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Microglia-glioma cross-talk: a two way approach to new strategies against glioma

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TABLE OF CONTENTS

1. ABSTRACT

Glioblastoma (GBM) is the most malignant and aggressive among primary brain tumors, characterized by very low life expectancy. *In vivo*, glioma and glioblastoma in particular contain large numbers of immune cells (myeloid cells) such as microglia and tumour-infiltrating macrophages (or glioma associated macrophages). These glioma-infiltrating myeloid cells comprise up to 30% of total tumor mass and have been suggested to play several roles in glioma progression including proliferation, survival, motility and immunosuppression. Although tumor microglia and macrophages can acquire proinflammatory (M1) phenotype being capable of releasing proinflammatory cytokines, phagocytosing and presenting antigens, their effector immune function in gliomas appears to be suppressed by the acquisition of an anti-inflammatory (M2) phenotype. In the present work we review the microglia-glioma interactions to highlight the close relationship between the two cell types and the factors that can influence their properties (chemokines, cytokines, S100B protein). A future therapeutic possibility might be to simultaneously targeting, for example with nanomedicine, glioma cells and microglia to push the microglia towards an antitumor phenotype (M1) and/or prevent glioma cells from "conditioning" by microglia.

2. INTRODUCTION

The brain tumor incurability, particularly in the case of glioblastoma multiforme, is substantially determined by the microenvironment created by the tumor, to the advantage of tumor progression. The traditional therapeutic approaches are aimed at curbing cancer cells' proliferation and invasiveness, leaving out the functional relationships stabilized with the other tumor niche elements. High-grade gliomas very effectively attract microglia/macrophages (1-3) and subsequently control their behavior (1-3), eliciting an

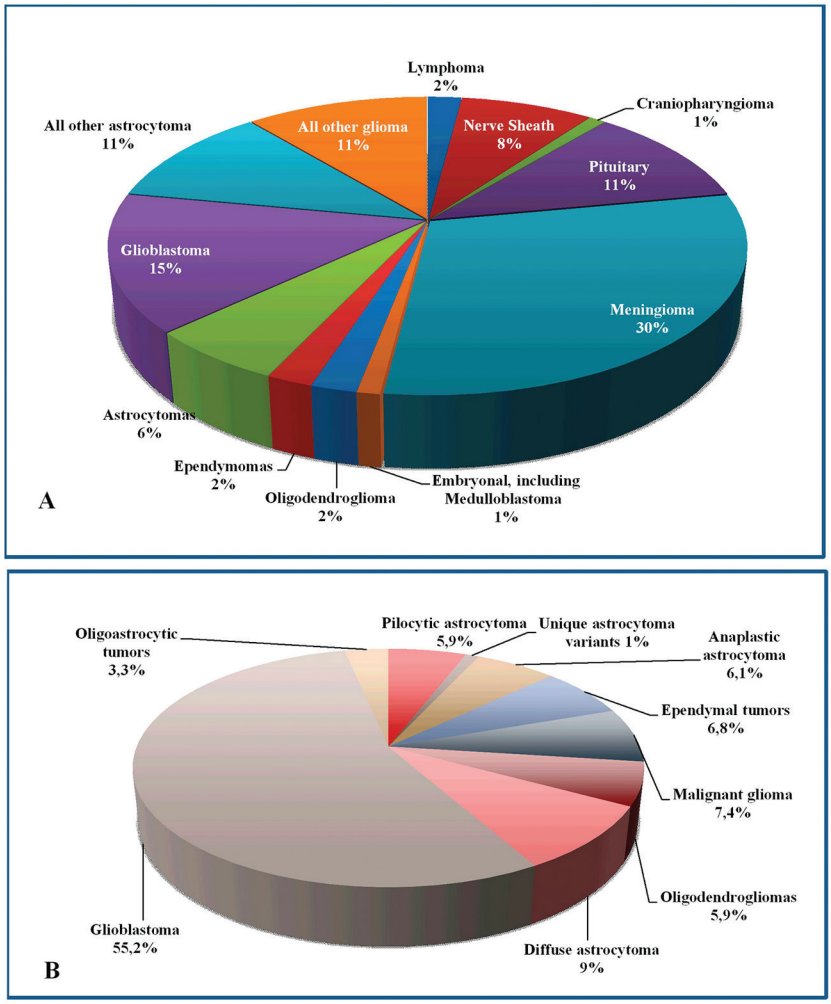

Figure 1. A. Tumors of the central nervous system. Tumors of the central nervous system can be grouped into seven different types, with different clinical and histological features: tumors of the neuroepithelial tissue, tumors of cranial and paraspinal nerves, tumors of the meninges, lymphomas and hematopoietic neoplasm, germ cell tumors, tumors of the sellar region (e.g. craniopharyngioma, pituicytoma), and metastatic tumors (not represented). B. Tumors of neuroepithelial origin. The majority (about 40%) of malignant brain tumors are of neuroepithelial origin and represent the so-called gliomas. They show a clear derivation from the cellular elements that make up the glia: astrocytes, oligodendrocytes and ependymal cells. From the histological points of view different types of glioma with a prevalence (about 50%) of glioblastomas can be identified.

immunosuppressive and protumoral phenotype. Thus, correct therapeutic strategy should take the different cellular types present in the tumor microenvironment and their reciprocal functional relationship into account. In this review, we'll focus on the relationships between the various components of the niche in glioma. In particular, we'll highlight the relationship between glioma cells and microglial cells/macrophages, the roles of myeloid derived suppressor cells (MDSCs), alterations of the brain-blood-barrier (BBB) in tumor settings, and the roles of soluble factors conveyed through the exosomes (EV).

3. GLIOMA

3.1. Classification and standard care

The brain tumor is considered a rare desease with a high mortality rate, and with half of the patients having in fact an incurable desease. Tumors of the central nervous system (CNS) can be grouped into seven different types, with different clinical and histological features: tumors of the neuroepithelial tissue, tumors of cranial and paraspinal nerves, tumors of the meninges, lymphomas and hematopoietic neoplasm, germ cell tumors, tumors of the sellar region, and metastatic tumors (Figure 1A). The majority (about 40%) of malignant brain tumors are of neuroepithelial origin (Figure 1A) and represent the so-called gliomas. They show a clear derivation from the cellular elements that make up the glia: astrocytes and oligodendrocytes. From the histological points of view different types of glioma with a prevalence (about 50%) of glioblastomas can be identified (Figure 1B). Considering clinic aspects and malignancy levels, gliomas are divided into four grades (I-IV), according to the World Health Organization (WHO) (4).

The grade I glioma (e.g. anaplastic astrocytoma, subependymoma, myxopapillary ependymoma (Figure 1B)), rarely progresses to more malignant phenotypes. It is most frequently localized to the cerebellum and is the most common in children 8- to 13-years old. The tumor presents well-circumscribed edges, mostly fusiform and bipolar glial cells which express fibrillary acidic protein positive (GFAP), an astrocyte marker. The grade II glioma (e.g. pilomyxoid astrocytoma, oligodendroglioma, oligoastrocytoma, ependymoma (Figure 1B)) frequently affects adults. The cells do not exhibit significantly elevated rates of proliferation, but can seep into adjacent areas of the brain tissue, altering the structure. GFAP is expressed to appreciably high levels. The glioma grade III, referred to as anaplastic astrocytoma (Figure 1B), is the most frequent variant, is characterized by high pleomorphism, nuclear atypia and elevated mitotic activity.GFAP expression is variable. The grade IV glioma, represented substantially by GBM (Figure 1B), is the most malignant and more frequent glioma, being characterized by a fairly short clinical course is with a median survival of only 14.6. months. This tumor includes spindle or rounded cells and typical multinucleated giant cells, whose extremely immature morphology is accompanied by a significant decrease in the GFAP expression. GBM shows a high migration capacity and cell proliferation rate in agreement with the high degree of its malignancy. These characteristics are maintained *in vitro* and cultures of glioma cells of different degrees of malignancy are good preclinical experimental models to study the characteristics of these tumors (5-7). Because of its high invasiveness, surgical excision, even if large, is far from curative, and infiltrating cells almost always generate recurrences in the tumor site or remotely. As to the origin of GBM, several experimental evidences have shown that they contain a subpopulation of cells, the glioblastoma stem cells (GSCs), endowed with high tumorigenicity. These cells possess the typical stem cells properties, including self-renewal and ability to differentiate into different lineages, besides generating tumors. GSCs are relatively quiescent and thus escape the traditional radio and chemotherapy approaches, ultimately resulting in tumor relapse. GBMs are classified as primary or secondary. Primary GBMs arise de novo, whereas secondary GBMs arise from the lower grade gliomas, low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III). Primary GBMs have a worse prognosis and generally affects younger individuals (mean age = 40 years), compared to secondary gliomas (mean age = 55 years). Indistinguishable from a histopathological point of view, it is now clear that GBMs originate from precursors with different genetic alterations and have different gene expression patterns. A further classification was proposed for GBMs by the Cancer Genome Atlas Network (TCGA) and other (8-10). Recurrent and specific genetic alterations give rise to four different subtypes of GBM: classical, mesenchymal, proneural and neural.

Gliomas are the most common brain tumors. Their treatment is difficult and complex because of their possible proximity to vital structures and their high infiltration capacity and weak radiosensitivity. The prognosis is generally unfavorable, especially for GBMs. Standard treatment includes surgery as broad as possible, chemotherapyand radiotherapy according to the Stupp protocol (radiotherapy 60 Gy in 30 fractions for 6 weeks, day 1, 2, 3, 4, 5 and alkylating agent temozolomide (TMZ), at a dose of 75mg / m2 / day for 42 consecutive days, more than 6 cycles of 150-200mg/m2/day, day1-day5 every 28 days) (11). In clinical practice, adjuvant treatment with TMZ is often extended beyond six cycles. Surgery alone results in a median survival of six months, whereas the combined approach of surgery and radiation therapy extendes median survival by little more than 12 months, and the addition of TMZ to about 14.6. months. The probability of survival at five years is 9.8.% in the case of radiotherapy and chemotherapy against 1.9.% with radiotherapy alone (12).

Although far from conclusive, the purpose of the surgery is to achieve maximum tumor resection, if possible, without compromising the neurological functions. Advances in surgical imaging techniques, such as awake craniotomy, stereotactic guidance, intraoperative magnetic resonance imaging, and fluorescent-guided resection, allow to better delineate the tumor limits and thus optimize cancer surgery (13). Particularly interesting is the use of fluorescent techniques that include pharmacological agents which localize to the tumor mass and emit fluorescence when exposed to a specific wavelength light. This fluorescence technique is used for the detection and resection of tumor tissue that appears normal. Comparative studies between fluorescence guided resection and standard resection have shown that patients undergoing fluorescence guided resection, are more likely to have a total resection and therefore more likely to be free from disease progression at 6 months (13,14).

The system of biodegradable wafers impregnated with carmustine is a treatment approved by Food and Drug Administration that allows the release of carmustine, an alkylating agent, in the resection cavity (15). The release of carmustine occurs in the surrounding brain tissue immediately after tumor resection and persists for several weeks. Unfortunately, the use of carmustine wafers in combination with TMZ and radiation showed a modest increase in survival, with possible complications. Hence, this treatment is not routinely used.

Experimental therapies directed toward specific molecular targets support numerous in progress clinical trials. Considering the different and complex genetic alterations involving tyrosine kinase receptor (TKR) and transduction pathways downstream of these, a first strategy has been to inhibit the signaling

pathway of epidermal growth factor (EGF). Small EGF receptor (EGFR) tyrosine kinase inhibitor molecules (TKIs) have been used to target this pathway. Among these, erlotinib (16) and gefitinib (17) by binding to the intracellular portion of the EGFR, inactivate the downstream pathway. Preclinical models have shown an important anti-proliferative and anti-invasive effects of TKIs, however not confirmed in clinical trials. Although associated with radiotherapy and chemiotherapy have shown little or no benefit in clinical outcomes. Using a different approach, cetuximab (18), a monoclonal antibody, has been used to block EGFR. Also in this case, clinical trials have shown limited therapeutic benefits to patient. Overexpression of platelet derived growth factor receptor (PDGFR) is another distinctive feature of GBMs. Even in this case, small TKIs, such as imatinib (19) that binds to and inactivates PDGFR and other TKRs, have been used against GBMs, but again clinical trials do not show therapeutic efficacy.

Given the high vascularity of high degree gliomas (20) and particularly GBMs, therapeutic effects of antiangiogenic molecules also have been evaluated. Vascular endothelial growth factor (VEGF) is a critical mediator of angiogenesis (21) and is highly expressed in GBMs (22). Avastin is a human monoclonal antibody that binds to and neutralizes the VEGF Receptor (VEGFR), preventing its activation. The use of Avastin in clinical trials showed benefits with an increase in progression-free survival. However, recent results of phase III clinical trials in which bevacizumab was used in combination with RT and TMZ showed, compared to treatment with RT and TMZ, a modest non-significant increase in progression-free survival in patients diagnosed with new GBM. Other angiogenesis inhibitors have been used without any clinical benefit (23).

Many signaling pathways downstream of TKR, including EGFR and PDGFR, converge on the complex systems, Ras/Raf/MEK/Erk and PI3K/mTOR/Akt. These intracellular signaling pathways are very often involved and dis-regulated in gliomas, resulting in hyperactivation. Neurofibromin 1 (NF1) and phosphatase and tensin homolog (PTEN) are physiological inhibitors of Ras and Akt pathways, respectively, and are often mutated or not expressed in glioblastoma, as probably are other regulatory elements of these pathways. Intracellular inhibitors directed towards fundamental intermediates of these pathways have been used in clinical trials. Farnesyl transferase inhibitors, such as tipifarnib and lonafarnib (24,25), have been used to block the Ras/Raf/MEK/Erk axis, but despite preclinical studies have demonstrated a reduced Ras signaling, clinical trials have shown no benefit (21,22). Similarly, the multikinase inhibitor, Sorafenib, directed to Ras, VEGFR, PDGFR and other kinase, was well tolerated, but with limited therapeutic benefit (26). mTOR inhibitors such as rapamycim, temsirolimus, everolimus, and sirolimus proved well tolerated, but of little clinical efficacy (24,27,28).

Immunotherapeutic approaches have also been attempted for the treatment of gliomas. Two approaches were considered: active immunotherapy and passive immunotherapy. The active immunotherapy corresponds generally to vaccines and includes a patient's immune response against cancer cells following exposure to GBM-related antigens. The therapies can be peptide-based therapies and cell-based therapies (29). In the first case specific antigens are administered directly to patients. These antigens are small molecules, about 9 amino acids long, capable of inducing activation of cytotoxic T lymphocytes (CTLs). Rindopepimut is an injectable vaccine of this type, designed to stimulate an immune response against a mutated form of EGFR, EGFRvIII (30), present in 30% of GBMs. This vaccine has shown promising and clinical trials are underway to confirm these expectations. In the case of cell-based therapies, antigen presenting cells (APC), generally dendritic cells (DC), are obtained from peripheral blood mononuclear cells of patients, cultured in the presence of specific growth factors, activated *ex vivo* with tumor-related peptides, and then administered to the patient (31). Although well tolerated, this approach has shown no therapeutic benefits (23,32). Passive immunotherapy involves the use of antibodies, immune cells and other immune system elements to attack cancer cells and does not require prior activation of the patient immune system (33). This kind of therapeutic approach is referred to as adoptive cell transfer. The two cell types most used are lymphocyte-activated killer (LAK) cells and CTLs (34). LAK cells are prepared from autologous peripheral lymphocytes cultured in the presence of interleukin (IL)-2, generating a polyclonal population of both T and natural killer (NK) cells (31), with specificity not only directed towards the tumor. CTLs are obtained from patients' peripheral blood mononuclear cells stimulated *ex vivo* with autologous tumor cells and generating activated, tumor specific CTLs (31,34). Some attempts have been made using both LAK cells and CTLs. Typically, these last two approaches were better tolerated by patients, probably for higher tumor cell specificity. In any case, therapeutic approaches that utilized LAK cells or CTLs showed minimal efficacy with no effect on survival (23,33-35).

Another possible therapy against gliomas is gene therapy, with major technical problems in the transgene delivery system. A first approach is the so-called immunomodulatory gene therapy. Gliomas have long been known to develop mechanisms to evade the anti-tumor immune response. The purpose of this approach is therefore to stimulate an anti-tumor immune response. In GBM, cytokine-mediated gene therapy techniques and immune cell recruitment strategies have been tested. Cytokine-mediated gene therapy involves

the use of transgenes for various immunostimulatory agents that, released in the tumor environment, induce a more potent immune response (36). Immune cell recruitment strategies attempt to recruit DC or other APCs in the tumor to trigger a more effective immune response (37). Combined approach of cytokine-mediated and cytotoxic gene therapies have shown good results in preclinical trials (37). Clinical trials using this approach have recently begun (37,38).

Suicide gene therapy also has been used as an anti-glioma treatment. Generally, in conditionally cytotoxic approaches a transgene coding for a non-toxic enzyme is released in cancer cells. Following non-toxic pro-drug administration, the enzyme converts the non-toxic pro-drug into a metabolite that induces tumor cell death (38). The toxic metabolite may also spread to nearby tumor cells, not reached by the transgene, through gap-junctions or intercellular space by amplifying therapy cytotoxicity (39). In GBM the most used system is the combination of herpes simplex virus-1 thymidine kinase (HSV1-TK) and ganciclovir (GCV) (38). In GBM clinical trials, replication-deficients retrovirus and adenovirus for the transgene release into the surgical resection cavity were successfully used. The adenoviral system appears however to be preferred for a greater capacity for gene transfer into GBM cells. Unfortunately, clinical trials have shown that, despite being well tolerated, HSV1-TK/GCV therapy has a limited efficacy (23,38,40).

In oncolytic viral therapy, virus toxicity is directly responsible for the death of the cancer cell. Viral vectors are engineered so as to selectively infect tumor cells, resulting in lysis, but not the surrounding healthy parenchyma. Following lysis, the viral particles will infect other tumor cells. The first oncolytic virus used against gliomas was the neurotrophic virus HSV1 (38). HSV1 attenuation is therefore a fundamental requirement for its use. HSV1 has been attenuated in several ways. One of the attenuated forms is G207, genetically engineered to replicate only in rapidly dividing cells (41). This HSV1 form maintains the HSV1-TK gene, so as to be responsive to GCV, increasing the cytotoxicity to tumor cells. Use of this form and other HSV1 attenuated forms in clinical trials has shown a good level of security, but limited therapeutic efficacy (42). Engineered adenovirus were also used (38). One of the advantages of using adenoviruses is that they are not naturally trophic for nerve tissue, thus having a greater safety profile compared to HSV1. ONYX-015 is a modified adenovirus that replicates only in p53 mutated cells (43); Ad5-Delta is an adenovirus that replicates in cells with Rb defective functions (44). Clinical trials have initiated and are currently underway; they showed a good safety profile. To overcome the problems relative to a possible immune response directed to the oncolytic virus, alternative delivery strategies have been suggested. At present the clinical trials involve the direct release of oncolytic viruses to cancer cells, and preclinical studies are evaluating the use of neural or mesenchymal stem cells as vectors to release the oncolytic viruses (45,46).

Directly cytotoxic gene therapy can be accomplished through toxins that, via overexpressed surface molecules, are internalized in the tumor cell, resulting in cell death. Toxins can be released through transgenic viral vectors for highly toxic proteins or through recombinant molecules. Immunotoxins, for example, consist of cancer specific monoclonal antibodies associated with toxins (47). The antibody, or more in general the ligand, binds to the surface molecule, determining the toxin internalization with a succession of events that leads to cell death. Some toxins used are the Pseudomonas exotoxin or diphtheria toxin (47), directed to over-expressed surface antigens such as EGFR, interleukin(IL)-13 and transferrin (23,48-50). Again, despite promising preclinical studies, clinical trials have not yielded the desired results (51). Another type of recombinant molecules are monoclonal antibodies conjugated to radionuclides, small molecule inhibitors and chemotherapeutic agents. Radiolabelled antibodies can be targetted to tumor cells through overexpressed surface antigens such as EGFR or integrin alphavbeta3, causing cell death by radiation damage to DNA. Clinical trials have not demonstrated benefits for the patient (52,53).

3.2. Molecular markers

From a genetic point of view, gliomas exhibit many complex alterations which make it is difficult to determine their exact progression. It is clear, however, that some of these alterations are early events in neoplastic transformation, whereas other alterations are late. Genetically GBM cells appear extremely altered, with loss or gain of functions involving numerous and important signal transduction pathways. The primary and secondary GBM show similar alterations and the low-grade gliomas transition towards secondary high degree GBMs is determined by addition of genetic alterations associated with malignancy changes. Yet, certain aberrations are more frequent in each group. In this respect, two points must be taken into account, that is the changes occurring in the progression from an initial low-grade astrocytoma (grade II) to anaplastic astrocytoma (grade III) and GBM (grade IV), and the peculiar alterations of primary GBM, compared to secondary GBM. Many studies have been done to clarify the genetic alterations in low-grade gliomas to GBM transition. It now seems clear that in gliomagenesis and glioma progression, certain events must be present such as alterations of p53 and retinoblastoma (Rb) functions and TKR signaling. It is also clear that these pathways can be altered at different levels (54,55).

The main events in the genesis of grade I and II astrocytomas are mutation of TP53 gene on chromosome 17p13, 17p loss and PDGFA/PDGFRalpha

overexpression (56). p53 responds to DNA damage either causing the arrest in G1 by inhibiting the formation of cyclin-cyclin dependent kinase (CDK) complex or, if the damage is extensive, causing apoptosis. p53 inactivation is observed with a similar frequency both in low-grade and high grade astrocytomas, indicating that this event occurs in a rather early stage of gliomagenesis. The PDGFA/PDGFRalpha overexpression is another molecular event that outlines the first steps of the neoplastic transformation towards more malignant gliomas. The PDGFR gene amplification or more rarely its structural alteration determines the synthesis of constitutively active forms of this tyrosine kinase receptor that in turn activates the Ras/MAPK and PI3K/Akt pathway, involved in cellular proliferation and migration. Isocitrate dehydrogenase (IDH) 1/2 mutations are also early genetic markers of WHO grade II/III gliomas. These mutations have an important prognostic significance (57).

The acquisition of other alterations, such as Rb gene mutations and 13q loss, amplification of specific loci of chromosoma 12, deletion of part of chromosome 9 with CDKN2A gene coding for p16INK4a/p14ARF, loss of chromosomes 1p and/or 19q, are the main alterations resulting in the transition to grade III gliomas (anaplastic astrocytoma) (56). The physiological function of Rb is to block cell division before the entry into S or G1 phase thus preventing wrong or harmful division. When Rb is altered some cells can continue to divide, giving rise or contributing to tumorigenesis. Ipophosphorylated Rb binds to and inhibits the E2F transcription factor crucial to introduce the cells into S phase. The cyclin D/CDK4/6 complex determines a first phosphorylation followed by cyclin E/CDK2 mediated phosphorylation, causing the Rb detachment from E2F trascriptional complex, that favors cell cycle progression. Rb is phosphorylated during the S, G2 and M phases of the cell cycle. The amplification of specific loci of chromosome 12, containing the tumor growth regulatory genes, is another key event in tumor progression; CDK4 overexpression supports Rb phosphorylation, as already mentioned, to the advantage of cell cycle progression. Overexpression of mouse double minute 2 homolog (MDM2, a ubiquitin ligase), often alternative to mutations of p53, regulates the p53 function, inactivating its transcriptional activity. The genes coding for CDK4 and MDM2 are contained in amplified loci of chromosome 12. Deletion of the 9p21 region leads to loss of function of the important cell cycle regulator, CDKN2A gene. This gene encodes two tumor suppressors genes, p16INK4a and p14ARF. p16INK4a is a CDK inhibitor and CDK4/6 blockade prevents Rb phosphorylation and cell cycle progression. p14ARF is expressed in response to signals of high mitotic activity mediated by Myc and Ras and positively regulates the p53 activity by inactivating MDM2. The loss of function (LOH) of these tumor suppressors induces a strong cell proliferation gain. LOH of 19q is another hallmark of progression to anaplastic astrocytomas although the double 1p/19q LOH is typical of oligodendrogliomas and it is considered as an important prognostic marker (58).

The last step of tumor progression to glioblastoma is determined by EGFR gene amplification and mutation, LOH of chromosome 10, PI3K mutated/amplified, VEGF overexpression (56,59). EGFR signaling may be activated in a ligand dependent or independent way, through overexpression of both the ligand(s) and the receptor, leading to an autocrine loop, and via genomic amplification of EGFR and/or mutation of the receptor, leading to constitutive activation in the absence of ligand. The oncogenic properties of EGFR are associated with a constitutive and uncontrolled increase in its phosphorylation (catalytic) activity. Ligand-activated receptors trigger downstream signal transduction pathways, including the Ras/Raf/MAPK pathway, the PI3K/AKT pathway, the protein kinase C pathway, and the STAT pathway, together with VEGF production, with an impact on cell proliferation, migration, invasion, resistance to apoptosis, and tumor neovascularization. The PTEN tumor-suppressor gene negatively regulates PI3K, but its function is frequently lost in GBM because of LOH at the 10q23.3. locus, causing a constitutive activation of the PI3K pathway and higher levels of activated AKT in glioma cells. Recruitment of PI3K to the cell membrane activates downstream effector molecules, such as AKT and mTOR, resulting in cell proliferation and increased cell survival by blocking apoptosis. Similarly, mutated/amplified PI3K determines high AKT activity with the same consequences.

With regard to the cytogenetic and molecular differences, both primary and secondary GBM contain similar aberrations, although certain aberrations are more prevalent in one subtype over the other (59,60). Primary GBM frequently show EGFR gene amplification and mutation on chromosoma 7, LOH of chromosome 10q PTEN gene, PTEN and telomerase reverse transcriptase (TERT) mutations, overexpression of MDM2, deletion of p16 gene. PTEN gene mutations have been reported in approximately 25% of all GBM, and exclusively in primary glioblastoma. TERT mutations as well as deletion of p16 gene (CDKN2A-p16INK4) are more frequent in primary than secondary GBM, whereas other alterations of CDKN2A (CDKN2A-$p14^{ARF}$) are common in secondary GBM (61). Alterations that are characteristics of secondary GBM include mutation of TP53, LOHs of chromosome 13q (Rb), chromosome 19q, chromosome 22q, partial LOH of chromosome 10, PDGFA/PDGFRalpha overexpression and/or amplification, IDH-I/2 mutation (61). Interestingly, secondary GBM show greater promoter methylation of several genes compared to primary GBM (MGMT, Rb, p16 CDKN2A-p16INK4, CDKN2A-p14ARF) (61).

While several genetic differences have been identified, hardly useful from a diagnostic and therapeutic point of view, the characterization of IDH1/2 gene mutation

has allowed a more reliable molecular differentiation between primary and secondary GBM (57). IDH1 is mutated in about 70% of grade II-III glioma as well as in secondary GBM that originate from these lowest degree glioma thus representing an important way of distinction between primary and secondary GBM. Only 3-6% of primary GBM shows mutations of IDH1 compared to about 65-80% of secondary GBM. From a clinical point of view this mutation was detected in a high percentage of younger patients and in those with secondary GBM and this was reflected in a greater overall survival. When associated with 1p-19p co-deletion and glioma CpG island hypermethylator phenotype (G-CIMP), IDH1/2 mutations are prognostic of a favorable outcome for anaplastic oligodendroglioma tumors and predictive of chemotherapic response (62). Mutant IDH 1/2 alters glioma metabolism, favoring the reduction of alpha-ketoglutarate to 2-hydroxyglutarate (2-HG), which in turn inhibits DNA and hystone demethylates and establishes a G-CIMP, featuring hypermethylation at a large number of loci. Moreover, 2-HG indirectly stimulates the degradation of hypoxia- inducible factor (HIF)1alpha, with the consequent downregulation of HIF 1alpha-induced glycolytic enzymes. Besides, in IDH 1/2 mutated glioma, lactate dehydrogenase (LDH) A is silenced by IDH mutant-dependent promoter methylation. LDH converts pyruvate to lactate, a key step in glycolysis, and is overexpressed in highly aggressive and treatment-resistant cancers. IDH 1/2 mutations, by downregulating essential glycolytic genes such as LDHA, may prevent the glycolityc switch (Warburg effect) and limit the rapid growth of secondary glioblastoma and low grade glioma (63).

The O(6)-methylguanine-DNA methyltransferase (MGMT) gene is frequently silenced in GBM by promoter hypermethylation. Currently, this represents one of the most relevant prognostic factors in GBM and a potent predictor of response to treatment with alkylating agents as TMZ (12). The use of alkylating chemotherapeutic drugs, such as TMZ, causes binding of an alkyl group to the O6-position of guanine, thereby inducing a DNA mismatch, DNA double-strand breakage, and ultimately apoptosis of proliferating cells. Thus, the MGMT protein counteracts the normally lethal effect of TMZ by repairing DNA damage. When a tumor has a hypermethylated MGMT promoter, transcription of the gene is blocked, leading to lack of MGMT mRNA and protein expression, while enhancing the cytotoxic effects of the alkylating drug(s). Previous studies have shown that patients with hypermethylation of the MGMT gene promoter may have longer survival rates when treated with both TMZ and radiotherapy instead of radiotherapy alone (12).

The EGFR gene is present in normal cells and is the most frequently overexpressed gene mainly in primary GBM. Its alterations are associated with high-grade glioma malignancy. However, controversial results exist regarding the prognostic significance of EGFR amplification in GBM. Although some studies reported that EGFR amplification is associated with a poor prognosis and a shorter survival of GBM patients, others claim that such association with survival would not be significant or that it could even be associated with a better outcome. In turn, some reports found a poor prognosis for GBM patients carrying amplification of the EGFR gene among all age groups, whereas others found EGFR amplification to be a predictor for prolonged survival only among older patients. Such discrepant results could potentially be associated with the specific underlying genetic lesion targeting the EGFR gene. In this regard, the prognostic impact of EGFRvIII (the most common EGFR mutant variant) has not been investigated as extensively as EGFR amplification. Despite this, controversial results have also emerged. Accordingly, the EGFRvIII variant was found not to be related to patient outcome, to be associated with an unfavorable prognosis, or even to be a molecular predictor of prolonged overall survival among conventionally treated GBM patients (61). Recently, TERT promoter mutation have been considered as predictive of poor outcome in grade III-IV gliomas (64).

Studies of gene expression profile and integrated genomic analyses have allowed to identify, despite the GBM high heterogeneity, four subtypes characterized by specific alterations. In this gene-expression based molecular classification, GBM can be classified into classical, mesenchymal, proneural and neural (8-10). Each of the subgroups presents different mutations, genomic and transcriptional characteristics and also differs in their response to treatment and overall survival. The classical subtype is characterized by a typical astrocytes gene expression pattern with overexpressed and altered EGFR activity, frequent loss of chromosome 10, and homozygous deletion of 9p21.3. region while TP53 and IDH1 mutations are less frequent. Neural precursor and stem cell markers are highly expressed in classical subtype. The mesenchymal subtype is characterized by hemizygous deletion of 17q11.2. containing the gene NF1 and by PTEN mutations both altering the PI3K/Akt/mTOR signaling pathway. Tumor necrosis factor super family and NK-kB pathways genes are highly expressed. The expression pattern is similar to that of mesenchymal and astrocytes cells, reminiscent of an epithelial to mesenchymal transition. EGFR overexpression is less frequent. The patients with the mesenchymal subtype show a poor overall survival. The proneural subgroup is characterized by alteration of PDGFRA and IDH1 mutation. p53 and PI3KCA/PI3KR1 mutations are also frequent in this sybtype as is 1p19q loss. The expression pattern shows a high expression of oligodendrocytic and proneural development genes, such as OLIG2 and Sox genes. The neural subtype is characterized by a gene expression pattern similar to the normal brain tissue with no subgroup-specific features, although mutations of the ERBB2 gene are frequently

observed. It is strongly enriched in the gene expression pattern seen in neurons, and expresses both astrocytic and oligodendrocytic markers.

Lastly, GBM without IDH1 mutation have been defined as classical, mesenchymal proneural and neural and are considered as primary GBM (59), while the majority of GBM with IDH mutation have a proneural pattern (8). Considering that secondary GBM shown IDH1 mutation in greater than 80%, it is correct to state that secondary GBM represent a more homogeneous group characterized by an IDH1 mutation and a proneural pattern of gene expression, whereas primary GBM are a more heterogeneous group with different genetic mutations and aberrant gene expression profiles. Accordingly, the proneural subtype is frequently diagnosed in younger patients (secondary GBM) with a better prognosis and an increased survival compared with other subtypes.

An interesting approach is to draw relationship between the electrophysiological properties of glioma cells and the degree of malignancy. Regarding the potassium currents, those mainly considered in glioblastomas are the rectification inwards (KIR), the adjustment in delay (DRK) and the Ca^{2+}-activated K (KCa) current. The expression of KIR currents in glioblastoma cells is however generally low or absent (65), in agreement with the highly depolarized membrane potential of glioma cells and immature astrocytes that equally display very low KIR currents (66).

Electrophysiological studies have shown the presence of DRK currents in primary cultures of human glioblastoma cells. In particular, the DRK current was observed in 70% of the cells, a percentage similar to that found in slices from fresh biopsies (65). Little information is available regarding the molecular nature of these currents. Glioblastoma biopsies as well as primary cultures were found to express Kv11.1. transcripts encoding the human ether-a-go-go-related gene (hERG) channel, whose expression level is positively correlated with the degree of glioblastoma malignancy (67). DRK currents mediated by Kv1.1. were important in the control of membrane potential in the rat C6 cell line (68).

The KCa channels can be divided into three classes: the large conductance (BKCa), intermediate conductance (IKCa) and small conductance (SKCa). The presence of a particular isoform of the BKCa channel, called gBKCa and specific to glioma cells, has been positively correlated with glioma malignancy (69). BKCa currents have been recorded by several groups both in slice preparations and in primary and stabilized glioblastoma cell cultures (70).

Recent work has reported the expression of IKCa currents in human glioblastoma cell lines (71) and demonstrated that the IKCa currents are involved in the modulation of glioma motility induced by SDF-1 (72) and in the invasion of brain parenchyma (73). Notably, it was reported that the IKCa channel (KCa3.1.) inhibition with specific antagonists or by shRNA silencing has beneficial effects in glioma-bearing mice, reducing the maximal distance reached by glioma cells through brain parenchyma (73,74). KCa3.1. channels can be activated by different signals that mobilize intracellular Ca^{2+} such as histamine, EGF etc. and support calcium influx through membrane hyperpolarization (75).

3.3. Glioma stem cells

What is the cell or group of cells giving rise to glioma and which are the characteristics of these cells? Three theories are typically considered for the genesis of glioma.The first one poses that mature glial cells dedifferentiate to acquire unregulated “stem cell (SC)” like properties through the acquisition of several and successive mutations and/or epigenetic lesions. According to a second theory, restricted neural progenitors, which have limited self-renewal potential, acquire mutations which lead to the gain of unregulated “stem-cell” like properties. The third theory is the so-called cancer stem cell (CSC) theory, which postulates that only a subset of tumor cells possess the ability to self-renew, continuously proliferate and give rise to clones containing variable genetic profiles. The heterogeneity within a tumor arises from proliferation of these genetically variable CSC (76). The glioma SCs (GSCs) originate from adult neural stem cells (NSCs) which normally have tight regulation over their proliferative and differentiation potential. The acquisition of several and progressive mutations changes the NSCs into GSCs. Owing to this, gliomas are thought to originate in the subventricular zone (SVZ) containing the highest density of adult NSCs, which may be more susceptible to malignant transformation (77) and may invade, along blood vessels and white matter tracts, the cerebral lobes. The stem cell theory was first introduced by Virchow in 1863 based on histological similarities between embryonic stem cell and cancer cells. The discovery of SCs in the adult CNS occurred in the early 1960’s when it was reported that adult neuronal cells could incorporate tritiated thymidine. Thirty years later, neural cells capable of regenerating were identified within the canary brain SVZ and finally NSC were described (78). Subsequently, CSC were identified in a wide variety of solid organ malignancies including brain tumors such as GBM and medulloblastoma (79).

Overcoming the differences in nomenclature (80), many studies have been done to define markers characterizing these cells and their functional properties. Most GSC markers have been derived from normal NSCs. Indeed, many transcription factors and structural proteins defined as NSC markers, are expressed in GSCs including Sox2, Musashi-1, BMI1 (81), Myc, Nestin, Nanog and others (80). The prognostic significance of these markers has been evaluated. In

gliomas, the results demonstrate a positive correlation between increasing Nestin expression and grade and survival. However, data limited to GBM did not support this association. Studies involving Musashi-1 show a positive correlation with grade. Among ESC markers, only Nanog has been shown to predict grade and survival both within and across glioma grades. Oct4 and Sox2 expression increases with grade but does not predict survival (80). Other studies have proposed S100B protein as a factor involved in the regulation of stem cell properties not only in neural cells (82,83), but also in muscle cells (84).

Because of their intracellular localization, these markers can not be used to isolate the CSCs, then a multitude of surface markers have been postulated. The first proposed marker was cluster of differentiation (CD) 133 (prominin-1), a surface transmembrane glycoprotein (79). Early experiments demonstrated that GSC exclusively within the CD133+ fraction gave rise to tumor cells phenotypically resembling parent tumor cells, and reproduced GBM when implanted into SCID mouse brain. Unfortunately, the complexity of this marker has led to less enthusiasm by subsequent data identifying CD133– GSC with similar functional characteristics (85). This does not necessarily detract from CD133 as a GSC marker, but suggests that it may not capture all GSC. Also, differences in cell and/or culture conditions may influence its expression and detection. A2B5, a marker of glial progenitor cells, has also been identified as a GSC marker (85). A2B5+ GSCs fulfill GSC criteria, generating tumors phenotypically similar to the original tumor, regardless of CD133 expression. While this can be viewed as evidence against CD133 as a GSC marker, A2B5 may encompass a broader population of GSC (85). Stage specific embryonic antigen-1 (SSEA-1), a carbohydrate adhesion molecule associated with glycoproteins and glycolipids whose expression was initially associated with NSC, was also identified as a GSC marker (86). Similar to A2B5+ cells, SSEA-1+ cells displayed the functional characteristics necessary to qualify them as GSC. SSEA-1 was expressed in both, early and late GSC passages, whereas CD133 expression diminished in late passages (86), suggesting that SSEA-1 may be a reliable marker *in vitro*. To date, there are no clinical studies correlating the presence of CSC and outcome. The hypothesis that gliomas with a greater degree of GSC marker expression should be higher grade and/or be associated with worse survival was validated by the correlation between increasing CD133 expression and tumor grade. However, when data was limited to grade IV glioma, results were contradictory (78). Other GSC markers received little attention. It seems clear that the use of a combination of markers could lead to a better cellular characterization and to a more linear relationship with prognostic and therapeutic aspects.

GSC markers, although useful to enrich populations of stem cells from nonstem cells, are not sufficient to define either population due to the lack of definitive markers. Functional validation is therefore necessary to determine the differences between GSCs and non-stem tumor cells (NSTC). GSCs are defined by functional characteristics that include self renewal, persistent proliferation, tumor initiation upon secondary transplantation and, as somatic stem cells, frequency within a tissue or tumor, and ability to generate progeny of multiple lineage. *In vitro* neurosphere formation is a test for both proliferation and self-renewal but fails to reproduce a typical tumoral cellular hierarchy and to recapitulate the tumor microenvironment. The gold standard for CSC determination remains the ability of a limiting dilution of cells to recapitulate the complexity of the original patient tumor when transplanted orthotopically. The ability to derive heterogeneity is essential because populations of transit-amplifying cells may form a tumor but will only give rise to cells from their specific lineage (80).

GSC regulation is performed in two ways, through own cell-autonomous mechanisms and mechanisms related to the tumor niche. In the first case, we can consider metabolic, genetic and epigenetic mechanisms. GSCs demonstrate a high metabolic plasticity in response to metabolic restrictions. The “Warburg” effect, a shift towards aerobic glycolysis with lactate accumulation, is the most important event which affects not only the GSC, but all tumor cells. Another metabolic shift is the use of the pentose phosphate pathway by GSCs (87). The presence of GSCs in hypoxic and acid environment and the preferential use of HIF-2alpha signaling compared to normal progenitor and NSTCs, promote the maintenance of GSCs selfrenewal, proliferation and survival (87). Moreover, in nutrient deprivation conditions, such as low glucose, GSCs compete with neighbors NSTCs for the absorption of glucose through preferential up-regulation of the high affinity glucose transporter isoform, type 3 (GLUT3) (88). In proneural GBM, IDH mutations are also involved in metabolic remodeling of cells and while in GSCs the function of these mutations is not well defined, this relationship is an interesting example of correlation between genetic alterations and metabolomic. Regarding the GSCs genetic and epigenetic mechanisms of regulation, we now have a comprehensive picture of the genetic mutations and structural variants present in GBM (8-10).These studies show the high degree of intertumoral heterogeneity present in GBM, which is further captured at both transcriptional and epigenetic levels (8-10), and also underscore the complexity of the clonal evolution and clonal diversity that occur during the genesis of GBM. Epigenetic GSC regulation converges on Myc, which occurs in the presence of Myc- mediated cancer cell survival and proliferation programs (89). Additional transcription factors have been identified as important for CSC identity, including STAT3, Sox2, FOXM1, Nanog and others (80). These transcription factors recruit necessary chromatin remodeling factors to promote the maintenance of the glioma CSC state.

A recent work has shown that increased Ras signalling, determines neural stem cells depletion and glial progenitors expansion in gliomagenesis. In this context, inhibition of Ets signalling downstream of Ras inhibits glioma formation by attenuating gliogenesis necessary for tumor propagation (90). By using epigenome-wide mapping of cellular chromatin state, Suvà *et al.* (91) identified a core set of four transcription factors, POUF3, Sox2, SALL2 and Olig2, in proneural GBM that are able to reprogram differentiated tumor cells into GSCs. These studies highlight the importance of understanding the dynamics of core transcription factors in maintaining stem cell state and the effect(s) these factors have on shaping the chromatin landscape of cells within the tumor hierarchy.

4. MICROGLIAL CELLS

4.1. Origin and location of microglia

Microglia are the resident innate immune cells of CNS and are the primary responders in a defense network that covers the entire brain parenchyma. The first studies on microglia dating back more than a hundred years ago, when eminent anatomists and psychiatrists, observing the histological changes of diseased brains, spotted a cell type with phagocytic activity. Nissl (1899) set up the homonymous coloring, Alzheimer (1904), and Cajal who in 1913 classified the cellular elements of the CNS, defined the mesoglia as 'a third element'. However it was the neuroanatomist Pio del Rio-Hortega in 1932 to earn the title of 'father of microglia'.

del Rio-Hortega first suggested that microglia originate from meningeal macrophages penetrating the brain during late embryonic development (92). Ever since, these small and versatile cells have been studied extensively, although today there are still many gaps. The microglial cells in fact, in addition to being myeloid elements are also CNS cells and retain characters of stem cells. The origin of microglia has been subject of debate for many years. Controversy about the possible origins of microglia from neuroectodermal cells, instead of myeloid progenitors, persisted well into the 1990 (93). Data accumulated in those years convincingly show that microglial cells belong to the hematopoietic system and display macrophage markers (94). The most convincing evidence favoring the myeloid origin of microglia came from studies of the purine-rich box1 transcription factor (PU.1). The differentiation of cells in the myeloid lineage (dendritic cells and macrophages) failed in mice that lacked the PU.1 transcription factor (PU.1–/– mice) and no microglia were detected in the CNS of these mice (95). However, time of appearance, site of origin and mode of penetration of microglia precursors into the brain rudiment were not clear. While del Rio-Hortega suggested that microglia originate from meningeal macrophages penetrating the brain during embryonic development (92), many authors claimed that brain parenchymal microglia derived from circulating blood monocytes originating from bone marrow. A major question was whether embryonic and adult microglia have distinct origins or whether adult microglia were the progeny of embryonic microglia.

Ginhoux and Jung (96) discovered that the microglia compartment is established well before birth from primitive macrophages that are generated during an early 'primitive' wave of hematopoiesis in the yolk sac between embryonic day E7.0. and E9.0. Moreover, these authors showed that after birth, microglia remain independent from input from bone marrow-derived monocytes and adult hematopoiesis, and maintain by longevity and limited self- renewal. Fate mapping analysis reveals that adult microglia derive from primitive macrophages (96).

Fate mapping studies conducted independently by Kierdorf *et al.* (97) confirmed the embryonic origin of microglia from CD117+ erythromyeloid precursors prior to the formation of blood–brain barrier and vascularization of the embryo (97). They showed that microgliogenesis was not only dependent on the transcription factor PU.1, but also required interferon regulatory factor 8 (Irf8), which is vital for microglial precursors CD45+, CD117−, chemokine (C-X3-C motif) receptor 1 (CX3CR1+), whereas other transcription factors such as cellular myeloblastosis transcription factor (c-Myb), a critical regulator of hematopoiesis, inhibitor of DNA binding 2 (Id2), basic leucine zipper transcription factor ATF-like 3 (Batf3) and kruppel-like factor 4 (Klf4) are not required. It is well known that the colony-stimulating-factor 1 receptor (CSF-1R) and its ligand, colony-stimulating-factor (CSF)-1, play a central role in macrophage development and maintenance. This is likely due to the fact that PU.1 transactivates the proximal promoter of the CSF-1R gene; indeed, CSF-1R expression was not detected in PU.1- deficient embryos (98). Ginhoux and collaborators (2010) (99), showed that the development of yolk sac macrophages and microglia, but not monocytes, is strongly dependent on CSF-1R and that IL-34, another CSF-1R ligand, recently found in mice (100), is critical in regulating microglia homeostasis after birth and in the adult.

Like the microglial cells, cells in other tissues such as the liver (Kupffer cells), skin (Langerhans cells), lung (alveolar macrophages), bone (osteoclasts), and kidney (kidney macrophages) come primarily from yolk sac and are maintained throughout adulthood. Like the microglia, their progenitor cells are independent of the transcription factor c-Myb, which is required for the development of hematopoietic stem cells (HSCs), suggesting that these populations of tissue-resident macrophages develop and persist independent of the contribution by HSCs (101) and are also independent of fms-related tyrosine kinase (FLT)3, a cytokine receptor expressed on hematopoietic multipotent progenitors cells (102).

In the mature brain, microglia are confined by the fully developed BBB and become an autonomous, long- living cell population that retains the ability to divide and self-renew throughout life without any significant input from circulating blood cells (103). Microglia and bone marrow derived macrophages thus represent two genetically distinct myeloid populations. These differences imply different functions for microglia and infiltrating macrophages, which are increasingly apparent in mouse models of disease (103,104).

Depending on the species, microglia account for 5–20% of the total glial cells present in the adult brain and are present in large numbers in all major regions of the brain but are not uniformly distributed. Densely populated areas include the hippocampus, basal ganglia, the olfactory telencephalon and the substantia nigra. In comparison, the less densely populated areas include fiber tracts, the cerebellum and most of the brainstem. The cerebral cortex, thalamus and hypothalamus have average cell densities. More microglia are found in gray matter than white matter (94). Microglia morphology varies considerably. While white matter microglia show elongated somata and processes preferentially oriented along fiber tracts, microglia in the circumventricular organs, a region characterized by a leaky blood–brain barrier, exhibit compact morphology with few short processes. In contrast, gray matter microglia exhibit many elaborate radially oriented arbors (105).

4.2. Microglia phenotypes

Microglia is still considered among the most mysterious cells of the brain. Ransohoff and Cardona (106) describe microglia as a unique type of mononuclear phagocytes that do not have dendritic-cell characteristics *in vivo*. Microglia are localized within the parenchyma of the CNS near the neurovascular unit but not directly associated with vessels. Microglia may form part of the perivascular glial limitans basement membrane and, compared to peripheral monocytes/macrophages (major histocompatibility complex (MHC) class II; CD45high), express cytosolic rather than membrane MHC class II (106) and relatively low levels of CD45 (CD45low) (107).

In the CNS, there are other mononuclear phagocytes such as meningeal macrophages, choroid plexus macrophages and perivascular macrophages, each with a distinct location and armed mainly with MHC class II molecules (106). Additional discrimination between monocytes/macrophages and microglia can be made using the surface marker lymphocytes antigen 6C (Ly6C) and expression of the chemokine (C-C motif) receptor (CCR)2 and CX3CR1 which are absent and present in microglia, respectively (108). The issue of the CCR2 receptor in microglia is much debated. Studies using CX3CR1(+/green fluorescent protein (GFP))/CCR2(+/red fluorescent protein (RFP)) knock-in fluorescent protein reporter mice demonstrated that microglia are CX3CR1+/CCR2- whereas the monocyte-derived macrophages are CX3CR1−/CCR2+, indicating that in the brain, microglia and monocyte-derived macrophages are different populations with distinct and specific surface antigens (109).

In healthy brain, microglia have a “down-regulated” phenotype represented by a ramified shape with short fine processes (Figure 2A) and thus increased surface area for tissue surveillance. Individual cells scan their own territory without any overlap or contact with neighbouring microglia, interacts physiologically with all cells in the brain parenchyma, respond promptly to brain damage or injury and clears cellular debris or accumulating metabolic products by phagocytosis (110).

Recent reports ascribe to microglial cells fundamental functions in the healthy developing brain and in adult brain, affecting synaptic connectivity, synaptic pruning, and programmed cell death and are involved in proper brain wiring and circuitry formation (111). Microglia regulates neuronal activity by direct microglia-neuron or indirect microglia-astrocyte-neuron interactions (Figure 2A), by communicating with astrocytes via an adenosine triphosphate (ATP)-dependent mechanism, in the second case (112, 113). It has been proposed a model in which activation of microglia induces a rapid production of ATP. Microglial ATP then recruits astrocytes to amplify ATP production and glutamate release. Thus, microglia might act as a genuine regulator of neurotransmission and upstream partner of astrocytes. Direct interaction with neurons ensures the maintenance of the microglial phenotype that can be defined quiescent, but alerted, tolerant and pro-homeostatic (114). Such cross-talk is mediated by several mutual ligand-receptor interactions (104) (Figure 2A) and in part is due to signals derived from neuron-released factors (115) (Figure 2A).

Whereas the gray matter microglia are in close contact with the cell bodies of neurons, in the white matter the interactions change. Goldmann *et al.* (116) identify ubiquitin-specific protease (Usp) 18 as a new critical negative regulator of microglial activation. Usp 18 is expressed mainly in white matter microglia and negatively regulates the activation of STAT1 and concomitant induction of interferon-induced genes, thereby terminating type 1 interferons (IFNs) signaling (116).

Astrocytes represent a highly communicative partner of microglial cells as well (Figure 2A). Their main task is to maintain the physiological homeostasis of neurons by providing a stable microenvironment and growth factors. Therefore, it is reasonable to assume that astrocytic information-processing can be influenced by microglial activation. The reverse is also true: astrocytes have an influence on microglial behavior, releasing soluble factors, many of which are still mysterious (117) (Figure 2A). Astrocytes produce glia maturation factor

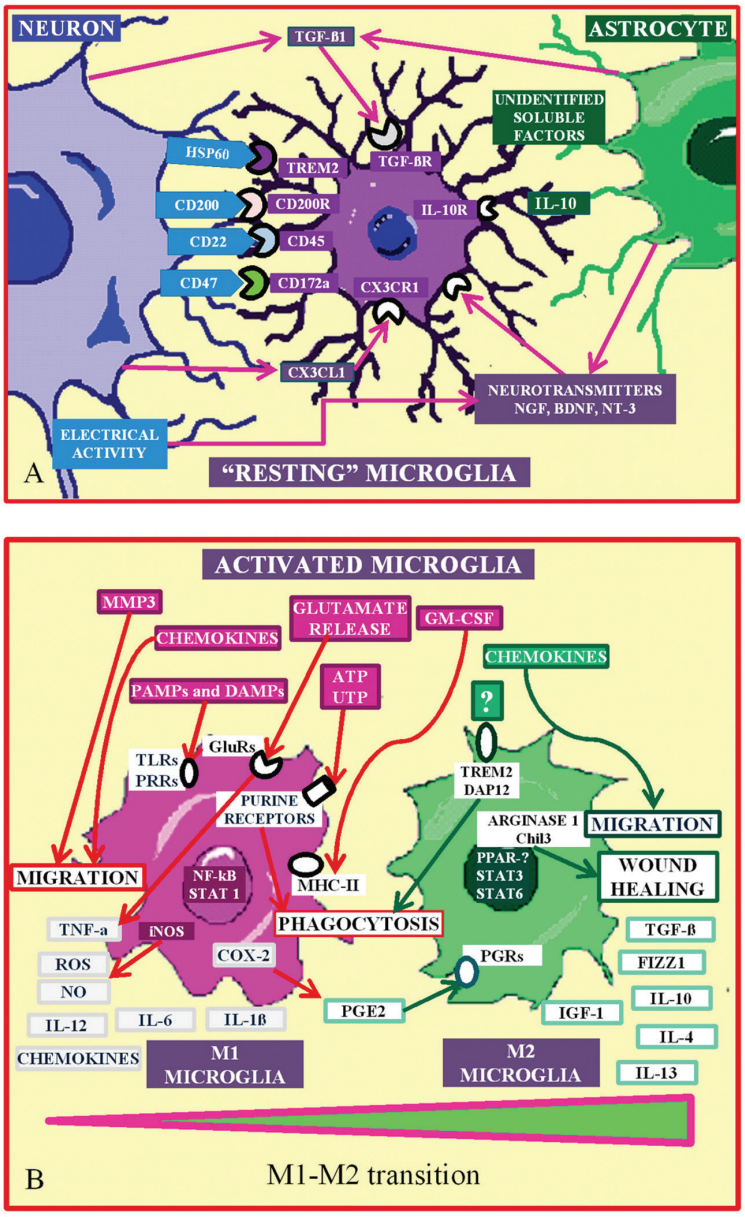

Figure 2. A Resting microglia. In healthy brain, microglia have a "down-regulated" phenotype. Individual cells scan their own territory without any overlap or contact with neighbouring microglia, interacts physiologically with all cells in the brain parenchyma. Microglia regulates neuronal activity by direct microglia-neuron or indirect microglia- astrocyte-neuron interactions, by communicating with astrocytes via an ATP-dependent mechanism, in the second case. Activation of microglia induces a rapid production of ATP. Microglial ATP then recruits astrocytes to amplify ATP production and release glutamate. Thus, microglia might act as a genuine regulator of neurotransmission and upstream partner of astrocytes. Direct interaction with neurons ensures the maintenance of the microglial phenotype that can be defined quiescent, but alerted, tolerant and pro-homeostatic. Such cross-talk is mediated by several mutual ligand-receptor interactions and in part is due to signals derived from neuron-released factors. Astrocytic information-processing can be influenced by microglial activation and the reverse is also true: astrocytes have an influence on microglial behavior, releasing soluble factors, many of which are still mysterious. Resident microglia is also heavily dependent on TGF-beta1 released by neurons and glial cells. These cells release low levels of these cytokines which are overexpressed in response to a wide range of neuronal insults and during aging. Microglia continuously monitors the potential presence of a broad repertoire of PAMPs and DAMPs molecules, by using TLR and PRRs. B. Activated microglia. Upon brain damage, microglia de-branch and rapidly change their phenotype and undergo different forms of polarized activation, classic or M1 and alternative or M2. *In vivo*, a spectrum of differentiation has been proposed, with M1 and M2 being at the ends. M1 microglia are essential for host defense, are generally considered potent effector cells that kill intracellular micro-organisms and tumor cells and produce copious amounts of pro-inflammatory cytokines, but also result in collateral damage to healthy tissues. M1 microglia produce high levels of oxidative metabolites and pro-inflammatory cytokines including IL-1beta, IL-6, IL-12 and TNF-alpha. The sustained production of proinflammatory cytokines and ROS and a dysregulated release of glutamate can be neurotoxic. Among all the diseases of the CNS, tumors are more sneaky, in that they bypass the attack of M1 microglia. Glioma cells secrete factors that suppress immune cells, thereby promoting an ‘‘alternatively’’ activated macrophage or "M2"-like phenotype and/or suppressing the M1 phenotype. M2 microglia, promote wound healing and suppress destructive immune responses. The microglial M2 phenotype prolongs neuron survival and restricts brain damage with high levels of Arg-1, Chil3, FIZZ1, IL-10, TGF-beta and IGF-1.

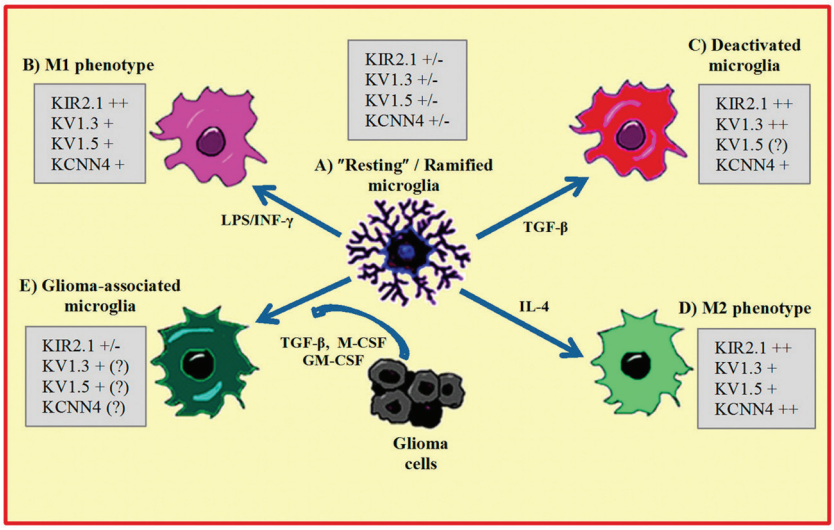

Figure 3. Pattern of potassium currents in different activation states of microglia. A. "Resting" /ramified microglia display very scant voltage dependent potassium currents, with expression changing during development. B. Acquisition of M1 activation state by LPS increases the expression of iward (mainly KIR2.1. subunit) and outward (mainly Kv1.3. and Kv1.5. subunits) currents. Sustained expression of KIR current is observed in deactivated microglia promoted by TGF-beta and in M2 phenotype promoted by IL-4. A marked upregulation of Kv1.3. and KCNN4 represents a feature of deactivated (C) and M2 activated (D) microglia, respectively. E. Absence or very scant expression of KIR currents is observed in glioma-associated microglia with an increased expression of DRK potassium current with respect to resting microglia, suggesting an "intermediate downregulated" activation process or a microglial immature phenotype. (+/-) absence or very scant expression, (+) moderate expression, (++) sustained expression and (?) no available data.

(GMF). GMF is not only necessary for the growth and maturation of neurons and glia cells, it can also induce the production of proinflammatory cytokines. Overexpression of GMF in astrocytes induces the production and secretion of granulocyte- macrophage-colony stimulating factor (GM-CSF), an activator of microglia, and the expression of proinflammatory genes including MHC-II, IL-1beta, and macrophages inflammatory protein (MIP)-1beta (118) (Figure 2B). Interestingly, increased GMF also inhibits growth of glioblastoma cells by inducing gap (G)0/G1 cell cycle arrest *in vitro* (119).

Recent studies demonstrated that resident microglia exhibit a distinct M0 expression profile that includes genes associated with nervous system development and is highly dependent on transforming growth factor beta (TGF- beta) (Figure 2A). They observed high expression of TGF-beta1 and the transforming growth factor beta receptor (TGF-betaR)1 in microglia, while neurons and glial cells express low levels which are upregulated in response to a wide range of neuronal insults and during aging (120).

To better understand microglia functions in the CNS, it is necessary to introduce the concept of immune privilege. The privilege is not simply the absence of immune reactions but rather a complicated network of brain tissue passive and active barriers. The BBB, formed by complex interactions between capillary endothelial cells (ECs), astrocytes end-feet, pericytes, and microglia, is the largest and most stringent barrier into the CNS that impedes the paracellular movement of ions, solutes, proteins, water, and leukocytes (121). Galea et coworkers (122) believe that the principal determinants of immune privilege include the fact that microglia, the only immunocompetent element, is not an APC. Moreover the specialization of the afferent arm of the adaptive immune response is skewed away from cell-mediated towards soluble factors (122). It follows that soluble factors (cytokines, chemokines, small proteins, lipids and ions) to and from the CNS are critical to immune reactions.

Microglia are provided with almost all of the receptors present in macrophages, dendritic cells, lymphocytes and even in neurons and astrocytes (123). Microglia continuously monitors the potential presence of a broad repertoire of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), by using toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) (115) (Figure 2B). Upon brain damage (infection, injury, or disease), microglial processes rapidly and autonomously converge on the site of injury without cell body movement, establishing a potential barrier between the healthy and injured tissue (124). Then, microglial cells de-branch and rapidly change their phenotype and undergo different forms of polarized activation, classic or M1 and alternative or M2 (Figure 2B). *In vivo*, a spectrum of differentiation has been proposed, with M1 and M2 being at the ends (125).

Considering the electrophysiological properties of microglia (Figure 3) in adult mice, ion currents recording from resting/ramified mice microglia in slice preparations shows high input resistance (in the GΩ range) and a

bimodal distribution of the resting membrane potential (around -50 and –30 mV), (126) that can reflect a heterogeneous expression of KIR and DRK channels (127). Four electrophysiological subpopulations can be found *in vivo* using the slice preparation from juvenile and adult mice (Figure 3): a) a subpopulation expressing neither inward nor outward K current, and defined “silent”, b) a subpopulation that expresses both inward and outward K currents in about equal amount, c) and d) subpopulations expressing both inward and outward K currents, but with marked prevalence of either one (127). It is not clear if the latter subpopulations represent partial activation states of microglia induced by the electrophysiological procedure (126), or portrays effective heterogeneous functional states (127,128). In microglia the molecular correlate of the KIR current is primarily the KIR2.1. channels (129), whereas Kv1.3., Kv1.5., and Kv1.2. are the main counterparts of DRK currents (130).The KIR current in microglia is characterized by strong rectification with small current flow at potentials positive to EK. Accordingly, the input resistance measured in microglia between -60 and -70 mV was normally in the GΩ range indicating the presence of strong rectification of the KIR current. This very high membrane impendance that nullifies the setting effect of KIR current on resting potential in some cases is associated to membrane potential instability such as voltage oscillations similar to those found in hair cells (131). In contrast, the resting membrane potential is regulated by the DRK current and set near its activation threshold, indicating that this current counterbalances a depolarizing current that insists at this voltage. The swelling activated chloride current and the voltage activated proton current could represent the inward depolarizing currents that together with the outward hyperpolarizing DRK current set the resting membranepotential in microglia (132).

4.2.1. M1 phenotype

M1 microglia (Figure 2B) are essential for host defense, are generally considered potent effector cells that kill intracellular micro-organisms and tumor cells and produce copious amounts of pro-inflammatory cytokines, but also result in collateral damage to healthy tissues. M1 microglia produce high levels of oxidative metabolites and pro- inflammatory cytokines including IL-1β, IL-6, IL-12 and tumor necrosis factor-alpha (TNF-alpha) (133) (Figure 2B). The sustained production of proinflammatory cytokines and reactive oxygen species (ROS) and a dysregulated release of glutamate can be neurotoxic, although the issue of neurotoxicity of activated microglia remains open. Some authors (134) propose that microglial cells are a beneficial protagonist to the resolution of most of the diseases of the CNS. They demonstrate that microglia become harmful subsequent to mutation or decreased expression of important receptors (CX3CR1, CD200R and triggering receptor expressed on myeloid cells (TREM)1 receptors (Figure 1A)), in the course of neurodegenerative diseases and in old age. That is, in all those situations in which microglia lose their natural properties and become hyperactive or tolerogenic (135,136).

In aged animals, the cellular ramification processes and the microglia motility decrease. This may lead to less efficient surveillance and perhaps attenuated protection from tissue damage. In addition, *in vitro* data indicate that microglia, in the aged brain, express more MHC II molecules and become less sensitive to regulatory signals, such as TGF-beta-1 or CSF 1 (137). During their life span, episodes of systemic inflammation and cytokine stimulation can instruct microglia and increase their reactivity. This mechanism of exposure to multiple noxious stimuli is called priming. Along with the priming, accumulation of mutations and deoxyribonucleic acid (DNA) damage with aging can lead microglia to gradually acquire a hypersensitive phenotype, expressing an increased immune responsiveness and resistance to regulation (138).

Activation of microglia by lipopolysaccharides (LPSs) leads to cytokine and nitric oxide (NO) release, and to reduced proliferation that has been suggested to depend on increased outward K(+) conductance associated to transcriptional upregulation of Kv1.5. subunit (139) (Figure 3) whereas the formation of peroxynitrate, but not NO, requires Kv1.3. activity (140). Proliferating microglia expresses predominantly Kv1.3., whereas Kv1.5. is typically recorded in non-proliferating microglia (130) (Figure 3). In microglia deactivated by TGF-beta, a functional upregulation of DRK currents associated to Kv1.3. channel subunit is observed (141). Interesting, the Ca(2+)-activated K(+) channel, KCa3.1. (KCNN4), has been shown to contribute to M1 activation of microglia (Figure 3). Consistently, KCa3.1. blockers such as TRAM-34 have been found to rescue retinal ganglion neurons after optic nerve damage *in vivo*, and to reduce p38 MAP kinase activation, production of reactive oxygen and nitrogen species, and neurotoxicity by microglia *in vitro* (142). In spite of these effects, LPSs do not induce KCa3.1. transcriptional and functional current, which in contrast was observed in IL-4-induced “alternative” activation state in JAK3 and Ras/MEK/ERK dependent way (143). IL-4 increased microglial migration, and this was reversed by KCa3.1. blocker TRAM-34 (143).

In neurodegenerative diseases, microglial TLR engagement by dead cell components can lead to the production of pro-inflammatory mediators (Figure 2B) such as TNF-alpha, IL-1beta, IL-6, ROS, and NO and these, in turn, can accelerate neuronal death. In this regard, the inflammation triggered by endogenous molecules, such as protein aggregates and dead neuron components, might promote the progression of neurodegenerative diseases such as Alzheimer’s disease (AD) and multiple sclerosis (MS) (144). However, many studies suggest that the effects of microglia

Table 1. Factors expressed and secreted by glioma cells involved in GAMs chemoattraction and M2 phenotype induction

Factors	Interactors	Activities	References
CCL2 (MCP-1)	CCR2	Recruitment of microglia and macrophage Tumor growth, neo-angiogenesis and invasiveness. Induction of IL-6 expression	(169, 172)
SDF-1 (CXCL12)	CXCR4	Microglia and macrophage chemoattraction	(173)
CX3CL1	CX3CR1	Microglia and macrophage chemoattraction Induction of MM2,9,14 expression	(174)
HGF/SF	c-MET	Microglia chemoattracion. Induction of VEGF expression	(176)
VEGF	VEGFR1, VEGFR-2	Microglia proliferation and migration. Endothelial cells proliferation and migration. New blood vessels formation Recruitment of Bone Marrow Derived Cell	(180-182)
TGF-beta2	TGFbetaR2	Downregulation of MHCII expression on microglia, deactivation of microglia as phagocitic cells Inhibition of microglia proliferation and proinflammatory cytokines synthesis Downregulation of NKG2D receptor on NK and CD8+Tcell	(184, 185, 187)
STAT3	Numerous interactions	Promotion of abnormal cell cicle progression, angiogenesis and tissue invasion. Inhibition of pro-inflammatory cytokine signaling Reduction of MHCII, CD80 and CD87 expression on microglia Increase of Treg cells	(188, 194)
FasL (CD5L)	Fas (CD95/APO-1)	Induction of T cells death Induction of ICAM-1. Induction of microglia cells death	(199, 200)
Prostaglandin E2 (PGE2)	Prostaglandin E2 Receptor (EP2)	Downregulation of LAK cells activity. Reduction of MHCII and HLA- DR expression on microglia and dendritic cells. Suppression of T cells activation. Proliferation of Treg cell	(204, 205)
Colony Stimulating Factor-1 (CSF-1)	CSF-1R	Induction of M2 microglia phenotyphe. Promotion of glioma invasiveness	(207, 208)
Vitronectin and Fibronectin	Integrins	Upregulation of cytokines production. Microglia and macrophages accumulation	(210, 211)
Osteopontin (OPN)	Intregrin and CD44	Induction of invasivenees and metastasis	(213)

Table 2. Factors contributing to the immune-suppressive milieu in glioma

Factors	Interactors	Activities	References
IL-10 (CSIF)	IL 10R	Inhibition of antigen presentation, T cells proliferation, pro-inflammatory cytokine synthesis. Promotion of glioma proliferation Enhancement of glioma invasiveness	(214)
IL-6	IL-6R (CD126)	Microglia polarization to M2 phenotype Production of MM-2. Promotion of glioma progression and invasiveness Inhibition of apoptosis	(219, 146)
FasL (CD5L)	Fas (CD95/APO-1)	Induction of T cells apoptosis	(221)
TGF-beta	TbetaRI/TbetaRII	Induction of glioma invasiveness	(222)
Metalloproteases	Matrix etracellular proteins	Induction of glioma growth and invasiveness	(224)
CTGF (CCN2)	ITGB1, TrkA, p75NTR	Induction of drug resistance. Induction of glioma progression	(228, 229)
STAT-3	Numerous nuclear interactors	Induction of IL-10 and IL-6 expression	(231)

are manifold. On the one hand, microglia promote neuroinflammatory and neurodegenerative events in MS by releasing inflammatory mediators and stimulating leukocyte activity and infiltration into the CNS. On the other hand, microglia cooperates in CNS repair through the production of neurotrophic factors and the clearance of inhibitory myelin debris. It has also been shown that M1 microglia may play a beneficial role in the regulation of neurogenesis through the production of neurotrophic mediators, such as insulin-like growth factor (IGF)-1 and TGF- beta (145).

Among all the diseases of the CNS, tumors are more sneaky, in that they bypass the attack of M1 microglia. Glioma cell studies indicate that tumors secrete factors that suppress immune cells, such as IL-10, IL-4, TGF-beta and prostaglandin E2 (146) (Tables 1, 2), thereby promoting an ‘‘alternatively’’ activated macrophage or “M2”-like phenotype and/or suppressing the M1 phenotype (147) (Figure 2B). Glioma cells recruit GAMs and MDSCs to the tumor site and block their maturation to generate an immunosuppressed niche for tumour expansion. This immunosuppressive

microenvironment is considered to be shaped through a complex multi-step interactive process between glioma cells, GAMs and MDSCs (148).

4.2.2. M2 phenotype

M2 cells (Figure 2B), which are sometimes further divided into M2a-c subtypes, promote wound healing and suppress destructive immune responses (147) (Figure 2B). The microglial M2 phenotype prolongs neuron survival and restricts brain damage with high levels of 1) arginase-1 (Arg-1) and a heparin-binding lectin, chitinasi 3 (Chil3), which prevent the degradation of extracellular matrix components; 2) found in inflammatory zone (FIZZ)1, which promotes deposition of extracellular matrix; 3) IL-10; and 4) TGF-beta and IGF-1 (149) (Figure 2B). The enzyme Arg-1 converts arginine to ornithine for wound healing and, by using arginine, which is the same substrate used by inducible nitric oxide synthase (iNOS), Arg1 can effectively compete with iNOS to downregulate production of nitric oxide (150). Thus, iNOS and Arg1 represent a relatively straightforward set of markers to follow M1 versus M2 phenotypes, respectively (Figure 2B).

Another way to classify the function and phenotype of M2 macrophages/microglia is based on the cytokines that induce this phenotype (149). In this respect, not all cytokines that determine the M2 phenotype in macrophages can do it in microglia. Certain macrophage M2 markers do not appear to be expressed in the CNS. The best example of this is the alternative macrophage marker, CD206, a mannose receptor, which is only seen in perivascular or choroid plexus-associated macrophages and not expressed by parenchymal microglia (151). Because the origins and responses of microglia and macrophages are different, the roles they play in mitigating or propagating pathology could be different as well. Recently Chen and Trapp (152) defined the microglia as a key to protect the CNS during development, in adulthood and during illness. They explain that the actions of microglia are modulated upon request and that microglia switch from the M1 to the M2 phenotype (Figure 2B), with the M2 phenotype being important to support healing (152). However, normally the immune responses are fine-regulated in the process of either initiation or resolution, so as to keep tissue homeostasis. In pathological conditions, however, the immune responses are uncontrolled and skew to either extreme.

5. GLIOMA-ASSOCIATED MICROGLIA/ MACROPHAGES (GAMs) AND MYELOID DERIVED SUPPRESSOR CELLS (MDSCs)

Macrophages are innate immune cells belonging to the monocitic lineage and are involved in host defence and maintenance of tissue homeostasis. In dependence of microenvironmental stimuli macrophages polarize into different subsets with different phenotypes; indeed, each subset displays a different expression profile of surface markers, cytokines and enzymes. Historically macrophages have been classified in classical M1 subset and alternative M2; Th1 cytokine IFN-gamma, LPS and TLR agonists are known inducers of M1 subset which is characterized by the expression of high levels of MHC-I and MHC-II and by secretion of pro-inflammatory factors such as IL-6, IL-12, IL-23 and TNF-alpha. Conversely M2 macrophages, which have anti-inflammatory and pro-tumorigenic activities, can be further subdivided into four different subsets named M2a, M2b, M2c and M2d. M2a subset has triggered by Th2 cytokines such as IL-4 and IL-13, whereas M2b and M2c subsets are induced respectively by the activation of TLRs, immune complex and IL-10. Macrophages observed into glioma masses are named GAM and recent reports have demonstrated that they resemble M2 macrophages and they have been classified as M2d subset. GAM are CD163+, CD200+, CD68+, F4/F8+ and lectin binding protein Iba-1+ and are characterized by IL-12low and IL-10high phenotype (153).

Recently, the electrophysiological properties of glioma associated microglia were investigated in acute brain slices of murine GL261 glioblastoma implanted into the cortex/striatum region of adult mice. Glioma associated microglia shows an amoeboid morphology and displays a higher electric capacitance and a membrane potentials ca. 12 mV more negative than control microglia. No apparent increase of KIR current density was observed in glioma associated microglia, whereas the DRK current was significantly increased with respect to control microglia. Similarly, primary human glioma associated microglia cultures did not display KIR current, whereas a third of cells expressed DRK current. Physiologically the percentage of DRK-positive microglia cells increases transiently over the first few weeks of maturation, whereas the KIR-positive cells increase with time of maturation during hippocampus development (127). An intriguing hypothesis is that glioma associated microglia represents a subpopulation conditioned by glioblastoma cells at early maturation stage.

The absence or very scant expression of KIR and the expression of DRK in glioma associated microglia can endow microglia with several specific properties. Recently the role of KIR in cell migration and proliferation has been studied in different activation stages of microglia (129). Using the selective KIR current blocker ML133 an increased proliferation rate was reported both in unstimulated and in IL-4-induced activated microglia. On the contrary, no effects were observed in acquired deactivated microglia induced by IL-10 treatment (129). Cell migration and ATP-induced chemotaxis were both greatly inhibited by selective pharmacological inhibition in unstimulated and IL-4 and IL-10-treated microglia. Although the mechanism through which KIR current controls cell migration and proliferation

in microglia remains to be defined, an important role appears to be played by those Ca^{2+} signals modulated by the KIR-dependent hyperpolarization (129). The upregulation of the delayed rectifier Kv1.3. current in microglia appears likewise to be important for regulating proliferation, cell volume and cytokine release (132). Several signals generated by glioblastoma can modify the microglia behaviour, and vice versa. Two types of signal are involved in this crosstalk: soluble factors and direct cell contacts (154). Among soluble factors, cytokines such as Macrophage-Colony Stimulating Factor (M-CSF), GM-CSF and TGF-beta can modify the electrophysiological profile of microglia. M-CSF increases the fraction of microglia cells that express KIR current, whereas GM-CSF increases the fraction of microglia cells that express DRK, as observed in *in vitro* cell development model (155). M-CSF and GM-CSF are identified, together with TGF-beta, as agents responsible for morphological and electrophysiological changes induced by astrocyte-conditioned medium (156). TGF-beta, the best characterized glioblastoma secreted factor, is able to oppose the proinflammatory action of microglia, and to increase the expression of the delayed rectifier Kv1.3. current in microglia. To claim that ion channels expression in glioma associate microglia are involved in the establishment of the activation status needs further investigation.

Interestingly, we have shown that *in vivo* treatment with the selective KCa3.1. channel blocker TRAM-34 inhibits glioblastoma cell growth in xeno-transplanted human glioblastoma, reduces CD68 expression, marker of microglia phagocytosis, and decreases microglia migration induced by glioblastoma-conditioned medium (73). These data show that several aspects of glioma associated microglia biology may be regulated by the KCa3.1. channel activity.

MDSCs are heterogeneous population of immature myeloid cells with the ability to suppress T cells activation. They belong to myeloid progenitor cells that are not yet commited to macrophages, dendritic cells or granulocytes lineages. In physiological conditions, MDSCs represent approximately 20-30% of bone marrow cells and 4% of nucleated cells in the spleen, while in pathological conditions such as infection, stress and cancer, a progressive accumulation of this cell type occurs in lymphoid organs.

In mice, MDSCs are identified through the expression of myeloid surface markers Gr-1 and CD11b and, according to the expression of Ly6G and Ly6C, are classified into granulocytic MDSCs (G-MDSCs) which resemble gralunolocytes and are CD11b+, Ly6G+ and Ly6Clow and monocytic MDSCs (M-MDSCs) which are similar to monocytes and CD11b+, Ly6G− and Ly6Chigh. Both subpopulation can also express CD115 (c-fms), CD16/32 (FcR), IL-4Ra (CD124) and low CD80 levels. In human, the presence of MDSCs was first observed in the blood of patients with head and neck cancer and identified as Lin−, CD133+, CD15+, CD34+ cells. To date, human MDSCs are classified as CD133+, HLADR low/neg. Two MDSCs subpopulations have been identified: M-MDSCs, which express CD14, and G-MDSCs which express CD15 (157).

In pathological conditions, including cancer, infections, trauma, bone marrow transplantation and some autoimmune diseases, MDSCs accumulates in peripheral lymphoid organs, blood and cancers microenvironment, being induced by overexpression of cytokines and growth factors. M-CSF (CSF-1) promotes macrophages growth and differentiation, but high M-CSF level, as observed in pathological conditions, can interfere with myeloid development, resulting in MDSCs generation (158). GM-CSF is a growth factor involved in macrophages and granulocytes differentiation from bone marrow precursors. In physiologial conditions GM-CSF levels are low and can enhance antigen presentation by DC cells, whereas high GM-CSF levels induce MDSCs generation (159). Moreover the presence of GM-CSF in tumor microenvironment has been associated with MDSCs development (160).

Granulocyte colony stimulating factor (G-CSF) has a molecular structure similar to GM-SCF and plays a critical role during neutrophil development; its receptor, known as granulocyte-colony stimulating factor receptor (G-CSFR or CSF3R), is primarly expressed on myeloid cells, but also on neuronal cells and cardiomyocytes located at the maternal-fetal interface (161). It is known that exogenous G-CSF is able to inhibit innate immune responses recluting MDSCs, and Waight *et al.* (162) showed a tight correlation between G-CSF levels and G-MDSCs numbers in tumor bearing-mice. They also demonstrated that reduced G-CSF expression trough siRNA causes a lowered accumulation of MDSCs and, as a conseguence, an attenuation of tumor growth (162). The bioactive lipid, prostaglandin E2, which is expressed at high levels in the tumor microenviroment, induces MDSCs differentiation of c-kit+ hematopoietic stem cells (163); similarly, the pro-inflammatory S100A8/A9 proteins and the complement component C5a are able to induce MDSCs accumulation (163).

Patients with glioblastoma multiforme harbor elevated MDSC levels and Unemura *et al.* (164) found that a high percentage of MDSCs in glioma mostly belong to the monocytic lineage rather than the granulocytic lineage (164). M-MDSCs over-express chemokine receptors although their preferential migration into the glioma parenchyma remains obscure. The kind of chemokyne released by glioma cells, hypoxia, low pH and/or other factors produced by glioma have been proposed to impede G-MDSCs survival in favor of M-MDSCs (165). Furthermore Rodrigues *et al.* (166)

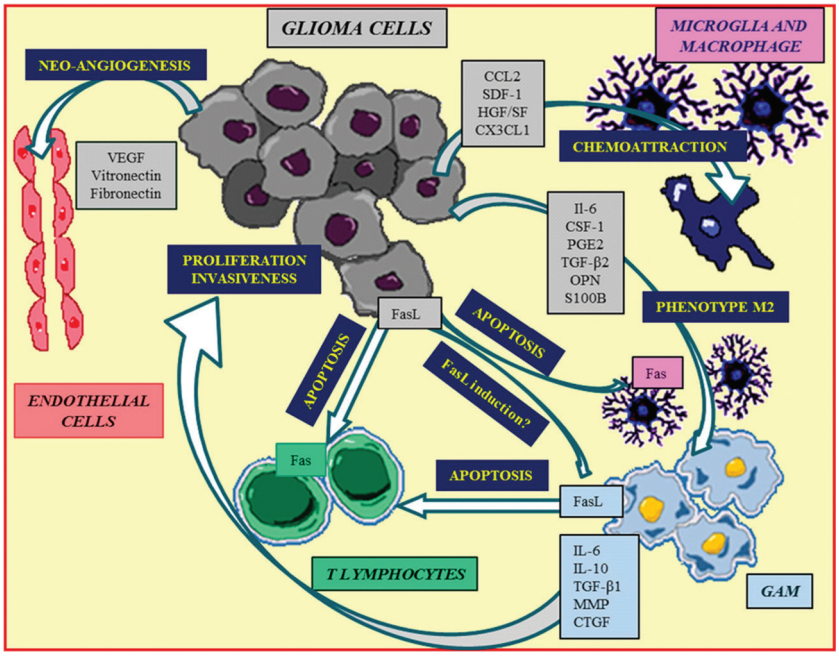

Figure 4. Microglia-glioma cross talk. Schematic representation of glioma microenvironment showing the cross-talk between glioma cells and glioma associated microglia/macrophage (GAM). Glioma cells secrete many soluble factors, such as CCL2 and SDF1 with chemoattractive properties on resident microglia and macrophage, and CSF-1 and TGF-beta with the ability to induce the M2 phenotype in GAM; on the other hand GAM produce cytokines (IL-10, IL-6, TGF-beta) and metalloproteases which support tumor growth and invasiveness. Moreover both glioma cells and GAM express FasL and can induce T lymphocytes death which express Fas on their membrane; also resident microglia expresses Fas on its membrane surface and could be susceptible to death signal derived glioma cells, but to date there is no evidence that this event occurs. It remains to be determined whether the glioma cells can induce the expression of FasL on GAM. Finally it is also shown that glioma cells produce growth factors such as VEGF and matrix etracellular proteins (vitronectin and fibronectin) which play a role in neo-angiogenesis.

demonstrated that normal monocytes exposed to glioblastoma multiforme assume the MDSCs phenotype, produce anti-inflammatory cytokine such as IL-10 and TGF-beta, decrease phagocytic activity and increase ability of activated lymphocytes to induce apoptosis (166) (Table 2).

6. INTERACTION BETWEEN GLIOMA CELLS AND GAMs/MDSCs

6.1. Glioma cells toward GAMs

6.1.1. Chemoattraction

GAMs represent approximately 30% of glioma tumor masses. GAMs are recruited by glioma cells through the secretion of many factors such as chemokines, cytokines and other proteins (Table 1). Among chemokines secreted by glioma cells, one of the first identified and powerful was chemokine C-C motif ligand 2 (CCL2) (Figure 4) (Table 1) also referred to as monocyte chemotactic protein-1 (MCP-1) (167). CCL2 expression is stimulated by ATP and recently Wang *et al.* (168) demonstrated that in a murine model the calcium binding protein S100B can enhance CCL2 production by glioma cells by interacting with the receptor for advanced glycation end products (RAGE) and that the STAT3 pathway is involved in this proccess (168). In human glioblastoma, hovewer, a tight correlation between S100B and CCL2 was demonstrated only in neural and proneural histotypes. Platten *et al.* demonstrated (169), *in vitro*, that CCL2 constitutively produced by glioma cells interacts with CCR2 expressed on glioma associated microglia and macrophage; this interaction indeed promotes the recruitment of resident microglia to the site of glioma (169). In contrast, Okada *et al.* (170) showed that the microglia and macrophage infiltration is due to MCP-3 rather than to MCP-1 but they didn't report which is the receptor transducing MCP-3 effects (170). Other studies highilighted that the expression of CCL2 is correlated with the grade of glioma malignancy (171) and that CCL2 has also a role in promoting tumor growth, neo-angiogenesis and invasivness, likely by stimulating microglia cells to produce IL-6 (172).

Stromal-derived factor-1 (SDF-1) (Figure 4) (Table 1) plays a role in microglia/macrophage chemoattraction; in a murine glioma model which express high-grade of SDF-1, Wang *et al.* (173) demonstrated that the inhibition of SFD-1 produces a higher microglia/macrophage density in nonhypoxic regions rather than in hypoxic regions. In light of this evidence the authors suggested that the production of SDF-1 by glioma cells is an important tool for the accumulation of microglia/macrophage and tumor invasiveness (173).

Chemokine C-X-C motif ligand 1 (CX3CL1) (Figure 4) (Table 1) is one of the most expressed chemokines in the CNS. There are two isoforms of CX3CL1, both expressed in glioma cells, a membrane bound form involved in cell-cell adhesion and a soluble form which contributes to chemotaxis. Held-Feindt *et al.* (174) demonstrated that microglia/macrophages infiltrating human glioma express CX3CR1 on their surface and that CX3CL1, aside from its role in promoting microglia/macrophage chemoattraction, enhances the expression of metalloproteases (MMPs) 2, 9 and 14 in these cells, which in turn promote glioma migration (174).

Another factor involved in microglia cells chemoattraction is the hepatocyte growth factor/scatter factor (HGF/SF) (Figure 4) (Table 1) which binds to and activates the tyrosine kinase receptor, c-Met. It is known that HGF/SF and c-Met are expressed by both glioma and microglia cells (175) and Badie *et al.* (176) showed that HGF/SF is able to attracts microglial cells *in vitro*. Furthermore, HGF/SF plays a role in glioma growth and angiogenesis; its expression levels increases with the grade of glioma malignancy (177). As an angiogenic factor, HGF/SF acts on brain tumor endothelial cells through the induction of VEGF expression and secretion (178).

VEGF (Table 1) is involved in vasculogenesis and angiogenesis (Figure 4). It is constitutively secreted by glioma cells and its production increases with glioma malignancy (179). The best studied and characterized function of VEGF in human glioma is its role in angiogenesis. Indeed, the endothelial cells near the glioma cells express VEGFR-1 and VEGFR-2 and react to VEGF secreted by glioma cells; in this way VEGF stimulates endothelial cells migration and proliferation and induces new blood vessel formation (180). Another possible mechanisms of VEGF action in promoting glioma growth demonstrated *in vitro* is the induction of migration and proliferation of microglia cells which express VEGFR-1 (181). Recently Iwamoto *et al.* have shown that VEGF is involved in the recruitment of bone marrow derived cells (182).

6.1.2. Promotion of tumor growth and invasion

TGF-beta was one of the first growth factors to be investigated as a possible mediator of microglia chemoattraction mediated by human glioma cells and nowadays represents the best characterized soluble cytokine with immunosuppressive properties in human glioma (Table 2). There are three different isoforms of TGF-beta, 1, 2 and 3. TGF-beta2 is the isoform mainly upregulated in glioma cells (Figure 4) (Table 1) and is involved in glioma immunosuppression (183). TGF-beta downregulates MHC-II expression in microglia cells and dampens microglial phagocytic activity (184). Moreover Crane *et al.* (185) demonstrated that TGF-beta is responsible for the downregulated expression of the natural-killer group 2, member D (NKG2D) receptor, which plays a role in the killing of transformed cells and is expressed on NK cells and CD8+ T cell membrane (185). On the other side, TGF-beta1 has a role in the stimulation of glioma cell motility. Indeed, TGF-beta1, relased by microglia/macrophage, promotes glioma cell migration and invasion through the upregulation of MMP-9 expression (186) (Figure 4) (Table 2). Finally, Wu *et al.* (187) showed that TGF-beta inhibits microglial cell proliferation and the production of proinfiammatory cytokines *in vitro*.

STAT3 is a transcription factor belonging to the STAT family and its activation induces genes involved in cell growth, proliferation, differentiation and apoptosis (188) (Table 1). IL-10, IL-6, EGF and FGF are known STAT3 activators; additionally, oncostatin M ehnances the STAT3-dependent expression of VEGF (189). The involvement of EGFR in glioma-associated STAT3 activation has recently emerged. Indeed, about 50% of human glioblastomas express a mutated EGFR (EGFRvIII) that laks a portion of the extracellular ligand-binding domain and is permanently autophosphorylated at a low levels (190). The continuous activation of the EGFR causes a persistent activation of downstream kinase pathways such as MAPK, Akt and STAT3 (191). Finally, the fibroblast growth factor (FGF) signaling cascade is another mechanism by which cytokines and growth factors promote STAT3 activation (192). STAT3 is constitutively activated in glioma cell lines as well as in human glioma tissue (193) and plays a pivotal role in tumorigenesis by promotion of abnormal cell cycle progression, angiogenesis, tissue invasion and immune sistem evasion (Table 1). Moreover, in several experimental models STAT3 promotes tumor growth undermining the immune sistem response (Table 2); it inhibits pro-inflammatory cytokine signaling, by blocking the anti-tumor activity of immune cells, reducing the expression of the MHC class II molecules, CD80 (B7- 1) and CD87 (B7-2) in glioma infiltrating microglia and promoting tollerogenesis by increasing regulatory T cells (Treg). In Treg cells, STAT3 activation supports proliferation and the expression of forkhead box P3 (FOXP3), TGF beta and IL-10 which in turn inhibit CD8+ T and dendritic cell proliferation and maturation. Indeed, CD8+ T cells in glioma are characherized as being neither activated nor proliferating (CD8+, CD25-) (194). Finally, it was reported that glioma Treg cells are deficient in suppressor of cytokine signaling-3 (SOCS-3) expression (195). SOCS-3 is a member of the SOCS protein family that in normal conditions attenuates STAT3 activity by ihnibiting the activation of the STAT3 upstream JAK (196). Blocking STAT3 activity in tumor cells causes an increase in pro-inflammatory cytokine/chemokine expression such as TNF-alpha, INF-gamma, IL-6, RANTES, and CXCL10 (also known as interferon gamma-induced protein 10, IP-10) (197,198).

Fas Ligand, also named CD95L is a transmembrane protein belonging to TNF family; it

interacts with Fas (CD95/APO-1) leading to the induction of apoptosis in Fas-expressing cells. Human glioma cells express on their surface Fas Ligand (Figure 4) (Tables 1, 2) and both microglia and T cells express Fas receptor (Figure 4) so presumably they are susceptible to death signal delivered by active Fas Ligand (199,200). Fas Ligand is responsible for T cells death when co-colturated with glioma cells *in vitro*; furthermore it has been shown that the downregulation of Fas Ligand expression causes an enhancement of T cells tumor infiltration and a reduced tumor growth *in vivo* (199). In contrast, to date there is no evidence that Fas Ligand induces cell death in microglia infiltrating glioma cells despite the expression of Fas on microglia cells (200) (Figure 4). Anyway it is well known that Fas Ligand is involved also in the regulation of glioma invasiveness; indeed, destroying Fas Ligand signaling causes an impariment of MMP-2 activity that results in a reduction of glioma motility and invasivness (201). Finally, Fas Ligand may be involved in angiogenesis because of ts ability to induce the expression of intracellular adhesion molecule 1 (ICAM-1) in human glioma cells (202).

Prostaglandin (PGE2) is a small soluble lipid molecule relased by immune-competent cells and tumor cells under the stimulus of the inducible membrane associated PGE2 synthase cyclooxygenase-2 (COX-2) and the microsomal PGE synthase (mPGES-1) (203). Glioma cells relase high amounts of PGE2 (Figure 4) (Table 1) and enhance in microglia and dendritic cells the levels of PGE2 responsible for downregulated activity of LAK cells and reduced expression of MHC class II and human leukocyte antigen (HLA)-DR (204). In addition, the increased PGE2 production by glioma cells causes the suppression of T cells activation and proliferation and induces Treg cells (205).

CSF-1 is a growth factor involved in macrophages proliferation and differentiation; it binds a tyrosine kinase receptor known as CSF-1R, expressed on macrophages membrane (206). More in detail, it was osberved that high levels of CSF-1 are expressed in high grade glioma (Figure 4), and its upregulation correlates with the expression of microglia M2 phenotype (207) (Table 1). Recently, Coniglio *et al*. have demonstrated that CSF-1, relased by glioma cells, induces microglia cells to promote tumor invasion (208); the same authors showed that the expression of M2 markers on microglia and macrophages is due only to soluble CSF-1; indeed if glioma cell lines are forced to express the CSF-1 membrane-bound form there is a reduction of tumor burden through macrophages-induced killing of glioma cells (209).

Another feature of high grade glioma is the aberrant production of extracellular matrix (ECM) (Table 1); it is known that glioma secreted matrix (Figure 4) induces microglia cells to produce pro-invasive factors. In fact, Yeh *et al*. (210) demonstrated that a rat glioma cell line expresses high fibronectin and vitronectin levels which in turn stimulate microglia cells to upregulate the production of several cytokine including IL-18 (210). These data support the research of Farber *et al*. (211) who showed that the treatment of a mouse glioma model with an inhibitor of alpha5beta1 integrin, a fibronectin receptor, prevents microglia and macrophages accumulation at tumor border and inhibits glioma cells invasion of the adjacent parenchyma (211).

Osteopontin (OPN) is a secreted glycoprotein belonging to the ECM proteins (Figure 4) which interacts with integrins and glycoprotein CD44 (Table 1); OPN constitutes one of the highest expressed genes in 90% of glioma patients; IHC analysis of patients samples revealed that OPN colocalizes with glioma infiltrating macrophages (GIMs) and tumor infiltrating neutrophilis (212), and its expression is associated with enhanced invasion and metastasis development in several cancers, included glioma (213).

6.2. GAMs toward glioma cells

6.2.1. Molecules contributing to the immune-suppressive milieu in glioma

IL-10, also known as cytokine synthesis inhibitory factor (CSIF), is a 17-20 kDa cytokine with immunosuppressive properties including inhibition of antigen presentation, T cells proliferation and pro-inflammatory cytokine synthesis in glioma infiltrating microglia and macrophages (214) (Table 2). Human glioblastoma show a higher IL-10 mRNA expression than do low grade astrocytoma, however human glioblastoma cell lines show low IL-10 expression levels. Both microglia and astrocytes can express IL-10 gene but the contribution of glioma infiltrating microglia is greater than that of glioma cells (215). The expression of IL-10 in glioma infiltrating microglia is regulated by stimulating factor 1 (USF-1) and STAT3 (216, 217). IL-10 promotes glioma cell proliferation and enhances glioma cell invasivness (218) (Figure 4).

In addition to IL-10, glioma cells also display high levels of IL-6, and this high expression correlates with glioma growth and invasiveness (219) (Figure 4) (Table 2). Glioma derived IL-6, along with other cytokines such as TGF-beta1, polarizes glioma infiltrating microglia toward the M2 phenotype which in turn produces and secretes IL-6 (143). It is konwn that IL-6 induces the expression of MMP-2 by glioma cells and recenty has been hypothesized that activated microglia cells produce IL-6 trough NF-kB activation; this pathway might induce the activation of STAT3 in glioma cells, contributing to glioma progression, migration and apoptosis inhibition (219).

Like glioma cells, glioma infiltrating microglia express FasL on the membrane surface. Glioma infiltrating

microglia are the major source of FasL in a glioma murine model (220). In light of the ability of microglia to induce apoptosis of activated T cells (221), which express Fas on their membrane, microglia may contribute to the immunosuppressive enviromnemt in glioma by inducing T cells apoptosis via the Fas-FasL pathway (Figure 4) (Table 2). In contrast, Hussain *et al.* (194) claim that human glioma infiltrating microglia do not express FasL on their membrane or express it at very low levels suggesting that apoptosis mediated by the Fas-FasL pathway may not be the predominant mechanism of immune evasion in human glioma. Therefore further investigations *in vivo* are required to elucidate the major source of FasL in human glioma.

TGF-beta is produced *in vivo* by both glioma and activated microglial cells. Recently Wesolowka *et al.* (222) have confirmed that microglia secrete TGF-beta1 and showed that there is an increase in TGF-beta1 secretion by microglia following exposure to glioma cells. In the same study they also demonstrated that glioma cells exhibit a downregulated expression of TbetaRII (one of the two TGF-beta receptors) and low, if any invasion promoting activity suggesting that glioma's invasion activity is mediated by microglia-derived TGF-beta1 (Figure 4).

Metalloproteases are proteolitic enzymes involved in degradation of extracellular matrix and exist in a membrane-bound and a secreted form. The activation of these enzymes is a key mechanism by which glioma cells invade nearby tissues (223) (Table 2). In this context MMP-2, also refered to as gelatinase A, has an important role; there is a dramatic increase of MMP-2 gene expression in glioma and its activity is closely related to glioma invasiveness and survival rate (224). MMP-2 is produced and relased by both glioma and microglial cells (Figure 4), but the enzyme secreted by glioma cells is in an inactive form (pro-MMP-2) and needs to be cleaved to become active. Glioma cells however do not produce the activator of MMP-2, known as membrane type 1-matrix metalloproteinase 1 (MT1-MMP), so they rely on microglial cells which represent the major though not exclusive source of MT1- MMP. In physiological conditions microglia secrete low levels of MT1-MMP exclusively in the white matter, however microenviromental microglia upregulate MT1-MMP expression in gliomas (225). In recent *in vitro* study, Markovic *et al.*(226) demonstrated that the attenuated expression of MT1-MMP throught minocycline causes a reduced glioma growth and invasion. They also showed that in microglial cells deletion of myeloid differentiation primary response 88 (MyD88), a TLR protein adapter, reversed the overexpression of MT1-MMP stimulated by glioma conditioned medium and suggested that in glioma associated microglia the expression of MT1-MMP is dependent on p38 MAPK which is downstream of MyD88 in TLR signaling (225).

Connective tissue growth factor (CTGF) (Figure 4), also known as CCN2, is a cysteine rich, matrix associated heparin binding protein which is secreted by microglial cells in pathological conditions (227). Its expression in glioblastoma multiforme is associated with drug resistance (228) (Table 2). CTGF binds to the cell surface protein beta1-integrin (ITGB1), tyrosine kinase receptor type A (TrkA) and neurotrophin receptor (p75NTR). These interactions are involved in cancer progression; a CTGF rich micoenvironment increases glioma invasiveness (229). Furthermore Xie *et al.*, observed a significant correlation between CTGF mRNA levels *vs* glioma grade; these results suggest that CTGF levels may have a prognostic significance and could be a promising therapeutic target (230).

Finally, compared with normal tissue glioma infiltrating microglia exhibit a high STAT3 activity (217) determined by immunosuppressive factors secreted by glioma cells, such as IL-10 and VEGF (198). Glioma cells constitutively express and secrete the calcium binding protein S100B (Figure 4) which interacts with RAGE expressed on microglia cells surface thereby activating STAT3 signaling (231). Therefore, secreted S100B might contribute to STAT3 induction in glioma infiltrating microglia. STAT3 signaling activation increases IL-10 and IL-6 expression in microglial cells (231) and the inhibition of STAT3 activity reverses IL-10 and IL-6 upregulation via TNF-alpha expression and enhanced expression of the costimulatory molecules CD80 and CD86 (231, 232).

6.3. Roles of MDSCs in glioma

MDSCs (Figure 5) contribute to tumor-induced immune suppression along with Treg cells, tumor-associated macrophages (TAMs), type 2 NK cells and mast cells. MDSCs phenotypic heterogeneity mirrors the heterogeneity of their molecular targets and suppressive mechanisms of action. MDSCs are involved in the regulation of anti-tumor immune response indirectly by targeting angiogenesis and tumor cell motility through the production of MMP and VEGF, and directly by perturbing innate immunity through the suppression of NK cell-mediated lysis and the polarization of macrophages to M2 phenotype. Neverthless, the best studied and well characterized mechanism of action of this cells is the suppression of T cells through the inhibition of antigen-dependent cytokine secretion, the apoptosis in activated T cells, the secretion of factors with immunomodulatory properties, the induction of enzymes involved in amino acid metabolism, the expression of Treg cells and the impaired T cells homing to lymph nodes.

It is well known that the metabolism of the amino acid L-arginine impaire T cells function; both MDSCs and T cells require L-arginine for protein synthesis, but MDSCs produce high levels of the arginase enzyme which catabolizes L-arginine, depleting the extracellular environment of L-arginine and limiting its availability to

T cells. T cells deprived of L-arginine cannot synthetize the CD3zeta chain and arrest the cell cycle in G0-G1 phase (157).

Another mechanism by which the amino acid metabolism can inhibit T cells activation has recently emerged. The amino acid cystein is required for protein synthesis by all cell types, and it is synthetized from intracellular pool of methionine through the enzyme cystathionase or in alternative it is imported from extracellular environment through the x(c)-cystine/ glutamate antiporter; T cells can not produce cysteine through both of theaformentioned mechanisms, mainly because they do not express either cystathionase or cystine transporter. Consequently, cystein is an essential amino acid for T cells, expecially for activated T cells which are dependent on exogenous source of this amino acid and import it via alanine-serine-cysteine transporter 1 (ASC). The extracellular sources of cystein are the APC which import cystine, reduce it to cysteine and then export cystein through the ASC transporter. Similarly to T cells, MDSCs do not produce cystathionase, import cystin trough cystin transporter and lack ASC transporter so they cannot export cystein. Despite the inability of MDSCs to synthetize cystein, these cells have an intracellular pool of this amino acid higher than APC; this evidence led to speculate that MDSCs sequester cystine and as a conseguence APC cells cannot import cystin and export cystein which is necessary for T cells activation and proliferation. This hypotesis has been demonstrated mesuring cysteine levels relased from macrophages co-coltured with increasing number of MDSCs (233).

Also tryptophan metabolism, originally associated with peripheral tollerance, maternal tolerance of fetus and regulation of autoimmune disease, has been linked with MDSCs immunosuppressive mechanisms. Indeed, tryptophan is highly metabolized in tumor microenvironment and it is known that the activation of the enzyme indoleamine 2,3-dioxygenase (IDO) in dendritic cells is responsible for tumor immune evasion (234).

Furthermore, MDSCs produce high ROS and reactive nitrogen species (RNS) levels which are able to impair DC maturation, whereas, conversely, they can induce MDSCs accumulation. In addition, ROS and RNS are involved in T cell inactivation; indeed, peroxynitrites production lead to post-translation protein modifications by nitrating tyrosine, cysteine, methionine and tryptophan. These molecular alterations induce a decrease in TCR binding to MHC I-peptide complexes in CD8+ T cells and reduce the ability of this cells to respond to antigen peptides. Moreover, Huang *et al.* (235) provided the first evidence that MDSCs can induce Treg cells development, both *in vitro* and *in vivo*, through the involvement of different pathways depending on TGF-beta, IL-10 and cell-cell contact (235). Finally, MDSCs are responsible for impaired T cells homing to lymph nodes; naive T cells enter secondary lymph nodes through the expression of L-selectine and become activated by antigen peptides coming from tumor sites following lymphatic vessels. MDSCs downregulate L-selectine expression on naive T cell surface as they constitutively express on their membrane ADAM metallopeptidase domain 17 (ADAM17) which is involved into the proteolytic cleavage and shedding of the L-selectine ectodomain (236).

M-MDSCs in glioma suppress antigen-independent T cells responses through the up-regulation of arginase and the production of immune-suppressive cytokine (165). Recently Raychaudhuri *et al* (237) have demonstrated that patients with glioma have higher serum MSDCs levels than age matched donors and that this value, on average, is the highest if compared with other cancers such as bladder carcinoma, melanoma and renal cell carcinoma (RCC). They also showed that, compared with healty donors, patients affected by glioma have increased serum G-CSF and arginase levels, which contribute to MDSCs expansion and T cells inhibition. Finally they proved that T cells of glioma patients produce less INF-gamma than those of heathy donors and that this phenomenon can be reversed by depleting MDSCs.

7. THE GLIOMA MICROENVIRONMENT

The physiological oxygen concentrations in the healthy brain varies between ~7.5.% and 0.5.% whereas high grade gliomas are characterized by foci with mild to moderate (2.5.% to 0.5.% O2) or severe (≤0.1.% O2) hypoxia. Thus, hypoxia characterizes high-grade gliomas and this lack of O2 gives important properties to the tumor microenvironment. Different cell types are an integral part of this environment, namely GSCs, glioma NSTCs, GAMs, MDSCs, and endothelial cells (Figure 5). All these cells play a major role in the genesis, maintenance and progression of glioma by cooperating and interacting with the GSCs in the so-called stem cell niche (Figure 5).

The molecular responses to hypoxia are mainly mediated by the hypoxia-inducible factor (HIF) family of transcription factors, in particular HIF-1 and HIF-2 (238, 239). HIFs are composed of two subunits, an oxygen- insensitive HIFbeta subunit and an oxygen-regulated HIFalpha subunit. Hypoxia stabilizes HIFalpha proteins by preventing its degradation. Stabilized HIFalpha forms a dimer with HIFbeta and activates the transcription of multiple target genes. Importantly, HIF-2alpha remains elevated under chronic hypoxia, whereas HIF-1alpha only becomes transiently upregulated. Several studies have shown that hypoxia directly promotes the expansion of the GSC pool and that this function is dependent on HIF-1alpha and HIF-2alpha. However, HIF-1alpha appears to play a more general, permissive role for GSC maintenance, possibly by enabling cell survival, whereas HIF-2alpha is directly involved in promoting the GSC phenotype. In contrast to HIF-1alpha, HIF-2alpha can

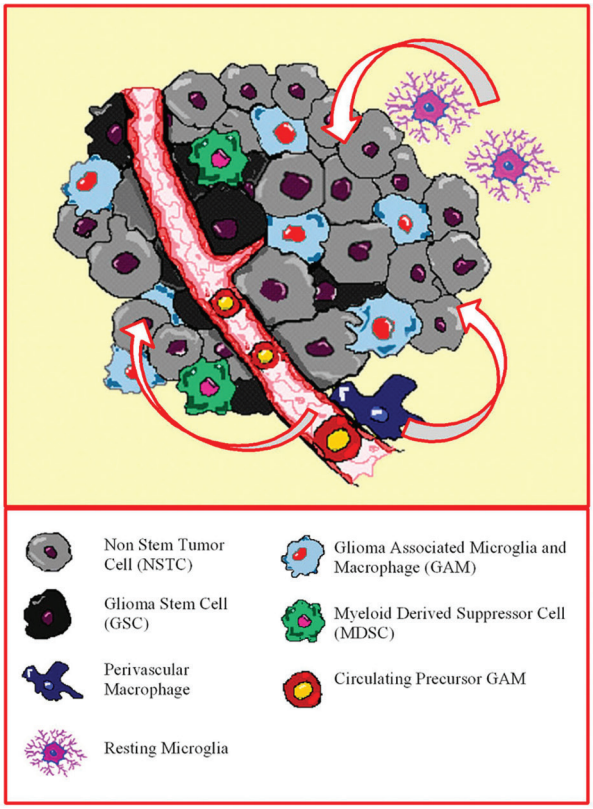

Figure 5. Glioma microenvironment. Different cell types are an integral part of tumor microenvironment, namely GSCs, glioma NSTCs, GAMs, MDSCs, and endothelial cells. All these cells play a major role in the genesis, maintenance and progression of glioma. Blood vessels play other important roles in tumor progression, e.g. by serving as conduits for the influx of various immune cells, as well as for the dissemination of tumor cells during the process of metastasis. Interestingly, a GSCs subset are located next to capillaries *in vivo* forming the so-called perivascular niche and associate with endothelial cells *in vitro*. Endothelial cells and GSCs influence each other. The extracellular matrix is another key component of the tumor microenvironment and the perivascular niche. Extracellular matrix proteins such a laminin can positively regulate the self-renewal and tumorigenicity of GSCs. Moreover, the perivascular niche appears to play a role in promoting GSCs radioresistance. The presence of a perivascular niche, with the GSCs as fundamental elements, results in the release into circulation of numerous factors that, among other things, recruit circulating immunocompetent cells, GAM, MDSCs and Treg. In this context the presence of BBB is basic. In early stages of glioma development there is no apparent disruption of the BBB, but, as glioma progresses, the BBB and the the normal microenvironment are altered.

specifically upregulate key genes involved in the induction of a pluripotent state, including Klf4 and the direct HIF-2 targets Sox2 and Oct4 (three out of the four Yamanaka's factors) (240). Moreover, HIF-2alpha activates c-Myc (the fourth Yamanaka's factor), suggesting that HIF-2alpha is a key regulator of the undifferentiated phenotype of GSCs in the hypoxic niche. Recent studies showed that in long-term hypoxia HIFs induced a non-stem cell population phenotypic shift towards a more stem-like state by upregulating the reprogramming inducers Oct4, Nanog, Sox2 and c-Myc and the neural stem cell marker nestin (240). These findings highlight the critical role of the microenvironment in regulating the differentiation status of tumor cells and its possible involvement in controlling the plasticity of the cancer stem cell hierarchy. Hypoxia also triggers a metabolic reprogramming towards a more glycolytic phenotype (Warburg effect) and this metabolic shift results in increased lactate production, leading to acidification of the tumor microenvironment (241) and upregulation of stem cell genes (242). Consistently, mice bearing tumors originating from GSCs cultured at reduced pH had a decreased survival and higher microvessel density.

Hypoxia-driven GSC expansion depends on the phosphatidylinositol 3-kinase (PI3K)/Akt and ERK1/2 pathways as their inhibition reduces the fraction of CD133+ GSCs (240). The Notch pathway is also activated in GSCs under hypoxic conditions. Blockade of Notch signaling with gamma-secretase inhibitors depleted the GSC population, reduced the expression of GSC markers such as CD133, nestin, Bmi1 and Olig2 and inhibited the growth of tumor neurospheres and xenografts, associated with decreased Akt and STAT3 phosphorylation (240). However, a molecular link between Notch and HIF has not been directly demonstrated in GSCs. Among its other functions, hypoxia is the main driving force of tumor vascularization by stimulating the growth of new blood vessels.

Blood vessels play other important roles in tumor progression, e.g. by serving as conduits for the influx of various immune cells (Figure 5), as well as for the dissemination of tumor cells during the process of metastasis. Interestingly, a GSCs subset are located next to capillaries (Figure 5) *in vivo* forming the so-called perivascular niche and associate with endothelial cells *in vitro*. Endothelial cells and GSCs influence each other. Soluble factors produced by endothelial cells can enhance the expression of GSC markers and in this context, Notch signaling is fundamental (243). Other reports have uncovered an important role of nitric oxide (NO) in driving the GSC phenotype, in part by regulating Notch signaling. GSCs produce NO endogenously via inducible nitric oxide synthase (iNOS/NOS2),which supports GSC growth and tumorigenicity (244). NO is also produced in the tumor vasculature by endothelial nitric oxide synthase (eNOS/NOS3). In adjacent GSCs, NO activates the cGMP/ PKG pathway, enhances Notch signaling and promotes the stem cell phenotype. Loss of eNOS suppresses Notch signaling *in vivo*, delays gliomagenesis and prolongs the survival of tumor-bearing mice (244). The perivascular niche (Figure 5) might additionally regulate GSCs by providing them with the ligand sonic hedgehog (SHH) (245). The extracellular matrix is another key component of the tumor microenvironment and the perivascular niche. Extracellular matrix proteins such a laminin can positively regulate the self-renewal and tumorigenicity of GSCs (246). Finally, the perivascular niche appears

to play a role in promoting GSCs radioresistance. CSCs localized in the vicinity of blood vessels (Figure 5) in the brain were resistant to radiation, owing to their ability to activate the PI3K/Akt/mTOR pathway and undergo a transient, PTEN and p53-dependent cell cycle arrest (247). Notably, NSCs also maintain close contact with blood vessels, which may promote their self-renewal and regenerative potential. GSCs play an important role in generating the perivascular niche. GSC-derived factors may stimulate the proliferation of endothelial cells and the sprouting of new vessels within the local tumor environment or can induce the homing of endothelial progenitor cells derived from bone marrow to the tumor and promote their differentiation into blood vessels. More importantly, recent evidence indicates that GSCs can transdifferentiate into endothelial-like cells that directly and substantially contribute to the formation of tumor blood vessels (248). In this case, analysis of chromosomal aberrations and mutations specific for tumor cells revealed that between 20% and 90% of glioblastoma blood vessel cells were of tumor origin.

The presence of a perivascular niche (Figure 5), with the GSCs as fundamental elements, results in the release into circulation of numerous factors that, among other things, recruit circulating immunocompetent cells (Figure 5). GAMs are a heterogeneous cell population with a broad spectrum of activities in gliomagenesis; they are actively recruited into tumor masses by glioma cells through the secretion of chemokines such as CCL2 and CXCL1 and cytokines, among which TGF-beta is the best characterized; GAMs play a pivotal role in tumor microenvironment since they induce the expression of cytokines such as IL-10 and IL-6, metalloproteases, MDSCs and Treg cell induction which in turn sustain tumor growth, invasiveness and tumor escape. These factors are actively secreted by GSCs, with consequent entry into the bloodstream and paracrine effects in the tumor environment (see chapter 6). In this context the presence of BBB is basic. In early stages of glioma development there is no apparent disruption of the BBB, but, as glioma progresses, endothelial cells derived from normal vessels are separated from the vessel main structure and form new angiogenic spots associated with the tumor site. As these cells must migrate when forming new vessels, they disrupt the normal vessel structure to arrive at the tumor site. Because tumors secrete many different molecules that alter the normal microenvironment, endothelial cell migration is impaired, and this impairment is reflected in the vascular architecture. In GBM, the morphological alterations of blood vessels involve the formation of fenestrations and tight junctions disruption. Besides, the thickness of the basal lamina is altered and perivascular space is increased as is the number of pericytes associated to the vessels (249). The disruption of the BBB can be detected through magnetic resonance imaging. Under normal conditions gadolinium is not able to cross the intact BBB. In the case of GBM, since there is a disruption of the blood brain barrier, gadolinium can diffuse into the tissue. BBB disruption also causes an increased permeability to extracellular vesicles with exosomes representing a class of these vesicles. Recently, considerable attention has been paid to the potential of circulating miRNAs for cancer diagnosis and prognosis. A pilot study demonstrated that miR-15b and miR-21 in CSF were good marker for gliomas (250). In GBM cells miRNAs, messenger RNAs and angiogenic proteins are released from cells contained within exosomes, microvesicles, apoptotic bodies or as protein-miRNA complexes. microRNAs detected in CSF of brain-cancer patients may be derived from brain-cancer cells as a consequence of BBB disruption and cancer treatments. Glioblastoma derived microvesicles are likely to represent one of the mechanisms by which tumor cells change the brain microenvironment and make it more permissive for growth and invasion (251).

8. SUMMARY AND PERSPECTIVE

Gliomas and glioblastoma tumors in particular are characterized by a high heterogeneity. This heterogeneity mirror the numerous and complex genetic alterations some of which are more common in certain types of glioma than others. These alterations, accompanied by changes in gene expression, give a gain of function in proliferation and typical properties of the tumor transformation. The cellular heterogeneity is caused by the presence of GSCs which, by acquiring further genetic alterations during the course of tumor progression, give rise to different tumor clones distributed hierarchically with GCS being at the top. From a diagnostic point of view, this heterogeneity results in the presence, within the same tumor mass, of histological patterns corresponding to different types of glioma. Despite the great mass of data on the genetics of gliomas, effective cures are lacking. The glioma and glioblastoma in particular, remain extremely serious disease with a median survival of around 15 months for GBM. Indeed, all experimental protocols and clinical trials directed toward specific pathway have proven largely ineffective. More recently, the approach to the study of gliomagenesis was directed to a broader view, considering the microenvironment in which the tumor generates and grows up, especially the GSC niche, rather than the glioma cell with its alterations. From this perspective the scenario appears radically different.

In this context, genetic subpopulations of tumor cell clones and immune/stromal factors co-operate to create a favorable microenvironment. Feedback from the microenvironment drives disease progression and a malignant phenotype. The complex tumor stroma, consisting of reactive astrocytes, microglial cells and immune infiltrate, aberrant microvascular proliferation and hypoxia, as well as cell populations at different developmental stages, may also enhance the proliferation of specific cancer subclones. As

meantioned earlier, the hypoxic perivascular niche has been shown to be critical for the self-renewal of glioblastoma cancer stem-like cells, and may be important as a driver of divergent tumor evolution and treatment resistance.

These considerations may lead to new treatment approaches for glioblastoma that might inhibit glioma growth through the modulation of its microenvironment, for example by enhancing the immune responses against the tumor. Thus, any immunotherapy would work better if the M2 phenotype of GAM would be (re)converted to an M1 phenotype. In addition, the BBB permeability could also be considered as a potential route to convey, through exosomes, therapeutic substances such as toxins, miR, antibodies preferably directed to the GSC. A combination of classical therapeutic approaches directed to the tumor cells and the most innovative approaches targeting one or more component of the stem cell niche might ultimately lead to new and interesting therapeutic options.

9. ACKNOWLEDEGMENTS

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Abbreviations: GMB: glioblastoma multiforme; MDSCs: myeloid derived suppressor cells; BBB: brain blood barrier; EV: exosomes; CNS: central nervous system; WHO: World Health Organization; GFAP: glial fibrillary acidic protein; GSCs: glioma/glioblastoma stem cells; TCGA: cancer genome atlas network; TMZ: temozolomide; TKR: tyrosine kinase receptor TKIs: tirosine kinase inhibitors; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; PDGFR: platet derived growth factor receptor; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; NF1: neurofibronim 1; PTEN: phosphatase and tensin homolog; LAK cells: lymphocyte-activated killer cells; CTLs: cytotoxic T lymphocytes; NK cells: natural killer cells; DC: dendritic cells; APCs: antigen presenting cells; HSV1-TK: thymidine kinase; GCV: gangiclovir; IL: interleukin; Rb: retinoblastoma; TKR: tirosine kinase receptor; CDK: cyclin dependent kinase; IDH: isocitrate dehydrogenase; MDM2: mouse double minute 2 homolog; TERT: telomerase reverse transciptase; LOH: loss of heterozygosity; G-CIMP: CpG island hypermethylator phenotype; 2-HG: 2-hydroxyglutarate; HIF-1alpha: inducible factor-1alpha; LDH: lactate dehydrogenase; MGMT: O(6)-methylguanine-DNA methyltranferase; KIR: rectification inwards; DRK: adjustment in dalay; KCa: Ca^{2+} activated K current; hERG: human ether-a-go-go-related gene BKCa: large conductance;IKCa: intermediate conductance; SKCa: small conductance; SC: stem cell; CSC: cancer stem cell; NSC: neural stem cell; SVZ: subventricular zone; SSEA-1: stage specific embryonic antigen-1; NSTC: non stem tumor cell; HIF: hypoxia-inducible factor; GLUT3: glucose transporter 3; SCID: severe combined immunodeficiency; PU.1: purine-rich box1 transcription factor; Irf8: interferon regulatory factor 8; CD: cluster of differentiation; CX3CR1: chemokine (C-X3-C motif) receptor 1; c-Myb: cellular myeloblastosis transcription factor; Id2: inhibitor of DNA binding 2; Batf3: basic leucine zipper transcription factor ATF-like 3; Klf4: kruppel-like factor 4; CSF-1R: colony-stimulating-factor 1 receptor; CSF: colony-stimulating-factor; HSCs: hematopoietic stem cells; FLT: fms-related tyrosine kinase; MHC: major histocompatibility; Ly6C: lymphocytes antigen 6C; CCR2: chemokine (C-C motif) receptor 2; GFP: green fluorescent protein; RFP: red fluorescent protein; ATP: adenosine triphosphate; Usp: ubiquitin specific protease; INF: interferon; GMF: glia maturation factor; GM-CSF: granulocyte- macrophage-colony stimulating factor; MIP: macrophages inflammatory protein; G: gap; TGFbeta: transforming growth factor beta; TGFbetaR: transforming growth factor beta receptor; TbetaRII: transforming growth factor beta receptor II; ECs: endothelial cells; PAMPs pathogen associated molecular patterns; DAMPs damage associated molecular patterns; TLRs; toll-like receptors; PRRs: pattern recognition receptors; TNF-alpha: tumor necrosis factor alpha; ROS: reactive oxygen species; TREM: triggering receptor expressed on myeloid cells; DNA: deoxyribonucleic acid; NO: nitric oxide; LPSs: lipopolysaccharides; AD: Alzheimer's disease; MS: multiple sclerosis; IGF-1: insulin-like growth factor; Arg-1: arginase-1; Chil3: chitinasi 3; FIZZ1: found in inflammatory zone; iNOS: inducible nitric oxide synthase; MCP-1: monocyte chemotactic protein- 1; CCL2: chemokine C-C motif ligand 2; RAGE: receptor for advanced glycation endproducts; SDF-1: stromal-derived factor-1; CX3CL1: Chemokine (C-X-C) motif ligand 1; MMPs: metalloproteases; HGF/SF: hepatocyte growth factor/scatter factor; NKG2D: natural-killer group 2, member D receptor; FGF: fibroblast growth factor; Treg cells: T regulatory cells; FOX: forkhead box; SOCS-3: suppressor of cytokine signaling-3; RANTES: chemokine C- C motif ligand 5; FasL or CD95L: fas ligand; ICAM-1: intracellular adhesion molecule-1; PGE2: prostaglandin; HLA-DR: human; leukocyte antigen; COX-2: cyclooxygenase 2; mPGES-1: microsomal PGE synthase; ECM: extracellular matrix; CSIF: cytokine synthesis inhibitory factor; USF-1: upstream stimulating factor-1; MT1-MMP: membrane type 1-matrix metalloproteinase 1; MyD88: myeloid differentiation primary response 88; CTGF or CCN2: connective tissue growth factor; ITGB1: integrin beta-1;TrkA: tyrosine kinase receptor type A; p75NTR: neurotrophin receptor; TAMs: tumor associated macrophages;

IDO: indoleamine 2,3-dioxygenase; RNS: reactive nitrogen species; TCR: T cells receptor; M-CSF: macrophage colony stimulating factor; G-CSF: granulocyte colony stimulating factor; G-CSFR or CSF3R: granulocyte colony stimulating factor receptor; ASC: alanine-serine-cysteine transporter 1; GIMs: glioma infiltrating macrophages; ECM: extracellular matrix; OPN: osteopontin; ADAM17: ADAM metallopeptidase domain 17; RCC: renal cell carcinoma; G-MDSCs: granulocytic myeloid derived suppressor cells; G-MDSC: monocitic myeloid derived suppressor cells.

Key Words: Glioma, Microglia, GAM, Cancer Stem Cells, Tumor Niche, Review

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