

Characteristics of Phenotype and Pro-Tumorigenic Roles of Glioma Infiltrating Microglia/Macrophages

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Abstract

Recent clinical and experimental studies show an important role of tumor-infiltrating macrophages in tumor growth, metastasis and response to cancer treatments. Tumor-associated macrophages are attracted by tumor-released molecules which induce reprogramming/differentiation of macrophages/myeloid derived cells into anti-inflammatory cells known as alternatively activated, M2-type macrophages, in contrast to inflammatory M1-type. The stromal signals and components of immune microenvironment influencing glioma progression are poorly known and are likely distinct from those implicated in non-nervous system cancers. This review summarizes recent findings on characteristics of immune microenvironment of gliomas. Various functional properties of microglia in the normal and pathological central nervous system are now being revealed because of combinations of bone marrow transplantations and experimental disease models. Here, we describe some of the latest findings on the heterogeneity of glioblastoma-infiltrating brain macrophage, their alternative, immunosuppressive phenotype and contribution to glioma pathology. Recent attempts to determine a profile of cytokine/chemokine production and genes expression profiling on sorted CD11b⁺ cells from tumor tissues, revealed that many genes induced in M2-type macrophages from other tissues or in the context of helminth infections, are not up-regulated in CD11b⁺ cells isolated from gliomas. On the other hand, some genes characteristic for the alternative and pro-invasive phenotype: *arg-1*, *mt1-mmp* and *cxcl14* were consistently activated in those cells. Furthermore, we discuss the potential for targeting interactions between glioma and brain macrophages in therapeutic interventions. Small molecule inhibitors of MAPK signaling with immunosuppressive properties, such as cyclosporine A and minocycline were shown to block infiltration and activation of microglia/macrophages *in vitro*, in organotypic brain slices and *in vivo*. Small molecule inhibitors with anti-inflammatory properties significantly reduced infiltration of brain macrophages, angiogenesis and tumor growth in mice, demonstrating that blockade of their pro-invasive functions could be a novel therapeutic strategy in malignant gliomas

Keywords: Glioma; Microglia/macrophages; M2 phenotype; Gene expression; Pro/anti-inflammatory cytokines; Tumor angiogenesis; cyclosporine A

Abbreviations: AMCCase: Acidic Mammalian Chitinase; β IG-H3: Transforming Growth Factor-Beta-Induced Protein; FIZZ1: Found In Inflammatory Zone; IFN: Interferon; FcR: Fc-receptor; IL-1: Interleukin 1; IP-10: IFN γ -inducible protein 10; iNOS: inducible Nitric Oxide Synthase; MMP: Metalloproteinase; PD-L1, Programmed Cell Death 1 Ligand 1; PRR: Pattern Recognition Receptors; TGF: Transforming Growth Factor; TLR: Toll Like Receptor; Ym1: Chitinase-Like Protein

Introduction

Recent clinical and experimental studies suggest that the tumor microenvironment plays an important role in tumor growth, metastasis and the response to cancer treatment. Numerous non-neoplastic cells such as macrophages, lymphocytes, neutrophils, mast cells, pericytes and endothelial cells, which accumulate within tumors, produce various pro-angiogenic cytokines, proteolytic enzymes and growth factors [1,2]. It was initially thought that the organism recruits immune cells to tumors to defend itself against developing tumor. However, the opposite is true because infiltrating macrophages instead of initiating anti-tumor responses, enhance tumor cell migration and invasion through their secretion of chemotactic factors, enzymes, cytokines. In many human cancers, e.g. breast and ovarian cancers, glioblastomas, the abundance of macrophages in the tumor microenvironment correlates with poor prognosis. Accumulating evidence show that tumor-infiltrating macrophages called tumor-associated macrophages (TAM) promote tumor growth by enhancing vascularization, invasion, metastasis [1] and resistance to chemotherapy. Experimental studies in murine tumor models have identified and characterized various

TAM populations by their gene expression signatures and specific localization in distinct tumor regions [3-5]. Tumor-infiltrating cells include macrophages, myeloid-derived suppressor cells (MDSCs), mesenchymal stromal cells (MSCs) and TIE2-expressing monocytes (TEMs). MDSCs comprise a phenotypically heterogeneous population of cells of myeloid origin found in tumor-bearing mice and in patients with cancer and together with regulatory T cells (Tregs) promote an immunosuppressive environment in tumor-bearing hosts. The phenotype of MDSCs differs in humans and mice, and the exact mechanisms of their suppressive function are still controversially discussed [6]. The TIE2-expressing macrophages physically interact with tumor blood vessels and promote angiogenesis in mouse tumor models. Recent investigations noticed the up-regulation of some M2-associated genes such as CD163, Fc fragment of IgG, C-type lectin domains and heat shock proteins [7]. Compelling evidence suggests that tumor infiltrating macrophages can be phenotypically polarized in the tumor microenvironment to set up specific functional programs.

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A number of tumor-derived chemoattractants have been implicated in macrophage recruitment, including colony-stimulating factor-1 (CSF-1 also known as M-CSF), the CC chemokines, CCL2, CCL3, CCL4, CCL5, and CCL8, and vascular endothelial growth factor (VEGF) [8]. The levels of many of these proteins in human tumors correlate positively with the numbers of TAMs present in those tumors.

Glioblastomas are considered to be one of the most difficult human malignancies to manage, due to frequent dysfunctions of tumor suppressors and oncogenes [9,10]. Although glioblastomas rarely metastasize beyond the central nervous system, these highly infiltrative tumors often invade into normal brain tissues preventing surgical resection. The mean survival of patients with glioblastomas has only increased slightly and generally ranges between one and two years in spite of recent therapeutic advancements. Treatment of glioma patients remains a paramount challenge for clinicians and researchers [11]. Major signaling pathways that have been identified as playing important roles in glioblastomas are: the PTEN/PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK signaling cascades, which support cell proliferation, survival and invasion, and prevent apoptosis. Similar signaling pathways could be responsible for production of cytokines, chemokines and growth factors attracting monocytes and bone marrow cells to the tumor site. Histopathologic and flow cytometry studies of human glioma tissue have shown high levels of infiltrating microglia. The stromal signals that influence glioma formation and growth are poorly known and are likely distinct from those implicated in non-nervous system cancers.

Brain macrophages: origin and phenotypical heterogeneity

The term “brain macrophage” is used to encompass not only microglia but all types of macrophages infiltrating the brain, pericytes and perivascular macrophages. The consensus phenotypic profile for the microglial cell is: CD68⁺, CD45^{low}, CD11b⁺, CD11c^{high}, MHC class II⁺, CD14⁺ [12]. Based on their morphology and immunophenotype, perivascular macrophages appear to be very similar to blood-derived macrophages. Microglia are the central nervous system (CNS) resident macrophages that are the main innate immune cells which respond to pathogens or injury in and display the immune-effector functions. Once activated, they become immune effector cells mediating both innate and adaptive responses [13-16].

Microglial cells, in contrast to other central nervous system cell types such as neurons, astrocytes and oligodendrocytes, are of myeloid origin. Microglial progenitors, derived from extramedullary sources of hematopoiesis, invade the embryonic and fetal CNS. The second wave of microglial progenitors is formed by bone marrow-derived monocytic cells that colonize the CNS during the early postnatal period (P0-P15) in rodents, or before birth in human [17]. The traffic of leukocytes from blood to CNS parenchyma is exquisitely controlled by the blood-brain barrier. Perry and Gordon [18] have demonstrated that perivascular macrophages, pericytes, and probably microglia “turn over” from circulating monocytes. The rates of turnover vary considerably in mice: several months for perivascular macrophages and pericytes; years for microglia. It is not clear to what extent it reflects turnover in humans, as the issue of microglial renewal and monocyte infiltration under normal or pathological conditions has been controversial. Several lines of evidence indicate that a limited traffic of leukocytes to CNS occurs under specific circumstances, e.g. bone marrow transplantation to irradiated donors. Brains from female recipients of sex-mismatched donor marrow cells contain Y-chromosome⁺ cells that are present as perivascular macrophages or microglia. In these patients, the

recruitment is a fast process because up to 1% microglia is of donor origin 3 weeks after transplantation [19].

In mice, results from bone marrow (BM) chimera studies indicated that microglia are slowly replenished by bone marrow-derived cells and microglial turnover could be greatly accelerated under neurological conditions. However, recent works questioned the use of irradiation/reconstitution experiments and re-evaluation of microglia origin in the inflamed CNS has been suggested [20]. Moreover, studies of the recruitment and microglial transformation of BM-derived cells in irradiated BM-chimeric mice and rats after transient global cerebral ischemia, revealed interspecies differences. Both species displayed microglial hyperplasia and cell transformation in the hippocampal CA1 region: but in mice, lesion-reactive microglia originated from transformed BM-derived cells, while no such recruitment or microglial transformation was observed in BM-chimeric rats. These results suggest that reactive microglia in rats originate from resident microglia, whereas they have a mixed BM-derived and resident origin in mice, depending on the severity of ischemic tissue damage [21].

One of the most remarkable features of microglia is their high level of morphological and functional plasticity in response to activating stimuli. Thus, in their resting state, microglia displays a ramified morphology. However, under number of pathological conditions, quiescent ramified microglia, also called resting microglia, will activate and engage a series of morphological alterations that leads to a hypertrophy of microglia cell body and a retraction of their ramifications [14,22]. Fully activated (reactive) microglia, harbor a similar morphology than any activated macrophage. Some evidence suggests that microglia is not fully differentiated and express some features of progenitor cells. Several molecules, among them interleukin (IL)-3, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), and colony stimulating factor (CSF-1), have been demonstrated to stimulate microglia proliferation *in vitro* and for all of these cytokines, microglia express the appropriate receptors; other tissue-resident macrophages do not proliferate. Under some pathological conditions, activation of microglia is coupled with their proliferation, which leads to the focal accumulation of activated microglia [20,23].

Accumulation of microglia/macrophages in glioblastomas

Histopathologic and flow cytometry studies of human glioma tissue have shown high levels of infiltrating microglia. Intratumoural microglial density increases during glioma progression and correlates with the grade of malignancy [24-26]. A preliminary study by Wierzbak-Bobrowicz et al. [25] demonstrated the presence of both the ramified and amoeboid microglia in the protoplasmic and fibrillary astrocytomas, while in glioblastomas and anaplastic astrocytomas the greatest number of amoeboid microglia and very rarely ramified microglial cells were found. In the non-anaplastic tumors such as gemistocytic astrocytomas numerous but mostly ramified microglia have been observed. Further studies using immunohistochemical markers for the monocyte/macrophage lineage (Ki-M1P, HLA-DR, KP1, My4, My7, Ki-M1, Ki-M6, EB11) showed that among the different tumors, glioblastomas and anaplastic gliomas showed the largest number of macrophages and ramified and amoeboid microglia. Fewer, predominantly amoeboid, microglia were found in glial tumors of low malignancy. Neuronal tumors showed only a mild increase of microglia [26] suggesting some type of specificity for microglia-glioma interactions.

Gemistocytic astrocytomas contain unusually high numbers of microglial cells and aberrant MHC Class II expression by tumor cells

correlates with a loss of immune-competent microglia. Although gemistocytic astrocytomas are graded as World Health Organization II astrocytomas and their proliferative potential is low, they behave aggressively. A poor prognosis of gemistocytic astrocytomas could be due to pro-invasive role of microglia [27]. An immunohistochemical double-labeling study of pilocytic astrocytomas and astrocytomas WHO grade II-IV using the antibodies Ki67 (as proliferation-marker) and CD68 (as microglia marker) demonstrated that microglial cells in astrocytic brain tumors proliferate with the highest rates of proliferating microglia especially in pilocytic astrocytomas. The proliferation indices of microglia were lowest in fibrillary astrocytomas [28].

Flow cytometry has been used to identify microglial cells within the central nervous system. Highly purified populations of CD45^{low}CD11b/c⁺ microglia and CD45^{high}CD11b/c⁺ macrophages have been obtained directly from the adult CNS [29]. Using this method Badie and Schartner [30] determined infiltration of immune cells into experimental gliomas (intracranially implanted rat C6, 9L, and RG-2 tumor cells) based on detection of CD11b/c, CD45, and CD8a antigens by flow cytometry. The extent of microglia (CD11b^{high}, CD45^{low}), macrophage (CD11b^{high}, CD45^{high}), and lymphocyte (CD11b^{negative}, CD45^{high}) infiltration into tumors, tumor periphery, and contralateral tumor-free hemispheres was measured for each glioma type. They found that microglia account for 13 to 34% of viable cells and were present in the tumors, tumor periphery, and contralateral tumor-free hemispheres. In contrast, macrophages were less prominent within the tumors and tumor periphery (4.2-12%) and were rare in the contralateral tumor-free hemispheres (0.9-1.1%). Among the tumor types, RG-2 gliomas had the least microglia/macrophage infiltration. The distribution pattern of lymphocytes varied among tumor models: whereas lymphocytes accounted for more than one-third of the cells in C6 and 9L tumors, they represented only 1% of cells in RG-2 gliomas.

Using immunomagnetic sorting of CD11b-positive cells followed by detection of CD45 antigens by flow cytometry, we demonstrate an early accumulation of activated microglia followed by accumulation of peripheral macrophages in experimental murine EGFP-GL261 gliomas. Quantification of glioma-infiltrating microglia/macrophages revealed a ~2.6-fold increase in the number of microglia and a ~30-fold increase in the number of macrophages 15 days after tumor implantation [31]. Iba1 staining of tissue sections revealed only a few ramified Iba1-positive cells detected in the intact brain. A strong increase in Iba1-positive cells was observed at day 8th after tumour implantation. At the day 15 the tumor was heavily infiltrated with amoeboid, Iba1-positive cells that accumulated within and around the implanted glioma cells. Interestingly, Iba1-positive cells in the close vicinity of the tumor were evidently more activated and amoeboid than the cells located distantly. Ramified microglia, with thin branching processes and a small cell body were detected in the tumor-free parenchyma (Figure 1) [31]. Further studies of glioma-infiltrating microglia/macrophages in chimeric mice with the reconstituted green bone marrow showed a significant infiltration of peripheral macrophages into experimental murine DsRed-GL261 gliomas (Sielska et al. unpublished).

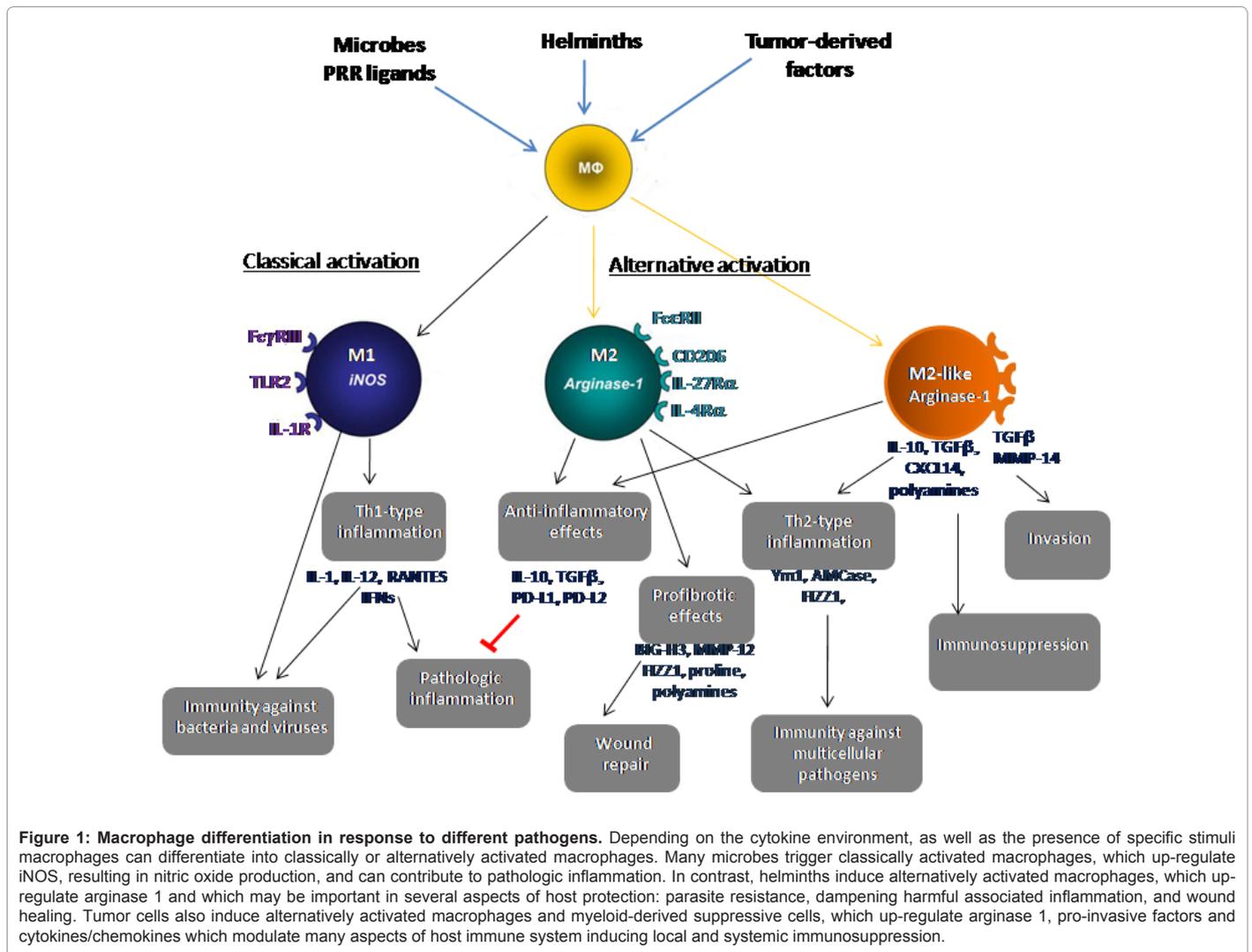
Polarized macrophages - phenotypical and functional characteristics

Polarized macrophages can be broadly classified in two main groups: classically activated, inflammatory macrophages (M1 type), whose activating stimuli are interferon gamma (IFN γ) and lipopolysaccharide (LPS) and alternatively activated macrophages (M2 type). The latter could be further subdivided depending on stimuli. In

general, M1 type macrophages exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses, whilst M2 support Th2-associated effector functions [32,33]. M2 polarized macrophages play a role in resolution of inflammation through high endocytic clearance capacities and trophic factor synthesis, accompanied by reduced pro-inflammatory cytokine secretion. Chronic evolution of infectious diseases such as brucellosis, mycobacterial infections, Buruli disease, lepromatous leprosy and helminthic parasite infections frequently leads to development of a M2 profile. Some bacterial pathogens or parasites have evolved multifaceted strategies to prevent M1 polarization, neutralize microbicidal effectors of macrophage, or promote M2 polarization [34]. Recent studies suggest a role of M2 macrophages in autoimmune diseases. Monocytes/macrophages in granulomas of muscle tissue from patients with sarcoidosis show M2 activation based on their expression of CD206, CD301, arginase-1, and suppressor of cytokine signaling-1, CCL18 with poor inducible nitric oxide synthase and cyclooxygenase-2 expression [35].

The various forms of M2 macrophages share an interleukin IL-12^{low} and IL-23^{low} phenotype, generally display high levels of scavenger, mannose and galactose-type receptors, and arginine metabolism is shifted to production of ornithine and polyamines via arginase [36-38]. Arginase-1 is up-regulated in alternatively activated macrophages and, due to its higher affinity for arginine, better competes with inducible nitric oxide synthase (iNOS), which metabolizes arginine. Therefore arginase-1 and its metabolic products, including urea and proline, are indicative of M2 differentiation. Previous studies have addressed the issue of profiling gene expression in M1 or M2 macrophage activation leading to the identification of new molecules expressed in polarized murine macrophages (e.g., Ym1, Fizz1, MRC1) [39]. Alternatively activated macrophages during nematode infection express certain chitinase and FIZZ (found in inflammatory zone) proteins, including: FIZZ1/RELM α , Ym1, and acidic mammalian chitinase. Other described moieties include: mouse macrophage galactose-type C-type lectins, IL-27R α , matrix metalloproteinases (MMPs) and extracellular matrix protein β IG-H3 [40]. Data on human mononuclear phagocytes are rare and highlight interspecies differences in key molecules, such as arginase 1 and inducible NO synthase.

There is an increasing interest in immunosuppressive cells of myeloid origin, myeloid-derived suppressor cells (MDSCs). MDSCs can be defined as a population of myelomonocytic cells normally lacking the markers of mature myeloid cells and commonly expressing both Gr-1 and CD11b in mice, with a high potential to suppress immune responses *in vitro* and *in vivo*. Accumulation of these cells has been reported under pathological conditions including bacterial and parasitic infections, acute and chronic inflammation, and in cancer. MDSCs accumulate in the blood, lymph nodes, and bone marrow and at tumor sites in most patients with breast, lung, prostate, kidney, head and neck and other types of cancer and experimental animals with cancer and inhibit both adaptive and innate immunity. In cancer patients they were detected in the blood. Mononuclear MDSC (considered "monocytic") typically are CD11b⁺Ly6G⁺/Ly6C^{high}, whereas those with multilobed nuclei are "granulocytic/neutrophil-like" and have a CD11b⁺Ly6G⁺Ly6C^{low} phenotype. In both patients and experimental animals MDSC levels are driven by tumor burden and by the diversity of factors produced by the tumor and by host cells in the tumor microenvironment. MDSC indirectly effect T cell activation by inducing T regulatory cells (Tregs), which in turn down-regulate cell-mediated immunity. Depending on the subpopulation of MDSC, Treg induction requires MDSC production of IL-10 and TGF β or arginase



and is independent of $TGF\beta$ [41]. MDSC also perturb tumor immunity by distorting it toward a tumor-promoting type 2 phenotype via production of IL-10 and by down-regulating macrophage production of IL-12 [42].

Although the precise nature of the suppressor cell population depends on tumor-derived factors, they share some common characteristics: lack or reduced expression of markers of mature myeloid cells, expression of both Gr-1 and CD11b molecules in mice, inability to differentiate into mature myeloid cells in the presence of tumor-derived factors, high levels of reactive oxygen species, activation of arginase I and other molecules [43,44]. In mice, MDSC are uniformly characterized by the expression of the cell surface molecules detected by Abs to Gr1 and CD11b. Gr1 includes the monocyte and neutrophil markers Ly6C and Ly6G, respectively, whereas CD11b is characteristic of macrophages. A number of different surface molecules: the α -chain of the receptor for IL-4 and IL-13 (IL-4R α), macrophage marker F4/80, M-CSF receptor or *c-fms* (CD115), PD-L1, PD-L2, Ly6C and Ly6G and the costimulatory molecule CD80 (B7.1) were suggested to define different immune suppressive myeloid cells [44,45]. Because the expression of many markers is restricted to Gr1⁺CD11b⁺ cells present in only a subset of tumors, aside from Gr1 and CD11b, there

are no unambiguous cell surface markers that define all mouse MDSC populations.

M2 polarized microglia/macrophages and myeloid-derived suppressor cells in gliomas

A comprehensive analysis of immune cells infiltrating glioblastomas revealed that microglia/macrophages were the predominant population of immune cells (approximately 1% of total cells); others identified were myeloid and plasmacytoid dendritic cells, and T cells. The immune functions of CD11b/c⁺CD45⁺ glioma-infiltrating microglia/macrophages from postoperative tissue specimens of glioma patients have been reduced, as they did not produce pro-inflammatory cytokines (TNF α , interleukin 1 β , or interleukin 6), and do not mediate T-cell proliferation. They express major histocompatibility complex class II, but they lacked expression of the co-stimulatory molecules CD86, CD80, and CD40 that are critical for T-cell activation. The presence of regulatory T cells may also contribute to the lack of effective immune activation against malignant human gliomas [46].

Positively stained for CD163 and CD204, which are believed to be markers for “M2” macrophages [47], as well as myeloid-derived suppressor cell (MDSC)-like cells have been detected in glioma patients

[48]. The ratio of CD163 and CD204 positive cells among glioma-infiltrating microglia/macrophages correlates with the histological grade of glioma [47]. Patients with glioblastoma have increased MDSC counts (CD33⁺HLADR⁻) in their blood that are composed of neutrophilic (CD15⁺; >60%), lineage-negative (CD15⁻CD14⁺; 31%), and monocytic (CD14⁺; 6%) subsets. T cells from patients with glioblastoma had suppressed IFN- γ production after stimulation and removal of MDSCs by pre-incubation with anti-CD33/CD15-coated beads significantly restored T cell function. Significant increases in arginase activity and G-CSF levels were observed in plasma specimens obtained from patients with glioblastoma [49].

Increased tumor infiltration by MSDC has been reported in rat glioma models [50,51]. Animal studies on GFAP-V(12)HA-ras mouse astrocytomas demonstrate that immune infiltration at the tumor site in mice is dominated by immunosuppressive cells from the early stages of tumor development, even at very early asymptomatic stages [52]. Phenotypical characterization of MSDC in GL261 murine glioma suggests that tumor-infiltrating myeloid-derived suppressor cells are pleiotropic monocytes/macrophages that bear M1- and M2-type characteristics. Over 90% were of the CD11b⁺F4/80⁺ monocyte/macrophage lineage and displayed a MDSC phenotype - CD11b/c⁺Gr-1^{low}IL-4Ra⁺ phenotype and suppressed the proliferation of activated splenic CD8⁺ T cells. These MDSCs expressed both M1 and M2 activation markers: CD206, CXCL10, IL-1 β , TGF- β and TNF- α mRNAs, and CXCL10, CD206 proteins. In addition, the cells expressed CX (3) CR1 and CCR2 simultaneously, which are the markers of an inflammatory monocyte [53].

Soluble factors secreted by glioblastoma cells have been shown to stimulate cultured peripheral blood monocytes to differentiate into the M2-like or MSDC-like cells, characterized by increased CD163 and CD204 staining, reduced IL-12 and TNF α production [47] and up-regulation of IL-10, and TGF- β [48]. Peripheral blood monocytes co-cultured with glioblastoma cells (U87 and U251) acquired immunosuppressive, MDSC-like features, including reduced CD14 (but not CD11b) expression, increased immunosuppressive interleukin-10, transforming growth factor-beta, and B7-H1 expression, decreased phagocytic ability, and increased ability to induce apoptosis in activated lymphocytes. Direct contact between monocytes and glioblastoma cells was necessary for complete induction of such phenotype [48]. These results support a hypothesis that normal human monocytes exposed to malignant glioma cells would adopt an MDSC-like phenotype and MDSC are increased in glioblastoma patients. Local and/or systemic immunosuppressive effects of glioma-infiltrating macrophages/microglia, MSDC and circulating regulatory T cells have been described. Thus, multiple, immunologically active cell types are present in malignant glioma patients that may all contribute to immunosuppression. Interestingly, a recent study has demonstrated immunosuppressive properties of glioma cancer stem cells which produce soluble CSF-1, TGF- β 1 and macrophage inhibitory cytokine (MIC)-1, thus inducing recruitment and polarization of macrophages/microglia into immunosuppressive cells [54].

Pro-invasive functions of glioblastoma-infiltrating microglia/macrophages

Recent data support the notion that microglia accumulation in diffuse glial tumors reflects participation of these cells in promoting and supporting the invasive potential of gliomas. Microglia and macrophages can secrete various cytokines and growth factors that may contribute to the successful immune evasion, growth, and

invasion of brain neoplasms. They can also release many factors, including extracellular matrix proteases which directly or indirectly may influence tumor migration/invasiveness and proliferation [55-58].

Experimental studies using brain organotypic slices, microglia-glioma co-cultures and genetic models, in which microglial cells are ablated in the tumor, strongly support pro-invasive role of glioma-infiltrating microglia [5961]. The invasion of GFP labeled GL261 glioblastoma cells that were depleted of microglia by treatment with clodronate-filled liposomes was significantly decreased. Inoculation of exogenous microglia together with glioma cells into cultured brain slices increased the infiltrative behavior of the tumor depending on the microglia/glioma cell ratio and increased activity of metalloprotease-2. It has been shown that soluble factors released from glioma cells strongly stimulate metalloprotease-2 activity in microglia [59]. Membrane bound MT-MMPs, in particular membrane-type MT1- and MT2-MMP, play a major role in activating MMP-2. Newly synthesized MMP-2 is secreted as an inactive pro-enzyme, which is cleaved on the cell surface by membrane-type MT1-MMP complexed with TIMP-2. Metalloproteinase MT1-MMP secreted by glioma-exposed microglia activates pro-MMP-2 in glioma cells that promotes tumor invasion, as was shown using brain slices from MT1-MMP-deficient mice and in a microglia depletion model [59]. Glioma-released factors induce the expression and activity of MT1-MMP via microglial toll-like receptors and the p38 MAPK pathway, as deletion of the toll-like receptor adapter protein MyD88 or p38 inhibition prevented MT1-MMP expression and activity in cultured microglial cells. Microglial MT1-MMP in turn activates glioma-derived pro-MMP-2 and promotes glioma expansion, as shown in an *ex vivo* model using MT1-MMP-deficient brain tissue and in microglia depleted mice [60].

Activated microglia release cytokines which enhance tumor cell invasion [62]. Microglial cells exposed to glioblastoma cells secrete active TGF- β 1, which through a paracrine loop stimulates glioblastoma invasion. We developed plasmid-transcribed small hairpin RNAs (shRNAs) to down-regulate the TGF- β type II receptor (T β IIR) expression, which effectively inhibited TGF- β -dependent signaling pathways and transcriptional responses in glioblastoma cells. Microglia strongly enhanced glioma invasiveness in the co-culture system, but this activity was lost in glioma cells depleted of TGF- β type II receptor indicating an important role of microglia-derived TGF- β . Moreover, tumorigenicity of glioblastoma cells depleted of TGF- β IIR in nude mice was reduced by 50%.

Fas (CD95/APO-1) is a cell surface "death receptor" that mediates apoptosis upon engagement by its ligand - FasL but can also promote tumor invasion when apoptosis is compromised. Kleber and co-workers (2009) [63] demonstrated that interaction of glioma cells with the surrounding brain tissue induces expression of FasL in both tumor and host cells. FasL modulated glioma invasion via PI-3K/MMP-dependent mechanism and neutralization of Fas activity blocked migration of glioma cells in a mouse syngenic model of intracranial glioblastoma [63]. We demonstrated that a recombinant FasL Interfering Protein (FIP), which interferes with Fas signaling in C6 glioma cells, impaired cell motility and invasiveness of glioma cells *in vitro*. Blockade of Fas signaling reduced MMP-2 activity in glioma cells. Interestingly, reduction of MMP-2 activity was not due to down-regulation of *mmp-2* and *mt1-mmp* expression by Fas signaling dependent modulation of *timp-2* mRNA and TIMP-2 protein levels [64]. FasL expression is higher in many glioblastoma cell lines in comparison to its expression in non-transformed astrocytes and is up-regulated in microglia

exposed to glioma-conditioned medium [64]. Microglia accumulating within tumors account for a half of the FasL expression in the murine intracranial tumors [65].

Signals, signaling pathways and molecular mechanisms underlying “switch” or “re-education” of glioma-infiltrating macrophages into pro-invasive cells are currently unknown. We have previously demonstrated that glioma-derived factors transform rat primary microglial cells into amoeboid cells, without inducing production of the inflammation mediators nitric oxide and interleukin 6 [61]. Further studies on cultured microglial cells demonstrate its remarkable plasticity and ability to adapt different fate or differentiation route depending on a stimulus. Rat microglial cultures exposed to glioma-conditioned medium (G-CM) undergo morphological alterations, become motile and highly phagocytic. High-throughput screening of gene expression in LPS- or G-CM- stimulated microglial cultures followed by computational analysis of gene expression pattern in differentially stimulated microglia revealed activation of alternative genetic programs. Most genes characteristic for innate and inflammatory immune response, which are commonly up-regulated during classical inflammation, were not induced by G-CM stimulation. The analysis of signaling pathways activated in microglia after G-CM demonstrates defective NF κ B and STAT 1 activation, resulting in failure to mount production of inflammation mediators (IL-1 β , iNOS, Cox2). Furthermore, increased expression of strong negative and positive modulators of transcription in G-CM activated microglial cultures could be responsible for its sustained proliferation and acquisition of the alternative phenotype (Wisniewski et al., unpublished).

Targeting glioma-infiltrating microglia/macrophages as a novel therapeutic strategy

Recent studies from our laboratory demonstrated that pharmacological inhibition of infiltration and activation of brain resident microglia and peripheral macrophages to the glioma effectively considerably impairs tumor growth in mice [31,61]. Cyclosporine A (CsA), a widely used immunosuppressive drug and atypical MAPK signaling inhibitor reduced morphological transformation of microglial cell into amoeboid cells and microglia-dependent invasion of glioma cells *in vitro* and in organotypic brain slices [61,66]. A systemically applied CsA inhibits microglia/macrophages infiltration, blocks expression/activity of enzymes (MMP-2, MT1-MMP) and production of cytokines (IL-10 and GM-CSF), which are important for establishment of a pro-invasive phenotype of glioma-infiltrating microglia/macrophages [31]. Our data demonstrate that blockade of the alternative activation of brain macrophages blocks tumor infiltration, impairs their pro-invasive behavior considerably reducing tumor growth in mice.

In recent years, minocycline, a semisynthetic antibiotic of the tetracycline family, has emerged as a potent anti-inflammatory and microglia targeting drug that has been shown to be beneficial in animal models of many CNS disorders. For the reason of the good tolerance and penetration into the brain, minocycline has been clinically tested for some neurodegenerative diseases such as stroke, multiple sclerosis, spinal cord injury, amyotrophic lateral sclerosis, Huntington and Parkinson diseases [67]. The treatment with minocycline reduced the expression of membrane type 1 matrix metalloprotease (MT1-MMP) in tumor associated microglia *in vitro* and in organotypic brain slices. This reduction depended on the presence of microglia. Glioma

growth in an experimental mouse model was strongly reduced by the addition of minocycline to drinking water, compared to untreated controls. Coherently, MT1-MMP was abundantly expressed in glioma associated microglia in controls, but was strongly attenuated in tumors of minocycline treated animals [68]. This suggests that the clinically approved antibiotic minocycline is a promising new candidate for adjuvant therapy against malignant gliomas. However, minocycline and CsA treatments have some drawbacks because while blocking tumor-associated activation of microglia, they exert immunosuppressive action and inactivate many components of the immune system [69]. Minocycline represses MHC II expression in microglia (an event requisite for T cell reactivation) in an experimental model of autoimmune diseases via the inhibition of transcription factor CIITA expression [70].

Recent studies defined more specific, intracellular signaling pathways which could be targeted. Signal transducers and activators of transcription 3 (STAT 3) plays a suppressive role in antitumor immunity [71]. STAT 3 was activated in immortalized N9 microglial cells exposed to GL261 glioma conditioned medium that resulted in up-regulation of IL-10 and IL-6, and down-regulation of IL-1 β . Inhibition of STAT 3 by CPA-7 (the platinum (IV)-based anticancer drug) or specific siRNA reversed glioma-induced cytokine expression profile in N9 cells. Furthermore, inactivation of STAT 3 in intracranial GL261 gliomas by siRNA resulted in inhibition of tumor growth [72].

Glioblastomas are heterogenous, as many other tumors, contain glioma stem cells or glioma-initiating cells, a subpopulation of cells that possess the capacity for self-renewal and forming neurospheres *in vitro*, are capable of pluripotent differentiation, and could initiate tumors *in vivo*. Glioma-initiating cells have been shown to suppress adaptive immunity by affecting capacity to inhibit T-cell proliferation, triggering T-cell apoptosis, and induction of FoxP3 (+) regulatory T cells [73]. A recent study demonstrates that glioma cancer stem cells produce sCSF-1, TGF- β 1, and MIC-1, cytokines known to recruit and polarize the macrophages/microglia to become immunosuppressive phenotype. Glioma-initiating cells polarized human macrophages/microglia to an M2 phenotype, inhibited phagocytosis, induced the secretion of the immunosuppressive cytokines interleukin-10 (IL-10) and TGF- β 1, and enhanced their capacity to inhibit T-cell proliferation. The inhibition of phagocytosis and the secretion of IL-10 were reversed when the STAT 3 pathway in glioma stem cells was blocked with a pharmacological inhibitor WP1066 or specific STAT shRNA [54]. However, such approaches are in infancy, these findings illustrate that interactions between glioma and brain macrophages are potential, new targets in glioma therapy.

Current understanding the mechanisms responsible for high invasiveness of glioblastoma cells, which is even accelerated by interactions of the brain tumor with surrounding tissue, may lead to identification of specific targets for a future treatment. Therapies that will effectively target invasive glioblastoma cells may significantly improve therapeutic outcome. Thus, counteracting accumulation and activation of brain macrophages should be taken into account when considering the development of more effective therapy against malignant gliomas.

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