role in the modulation of gliomas. Within this context, we sought to examine the mechanistic and functional role KDR has in directing the hematopoietic fate of KDR-expressing BMDCs in protumorigenic immune cells.

We began by analyzing myeloid cell differentiation either in the presence or absence of KDR throughout distinct phases of tumor
progression. BM cells harvested from GFP* Rosa26-CreER<sup>T2</sup>Kdr<sup>fl/fl</sup> and GFP Rosa26-CreER<sup>T2</sup>Kdr<sup>+/−</sup> mice were mixed in a 1:1 ratio and transplanted into recipient mice. These mice were subsequently implanted with orthotopic GL261 tumors. Populations of myeloid cells, T cells, and B cells residing in the peripheral blood are shown in Figure 4, A–D. Populations of HSCs and myeloid progenitors residing in BM are shown in Figure 4, E–H. After BM engraftment, GFP* Rosa26-CreER<sup>T2</sup>Kdr<sup>fl/fl</sup> BM cells reconstituted 35%–40% of hematopoietic cells in both the BM and peripheral blood, whereas the remainder contained GFP* Rosa26-CreER<sup>T2</sup>Kdr<sup>+/−</sup> BM cells as well as the remaining host hematopoietic cells not eliminated by the radiation protocol (Figure 4, A and E). Compared with the control GFP population in peripheral blood, the GFP* population had fewer total CD11b<sup>+</sup> cells (Figure 4B and Supplemental Figure 8A), which contained fewer Ly6C<sup>+</sup> cells (Figure 4C and Supplemental Figure 8B). There was no significant difference in T cell (CD3<sup>+</sup>) and B cell (B220<sup>+</sup>) lineages between GFP* and GFP* populations (Supplemental Figure 8, G and H). Within the BM, the GFP* population had levels of lineage-negative cells and HSCs (Lin−c-Kit+Sca1+) that were comparable to those in the GFP population (Figure 4, F and G, and Supplemental Figure 8, C and D). Interestingly, within HPCs (Lin−c-Kit+Sca1), the GFP* population had significantly more common myeloid progenitor cells (Lin−c-Kit+Sca1 FcγR CD34<sup>+</sup>) than the GFP− population in peripheral blood, the GFP* population had significantly more committed BMDCs from ID2-competent or ID2-incompetent BMDCs (Supplemental Figure 8I).

To further define the KDR signaling network responsible for directing differentiation down a myeloid lineage, we performed gene-expression profiling of KDR-expressing (KDR<sup>GFP</sup>) HPCs (Lin−c-Kit+) using mRNA sequencing. Differentially expressed genes from KDR<sup>GFP</sup> versus KDR<sup>−/−</sup> HPCs were also clustered and displayed using a heat map (Figure 5A), and similar categories were grouped accordingly (Figure 5B). Candidate genes (P < 0.001, >2-fold change) were divided according to subsets with the highest expression, and categories with significant enrichment (P < 0.001) in Gene Ontology (GO) were derived using DAVID tools (22) (Figure 5C). Id2 was identified as a markedly upregulated gene in KDR<sup>GFP</sup>-expressing HPCs. As a well-defined transcriptional regulator controlling the timing of cell-fate determination and the differentiation of stem and progenitor cells, Id2 was a strong candidate for an upstream modulator of myeloid differentiation through KDR signaling. To validate the upregulation of Id2 in HPCs, we performed real-time PCR on KDR<sup>GFP</sup> HPCs and Ly6C<sup>−</sup>/Ly6G<sup>−</sup> myeloid cells from tumor-bearing or naive mice. KDR<sup>GFP</sup> HPCs from tumor-bearing mice had higher Id2 levels than did KDR<sup>−/−</sup> HPCs and HPCs from naive mice (Figure 5D). The expression of Id2 was generally lower in myeloid cells than in HPCs. We also examined the expression of Id2 in glioma patients. HPCs and CD11b<sup>+</sup> cells were isolated from both healthy controls and low- and high-grade glioma patients. HPCs and CD11b<sup>+</sup> cells from high-grade glioma patients had significantly higher levels of ID2 when compared with those from healthy controls and low-grade glioma patients (P < 0.0001, Figure 5, E and F).

Id2 functions by inhibiting the binding of E proteins to DNA (23). To investigate whether Id2 interacted with E proteins to regulate cell fate within a myeloid lineage, we analyzed the promoter region plus the 5′ UTR of Kdr for putative E protein-binding sites, known as E boxes. We identified a likely binding site for E2A (Supplemental Figure 9A) within the upstream promoter region of the Kdr gene. To conclusively establish the ID2/E protein/Kdr interaction, we performed a ChIP assay using E2A antibody in Id2<sup>+/+</sup>, Id2 overexpressing, and Id2<sup>−/−</sup> BM cells. As expected, the anti-E2A antibody was able to successfully pull down the upstream fragment of Kdr. Overexpression of ID2 abolished the binding of E2A to the promoter of the Kdr gene (Supplemental Figure 9B); however, the binding capacity of E2A was comparable between the Id2<sup>+/+</sup> and Id2<sup>−/−</sup> groups. We hypothesize that the basal level of ID2 in WT cells is not high enough to release E2A from DNA.Granulocyte-macrophage CSF (GM-CSF) and TGF-β induced expression of Id2 and Kdr in lineage-negative BM cells (Supplemental Figure 9C), similarly to conditioned medium derived from GL261 cells, blocking either reversed Id2 or KDR expression. These results support the hypothesis that glioma-secreted GM-CSF and TGF-β initiate protumoral myeloid cell differentiation, directing formation of the KDR<sup>+</sup> protumoral immune cell population, which plays a crucial role in tumor microenvironment and malignant glioma transformation. As shown in Supplemental Figure 9D, both total KDR and activated KDR were abolished by Id2 deficiency in BMDCs.

Id2/KDR axis directs proangiogenic function of myeloid cells. MDSCs are key players in antitumor immunity and angiogenesis in several reported malignancies (24–27), and the data from our patient cohort and animal models support a central role for these cells in the transformation of gliomas. We wanted to further understand the contribution of the ID2 signaling axis in regulating the differentiation of protumoral and angiogenic myeloid cells within the context of glioma immunomodulation. To begin this analysis, C57BL/6 mice that received Id2<sup>+/+</sup> or Id2<sup>−/−</sup> BMTs were orthotopically implanted with GL261 tumor cells. GL261 astrocytic tumors grew more slowly in mice with Id2<sup>−/−</sup> BM compared with those with Id2<sup>+/+</sup> BM (P < 0.01, Figure 6A). Fewer CD11b<sup>+</sup> myeloid cells and, remarkably, fewer CD11b<sup>+</sup> Ly6C<sup>−</sup> cells were recruited to tumors in mice with Id2<sup>−/−</sup> BM compared with those with Id2<sup>+/+</sup> BM (P < 0.01, Figure 6B). While the endothelial-derived VEGF/KDR signaling axis is well established within the regulation of tumor angiogenesis (28), its role in proangiogenic myeloid differentiation has not been previously established.

To determine candidate downstream targets of ID2 pathway signaling, we employed real-time PCR (RT-PCR), utilizing in vitro differentiated BMDCs from ID2-competent or ID2-incompetent tumor-bearing animals. Among target genes identified in this assay (Supplemental Figure 10A), we observed downregulation of matrix metallopeptidase 9 (Mmp9), hypoxia inducible factor 1α (Hif1α), and angiopoietin-2 along with Kdr in Id2<sup>−/−</sup> BMDCs compared with Id2<sup>+/+</sup> BMDCs (P < 0.01, Supplemental Figure 10, B and C). These molecules have been previously described as essential components of the tumor microenvironment and play an important role in vascular remodeling and angiogenesis (29–31). As shown in Supplemental Figure 10D, Mmp9, Hif1α, and angiopoietin-2 were upregulated in CD11b<sup>+</sup> blood cells of mice with high-grade tumors compared with those with low-
grade tumors. These proangiogenic factors are also significantly higher in KDR+ myeloid cells than those in KDR-myeloid cells (Supplemental Figure 10E). To further define the proangiogenic functions of the ID2/KDR pathway within our identified myeloid cell population, we performed in vitro tube-forming assays under different conditions. We coincubated GFP-brain endothelial cells (HCMEC) with previously in vitro–differentiated Id2+/+ BMDCs. In order to determine whether KDR overexpression would have a discernible effect on myeloid cells in the absence of ID2, Id2–/– BMDCs were also transduced by lentivirus over-expressing either a scrambled control or the WT Kdr gene. ID2-expressing BMDCs strikingly promoted tube formation; however, ID2-deficient BMDCs were unable to support the formation of endothelial tubules. KDR overexpression allowed BMDCs to regain their protubule-promoting capability (P < 0.0001, Figure 6C). In parallel, we performed an in vivo Matrigel plug assay utilizing the same BMDC conditions. Id2–/– BMDCs demonstrated potent induction of neovascularization in Matrigel plugs (P < 0.01, Figure 6D), whereas loss of ID2 compromised the proangiogenic functions of BMDCs. Once again, overexpression of KDR rescued the proangiogenic phenotype of BMDCs. Interestingly, while the overexpression of KDR in Id2–/– BMDCs showed enhanced proangiogenic capability, the blood vessels induced by Id2–/– BMDCs with overexpression of KDR were noticeably leakier than were those induced by WT BMDCs, as was evidenced by permeability of dextran. While we demonstrated that overexpression of KDR in BMDCs convincingly rescues the angiogenic deficiency induced by loss of the Id2 gene, the histoarchitecture remains imperfect and warrants further investigation.

Discussion

KDR has been previously described as a marker for endothelial progenitor cells and has been considered a specific marker for endothelial progenitors within BMDCs (1, 18, 32–34). Recently, however, KDR was identified in plasmacytoid dendritic cells during innate immune responses, suggesting additional roles for KDR in physiological processes (19). Using multiple complimentary approaches, we have demonstrated the expression and unique role of KDR in myeloid cells. Despite its lower expression levels in BMDCs compared with endothelial cells, KDR in BMDCs controls the differentiation of HPCs into proangiogenic myeloid cells, thereby directly affecting the composition of the tumor microenvironment. The majority of KDR-expressing BMDCs are CD11b+Gr–1+ (Ly6C+Ly6G+) cells, also known as MDCS (6, 35). Besides the canonical immunosuppressive role of MDCS, we observed distinct phenotypes on tumor-associated vascular development when KDR was selectively deleted from BM cell populations. Within the tumor microenvironment, macrophages and neutrophils have been shown to have robust proangiogenic capabilities in multiple disease states (4, 24, 36). Until now, the mechanisms by which MDCS differentiate into tumor-associated macrophages or neutrophils have not been convincingly demonstrated (26, 37); however, in our study, elimination of KDR-expressing BMDCs yielded fewer myeloid cells and normalized blood vessels within the tumor microenvironments. Our results suggest that KDR-mediated proangiogenic myeloid cells are crucial for malignant progression in fibrillar astrocytomas.

Previous research has demonstrated that KDR can also be found in the cytosol and nucleus in addition to its known localization on the cell membrane. KDR translocates to the inside of cells upon VEGF stimulation in a process that requires phosphorylation of the receptor (28, 38, 39). It has become clear that endocytosis and intracellular trafficking of KDR is key to the activation of VEGFR signaling (40–42). In connection with their proangiogenic functions, tumor-associated myeloid cells and myeloid progenitors serve as a major source of VEGF in many types of malignant lesions (4, 43). Given this information, we hypothesize that high concentrations of VEGF within myeloid cells restrain its receptor, KDR, from being localized on the cell surface. Instead, the majority of KDR remains within intracellular compartments of myeloid cells in an activated state.

Recent studies have demonstrated that malignant lesions polarize macrophages and neutrophils within the tumor microenvironment and redirect them toward tumor-promoting phenotypes (24, 44, 45). Prior to arriving at and populating tumor tissues, BMDCs receive soluble tumor-directed signaling (7, 18, 46). As they progress, malignancies evade immune surveillance by promoting immunosuppressive cells and suppressing antigen-presenting cell functions (26, 47). Primary tumors also prime BMDCs to facilitate neovascularization, allowing for exponential growth. Infiltration of several types of proangiogenic BMDCs, such as Tie-2–expressing monocytes and c-Met+ hematopoietic progenitors, increases as primary tumors progress (7, 46). Since these proangiogenic myeloid cells appear to have a direct role within malignant transformation, they may serve as prognostic markers of early transformation in cancer patients with low-grade gliomas (48–50). For the majority of solid tumors, staging and prognosis are based upon direct tumor biopsies, which lack meaningful information about the tumor microenvironment (50). After surgery, most patients receive radiation therapy, chemotherapy, and/or targeted therapy, from which the tumor microenvironment may be affected dynamically and extensively. Considering the high incidence of tumor transformation, progression, and recurrence within patients with low-grade gliomas, it is important to consider real-time monitoring approaches in addition to MRI imaging in glioma patients.

Inhibitors of DNA-binding proteins have been shown to play important roles in mediating hematopoietic differentiation in physiological settings (23, 51). It has been shown, for example, that ID2 serves as a suppressor of lymphoid and B cell development (52, 53); however, the role of ID2 in myeloid differentiation within malignancies has not been as well described in the literature (6, 7). In this study, we have demonstrated that E2A, the binding partner of ID2 (23, 53), directly binds the Kdr promoter region, illuminating a crucial link between ID2 and myeloid differentiation. We have shown that enhanced ID2/KDR signaling, induced by primary glioma, promotes the differentiation of MDSCs, particularly along a monocytic lineage. These results have expanded upon the previous understanding of KDR as simply an endothelial activator and elucidated a role for KDR as a molecular regulator responsible for directing proangiogenic myeloid cell differentiation. This suggests that ID2 may play an important part in promyeloid MDSC formation. Targeting the ID2/KDR axis within myeloid-derived cells may serve as an extratumoral therapeutic approach for suppressing glioma progression through immunomodulation of the tumor microenvironment.
Methods

Cell lines and mouse lines. C57BL/6, Rosa26-CreERT2, Id2-GFP–housekit, and Ubc-GFP mice were obtained from The Jackson Laboratory. Kdr (Vegfr2)fl/fl, KDR-GFP–housekit, Rosa26-CreERT2, KdrΔ6 (Vegfr2Δ6), and Ubc-GFP mice on a C57BL/6 background were obtained from Shahin Rafii. Littermate controls were used in all animal experiments. Rosa26-CreERT2 mice were crossed with KdrΔ6 mice to generate Rosa26-CreERT2KdrΔ6 mice. The littermates of Rosa26-CreERT2KdrΔ6 mice served as controls. GL261 cells were obtained from David Zagzag (Department of Pathology, New York University, New York, New York, USA). HCMEC cells were obtained from Babette Wexler (Department of Medicine, Weill Cornell Medicine).

RCAS-TVA glioma model and symptom-free survival analysis. RCAS-hPDGFb-HA-SV40 containing human PDGFb with partially deleted 59UTR, RCAS-hPDGFb-HA, and RCAS-Cre vectors were transfected into DF-1 chicken cells (ATCC). 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 n-tva mice on a heterozygous Ink4a/Arf+/Pten+/− background (obtained from Eric Holland) at PO–P2. The mice transgenically expressed the RCAS-binding tva receptor after the nestin promoter. For BMT study, the RCAS/TVA mice at week 2 were subject to 9.5 Gy irradiation with their heads shielded and transplantation of donor BM cells through retroorbital injection as well as the procedures described in the BMT section. Upon appearance of brain tumor symptoms (e.g., poor grooming, lethargy, macrocephaly, hydrocephaly, hemiparesis, substantial weight loss), mice were anesthetized by intraperitoneal injection of 1 mg/kg Nembutal solution or a ketamine (150 mg/kg)/xylazine (15 mg/kg) cocktail and underwent transcardiac perfusion with 10 ml ice-cold heparin in normal saline, followed by 10 ml ice-cold 4% paraformaldehyde. Brain tissue was extracted and postfixed for 30 minutes in ice-cold 4% paraformaldehyde and transferred to 30% sucrose at 4°C for cryoprotection. For a subset of animals sacrificed using CO2, brain tissue was extracted and fixed for 3 days in 4% paraformaldehyde and subsequently processed using standard paraffin embedding and processing. Kaplan-Meier curves for symptom-free survival were made using Prism software and analyzed with a standard log-rank test. The end-point was defined as the time mice had developed symptoms such as hydrocephaly and lethargy.

Genotyping. Genotyping methods, primer sequences, and PCR conditions were as follows: Rosa26-CreERT2: WT forward, 5′-AAAGTC-GCTCTGATGGTGTAT-3′; WT reverse, 5′-GGAGGCGGGAGAATG-GATATG-3′; mutant reverse, 5′-CCTGATCTGGCAATTTCCG-3′; 94×30 seconds, 65×30 seconds, 72×60 seconds, 35 cycles, WT band 650 bp, mutant band 825; KdrΔ6: forward, 5′-CCACAGAACAACTCAGG-GATAATG-3′; reverse, 5′-GGAGGCGGGAGAATG-GATATG-3′; 94×30 seconds, 60×30 seconds, 72×60 seconds, 35 cycles, WT band 158 bp, with LoP site 231 bp; Id2-GFP–housekit: primer 12341, 5′-CTTCCTCTCAGAGGAGCAT-3′, primer 12342, 5′-CTACCTCTGAAGAGCATGAT-3′, primer 12343, 5′-GCTCTCTGAGAGCTAAG-3′; 94×30 seconds, 59×60 seconds, 72×60 seconds, 35 cycles, WT band 371 bp, mutant band 750 bp; GFP: forward, 5′-CTCAGCTGCCGACACACCA-3′; reverse, 5′-TCCAGCAGGACCATGATGAC-3′.

Intracranial injections and MRI/bioluminescence imaging. Luciferase-labeled GL261 cells (1 × 106) were stereotactically inoculated into the right hemispheres of 8-week-old mouse brains (1.5 mm posterior to the bregma and 1.5 mm to the right of the sagittal suture to a depth of 2.5 mm below the surface of the skull using a 1 μl Hamilton syringe). Mice were euthanized when they presented with neurological symptoms or substantial weight loss. Mice were examined and weighed daily. Bioluminescence and MRI were performed to monitor the progression of tumors. Bioluminescence was performed under the IVIS-100 (Xenogen) system, and images were acquired and analyzed by Living Image (Caliper). High-resolution MRI images were acquired on a 7-T animal scanner system (Bruker Biospin). 200 μl of 40 mM Gd-DTPA or Gd-albumin was injected by tail vein to measure brain tumor contrast enhancement. During imaging, animals were under isoflurane anesthesia and monitored for respiration and heart rate; the temperature was kept at 37°C. Images were analyzed by an OsiriX DICOM viewer.

Tamoxifen administration. To induce KDR gene ablation, at day 3 after injection, mice with intracranially implanted tumor cells were treated with tamoxifen (20 mg/kg intraperitoneally) for 6 days, interrupted for 3 days after the third dose. After 3 days of respite, the fourth dose was reinstituted for an additional 3 days, resulting in Rosa26-CreERT2Kdrlox/fox KdrΔ6 mice that were deficient in KDR at both alleles. The control animals were Rosa26-CreERT2KdrΔ6 mice.

BMT and competitive BMT. Recipient mice were lethally irradiated with a single dose of 9.5 Gy applied to their whole bodies with their heads lead shielded; 24 hours after irradiation, 5 × 106 total BM cells were injected by tail vein. The BM of donor mice was harvested by flushing femurs and tibias with cold sterile EDTA/PBS. Engraftment was confirmed by complete blood count using a Hemavet 850 blood cell counter (Drew Scientific) and flow cytometry to ensure adequate hematopoiesis. For competitive BMT, we mixed donor BM cells from Ubc-GFP Rosa26-CreERT2 KdrΔ6 mice and Rosa26-CreERT2KdrΔ6 mice at a 1:1 ratio and injected 5 × 106 total cells into recipient mice; 3 days after tumor implantation, we treated mice with tamoxifen to induce the deletion of the Kdr allele.


Cell preparation and flow cytometry (intracellular staining). Hema-topoietic cells isolated from buffy coats were subjected to trypsin blue staining or live/dead cell staining (Fixable Dead Cell Stain Kits, Invitrogen). Cells were then fixed in 0.05% formaldehyde for 10 to 15 minutes followed by disruption of membrane by detergent Tween 20 at 0.5% v/v in PBS for 15 minutes. Cells were washed by PBS (containing 0.1% Triton X-100 or other permeabilizing detergent) and centrifuged at 300 g for 5 minutes; supernatant was discarded. Cells were resuspended in the volume remaining. Antibodies were given according to manufacturer directions. Antibodies were prepared in permeabilization buffer to ensure that cells remained permeable. Cells were counted, and 1 × 106 cells from each sample were subject to flow analysis using FACS Calibur (BD). All cells were stained at 4°C in PBS with 2% FCS. The following antibodies were used for staining: APC anti-
human KDR/isotype control, PEcy7-Ly6C, PE-Ly6G, PE-CD33, Bio-tin Mouse Lineage Panel (559971, BD Biosciences — Pharmingen), FITC/eF450 CD45 (clone 30-F11), PE/APC-c-Ki (clone 2B8), PE/PEcy7–Sca-1 (clone D7), FITC/eF450-CD11b (clone M1/70), PE-Gr1 (clone RB6-8C5), PEcy7-HLA-DR (clone LN3), AF-700-APC-CD16 (clone CB16), APC/PEcy7–CD14 (clone 61D3), eF5.5-FcRII (AT130-2), Pacific Blue/FITC-streptavidin (11-4317, eBioscience), and Pacific Blue/Mouse Lineage Panel (133310, BioLegend). Data were analyzed by Flowjo software. Analysis was done within single gate after the exclusion of debris and dead cells.

RNA sequencing analysis. CD11b’Gr-1’ KDR-GFP+ myeloid cells were sorted from peripheral blood, and c-Ki-Sca-1 KDR-GFP+ progenitor cells were sorted from BM cells by FACS (BD) directly into cell lysis buffer provided in the RNeasy Micro Kit (QIAGEN). Total RNA BM progenitor cells in each group were extracted with an RNeasy Microkit followed by cDNA amplification; 3 or 4 independent samples from each group were subjected to RNA-sequencing (Illumina HiSeq 2000). Gene expression data were analyzed using the Limma package in R/Bioconductor software, using a P value of less than 0.001 and fold change greater than 2. By unsupervised analysis, these samples were clustered based on their expression profiles. Z scores were used in the cluster analysis. All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE94271).

In vitro culture of HPCs. Whole BM was harvested from 7- to 10-week-old donor mice (C57BL/6 or F100 ng/ml human Fms-like tyrosine kinase 3, 100 ng/ml human IL-11, and 20 ng/ml murine IL-3). All cytokines were purchased from Pepro-tech. The Lin− BM cells were transduced with the appropriate lentiviral particles at an MOI of 10. At 18 to 24 hours after transduction, the transduced Lin− BM cells were harvested and transplanted into 2 wells of a 24-well culture plate supplemented with standard methylcellulose medium containing 10 ng/ml murine IL-3, 10 ng/ml human IL-6, and 50 ng/ml murine SCF (StemCell Technologies). Granulocyte, macrophage, and granulocyte-macrophage CFU were counted after 7 days of culture.

Endothelial tube formation. GFP-labeled HCMEC/D3 (5 × 104/ well) cells were seeded on 24-well plates coated with Matrigel with reduced growth factors (200 μl/well, BD Biosciences) and incubated for 6 hours followed by coculture with BMDCs. Spatial organization and sprout configuration were assessed after 12 hours using in situ fluorescence imaging. Cumulative sprout lengths of tubes represented by area of GFP+ field were quantitatively analyzed using ImageJ (NIH).

Matrigel plugs. Growth factors were thawed and Matrigel (BD Biosciences) was reduced on ice overnight at 4°C. The Matrigel was mixed with specified BMDCs on a vortex and placed on ice. A syringe with each solution was used to inject 0.5 or 1 ml subcutaneously into the ventral area of each mouse. After 10 days, mice were injected with 100 μl (10 ng/ml) 70-kD DsRed-dextran by tail vein. Ten minutes later, animals were sacrificed, followed by 4% paraformaldehyde perfusion, and plugs removed. Plugs appeared as bumps on the ventral side of the animal. Whole plugs were placed in gradients of methanol from 25% to 100% and then into benzyl alcohol benzyl benzoate (Sigma-Aldrich) at a 1:2 ratio. Plugs were then mounted for fluorescent imaging.

Zymography. Supernatant was harvested from specified cells, concentrated with 10-kD centrifugal filter columns (Millipore), and loaded without a reducing reagent to 12% gelatin gel (Invitrogen). Zymography development was done with Novex precast zymography buffers (Invitrogen) by following the manufacturer’s instructions. Following electrophoresis, the gel was incubated with 100 ml of renaturing buffer under gentle agitation for 30 minutes. Renaturing buffer was removed, 100 ml of developing buffer was added, and the gel was incubated under agitation for 30 minutes. Developing buffer was then removed and another 100 ml of developing buffer was added. The zymogram was then placed in the humidified incubator set to 37°C and incubated overnight. Developing buffer was removed and replaced with 100 ml of deionized water. The zymogram was washed 3 times under gentle agitation for 5 minutes, stained with 0.5% Coomassie G250 in 30% ethanol and 10% acetic acid for 30 minutes, and then destained in 30% ethanol/10% acetic acid. Destaining was quenched by transferring the gel to 2% acetic acid when the clear bands were observed.

CHIP assay. BM progenitor cells were collected and cultured as described above. Protein-DNA complexes were crosslinked by incubating the cells with 37% formaldehyde diluted to a 1% final concentration and incubating with cells on a rocking or shaking device for 15 minutes at room temperature. Formaldehyde was quenched by adding 1 M glycine diluted to a final concentration of 125 mM. Cells were rocked for 5 minutes at room temperature and pelleted; medium was removed. Protease inhibitors were added to the Lysis buffer (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). The cell pellet was resuspended in 500 μl of lysis buffer per 5 × 106 cells and incubated on ice for 10 minutes. Samples were sonicated to shear chromatin to an average length of about 1 kb. 500 μl of each
sample was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 10 minutes using a refrigerated ultracentrifuge at 12,000 g. Supernatant was collected in a clean tube and the pellet discarded. Supernatant was diluted by adding 1 ml of dilution buffer and 5 μg of the E2A antibody (Santa Cruz Biotechnology Inc., sc-763) or normal IgG to the samples and incubated overnight at 4°C on a rotating device. Secondary biotinylated antibody was added at 5 μg and incubated for 1 hour at 4°C on a rotating device. We added 100 μl of streptavidin magnetic beads to the samples and rotated for 30 minutes at 4°C. The beads were collected by leaving the tube in the magnet for 2 minutes and were washed with prewashed wash buffers. After the last wash, 100 μl of chelating resin solution was added directly to the beads and pipetted up and down for about 10 seconds. The sample was boiled for 10 minutes using a heat block or a temperature-controlled water bath and microcentrifuged at 12,000 g for 1 minute at room temperature before transferring the supernatant to a clean microcentrifuge tube. The DNA preparation was cleaned and concentrated using a DNA purification kit (QIAGEN) and resuspended in 30 μl of deionized or distilled water. 2 μl of the DNA sample was used in the real-time PCR reactions.

The probes for KDR promoter region were as follows: sense: CCTGTTTTGCGACCTGCTTCTG; antisense, TGTAACATGGT-

Statistics. All data are given as mean ± SD or ± SEM. n was used to show the number of biological replicates within figure legends. (a) For quantification of IHC, n represents the number of biological samples within each group. First, we randomly selected 5 slides from each sample/mouse. Nine fields from each slide were counted, and an average number per field was used to represent each slide. Then the mean of values from 5 slides was used to represent each sample/mouse and graphs were generated based on n independent samples/mouse. Two independent observers blindly measured or counted to determine intra- and interobserver variability. (b) n in animal experiments represents the number of mice in each group. (c) n in RT-PCR and cell count experiments represents the number of independent wells of treatment per condition, each of which represents the mean value from 3 experimental wells. In “Endothelial tube formation,” the value of each well is the mean of cell counts of six 100× fields randomly chosen within a well, and n is the number of independent wells of treatment per condition. Differences between the mean parameter values derived from 2 groups were compared using 2-tailed Student’s t tests and 1-way ANOVA, followed by a post-hoc test for comparison of multiple groups. Significance was determined as a P value of less than 0.05.

Study approval. All animal experiments were performed according to the guidelines set forth by the Institutional Animal Care and Use Committee at Weill Cornell Medicine. Human peripheral blood samples were obtained from subjects with grades I, II, III, and IV gliomas at Weill Cornell Medical College and were histologically confirmed as gliomas. Subjects with grade II gliomas were followed up over 12 months. Tumor progression was determined by either histology or radiography. All individuals provided informed consent for blood donation according to a protocol approved by the institutional review board.

Author contributions

YH, JB, DL, and JG designed the study. YH, PR, CH, JHK, EG, WC, EV, and RX conducted experiments. YH, WH, TZ, and JX acquired data. YH, WH, TZ, HP, JB, BD, SR, DL, and JG analyzed data. DP, JH, EH, BD, and SR provided important materials. YH, PR, CH, BC, RY, IM, DL, and JG wrote the manuscript.

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