Molecular interactions between tumor and its microenvironment in malignant gliomas

ABSTRACT

Growing evidence supports a critical role of the tumor-reprogrammed stromal cells in tumor growth and progression. Several extracellular communication networks are hijacked by the tumors to influence the surrounding tumor microenvironment. In malignant gliomas, tumor derived factors attract brain resident microglia and peripheral macrophages. These cells, instead of initiating antitumor responses, are re-educated by tumor cells and participate in matrix remodeling, support invasion and angiogenesis, and induce immunosuppression. Molecular underlining of these mutual and complex interactions in malignant gliomas is the main scope of this review.

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Abbreviations: CNS – central nervous system; CSC – cancer stem-like cells; ECM – extracellular matrix; GAM – glioma associated microglia/macrophages; GBM – glioblastoma; MDSC – myeloid-derived suppressor cells; MMP - matrix metalloproteinase; TME – tumor microenvironment

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INTRODUCTION

Cancer is driven by genetic and epigenetic abnormalities that accumulate in pre-malignant cells and lead to uncontrolled growth, resistance to cell death, invasion, reprogramming of energy metabolism, neoangiogenesis and evasion of immune recognition and destruction [1]. Moreover, a growing number of experimental and clinical data shows that numerous non-neoplastic cells such as macrophages, lymphocytes, neutrophils, mast cells, stromal fibroblasts, pericytes and endothelial cells accumulate within a tumor niche and contribute to tumor growth, progression and resistance to treatment [2-4]. This supportive stroma composed of various populations of cells surrounded by the extracellular matrix (ECM) creates the tumor microenvironment (TME) [1,5].

Composition and structure of tumor surrounding ECM, as well as functions of residing stromal cells and infiltrating immune cells are modified by the neoplastic cells. One of the main infiltrate are innate immune cells, collectively called tumor-associated macrophages (TAMs), which are the key responders to tumor-derived signals in many types of cancer. Instead of initiating anti-tumor responses, TAMs play an instrumental role in shaping a tumor niche by promoting angiogenesis, supporting tumor invasion, and by mobilization of different cells from circulation and a bone marrow [3,4,6,7]. In this review we focus on molecular interactions between various cells infiltrating glioblastoma (GBM, WHO grade IV glioma), the most common and aggressive primary brain tumor in adults [8]. Due to diffusive growth impeding complete surgical resection and poor responses to current therapies, GBMs invariably re-grow and are considered to be one of the most deadliest human malignancies. Despite application of surgery, radio- and chemotherapy the median survival of GBM patients is 15 months from diagnosis [9]. We describe cellular composition of GBMs, key signaling factors produced by GBM cells to attract and "re-educate" infiltrating cells, as well as mechanisms triggered in recruited immune and stromal cells that are implicated in facilitating tumor growth (summarized in figure 1). Communication networks in GBM can be formed by direct cell-to-cell contact via specialized structures, by shedding membrane vesicles (i.e. microvesicles, exosomes), secretion of soluble factors or binding to constituents of ECM by both tumor and host cells. The two latter types of interactions, which are based on ligand – receptor axes, are the major scope of this review. In the final section of the manuscript we provide examples of targeting these interactions by new therapeutics, which hold a promise to be affective against GBM.

TUMOR MICROENVIRONMENT OF GBM

Brain tumors, similarly to non-brain cancers, attract and change the phenotype of stromal cells, creating tumor-permissive TME. Brain tissue is composed of a variety of cells, including neurons, astrocytes, oligodrocytes and

microglia, all cells submerged in ECM. Brain is also physically protected from periphery by the blood-brain barrier, which does not allow penetration of peripheral immune cells under physiological conditions, and is preserved in early-stage brain tumors. However, in advanced tumors, such as GBM, the blood-brain barrier is often compromised allowing a robust infiltration of multiple immune cell types from circulation [7,10,11].

Microglia are the brain surveying macrophages and constitute the first line of innate immune defense in the central nervous system (CNS) [11,12]. Microglia develop from embryonic yolk sac progenitor cells, migrate to the CNS early in the development [13] and are not replenished postnatally by peripheral mononuclear hematopoiesis [14]. Although ontogenetically distinct, once attracted and activated by glioma cells, microglia can't be distinguished from infiltrating peripheral, bone marrow derived macrophages and are jointly characterized as glioma-associated microglia/macrophages (GAMs) [10,11,15]. Clinical and experimental studies, including ours, show that GAMs are a major component of leukocytic infiltrates in rodent glioma models and in human GBMs constituting up to 30% of the tumor mass [16-21]. Other identified cells include dendritic cells, myeloid-derived suppressor cells (MDSCs), Th-2 polarized and regulatory T-lymphocytes, as well as natural killer (NK) cells and CD8+ cytotoxic T cells [15]. Interestingly, despite accumulation of numerous innate immune cells, the proper antitumor responses in GBMs are not initiated. Usually upon stimulation microglia become immune effector cells and produce many factors activating other immune cells. Similarly to other tissue macrophages, microglia can acquire distinct functional phenotypes and depending on a type of stimulus, these cells can initiate either inflammation or immunosuppression/wound healing processes [12,22]. In response to tissue injury or microbial infection microglia polarize to M1, inflammatory cells, which also participate in tumor surveillance and carry on antitumor activity in benign gliomas [11,23]. They secrete inflammatory mediators and cytokines (nitric oxide, tumor necrosis factor (TNF)α, interleukin (IL)-12 and IL-23 that promote Th1 responses of T lymphocytes. "Alternatively activated" M2 microglia or macrophages play a role in resolution of inflammation by endocytic clearance, tissue repair and trophic factor synthesis, and support Th2-associated effector functions. 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 1 [12,24]. Functional polarization of different tissue macrophages to the M2 phenotype occurs under physiological conditions: e.g. ontogenesis and pregnancy, and in pathology: parasite infections, allergic reactions and chronic inflammation, tissue repair and remodeling, infection and cancer [25].

Formation of gliomas can be imitated in animal models, which are created by either implantation of glioma cells to a brain structure called the striatum or by genetic manipulations in transgenic mice introducing oncogenic genes into neural cells. The resulting tumors could be isolated from tumor-bearing hemispheres and after mechanical and enzymatic dissociation, specific cells could be labelled and sorted by flow cytometry to analyze tumor composition and properties of specific cells. Glioma infiltrating microglia/macrophages are recognized as CD11b expressing cells and could be further separated into subpopulations based on CD45 expression: with microglia being CD11b+CD45^{low} and macrophages being CD11b+ CD4^{5high}. One of the most frequently used methods is evaluation of gene expression profiles of those cells by RNA sequencing or microarrays, which provides a global picture of undergoing processes. We and other researchers demonstrated that GAMs isolated from rodent and human gliomas exhibit the immunosuppressive and pro-invasive phenotype [11,18,19,21,26]. Categorization of GAMs as having the M2 phenotype has been recently found inaccurate because of oversimplification of their highly complex and heterogeneous responses. Transcriptomic analyses of CD11b+ cells isolated from human GBMs and rodent experimental gliomas showed a small overlap between their expression profiles [21,27,28]. Moreover, computational comparison of genes significantly changed in GAMs from different experimental models and clinical samples revealed only a small set of common genes [29]. This could be due many reasons: technical differences in cell isolation, a presence of mixed subpopulations of cells with different functions, inadequacy of animal models poorly reflecting human pathology or imprecise categorization of M1/M2 markers. Although, these collected results are biased by differences in isolation procedure and cell identification criteria, the observed heterogeneity of responses may also result from co-existence of pro- and anti-inflammatory subsets of microglia and macrophages at specific phases of tumor evolution as well as dissimilar roles of microglia and peripherally recruited macrophages, which are yet to be understood [30,31]. Noteworthy, functional analysis on compared transcriptomic datasets from isolated GAMs shows similarities in upregulated cellular signaling pathways across all models [29]. It suggests that regardless of the observed differences, there is still an universal mechanism orchestrating polarization of microglia and macrophages within the tumor microenvironment. Thus, despite discrepancies between species and models, there is currently a consensus on pro-tumorigenic functions of these cells in GBMs.

TUMOR-DERIVED MOLECULES DRIVING RECRUITMENT AND POLARIZATION OF STROMAL AND IMMUNE CELLS

Signals that are responsible for recruitment of heterogeneous cell populations to glioblastomas and inducing the tumor-supportive phenotype in stromal and infiltrating cells are still inadequately understood. Due to deregulation of signaling pathways, distinct metabolism and behavior, tumor cells produce distinct metabolites, secrete factors and release microvesicles carrying different molecules (miRNA, RNAs, proteins). Numerous studies showed secretion of a plethora of chemotactic and differentiation factors that attract various populations of immune and stromal cells, and switch them to different phenotype in the immunosuppressive and pro-invasive

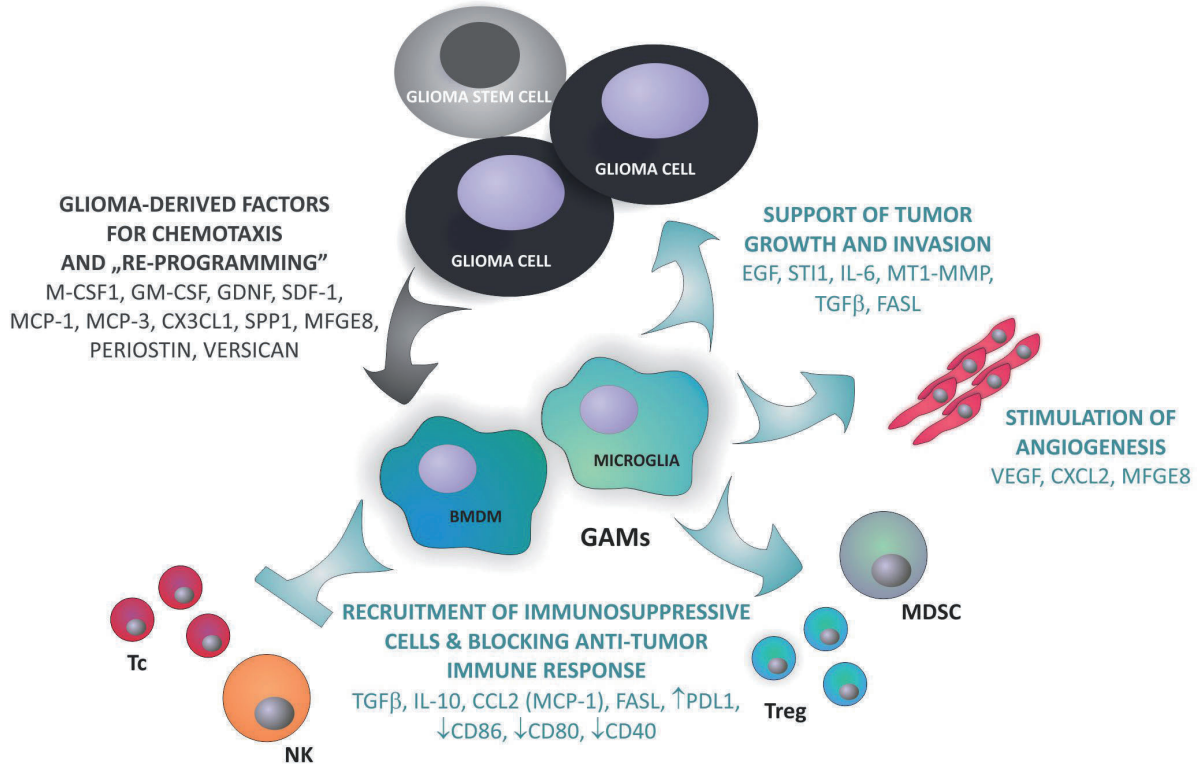

Figure 1. Communication network in TME of malignant gliomas.

Brain resident microglia and bone marrow-derived macrophages (BMDM) are attracted and polarized by glioma-derived factors to tumor supporting cells, called glioma-associated microglia and macrophages (GAMs). GAMs play a key role in shaping TME to promote tumor development. They support glioma cells proliferation and invasiveness, stimulate angiogenesis and contribute to immune evasion by recruitment of immunosuppressive cells, such as myeloid derived suppressor cells (MDSC) or T regulatory cells (Treg) and blocking the function of cytotoxic T cells (Tc) and NK cells.

environment [7,11,32]. Several glioma-derived factors have been implicated in controlling recruitment and/or polarization of host cells, mainly GAMs, to the glioma-supportive phenotype (Fig. 1).

CYTOKINES AND GROWTH FACTORS

Macrophage proliferation, differentiation and chemotaxis is regulated by several factors, including macrophage colony-stimulating factor (M-CSF/CSF-1), granulocyte macrophage colony-stimulating factor (GM-CSF/CSF-2), interleukin 34, chemokine CCL2, macrophage chemoattractant proteins (MCPs). The colony-stimulating factors: GM-CSF, M-CSF and G-CSF participate in granulocyte and myeloid hematopoiesis, and regulate many immune functions [33].

The macrophage colony stimulating factor 1 (M-CSF/CSF-1/) is required for differentiation, proliferation and survival of monocytes and macrophages and released by many types of cells (macrophages, fibroblasts, endothelial cells) [34]. M-CSF/CSF-1, but also IL-34, bind to CSF-1R receptor expressed on monocytes, tissue macrophages and monocyte-derived dendritic cells. Ligand-receptor interaction induces phosphorylation of the receptor and recruitment of Src kinase, PI3-kinase and Cbl adaptor protein followed by activation of downstream signaling pathways [35,36]. It has been shown that CSF-1 is over-expressed in human glioblastomas [17,37-39]. Inhibition of CSF-1R resulted in regression of established tumors in mouse glioma models and significantly increased animal survival. Depending on the mode of treatment, blocking of CSF-1R caused either depletion of GAMs or reduction of M2 phenotype markers in surviving GAMs [2,40,41]. Changing GAM phenotype had the greater inhibitory effect on glioma growth than GAM depletion [2]. By contrast, we found that *CSF-1* expression was barely detected in murine GL216 glioma cells and human glioma cells (at similar levels as in non-transformed astrocytes), and was not significantly up-regulated in glioma tissues [21,42]. Interestingly, growth of GL261 gliomas and accumulation of GAMs were not affected in mice having a non-functional *csf-1* gene [42]. These mice due the lack of Csf-1 have a reduced number of macrophages in various tissues, including the brain [43]. It suggested compensation of Csf-1 deficiency by tumor-derived factors or/and other cytokines with a similar activity.

We found the up-regulated Csf-2 levels in extracts from brains with implanted GL261 gliomas and the increased expression of *csf-2* in glioma cells compared to non-transformed astrocytes [21]. We developed clones of GL261 glioma cells stably depleted of Csf-2 that were implanted to the striatum of mice and found the reduced content of infiltrating GAMs and smaller tumors. We demonstrated that glioma-derived Csf-2 is responsible for recruitment/activation of microglia/macrophages and tumor progression. Invasion of glioma cells lacking Csf-2 was greatly reduced in organotypic brain slice cultures. If microglia were eliminated from the slices, glioma cells lacking Csf-

2 invaded brain slices as non-modified tumor cells. This pointed to the cytokine role in microglia-dependent tumor invasion [42].

Our finding that CSF-2 produced by tumor cells is a driving force for accumulation of $CD11b^+$ cells in glioblastomas may explain the observed heterogeneity of immune cells infiltrating tumors, in particular the presence of myeloid-derived suppressor cells (MDSCs). These cells are myelomonocytic cells lacking the markers of mature myeloid cells and are capable to suppress both adaptive and innate immune responses. MDSCs accumulate in the blood and at tumor sites in patients with breast, lung, prostate, kidney, head and neck tumors, glioblastomas, and in animals with experimental cancers [6,44]. CSF-2 (and G-CSF) synthesized by the tumor itself and by the brain damaged due to a growing tumor stimulates bone marrow to shift hematopoiesis toward production of cells of granulocytic/monocytic lineages. This shift produces immunosuppression and results in reduced lymphopoiesis characteristic for glioblastoma patients [45].

The relevance of our findings to human GBM pathology was also confirmed by the analysis of the clinical material. The expression of *CSF-2* was highly up-regulated in GBM biopsies, when compared to benign tumors and normal brain. Kaplan-Meier survival curves acquired from the Rembrandt depository showed inverse correlation between *CSF-2* mRNA levels and survival of glioma patients [42]. Other studies confirmed high expression of *CSF-2* mRNA in glioblastomas [46,47].

The other factor implicated in regulation of myeloid cell recruitment is the glial cell-derived neurotrophic factor (GDNF), which promotes differentiation and survival of many cells in the central nervous system (CNS). It signals through GDNF receptor-α1 (GFR-α1) and activates RET tyrosine kinase pathway [48]. GDNF is expressed by different cell types, such as neurons, astrocytes and is overexpressed by glioma cells [49,50]. Ku *et al.* demonstrated that GDNF secreted by mouse GL261 and human high grade glioma cells is a chemoattractant for microglia. Silencing of *Gdnf* in GL261 glioma cells reduced accumulation of GAMs and decreased glioma growth *in vivo* [49].

CHEMOKINES

Chemokines are small proteins with chemoattractant functions. They belong to a large superfamily of peptides produced and secreted by different cell types. Chemokines are classified in four groups (C, CC, CXC, and CX3C) according to the number and location of the conserved cysteine residues in the primary structure of these molecules. Chemokines are crucial autocrine and paracrine players in tumor development, and many chemokines from CXC and CCL groups have been implicated in GBM pathology.

The "CXC" group (in which one amino acid is present between the first two cysteines) includes 21 proteins (CXCL1-21) mostly encoded on human chromosome 4. CXC chemokines bind at least 7 receptors (CXCR1-7) and mediate neutrophil chemotaxis. The CXC group can be divided into two main categories based on the presence of the tripeptide Glu-Leu-Arg (ELR) before the CXC motif (N-terminal domain). Representative CXC chemokines include CXCL8/IL-8, among the ELR-containing peptides and CXCL9/monokine-induced by IFN-γ (MIG), CXCL10/IFN-γ inducible protein-10 (IP-10), and CXCL12/stromal cell-derived factor-1 (SDF1) as ELR negative molecules. CXCL12 (SDF-1), through its receptors CXCR4 and CXCR7, supports tumor progression by controlling cancer cell survival, proliferation and migration, and, indirectly, via angiogenesis or recruiting deleterious immune cells at tumor sites. CXCL12 is a potent microglia and macrophage recruiting molecule, especially for attracting these cells to hypoxic areas, where they support tumor neovascularization [51]. SDF-1 is one important factor in radiotherapy-induced tumor invasiveness, where it exerts its primary effect through macrophage mobilization. CXCL12/CXCR4 up-regulation was also observed after treatment with anticancer drugs, particularly after treatment with anti-VEGF antibodies [52].

Representative CC chemokines (structurally characterized by four cysteines) are CCL2 (also called monocyte chemotactic protein, MCP-1), CCL3 and CCL4 (macrophage inflammatory protein MIP-1*a* and MIP-1β), CCL5 (RANTES), and CCL11 (eotaxin). MCP-1was the first identified monocyte chemotactic factor. It is released by several cancer cells including glioblastoma [53-55]. MCP-1 signals through CCR2 receptor and induces migration of different immune cells such as monocytes, macrophages, T cells and NK. Rat glioma cells expressing MCP-1 protein *in vivo* generated threefold larger tumors with tenfold higher number of infiltrating GAMs, and this resulted in significant reduction of rat survival [53]. In humans, increased MCP-1 expression has been associated with high number of GAMs infiltrating tumor tissues, glioma malignancy and poor clinical prognosis [56]. Additionally, higher levels of MCP-1 are correlated with increased angiogenesis, tumor invasion and proliferation [55,57]. However, the importance of different MCP in glioma biology is still a matter of dispute. There is a stronger correlation between MCP-3 levels rather than MCP-1 and the density of tumor infiltrating GAMs. A more recent study shows that MCP-3 (but not MCP-1) is predominantly expressed in different glioma cells [58]. MCP-3 is a ligand of CCR-1, -2 (both present on monocytes) and -3 receptors, while MCP-1 binds only CCR2 [59].

The "CX3C" chemokines (three amino acids between the first two cysteines) are represented by a single peptide - chemokine (C-X3-C motif) ligand 1 (CX3CL1, fractalkine). In the normal brain, fractalkine is secreted by neurons, and its receptor CX3CR1 is predominantly expressed on microglia. The CX3CL1 – CX3CR1 signaling pathway is involved in communication between neurons and microglia, and migration of immune cells into the CNS during inflammation. CX3CL1 prevents microglia from excessive activation, maintaining cells in a quiescent state. Human glioma cells express both CX3CL1 ligand and CX3CR1 receptor [60,61], and both CX3CL1 and its receptor CX3CR1 are overexpressed on GAMs isolated from human GBMs

[62,63]. Moreover, treatment of patient derived GAMs cultured *in vitro* with CX3CL1 chemokine enhanced the expression of matrix metalloproteinase (MMP) 2, 9, and 14, and activated cell migration and adhesion [63].

INTEGRIN LIGANDS

Integrins are a large family of heterodimeric transmembrane adhesion receptors, which play a key role in interactions of a cell with a surrounding stroma [64]. Upon binding ligands or ECM components integrin dimers activate downstream signaling pathways, which regulate migration, invasion, proliferation and survival. Many tumor cells upregulate expression of specific integrins, for example brain tumor cells overexpress αvβ3 and αvβ5 integrins [65]. We analyzed by mass spectrometry a secretome of glioma cells and identified two αvβ3/αvβ5 integrin ligands, osteopontin and lactadherin, as factors responsible for polarization of microglia into tumor-supporting cells [66]. With the use of a blocking peptide (our in-house designed competitive inhibitor of ligand binding to αvβ3/αvβ5 integrins) we blocked glioma-microglia interactions *in vitro*. Moreover, silencing of either osteopontin or lactadherin in glioma cells implanted to the striatum resulted in significantly reduced tumor growth [66] . These results point to an important role of both ligands and αvβ3/αvβ5 integrin signaling for polarization of microglia/macrophages infiltrating a tumor.

Osteopontin (SPP1, secreted phosphoprotein 1) is a secreted glycoprotein produced by immune cells under inflammatory conditions. Osteopontin contains a RGD (arginine–glycine–aspartate) motif interacting with integrins αvβ1, αvβ3, αvβ5, αvβ6, α8β1, and α5β1, and a binding site for CD44, in particular for the isoform CD44v6-v7. Thrombin cleaves osteopontin at a conserved site (168RS169) and exposes a cryptic 162SVVYGLR168 motif interacting with a different set of the integrins α9β1, α4β1, and α4β7. The RGD and the cryptic sites are located in the N-terminal fragment of protein produced by thrombin cleavage, whereas the CD44 binding site is located in the corresponding C-terminal fragment. Osteopontin regulates recruitment of macrophages and T-cells, and the production of inflammatory mediators by these cells [67]. On the other hand, SPP1 modulates many functions of cancer cells: it stimulates cancer cell proliferation and invasion, and supports tumor angiogenesis [68]. *Spp1* knockdown in rat C6 glioma cells blocked the growth of intracranial tumors, reduced the number of pro-tumorigenic, arginase 1 expressing GAMs [66] and increased infiltration of tumor tissues by interferon producing, cytotoxic T lymphocytes (unpublished observations). The observation of different functions of osteopontin produced by glioma cells and non-transformed cells was puzzling. Differential posttranslational processing of osteopontin in glioma cells provides a plausible explanation of the contradictory action of osteopontin secreted by different cells. The thrombin-cleaved fragments of osteopontin have been found in malignant gliomas and conferred survival advantage for glioma cells [69]. We demonstrated that osteopontin produced by non-transformed cells activates a pro-inflammatory response in microglia, while sequential processing of this protein by thrombin and metalloproteinases MMP3 and/or MMP7 in glioma cells generated shorter peptides including a microglia activating form devoid of the inflammatory activity but retaining an ability to polarize microglia [66].

SPP1 mRNA and protein expression is highly elevated in tumor tissues and sera from GBM patients, and inversely correlates with patient survival [70,71]. Recent data suggest that high amounts of osteopontin detected in the tumors may originate not only from the tumor cells (including glioma initiating cells) but also from the host cells, such as microglia [27,28,72] or tumor-associated astrocytes [73]. Our data show upregulated *Spp1* expression in different glioma cells. However, we found upregulated *Spp1* mRNA levels in CD11b+ cells (microglia and infiltrating macrophages) in the rat brain under inflammatory conditions induced by stroke or in glioma infiltrating GAMs (unpublished). Consistently, Gabrusiewicz *et al.* (2016) found upregulated *SPP1* expression in CD14+ cells (cells of monocytic lineage) infiltrating GBM as compared to CD14+ cells isolated from nonmalignant brain tissue. The *SPP1* mRNA levels in GBM-infiltrating CD14+ cells were significantly higher than in matched CD14+ blood cells, thus suggesting that gene expression was upregulated upon interaction with glioblastoma [72]. Stromal astrocytes also may produce osteopontin in tumor microenvironment. In a mouse transgenic model of PDGFB-driven glioma, *Spp1* was the most up-regulated gene in tumor-associated astrocytes of the perivascular niche as compared to normal brain astrocytes [73]. Nevertheless, once produced in the tumor, osteopontin is subjected to proteolytic modifications in the extracellular space, thus the protein secreted by stromal cells may as well contribute to the pool of truncated osteopontin modulating the local immune responses. When released to circulation, osteopontin upregulates infiltration of neutrophiles and macrophages in glioblastoma [71].

Moreover, osteopontin secreted by glioma-associated astrocytes enhanced the cancer stem cell phenotype through interactions with CD44 [74]. Tumors contain a rare subpopulation of cancer stem-like cells (CSCs), which are multipotent, have an ability to self-renew and generate various more differentiated progeny. We demonstrated that glioma-derived SPP1 support self-renewal of glioma CSCs and expression of "stemness" markers, which indicate its role in maintaining "stemness" of human glioma stem-like cells. Osteopontin supported glioma CSCs via interactions with the receptor CD44 on glioma cells [75]. We demonstrated that *SPP1* is overexpressed in glioma CSCs due to the presence of stemness factors and restoration of the embryonic type regulation of *SPP1* expression. Altogether, the available data support the notion of overexpression of *SPP1* predominantly in tumor cells and its important role in supporting tumor growth and blocking initiation of proper antitumor responses.

The second identified glioma derived factor was lactadherin (milk fat globule-epidermal growth factor 8, MFG-E8) a glycoprotein secreted from various cells that enhances engulfment of apoptotic cells. MGF-E8 acts by

connecting phosphatidylserine on apoptotic cells and αvβ3/αvβ5-integrin on phagocytes [76,77]. Apart from the scavenging function, MFG-E8 can directly attenuate inflammation and regulate healing of injured tissues during intestinal inflammation and brain ischemia [77]. MFG-E8-mediated phagocytosis of apoptotic cells by macrophages *in vitro,* induced secretion of cytokines inducing regulatory T cell (Treg), which contributed to anti-inflammatory immune responses and development of immune tolerance [78]. Binding of MFG-E8 to αvβ3/αvβ5-integrin complexes on endothelial cells promoted vascular endothelial growth factor (VEGF)-dependent neovascularization [79]. MFGE8 promoted cell/cell and cell/extracellular matrix adhesion during physiologic migration of epithelial cells in the intestine [80]. Activation of all these processes is highly favorable for tumor growth, however the role of MFG-E8 in cancer has been largely overlooked. There are only a few published studies exploring its role in non-CNS tumors. In a mouse experimental melanoma MFG-E8 augmented tumorigenicity and metastatic capability, enhanced resistance of melanoma cells to apoptosis, induced epithelial-to-mesenchymal transition, and stimulated invasion and angiogenesis. MFG-E8 also contributed to local immune suppression by evoking Treg cell infiltration, suppressing Th1 reactions and cytotoxic effects of NK and $CD8^+$ T cells [81]. MFG-E8 has been also implicated in development of bladder carcinoma [82] and its upregulated expression was reported in 11 cancers including glioblastomas [82]. We demonstrated that MfgE8 knockdown in glioma cells reduced tumor growth and GAMs infiltration in orthotropic rat gliomas [66].

Periostin is yet another glioma-derived protein, which interacts with integrins on tumor infiltrating myeloid cells in murine gliomas. Periostin is a multidomain protein composed of a signal peptide (necessary for secretion), a small cysteine-rich motif (probably involved in the formation of multimers through cysteine disulfide bonds), four fasciclin-like domains (FAS1) that interact with integrins ($\alpha_v\beta3$, $\alpha_v\beta5$, $\alpha_6\beta4$), and a hydrophilic C-terminal region known to interact with other ECM proteins such as collagens, fibronectin, tenascin C, or heparin. A recent study showed that periostin is produced by glioma CSCs present in the perivascular niche. Periostin acts as a chemoattractant for brain macrophages through the integrin receptor αvβ3 [83]. Targeting these integrin receptors with the interfering RGD peptide (Arg-Gly-Asp-d-Phe-Lys) attenuated interaction between GAMs with GSC, reduced their recruitment and polarization into the tumor-supportive phenotype[83].

TUMOR MICROENVIRONMENT-DERIVED SIGNALS THAT SUPPORT GLIOMA GROWTH AND PROGRESSION

The interaction between tumor cells and components of its microenvironment is bidirectional. In response to tumor derived signals neoplastic cells receive support from stromal cells. As already emphasized, the key partners of glioma cells in these interactions are infiltrating microglia and macrophages – GAMs, which outnumber any other host cell type in the tumor. The supportive role of microglia in tumor invasion has been shown in the organotypic brain slices and in intracranial gliomas [84].Genetic [85] or pharmacological [21,86,87] depletion of GAMs reduced tumor growth in experimental murine gliomas. The crucial role of GAMs is supported by clinical observations. Accumulation of GAMs characterized as CD163+ CD204+ cells in human gliomas was correlated with higher tumor grades and worse prognosis [17]. As summarized in Figure 1, GAMs facilitate glioma progression mainly by secreting growth factors and extracellular matrix-degrading enzymes to promote proliferation and invasion, pro-angiogenic molecules to support formation of new blood vessels and immunosuppressive cytokines and ligands to hamper the immune response [7,11].

STIMULATION OF GLIOMA GROWTH AND INVASION BY GAMS

Microglia synthetize and release stress-inducible protein 1 (STI1), a cellular prion protein ligand that increases proliferation and migration of glioma cells *in vitro* and *in vivo* [88]. Epidermal growth factor (EGF) released from microglia upon binding to its receptor EGFR on glioblastoma cells stimulated downstream signaling and enhanced invasion of glioma cells in microglia-glioma co-cultures [40]. In response to tumor-derived CCL2 microglia released a cytokine IL-6, which in turn promoted invasion of glioma cells [55]. Moreover, CCL2 stimulated also its own expression and CD163+ infiltrating GAMs are recognized as a major source of CCL2 in GBM patients. As CCL2 participates in recruiting both Treg and MDSCs immunosuppressive cells to a tumor, GAMs were acting in this case as amplifiers of the received signal [89].

Matrix metalloproteinases (MMPs), enzymes that degrade ECM, are released as inactive pro-forms, that need to be cleaved to become active. The prominent enzyme for pro-MMP2 cleavage is the membrane-bound metalloprotease MT1-MMP [90]. Moreover, MT1-MMP degrades pericellular substrates, such as collagen networks, thus modulating cancer cell invasion. MT1-MMP expression in human glioma samples positively correlated with the increasing malignancy grade. *Ex vivo* and *in vivo* data suggest that microglia contribute to the majority of upregulated MT1-MMP pool in murine gliomas [91]. Increased production of MT1-MMP in microglia is induced by soluble factors released by glioma cells, including versican [92], which acts via microglial Toll-like receptor 2 (TLR2) [93]. The V0/V1 splice variants of versican, an endogenous ligand of TLR2, are highly expressed in mouse and human glioma tissues [92]. MMP2 and MMP9 expression may be induced in response to other tumor- or TME-derived factors, including transforming growth factor β1 (TGFβ1) [94] or CX3CL1 [63].

TGFβ1 controls various features of malignancy, from invasiveness (i.e. by upregulating MMPs and mesenchymal markers expression) and "stemness" to angiogenesis and immunosuppression [95]. TGFβ1 is produced mostly by tumor cells but also was found upregulated in glioma-activated microglia [24,94,96]. Addition of TGFβ1 to established and newly generated GBM cell cultures re-

sulted in induction of morphological changes, enhanced expression of mesenchymal markers, potentiation of migration and invasion, both *in vitro* and in an orthotopic mouse gliomas [97]. We demonstrated that co-culture of glioma cells with microglia doubles glioma invasion via secretion of TGFβ1, and blocking TGFβ1 signaling in glioma cells impairs tumor growth [96].

Components of Fas/FasL system are expressed in the majority of malignant glioma cell lines as well as in human GBMs [98]. FasL expressed in tumors contributes to evasion of immune surveillance by killing Fas expressing T cells. Other functions of Fas signaling in gliomas are not fully elucidated. Interaction of glioma cells with the surrounding brain tissue induced expression of FasL in both tumor and host cells [99]. GAMs accumulating within tumors may account for a half of the FasL expression in murine intracranial tumors [100]. In line with this observation, we found the increased *fasl* mRNA levels in microglial cells after exposure to glioma conditioned medium [101]. Kleber and co-workers [99] demonstrated that neutralization of Fas activity blocks migration of glioma cells in a mouse intracranial glioma. Our findings show that non-apoptotic Fas signaling activated in the autocrine manner or through microenvironment derived factors can regulate invasion of glioma cells via modulation of MMP-2 activation, likely by controlling TIMP-2 expression [101].

SUPPORTING ANGIOGENESIS

Signals generated by GAMs target not only tumor cells. Angiogenesis is mediated by pro-angiogenic growth factors, including vascular endothelial growth factor (VEGF), which induces proliferation, migration of endothelial cells and tube formation *in vitro* [102]. GAMs isolated from murine GL261 gliomas overexpress pro-angiogenic molecules, such as VEGF and CXCL2 [30]. ECM degrading enzymes produced by GAMs as described above, are also essential factors in angiogenesis. Intravital microscopy imaging of a murine orthotopic glioma *in vivo* revealed that microglia motility is highest within the perivascular niche compared to other areas of the tumor that suggests dynamic interactions of microglia with tumor blood vessels [103]. Accumulation of GAMs not only promotes angiogenesis but may contribute to the resistance or escape from anti-angiogenic therapies in glioblastoma [104,105].

INHIBITION OF ANTI-TUMOR RESPONSES

Anti-tumor responses of both innate and adaptive immunity cells are largely deactivated in patients with malignant tumors, including glioblastomas (for a review [15]). Apart from tumor immune evasion mechanisms, such as already mentioned FasL-mediated T cell death or overexpression of programmed death ligand 1 (PD-L1), GBMs suppress innate anti-tumor functions of GAMs, which are potent effectors of immunosuppression [11]. Microglia/macrophages isolated from brains of epileptic patients (polarized to the inflammatory phenotype) decreased proliferation of co-cultured tumor cells, in contrast to GAMs supporting tumor growth [106]. The immune functions of GAMs from postoperative GBM were reduced, as these cells did not produce pro-inflammatory cytokines (TNFα, I1β, or IL6, IL-2, IL-12), and did not induce T-cell proliferation [19]. These cells expressed major histocompatibility complex class II proteins but lacked the expression of the co-stimulatory molecules CD86, CD80, and CD40 critical for T-cell activation [18]. GAMs secreted immunosuppressive cytokines, such as IL-10, TGFβ, IL6 or CCL2, favoring the accumulation of suppressor T cells, MDSCs and blocking the cytolytic action of $CD8^+$ T cells and NK cells. This resulted in local immunosuppression preventing detection and eradication of tumor cells [7,15,18-20,22].

We demonstrated that glioma-derived factors induce in primary rat microglial cultures selected signaling pathways and a gene expression program associated with upregulation of genes coding for proliferation regulators, such as ID (inhibitor of DNA binding) 1/3 and c-Myc, Arg1, MT1-MMP, CXCL14, and numerous cytokines/chemokines implicated in immune cell trafficking. Microglial cultures, when stimulated with an immunomodulatory lipopolysaccharide, induced classical inflammation-related genes and signaling pathways related to inflammation (p38 MAPK, JNK and NFkB) [24]. Transcriptomic analyses of rat glioma-bearing hemispheres revealed overexpression of invasion and immunosuppression-related genes, reflecting the immunosuppressive microenvironment. This was associated with accumulation of amoeboid, pro-tumorigenic GAMs and Treg cells combined with the reduced presence of Tc lymphocytes [28]. GAMs isolated by flow cytometry as $CD11b^+CD45^{low}$ cells from experimental rat gliomas [28] or magnetically-sorted CD11b+ cells from experimental murine EGFP-GL261 gliomas [21], displayed the pro-invasive and immunosuppressive type of activation. Detailed studies of gene expression in GAMs sorted from benign and malignant gliomas showed downregulation of *IKKβ* expression in the latter. IKKβ is a kinase, which phosphorylates IκB proteins (inhibitors of NFκB) and represents a convergence point for many signaling pathways leading to NFκB activation. Activation of NFκB is instrumental for activation of inflammation-related genes. The observed downregulation of *IKKβ* expression and reduced activation of NFκB in GAMs from malignant gliomas may explain the lack of inflammatory responses in these tumors. Computational analyses of public datasets on gene expression in GBMs showed defective expression of immune/inflammatory response genes in malignant versus benign gliomas [26]. Thus, although various immune effector cells are recruited to the tumor site, their anti-tumor functions are downregulated. This is instigated by tumor polarized GAMs, which orchestrate local immunosuppression, and contribute to systemic immune deficits in GBM patients.

TUMOR MICROENVIRONMENT-GLIOMA INTERACTIONS AS TARGETS FOR ANTI-TUMOR THERAPIES

Due to the lack of efficient treatment against glioblastoma, strategies that combine anti-tumor agents with targeting the tumor microenvironment gained more attention

recently. One of the best example is combining cytotoxic agents with anti-angiogenic therapies or T-cell based therapies with immune checkpoint inhibitors. Some of the newly proposed therapeutic approaches are focused on modulating the functions of GAMs either by blocking chemoattractant receptors or their ligands to reduce recruitment of GAMs and/or by changing the phenotype of GAMs to transform an immunosuppressive tumor environment into anti-tumorigenic one with restored immune responses [107]. Several studies showed that GAMs promote angiogenesis and may participate in the escape from anti-angiogenic therapies [105]. To ensure success of the checkpoint inhibitors, CD8+ T cells, the main target of this treatment, should exert full cytotoxicity against tumor cells. Recent findings emphasize contribution of tumor-associated macrophages to the insufficiency of checkpoint therapies [108,109]. As described earlier, GAMs can impede anti-tumor immune responses by different mechanisms [7,15]. Given the abundance and leading role of GAMs in the tumor microenvironment, there is a rationale in combining other treatments with intervention targeting these cells.

One of the strategies to target GAMs is the blockade of CSF-1R that is essential for the recruitment, differentiation, and survival of macrophages associated with different types of tumors [109]. Blocking the CSF-1R signaling with anti-CSF-1R antibodies (Emactuzumab or Pexidartinib) or small molecule inhibitors resulted in reduced tumor infiltration by GAMs and/or changes in the functional phenotype of GAMs [40, 110]. Despite the observed reduction of tumor growth and increased animal survival in preclinical studies, anti-CSF-1R antibodies were not efficient as a monotherapy in patients with recurrent GBMs [111]. This resistance to GAMs depletion with the use of CSF-1R inhibitor was ascribed to compensation by other glioma derived factors such as CSF-2, IL-4, IFN-γ [41,42,112]. Nevertheless, as CSF-1R blockade resulted in increased CD8+/CD4+ T cell ratio suggesting restoration of some anti-tumor immunity capacities [110], there was a rationale for combining CSF-1R antagonists with immune checkpoint inhibitors. Recently, the phase I clinical study was initiated combining Emactuzumab with an anti-PD-L1 antibody.

Another example of attempted blockade of GAM recruitment is inhibition of CXCR4, a receptor of SDF-1/CXCL12 -, by synthetic peptides, such as AMD3100 or peptide R [113]. SDF-1 is an important factor in radiotherapy-induced tumor invasion facilitated by macrophages. Three noncytotoxic drugs: minocycline (an antibiotic targeting GAMs and MMPs), telmisartan (an antihypertensive drug) and zoledronic acid (a bisphosphonate) have been recently tested as addition to standard radiotherapy and temozolomide for GBM patients in a clinical trial. All three drugs exert an inhibitory activity on MCP-1/CCL2 synthesis. Since CCL2 attracts circulating monocytes to the tumor, the drugs have been re-purposed to inhibit or reverse GAM-mediated immunosuppression, angiogenesis and tumor growth [114].

Preclinical studies, including ours, point to important role of αvβ3/αvβ5 integrin signaling for recruitment and polarization of GAMs. We used the blocking peptide (our in-house designed competitive inhibitor of tumor-derived ligands binding to αvβ3/αvβ5 integrins) to interfere with glioma-microglia interactions *in vitro* and we blocked polarization of microglia to the pro-invasive phenotype [66]. Zhou *et al.* demonstrated that blockade of periostin-integrin signaling with the RGD peptide inhibits recruitment of tumor infiltrating myeloid cells [83]. A cyclic pentapeptide called Cilengitide (EMD 121974, cyclo-(Arg-Gly-Asp-DPhe-NMe-Val) was identified as a potent and selective αvβ3/αvβ5 integrin antagonist. In preclinical studies, cilengitide effectively inhibited the growth of orthotopic glioblastoma, however the primarily identified mechanism was the inhibition of angiogenesis. Cilengitide entered clinical trials and showed anti-tumor activity against malignant gliomas, when was given alone or in combination with chemotherapy [115]. Recently published results of the randomized phase III CENTRIC and phase II CORE clinical trials did not show consistent effects on GBM patient outcomes [116]. However, the detailed analysis of the CORE study discovered that improved progression-free survival and overall survival in patients treated with cilengitide correlated with higher αvβ3 levels in tumors [117]. Data on the intra-tumor immune infiltration or phenotype of GAMs in response to cilengitide treatment are not available.

Beside the approaches to interfere with extracellular ligand-receptor interactions, there is an attempt to overpass the pro-tumorigenic microenvironment by targeting GAMs via inhibition of STAT3 transcription factor. STAT3 is a key factor for glioma growth as a regulator of proliferation promoting genes [118] and suppressor of expression of CD80, CD86 and MHCII molecules in GAMs [119]. STAT3 downregulation by short interfering (si)RNA in GL261-bearing mice contributed to anti-tumorigenic activation of GAMs with increased TNFα expression, reduced glioma growth and increased animal survival [120]. The STAT3 pharmacological inhibitor - WP1066- is currently in the phase I clinical trial for glioma as well as melanoma brain metastases.

The present study shows a crucial role of tumor microenvironment, in particular of tumor infiltrating microglia/macrophages in growth and progression of malignant gliomas. Uncovering novel mechanisms, by which glioma cells exploit microglia and other types of cells in the tumor microenvironment to increase their growth, invasion and resistance to anti-tumor treatments can indicate potential new therapeutic targets and help to develop new therapies for the currently incurable malignant glioblastomas.

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Molekularne podłoże oddziaływania komórek nowotworowych z mikrośrodowiskiem w glejakach złośliwych

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STRESZCZENIE

Rosnąca liczba danych doświadczalnych i klinicznych wskazuje na aktywację prawidłowych komórek znajdujących się w guzie i ich istotną rolę w progresji nowotworu. Komórki nowotworowe wykorzystują ścieżki komunikacji między komórkami do oddziaływania na otaczające je mikrośrodowisko i do wspierania swojego wzrostu. W glejakach złośliwych, czynniki wydzielane przez nowotwór wywołują liczne zmiany, w tym powodują napływ występujących w mózgu komórek mikrogleju oraz makrofagów z krwi obwodowej. Komórki te zamiast zwalczać nowotwór, ulegają przeprogramowaniu i aktywują nowe programy transkrypcyjne i syntezę białek regulujących przebudowę macierzy zewnątrzkomórkowej, inwazyjność komórek nowotworowych, proces tworzenia nowych naczyń krwionośnych. Jednocześnie czynniki z komórek glejaka oraz te produkowane przez zmieniony mikroglej/makrofagi blokują odpowiedź przeciwnowotworową komórek układu odpornościowego. Molekularne podłoże tych wzajemnych złożonych interakcji jest głównym przedmiotem niniejszego artykułu.