Editorial

Defining differential roles for microglia and infiltrating macrophages in the growth and neovascularization of glioma

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Glioblastoma multiforme, the most common primary malignancy of the central nervous system (CNS), is a highly aggressive and fatal disease. A hallmark of the disease's pathology is aberrant neovascularization due to inappropriate upregulation of pro-angiogenic signaling pathways within the tumor microenvironment. Signaling via vascular endothelial growth factor (VEGF) is one of the primary aberrant pro-angiogenic pathways in glioma (1). Clinical trials have evaluated the blockade of this pathway with the drug bevacizumab, but failed to produce substantial improvements in patient life expectancy or disease-free progression (2). Due to the complex nature of pathogenic neoangiogenesis in glioma, a primary concern underlying bevacizumab's lack of efficacy was upregulation of alternative pro-angiogenic pathways within tumors.

While clonal tumor-derived cells constitute the bulk of gliomas and secrete large amounts of growth and angiogenic factors themselves, around 30% of the additional cellular content may be comprised by tumor associated macrophages (TAMs) (3). TAMs have garnered a great deal of interest in research pertaining to the tumor microenvironment and have been implicated in increasing the invasiveness of cancer cells, supporting immunosuppression and increased angiogenesis, among other things (4). Microglia, the resident macrophages of the CNS have potentially distinct behaviors, differing over those of peripheral monocytes, which invade tumoral tissue. Glioma associated microglia and macrophages (GAMs) have been implicated to play crucial roles for the appropriate establishment and survival of glioma in various animal models. For example, local depletion of microglia and macrophages from glioma via treatment with CSF1R inhibitors has been shown to significantly reduce tumor burden and prolong life expectancy in mice (5).

Brandenburg *et al*. present intriguing observations about the nature of GAMs, in their paper, Resident microglia rather than peripheral macrophages promote vascularization in brain tumors and are source of alternative pro-angiogenic factors (6), offering evidence for distinct roles of microglia and macrophages. Microglia have been implicated in playing major roles in pathogenic angiogenesis in other diseases, such as diabetic retinopathy, due to the release of pro-angiogenic factors after hypoxic insult (7). Studies have also indicated that TAMs contribute significant amounts of VEGF-A to influence tumor neovascularization in non-CNS and CNS tumors (8,9). Yet in most cases where mono-therapies targeting VEGF-mediated signaling are employed, neovascularization is initially delayed but eventually rebounds due to upregulation of complementary pathways within the tumors (10). Brandenburg *et al*. present the argument that one of these critical pathways, which influences neovascularization in glioma, involves signaling via CXCL2 (6), a chemokine with well established roles in the recruitment of monocytes to injured tissue via its receptor, CXCR2 (11). The upregulation of CXCL2 has been documented in diseases such as multiple sclerosis, traumatic brain injury, and glioblastoma in the past, where it promotes local neuro-inflammation via the recruitment of microglia and other immune cell infiltrate (12). Additionally, CXCR2 expression has been correlated with

poor prognosis and recurrence in high grade glioma and its inhibition *in vitro* has been shown to have modest effects on the migratory activity of a glioma-derived cell line (13). Its capacity to influence angiogenesis in glioma is poorly understood.

Brandenberg *et al*. employed the use of whole body irradiation to head-protected mice, which then received β-actin GFP allografts to de-lineate effects of microglia (GFP–) or infiltrating macrophage populations (GFP+) in the development of the well-documented GL261-based glioma disease course (14). The authors report considerable iba1+ infiltrates (GAM) into tumors throughout the disease course at days 7, 14, and 21, as well as peritumoral neovascularization, determined by CD31+ vessel density, remaining consistently high through the disease course. They also report that peritumoral GAMs show considerable incorporation of BrdU with an upward trend as the disease progresses, demonstrating that GAMs constitute a large, highly proliferative population of cells within the tumors. However, the percentage of the BrdU incorporating cells that were microglia or macrophages is not stated.

Many GAMs associated with the tumor were observed in the perivascular niche in direct contact with CD31+ vessels, a finding not too often described with normal vasculature in non-diseased brain tissue. This finding is consistent with reports from many groups, who have monitored similar interactions between microglia and the aberrant vasculature and found that microglia often traffic through tumors along the vasculature (15). Employing the allografted mice, the investigators were able to determine that both microglia and peripheral macrophages made direct contacts with these vessels both in the periphery of and within the tumor. However, it would have been interesting to quantify the percentage of microglia and macrophages, which made contacts with the vessels.

To further dissect the implications of the spatial associations that GAMs have with the aberrant vasculature, the group employed an *in vitro* endothelial cell tube forming assay approach where they co-cultured an endothelial cell line on matrigel with either naïve microglia/macrophages or microglia/macrophages isolated from glioma tissue. Eighteen hours after the initiation of co-cultures, significant, albeit modest, increases in tube length, tube number, and tube branching points were observed in co-cultures with tumor-derived microglia and macrophages. While differences were modest, phenotypic alterations in the behavior of these cells after isolation would be expected based on the change in environmental context that occurs when purifying a cell population from a tumor and placing them in an artificial experimental setup.

To understand differences between naïve and tumor-derived microglia and macrophages, the group performed qRT-PCR for a panel of chemokines and angiogenic factors in CD11b+ cells isolated from healthy, naïve mice and those isolated from the tumor-containing hemispheres of diseased mice. Significantly elevated levels of CCR2, CXCR4, CCL2, CCL5, CXCL2, CXCL10, CXCL14, VEGF, and VEGFR1 as well as a few additional transcripts were found in microglia and macrophages isolated from tumor-bearing animals. These findings were very similar to previously observed trends in the upregulation of these genes in GAMs in GL261-based tumors (16). Inter-animal variation could not be assessed as tissue from 6 animals was pooled for each group, likely due to the low yield of cell numbers. Although these results are interesting and pertinent to the paper's conclusions, it would have been exciting if they had dissected out transcriptional differences between the infiltrating macrophages and the resident microglia isolated from tumors. Using the approach of sorting the CD11b+ cells based on GFP positivity (transplant-derived macrophages) and negativity (host microglia) after tissue dissociation, they could have further evaluated potential differences between the two distinct macrophage populations. Whether microglia are responsible for greater production of these factors relative to invading macrophages remains an unanswered question.

Concentrating on VEGF as a well characterized angiogenic factor and CXCL2 due to its poor characterization, tube formation assays were performed using recombinant factors, demonstrating that tube count, length, and complexity increased in the presence of either factor, although CXCL2 caused a more robust increase in all the parameters measured. To correlate this *in vitro* finding to an effect on the glioma disease course, the group then evaluated the direct administration of α-CXCR2 antibody to the CNS of diseased animals, reporting significantly lower glioma volumes at day 14 post tumor implantation. This result is interesting, but unfortunately no further evaluation of the effects of drug administration on the vascularization of the tumor or its effects on the phenotypic variation of GAMs was performed. While the *in vitro* assays point to direct effects on endothelial cells, they do not evaluate the antibody's activity *in vivo* any further. It is conceivable that treatment may have additional effects and/or does not 'directly' decrease glioma growth due to the inhibition of migratory activity of immune cells. CXCL2 expression by

glioma-derived cancer cells is well documented as well, and could constitute a significant pool of the chemokine that influences neovascularization (17).

Further elaborating upon the potential for peripheral macrophages and microglia to differentially affect the disease course, the investigators employed the use of whole body lethal irradiation of head-protected CD11b-HSVTK mice, and then introduced marrow from β-actin GFP mice. In this clever experiment, mice were administered ganciclovir, which ablated populations of microglia within the brain but had negligible effects on GFP+ macrophages which lacked the transgene. Gliomas were then induced in these mice. Tumor volume was significantly reduced at 10 days in mice in which HSVTK+ microglia had been ablated. Vessel density was found to be significantly reduced intratumorally and peritumorally in these mice as well. Our group previously demonstrated that GL261-based gliomas develop more slowly and show reductions in vascularity in mice in which local ablation of CD11b-HSVTK expressing microglia and macrophages is achieved via concominant administration of ganciclovir, yet our approach lacked the finesse of selectively targeting a single macrophage population (18).

The authors concluded that their findings put forth evidence that microglia are the primary subclass of macrophages responsible for the vascularization of gliomas. However, this claim would seem more substantiated if additional time points past 10 days following disease induction had been evaluated histologically. Additional phenotypic analysis of macrophages populating tumors devoid of microglia would have been interesting to explore. Similarly to experiments concerning the administration of α-CXCR2 antibody to glioma-bearing mice, additional experimental analyses could have explored the 'division of labor' between microglia and invading macrophages within the tumor microenvironment. While microglia appear to play an important role in the initial neovascularization of gliomas, it may be that macrophages have a compensatory role later on in the disease course once a significant pool has infiltrated the tissue. Examining the phenotypes of microglia and macrophages over time, potentially via their segregation based on GFP positivity, could provide insights into their differential roles in the glioma microenvironment. On a similar note, the efficacy of CXCR2 blockade could be further evaluated to determine how long blockade can actually slow tumor progression and tumor vascularization. Despite showing a significant increase over VEGF's capacity to stimulate endothelial cell tube formation *in vitro*, the authors do not provide further evidence why targeting the CXCL2-CXCR2 signaling axis would provide long term efficacy or be a suitable target in the treatment of glioma. Although the thesis of the paper is that microglia rather than macrophages are responsible for the bulk of neoangiogenesis, there is no concrete evidence that CXCL2 derived from these cells is directly or principally responsible for significant vascularization *in vivo*.

Brandenburg *et al*. provide intriguing insights into potential distinct functions of microglia and macrophages within a glioma. With this in mind, the possibility for microglia to differ in neoangiogenic and immunomodulatory activity from peripheral macrophages is likely not limited to glioma, but other malignancies of the CNS. It has become increasingly apparent that microglia and macrophages behave differently in many other neuro-inflammatory disorders where they were once simply lumped together as one large homogenous population of cells. This work could initiate approaches that would differentially interfere with the activities of the two cell types to gain new insights into various neuroinflammatory disorders.

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