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TITLE: Characterizing and Targeting Bone Marrow-Derived Inflammatory Cells in Driving the Malignancy and Progression of Childhood Astrocytic Brain Tumors

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14. ABSTRACT In this study, we have utilized glioma patients along with two unique murine glioma models: RCAS glioma model and Gl261 model to study various lineages of BMDCs during different stages of glial tumors. Importantly, we identified the unique the population VEGFR2+MDSCs in both patients and mice, which might be used as a surrogate marker for glioma diagnosis and prognosis in future. We have validated the changes of myeloid lineage and endothelial lineages during the progression of gliomas, and We observed the increased population of myeloid derived suppressor cells and endothelial progenitor cells in murine glioma models. We have created inducible VEGFR2 knockout system in glioma bearing mice. Taking advantage of this transgenic model, we demonstrated that bone marrow derived VEGFR2 signaling plays an important role in myeloid differentiation, and infiltration into tumor tissues. Deficiency of VEGFR2 in BMDCs led to impairment of tumor associated myeloid cells and delayed progression of low-grade glioma. All of these findings may have implications to suppress the switch of low-grade to high-grade transformation, and predict the long-term survival.					
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Table of Contents

	<u>Page</u>
1. Introduction.....	2
2. Keywords.....	3
3. Overall Project Summary.....	4
4. Key Research Accomplishments.....	15
5. Conclusion.....	16
6. Publications, Abstracts, and Presentations.....	17
7. Inventions, Patents and Licenses.....	17
8. Reportable Outcomes.....	17
9. Other Achievements.....	17
10. References.....	18
11. Training & Professional Development	.19

1. Introduction

Brain tumors are most frequent solid cancer among all kinds of childhood cancer. Heterogeneity, invasiveness, and complex microenvironment are making therapies as well as research on astrocytic brain tumor particularly challenging^{1,2}. Low-grade gliomas are curable and most patients could live without further progression and severe condition for many years. However, once the glioma progress to high grade, the quality of life and survival of patients are very poor¹. Based on previous work, we hypothesized the bone marrow derived cells (BMDCs) could serve as a mediator of transition of low grade to high-grade tumor³⁻⁵. The proposed project aims to characterize the various lineages of glioma associated BMDCs including hematopoietic, endothelial, and mesenchymal lineages in both low grade and high grade stages of glioma. Studying the effect on glioma transition from low grade to high grade by depleting distinct populations of bone marrow derived inflammatory cells including monocyte, granulocytes, endothelial progenitors, and mesenchymal progenitors. Dissecting molecular mechanism/signaling of differentiation of glioma associated BMDCs, and screening the key factors or targets through the entire regulatory pathway. It would contribute to develop therapeutic strategies to target a specific population of BMDCs and their subsequent recruitment, in order to suppress the malignant transformation of gliomas. In this project, we have initiated the study of BMDCs with RCAS and GL261 murine glioma models as well as glioma patients. We also used transgenic tools to deplete certain populations of BMDCs to study functional contribution of BMDCs for glioma progression.

2. Keywords

Glioma, Pediatric, bone-marrow-derived-cells, endothelial, mesenchymal, myeloid, hematopoietic, differentiation, malignant, transformation.

3. Overall Project Summary

The project focuses on studying the microenvironment and functions of bone marrow derived cells within pediatric astrocytic tumor. In this study, two major glioma models will be used to investigate the role of BMDCs primarily. One is a transgenic mouse using the RCAS/Tv-a system created by Holland and Varmus that develops low-grade gliomas which progress to high-grade tumors over the course of twelve weeks^{5,6}. The other model is syngeneic orthotopic glioma model. GL261^{7,8}, a C57/BL6 derived glioma cell line with different markers such as GFP or luciferase, was intracranial injected to C57/BL6 mice to create allograft glioma. In addition, xenograft models with human glioma cell lines are also utilized. Furthermore, we also have used glioma patients' blood samples to analyze various lineages of BMDCs⁹⁻¹².

Task 1. Characterizing the various lineages of glioma associated BMDCs including hematopoietic, endothelial, and mesenchymal lineages in both low grade and high gradestages of glioma. (70% complete)

We have analyzed the myeloid lineage and endothelial lineage of BMDCs in both patients' blood samples and murine glioma models at low-grade or high-grade stage.

1a. Assess the frequency and absolute numbers of circulating BMDCs in glioma patients. Using flow-cytometry of hematopoietic, as well as endothelial and mesenchymal markers, we will investigate whether the frequency of HPCs, VEGFR2+ EPCs and CD105+ MSCs correlates with glioma transformation in patients. Human subjects involve this study will be radiographically-suspected or biopsy prove low-grade astrocytoma (WHO grade I and II) versus histology proven high grade gliomas (WHO grade III and IV). Blood samples will be collected at the time of diagnosis. Subjects selected for this study may be between 2 years and 16 years of age. We plan to enroll 160 human subjects, and quarterly enrollment is 20. The identifications of human subjects will not be accessible for research team, and only information of human subjects that research team is aware of is the patients' diagnosis and diseases' history.

So far, we have recruited 82 glioma patients with low-grade or high-grade glioma, plus 20 healthy volunteers as control into this study. Patients or healthy volunteers' peripheral blood has been analyzed with various lineages makers as showed in the figures below:

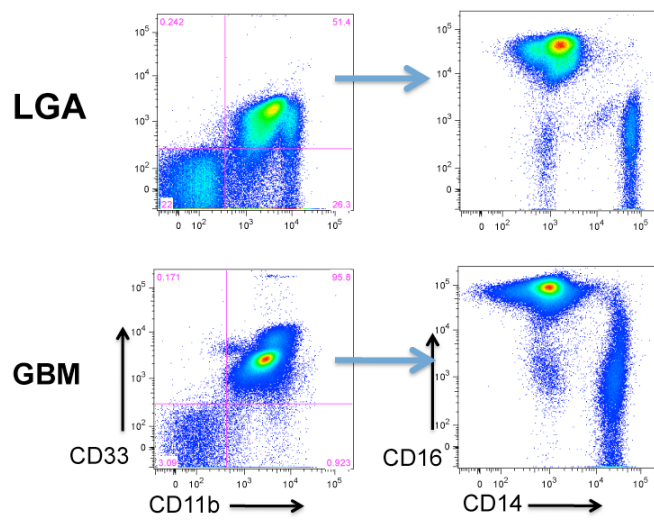


Figure 1. Characterizing myeloid lineage of BMDCs in patients (CD11b, CD33, CD14, and CD16) by flow cytometry in peripheral of low-grade astrocytoma patients (LGA) vs glioblastoma patients (GBM).

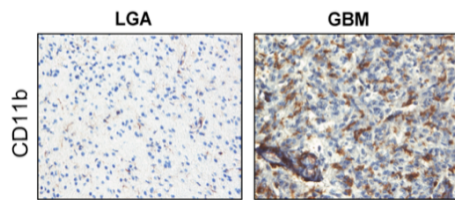


Figure 2. IHC of CD11b (infiltrated myeloid cells) on archived paraffin embedded tumor tissue from low-grade astrocytoma patients (grade II) vs glioblastoma patients (grade IV).

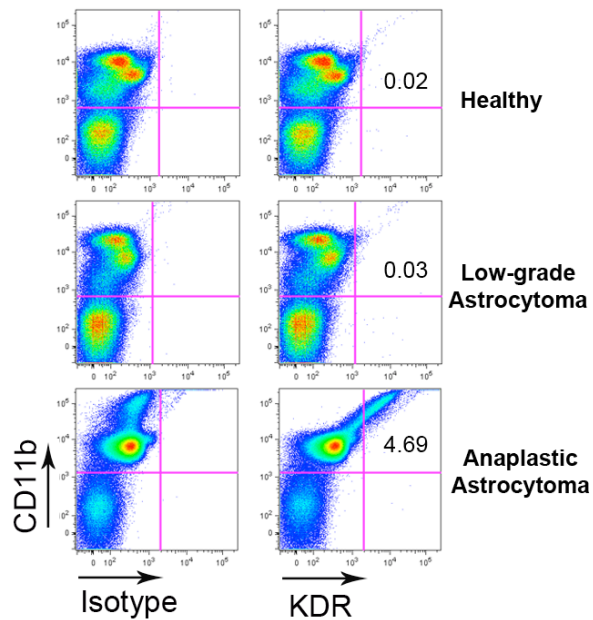


Figure 3. Characterizing endothelial/myeloid lineage of BMDCs in patients by CD11b, KDR (VEGFR2) in peripheral of low-grade astrocytoma patients (LGA), glioblastoma patients (GBM), and healthy volunteers.

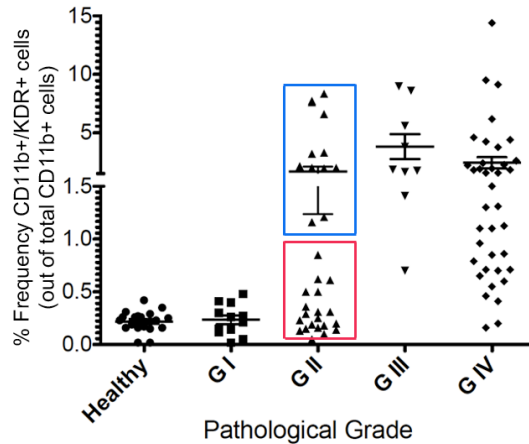


Figure 4. Statistic dots-plot on frequency of CD11b+/VEGFR2(KDR)+ cells out of total CD11b+ cells in patients with different stages of disease. Healthy donor served as a control. GIII/GIV vs healthy, One way ANOVA, $P < 0.001$.

During the study of BMDCs with glioma patients' blood samples, we have found that the number of myeloid derived support cells (MDSCs) within myeloid lineage increased following the progression of diseases. The MDSCs are heterogeneous regarding the expression of CD14/CD16, representing monocytic or granulocytic sub-lineages (Figure 1). While the tumor progressed, we also observed more infiltrated myeloid cells within tumor tissues (Figure 2). Interestingly, when we examine the expression of VEGFR2 to study endothelial lineage of BMDCs, we found the majority of VEGFR2 expressing cells are CD11b positive as showed in Figure 3. It suggested that there is overlap or interaction between myeloid lineage and endothelial lineage of BMDCs.

1b. Evaluate the frequency and number of the same BM-derived populations in blood, bone marrow and metastatic organs of murine models of glioma during low grade, transformation grade and high grade phases. Specifically, we will investigate the mobilization of HPCs, EPCs, and MSCs by flow cytometry. The cell line G1261, a C57/BL6 mice derived glioma cell line, is obtained from NYU medical center. Total number of mice used for this study will be 60.

We have analyzed the HPCs, EPCs, and MSCs in both RCAS and G1261 murine glioma models. The 4~6 weeks post-injection of RCAS model were consider as the low-grade stage, and 6~9 weeks were considered high-grade stage. The G1261 model was considered as high-grade glioma model.

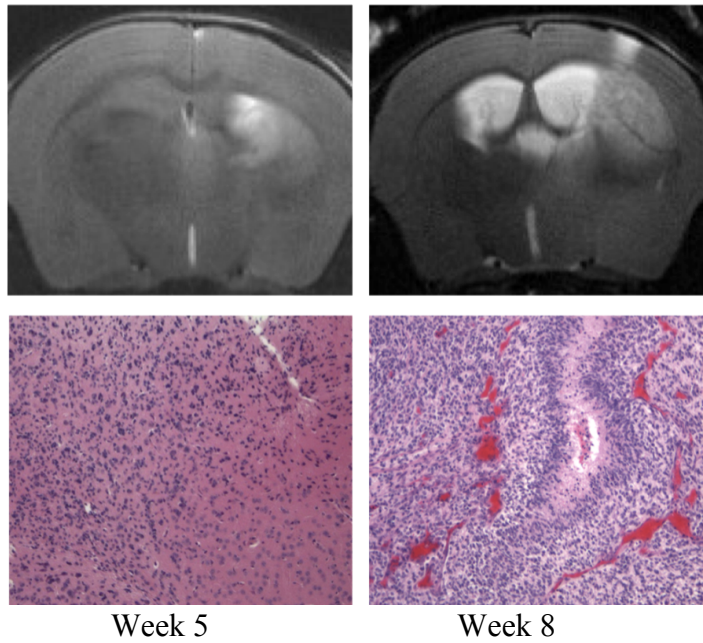


Figure 5. RCAS-tva murine glioma model on low-grade stage (week 5) and high-grade stage (week 8). The MRI or H&E staining from representative mice were showed.

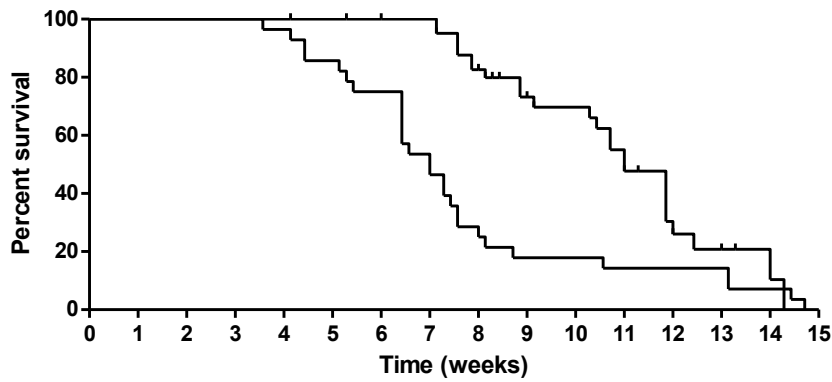


Figure 6. The Kaplan-Meier Survival curves of RCAS-tva tumor bearing mice with or without bone marrow transplantation (BMT). The median survival time for RCAS mice without BMT is 7 weeks, and with BMT is 11 weeks.

We characterized the RCA-tva murine glioma model in our experimental setting, and figured out their low-grade stage and high-grade stage evidenced by MRI and histology. Additionally, we showed the RCAS mice with bone marrow transplantation have delayed progression of tumor, which is important for our next step of study.

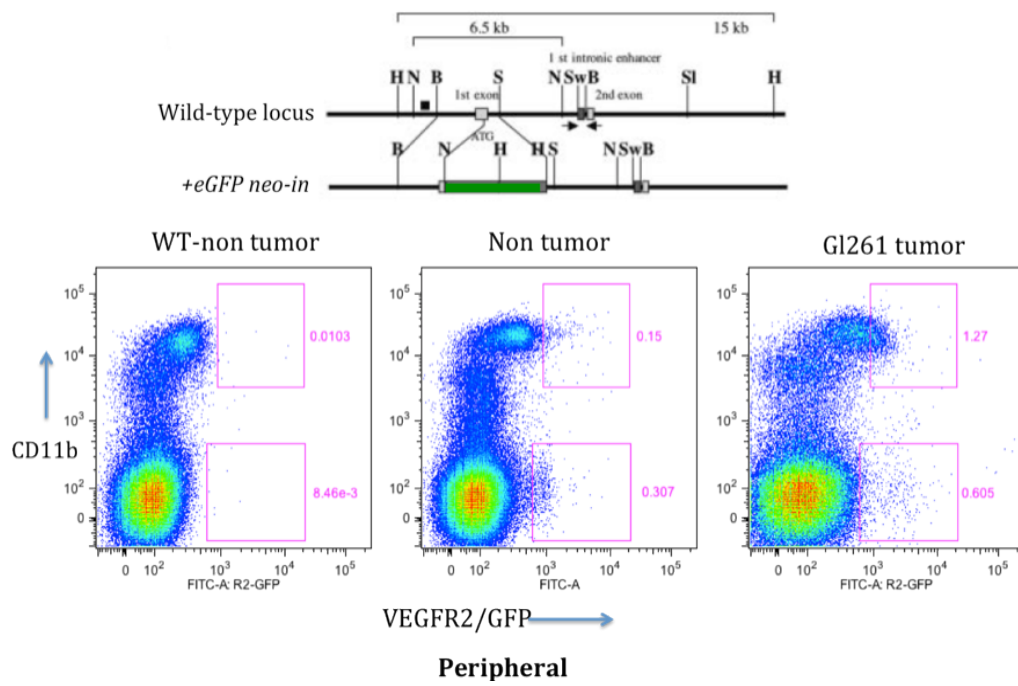


Figure 7. The expression of VEGFR2 on BMDCs in Gl261 model. Upper panel of schematic model showed the VEGFR2-GFP knock in mice for studying expression pattern of VEGFR2. Lower panel of flowcytometry graphs indicate the expression VEGFR2 on CD11b+ or CD11b- population.

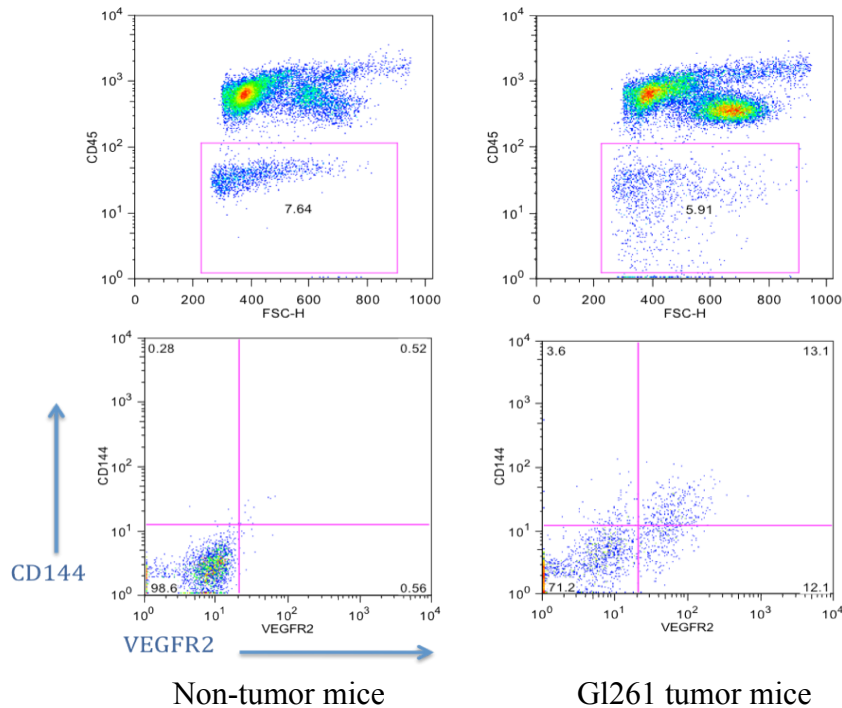


Figure 8. EPC in Gl261 glioma model. Flowcytometry graph of lineage negative CD144+ VEGFR2+ EPC in Gl261 tumor bearing mice.

We have studied the myeloid lineage and EPC with Gl261 glioma model. We observed that both VEGFR2+ myeloid cells and EPCs were elevated by Gl261 tumor, indicating potential interaction of BMDCs differentiation with primary glioma.

Task 2. Studying the effect on glioma transition from low grade to high grade by depleting distinct populations of bone marrow derived inflammatory cells including monocyte, granulocytes, endothelial progenitors, and mesenchymal progenitors. (50% complete)

We have set up all trans-genetic mice lines including ITGAM(CD11b)-DTR/EGFP mice, RosaCreERT2/PDGFR α loxP/loxP mice, and RosaCreERT2/VEGFR2loxP/loxP mice in suitable genetic background for bone marrow transplantation experiments. We performed the lineage depletion experiments with ITGAM(CD11b)-DTR/EGFP and RosaCreERT2/VEGFR2loxP/loxP mice for myeloid or endothelial lineages in both RCAS and Gl261 tumor models.

1a. We plan to transplant the bone marrow from ITGAM(CD11b)-DTR/EGFP mice into the RCAS and Gl261 glioma models, and use diphtheria toxin to induce depletion of myeloid cells in this RCAS glioma model. The total number of mice will be used is 20.

We have developed the ITGAM(CD11b)-DTR/EGFP bone marrow transplanted mice and implanted mice with Gl261 tumor after bone marrow was engrafted. We have tried to deplete the CD11b positive cells in tumor bearing mice once with 5 mice in each group. However, we only obtained approximately 10% deduction of CD11b cells compared with control group. In this case, we didn't see effect on tumor growth. We would optimize the administration and dosage of DT next step to acquire more efficiency of CD11b cells depletion, and then study the influence of myeloid cells on glioma.

1b. Bone marrow from RosaCreERT2/VEGFR2loxP/loxP mice will be transplanted to both RCAS glioma and Gl261 bearing mice to deplete endothelial lineage of bone marrow derived cells by knocking out the VEGFR2 gene. The total number of mice will be used is 30.

We have successfully knockout VEGFR2 in both RCAS model and Gl261 murine model. We have studied the effect of VEGFR2 deficiency on glioma progression.

As Figure 9 showed, we could obtain around 90% knocking out efficiency with our RosaCreERT2/VEGFR2loxP/loxP system, without affecting counts of blood cells (CBC) of mice.

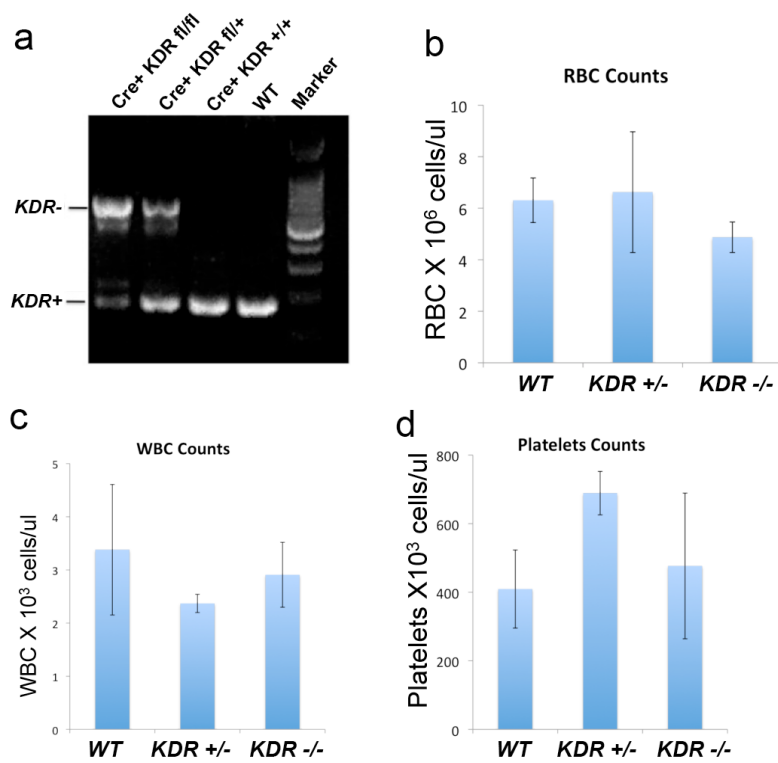


Figure 9. Validation of depletion of VEGFR2 (KDR) allele in bone marrow cells. (a) Amplification of KDR+ or KDR- allele on bone marrow cells of mice bearing indicated genetic background. Tamoxifen was applied to mice one week before testing. Complete blood counts on mice with WT, KDR+/-, or KDR-/- bone marrow (b) Red blood cells, (c) white blood cells, and (d) Platelets.

When we knockout VEGFR2 from BMDCs in Gli261 models, we observed that tumor progression was suppressed as shown in Figure 10. In the tumor tissue, we found much less tumor-associated myeloid cells (CD11b+) in the VEGFR2 KO group compared with the control group (Figure 11). With the RCAS system, we found the similar phenotype; after we performed the bone marrow transplantation and induced VEGFR2 knockout, we observed that tumor progression was delayed and median survival time (MST) was significantly elongated (Figure 12).

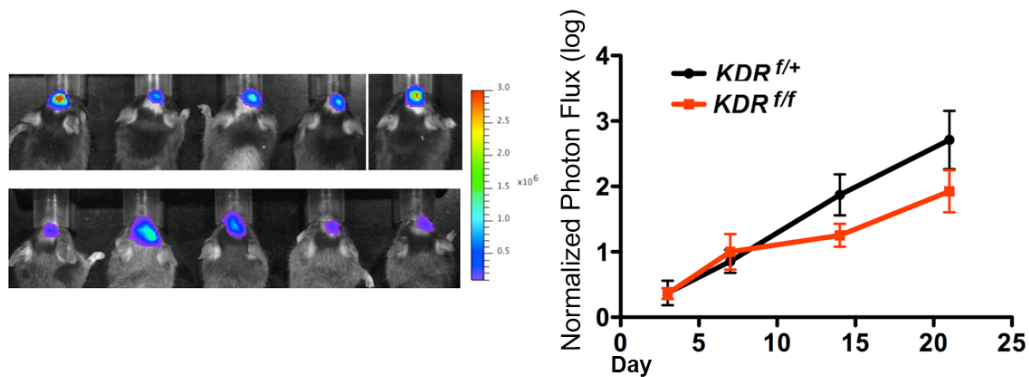


Figure 10. Knocking-out VEGFR2 (KDR) in BMDCs suppress Gl261 tumor growth, tumor-associated myeloid cells, and vasculatures. Chimeric C57/bl6 mice transplanted with rosa26ERT2-cre/KDR^{fl/fl} bone marrow cells (labeled as BM-KDR KO, and BM-KDR control is rosa26ERT2-cre/KDR^{fl/+}) were implanted with luciferase labeled-Gl261 tumors intracranially. Tamoxifen were applied at day 3 post- implantation. The tumors were monitored by bioluminescence. The quantification of bioluminescence based tumor growth.

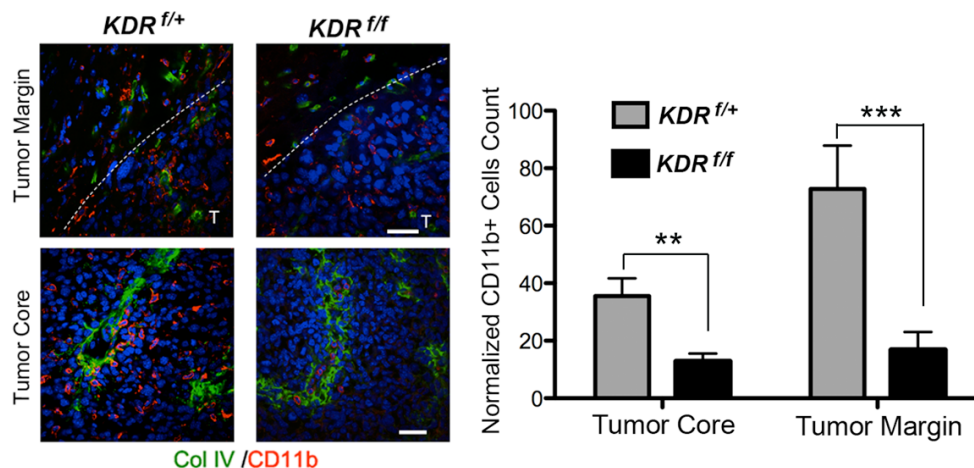
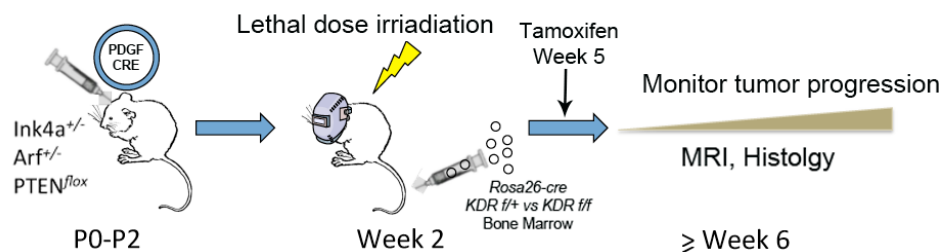


Figure 11. Immuno-staining of vascular basement (Collagen IV) and myeloid cells (CD11b) in the Gl261 tumors from each group. Scale bar, 20 μ m. The number of CD11b cells in tumor core or tumor margin was quantified.



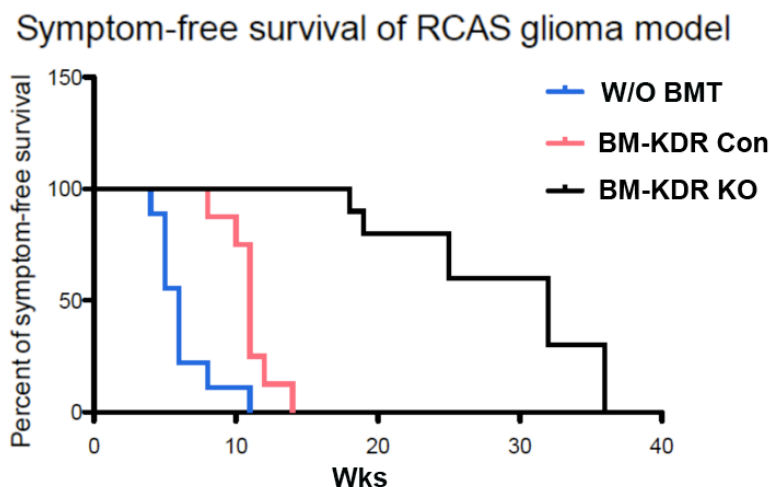


Figure 12. Knocking-out VEGFR2 (KDR) in BMDCs suppress spontaneous malignant transformation of RCAS gliomas. (a) Flow chart of experimental design. Oncogenes were transduced to P0-P2 pups in the beginning. Mice received lethal dose irradiation and received bone marrow transplantation at week 2, and then tamoxifen was applied to mice at week 5 to induce the ablation of target gene. Tumors were monitored by MRI over the process. (b) Kaplan-Meier symptom free survival curve for RCAS mice transplanted with *rosa26ERT2-cre/KDR^{fl/fl}* bone marrow cells (BM-KDR KO), *rosa26ERT2-cre/KDR^{fl/fl}* bone marrow cells (BM-KDR Con), or without irradiation/transplantation (W/O BMT).

1c. Bone marrow cells from *RosaCreERT2/PDGFR α loxP/loxP* mice will be transplanted to each genetic and orthotopic glioma generating mice. *PDGFR α* is expressed on bone marrow derived mesenchymal stem cells and *PDGF*- *PDGFR α* signal axis is very critical for maintenance of mesenchymal lineage. By knocking out *PDGFR α* gene, we will study the influence of defection of mesenchymal differentiation on the progression of low-grade glioma. The total number of mice will be used is 30.

We have completed the crossing donor *RosaCreERT2/PDGFR α loxP/loxP* mice, and the bone marrow transplantation experiments along with tumor study are ongoing.

Task 3. Dissecting molecular mechanism/signaling of differentiation of glioma associated BMDCs, and screening the key factors or targets through the entire regulatory pathway. (20% complete)

We will proceed to study which genes in certain lineages of BMDCs could play a critical role in promoting the invasion of glioma. We will isolate specific population from BMDCs from the tumor tissue, blood, and bone marrow. Then we utilize microarray/next generation sequencing, antibody array, LC-TOF-TOF to detect gene and protein expression. To determine the functional contribution of certain genes of interest, or certain subpopulation of BMDCs, we plan to utilize a series of in vitro/in vivo experiments, including basement invasion assay and knock specific genes bone marrow cells and then transplant them into glioma producing mice, to elucidate their specific roles in promoting invasion of glioma cells and infiltration of glioma. Total number of mice will be used for this study is 30.

We have been working on optimizing sorting different lineages of BMDCs by FACS or MACS and tested a few samples by RNA sequencing. The data suggested that ID2/VEGF2 signaling was playing important role in myeloid differentiation.

In order to further delineate the signaling network driving myeloid/endothelial lineage differentiation, we performed gene expression profiling of VEGFR2-expressing hematopoietic progenitor cells (Lin-C-kit+) by mRNA sequencing. Differentially expressed genes from VEGFR2+ versus VEGFR2- HPCs were clustered and arranged in a heatmap. Differentially expressed genes were also clustered and displayed (Figure 13). Candidate genes ($P < 0.05$, > 1.5 -fold change) were divided according to subsets with the highest expression and analyzed for categories with significant enrichment ($P < 0.05$) of categories in Gene Ontology (GO) biologic processes using DAVID tools. Similar categories were grouped accordingly (Figure 14). Inhibitor of DNA binding proteins 2 (ID2) was identified as a significantly up-regulated gene in VEGFR2+ HPCs a strong candidate to be an upstream molecule mediating myeloid endothelial differentiation.

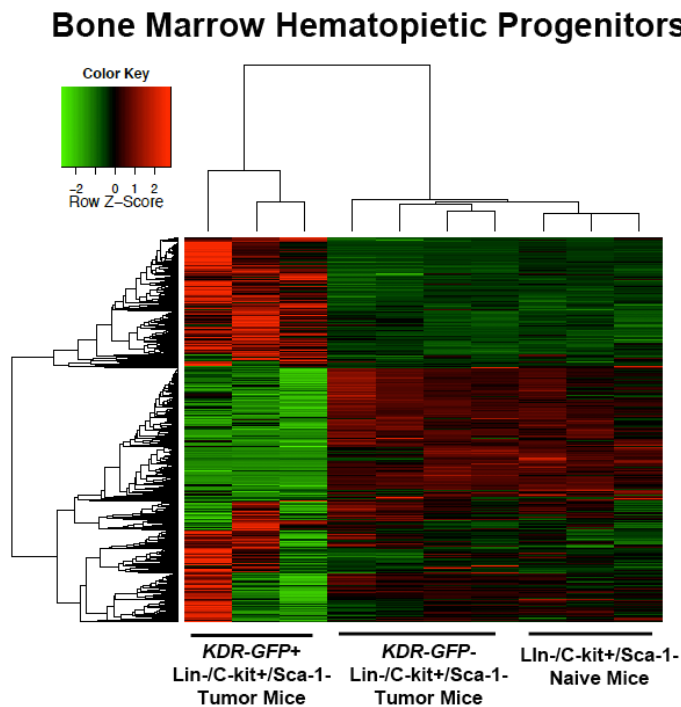


Figure 13. RNA-sequencing based gene profiles on KDR+ (VEGFR2) peripheral and bone marrow cells. The heatmap of significantly altered genes in Lin-c-kit+Sca-1- from naïve mice, Lin-c-kit+Sca-1- KDR-GFP+ cells and Lin-c-kit+Sca-1-KDR-GFP- cells from bone marrow of tumor bearing mice.

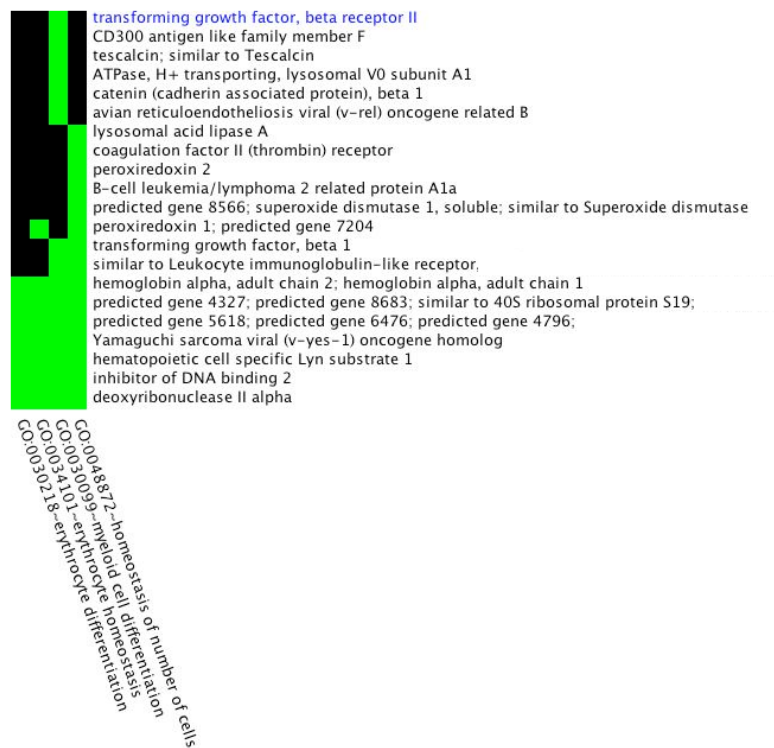


Figure 14. The candidate genes ($P < 0.05$, > 1.5 -fold change; Lin-c-kit+Sca-1-KDR(VEGFR2)-GFP+ vs Lin-c-kit+Sca-1-KDR-GFP-) were divided according to the subset with the highest expression and analyzed for categories with significant enrichment ($P < 0.05$) of categories in Gene Ontology (GO) biologic processes using DAVID tools. Similar categories were grouped accordingly.

4. Key Research Accomplishments

- I. We have demonstrated that myeloid derived suppressor cells increased following the progression of astrocytic tumor in both patients and murine models.
- II. We identified a specific population across endothelial and myeloid lineages, which is VEGFR2+CD11b+ population in patients and tumor bearing mice.
- III. We successfully performed knock out VEGFR2 within BMDCs in murine glioma models, and observed that bone marrow derived VEGFR2 contribute to tumor progression and animal survival.
- IV. We have performed RNA-sequencing on tumor associated myeloid progenitors, and identified inhibitor of DNA binding proteins 2 was related with pro-tumoral myeloid differentiation.

5. Conclusion

In this study, we have utilized glioma patients along with two unique murine glioma models: RCAS glioma model and GL261 model to study the BMDCs during different stages of glial tumor. Importantly, we identified the unique the population VEGFR2+MDSCs in both patients and mice, which might be used as a surrogate marker for glioma diagnosis and prognosis in future. We have validated the changes of myeloid lineage and endothelial lineages while the progression of gliomas, and observed the increased population of myeloid derived suppressor cells and endothelial progenitor cells in murine glioma models. We have created inducible VEGFR2 knockout system in glioma bearing mice. Taking advantage of this transgenic model, we demonstrated that bone marrow derived VEGFR2 signaling plays an important role in myeloid differentiation, and infiltration into tumor tissues. Deficiency of VEGFR2 in BMDCs led to impairment of tumor associated myeloid cells and delayed progression of low-grade glioma. All of these findings may help to find the approach to suppress the progression of low-grade glioma into high-grade form, and have implications to predict the long-term survival of glioma patients^{13,14}.

In the following study, we will keep exploring the various lineages of BMDCs in both low-grade and high-grade glioma patients. We would further validate functional role of VEGFR2+ BMDCs in malignant transformation, and investigate the connection of myeloid differentiation with tumor associated macrophages/neutrophils. We would dedicate our effort to delineate the signaling pathways, which affect pro-tumoral myeloid cells, and further characterize the downstream signaling of inhibitor of DNA binding proteins 2 in BMDCs^{15,16}.

6. Publications, Abstracts, and Presentations

1, Abstract/Oral presentation

ID2/KDR drives the differentiation of pro-malignant myeloid derived suppressor cells in glioma. Yujie Huang, Prajwal Rajappa, Jacqueline Bromberg, David Lyden, Jeffrey Greenfield. Cold Spring Harbor-Asia Meeting (International) "FRONTIERS OF IMMUNOLOGY IN HEALTH & DISEASES" September 2–September 6, 2014

7. Inventions, Patents and Licenses

None

8. Reportable Outcomes

None

9. Other Achievements

None

10. References

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11. Training & Professional Development

In the past year, I have received extensive trainings and related proceedings from various workshops, meetings, and hands-on practices, in addition to regular mentorships from weekly lab meeting and journal club. To follow the frontiers of tumor immunology and tumor microenvironment research, I have attended a workshop on tumor microenvironment (TME) organized by national cancer institute in April 2014. I have communicated several renowned experts on TME with their study and our proceedings. Two weeks ago, I also delivered a talk about our work in Cold Spring Harbor-Asia meeting focusing on immunology in diseases, and it was well received. Additionally, to update my knowledge on biomedical and genomic fields, I have enrolled some courses and workshops such as “Genomic workshop”, “Next generation sequencing analysis”, and “Medical genetic in translational research”, which are provided by Clinical Translational Science Center in Weill Cornell Medical College. All the training opportunities armed me for better bench-side research and long-term career development.