Editorial

Macrophages/microglia in glioblastoma: a Zelig-like story of changing phenotypes

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In the framework of an increasing number of human cancers being effectively tackled by novel and innovative therapeutic approaches, high-grade glioblastoma (GBM) multiforme stands as a most difficult task for our hopes of effective therapies, or cures. GBM usually localizes at the level of subcortical white matter, in the cerebral hemispheres. Probably because the tumor arises within an immunological sanctuary, immune responses elicited by GBM appear to involve mostly the innate immune system: up to 50% of human GBM tumor mass is represented by tumor-associated macrophages (TAMs), which include both resident microglia and bone marrow-derived macrophages. The source of TAMs, the relative contribution of resident microglia and systemic macrophages, as well as the putative role of TAMs in tumor growth are currently a matter of thorough investigation and dispute.

Chen and colleagues set up a sophisticated experimental model to address some of the above issues (1). They injected transfected glioma cells in the brains of two genetically engineered mouse models, where the investigators were able to observe the time-course of the monocyte/microglia selective CX3CR1 and CCR2 chemokine gene expression, with the $CX3CR1^{Lo}CCR2^{Hi}$, $CX3CR1^{Hi}CCR2^{Lo}$ and $CX3CR1^{Hi}CCR2^{-}$ profiles identifying the monocyte, macrophage and microglia-like cell types, respectively. After a series of elegant experiments, these authors were able to draw these conclusions: in this experimental model, about 85% of TAMs are made of infiltrating monocytes/macrophages, which distribute preferentially in the perivascular area, whereas the remaining 15% of resident microglia is found mainly in peritumoral regions; interaction with the tumor drives monocytes toward a macrophage/microglia phenotype; overall, monocyte infiltration appears to favour tumor growth.

There is little doubt that such a precise spatial and temporal analysis is only possible through a highly sophisticated experimental approach. In this setting, the very early phase of tumor growth is mimicked by the tiny volumes of glioma cells injected in mice brains; still, tumor implant per se represents a systemic bias, as it causes monocyte/macrophage infiltration at the injection sites within the parenchyma, i.e., a pattern of infiltration that seemingly is not occurring in human GBM. Overall, a thorough reflection is needed on the 'distance' existing between experimental models and human pathology, which is admittedly an old story, but a story we should never be tired to hear.

Talking about 'distance', the long-standing discussion about the role of microglia/macrophages in GBM growth, whether do these cells favour or oppose tumor growth, usually focuses on the activation status of these cells, and the attempts to classify such activation status into two broad paradigms, i.e., M1 and M2 phenotypes, which commonly apply to both macrophages and microglia. Immune activation of microglial cells appears more complex than originally described based on cell morphology (resting/ramified *vs.* activated amoeboid cells). Current evidence—mostly from the nonclinical setting—indicates that

macrophages and microglia display response to various stimuli by adopting specific phenotypes of activation (2). Both macrophages and microglia can acquire an activated M1 phenotype, characterized by the ability to release pro-inflammatory cytokines/mediators. Alternatively, they can express a M2 phenotype, which is associated with the ability to produce anti-inflammatory and immune suppressive factors. Recently an M0 phenotype (also referred to as resting microglia) has also been reported; M0 cells are considered to possess an 'attenuated' M2 phenotype.

Looking at TAM profile, in a preclinical model, Gabrusiewicz and collaborators showed that a continuum exists between the M1 and M2 like phenotypes; in the apparent difficulty to distinguish between M1 and M2 phenotypes, these authors concluded that GBM-infiltrated innate immune cells resemble M0 phenotype (3). In the last year, using *in vitro* models we focused our studies on microglia role in GBM pathology. In particular, we have investigated the interaction between rat microglia and rat C6 glioma cells (4,5). Exposure to conditioned media obtained from C6 cells taken under baseline conditions induced a predominant M2-like phenotype in rat microglia. Conversely, if C6 cells were exposed to a medium containing pro-inflammatory agent, the subsequent exposure of microglia to such medium was followed by a shift toward a M1-like phenotype (4). We interpreted these findings as the result of a positive-loop feed-back occurring between microglia and tumor cells: the exposure to inflammatory mediators causes tumor cells to release factor(s) able to shift the polarization state of microglia (4). In this context, we studied the role of mTOR Kinase and CCR5 receptor (5,6). Overall, our findings suggest than the presence of one phenotype rather than another appears to be related to the stage of disease.

In addition, we recently published that CD163 expression (M2 marker) is higher within the tumor than in surrounding periphery in both male and female patients; while iNOS (M1 marker) is higher within the tumor, no significant difference was found for ARG-1 (M2 marker). Furthermore, CD163 expression was significantly and inversely correlated with mean survival times. In contrast, no significant association was found between survival time and ARG-1 or iNOS expression (7). Based on our experience we may conclude that, while the classification of macrophages or microglial cells into the M1 or M2 polarized state is a well-established approach in most preclinical models, the same is not true in the clinical research setting, because of a high-degree of diversity and plasticity shown by these cell types in human pathology. TAMs were found to express both M1/M2 polarization markers in human GBM specimens (8,9). Cells within the tumor often display a complex pattern of phenotypes, up-regulating both M1 and M2 molecular markers, and no clear distinction exists between these phenotypes in many disorders. In light of such apparent difficulty in applying the M1/M2 paradigm to the CNS, it has been convincingly postulated that the notion of stimulus-dependent microglia phenotype should substitute that of microglia polarization (10,11).

Chen and colleagues also reported that the majority of GBM-associated macrophages are bone-marrow derived cells and that a continuous transformation of infiltrating monocytes into macrophages takes place during tumor progression (1). Moreover, the authors found that inhibition of perivascular monocyte infiltration increases the survival of GBM-bearing mice. These findings open up new perspectives for therapeutic strategies to reduce GBM aggressiveness by inhibiting the mobilization of bone marrow-derived myeloid cells that generate TAMs. One such approach might be for instance represented by the blockage of the vascular endothelial growth factor-1 (VEGFR-1), which is known to be expressed in hematopoietic progenitor cells. To this regard, we have recently generated an anti-VEGFR-1 monoclonal antibody (D16F7) characterized by a novel mechanism of action since it hampers the tyrosine kinase receptor activation and signal transduction without avoiding ligand binding. Interestingly, in an *in vivo* murine melanoma model D16F7 decreases myeloid progenitor mobilization as well as tumor infiltration by monocyte/macrophage (12). Actually, the VEGFR-1 ligands VEGF-A and placenta growth factor (PlGF) are produced by GBM where they can not only stimulate angiogenesis but also induce accumulation of VEGFR-1-positive bone marrow-derived myeloid cells in glioma tissues. Interestingly, D16F7 is able to inhibit angiogenesis and extracellular matrix invasion by tumor cells in response to VEGFR-1 ligands. The VEGFR-1 is expressed in endothelial cells of tumor-associated vessels as well as in human GBM cells or GBM stem cells and D16F7 inhibit GBM invasiveness (13). Since GBM is an infiltrative and highly angiogenic tumor, the advantage of blocking VEGFR-1 for GBM treatment if three-fold: inhibition of monocyte recruitment to the tumor mass, tumor-associated neovessels and tumor cell invasion in the brain parenchyma.

Even though the murine model might not closely reflect the *in vivo* situation of human GBM, the results obtained by Chen and colleagues certainly strengthen the rationale

for a multi-targeted approach in GBM treatment possibly including blockage of bone marrow-derived myeloid cells mobilization and monocyte extravasation into GBM.

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