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Review

Mechanisms of intimate and long-distance cross-talk between glioma and myeloid cells: How to break a vicious cycle

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ABSTRACT

Glioma-associated microglia and macrophages (GAMs) and myeloid-derived suppressor cells (MDSCs) condition the glioma microenvironment 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. Glioma cells recruit GAMs and MDSCs to the tumour site and block their maturation. Glioma cell-derived factors subsequently skew these cells towards an immunosuppressive, tumour-promoting phenotype. Finally, GAMs and MDSCs enhance immune suppression in the glioma microenvironment and promote glioma growth, invasiveness, and neovascularization. The local and distant cross-talk between glioma cells and GAMs and MDSCs is regulated by a plethora of soluble proteins and cell surface-bound factors, and possibly via extracellular vesicles and platelets. Importantly, GAMs and MDSCs have been reported to impair the efficacy of glioma therapy, in particular immunotherapeutic approaches. Therefore, advancing our understanding of the function of GAMs and MDSCs in brain tumours and targeted intervention of their immunosuppressive function may benefit the treatment of glioma.

Contents

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1. Introduction

Malignant gliomas form a family of highly aggressive brain tumours. Amongst these, glioblastoma represents the most frequent and most malignant type [1]. Despite great efforts to improve treatment, the median overall survival of patients treated with aggressive therapy is still below 15 months [2]. In 2005, the standard treatment for newly diagnosed glioblastoma changed from radiotherapy alone to a combination treatment of radiotherapy and temozolomide [3]. However, the median survival of patients treated with the combination therapy over surgery only increased from only 12.0 to 14.2 months [3]. It is well established that natural immunity is not effective in suppressing glioma growth. Immunotherapeutic approaches aimed at provoking anti-tumour immune responses achieved significant survival advantages for other tumour types, including prostate cancer for which it is currently used as standard treatment [4,5]. In the past decades, different immunotherapeutic strategies were tested in glioma patients participating in clinical trials, including passive approaches based on either cytokines or antibodies and adoptive cell transfer, and active immunotherapy using anti-tumour vaccines [6–8]. Most of the strategies involved administration of antigen-presenting cells (APCs), e.g. dendritic cell (DC) vaccines loaded with tumour peptides or whole tumour lysates [9]. However, although many of these approaches seemed to be promising *in vitro*, they demonstrated only limited success or significant side effects in animal models and clinical trials [7, 10–12].

Tumours of the central nervous system (CNS) raise challenges for immunotherapy because of the unique immune environment of the CNS. The CNS has long been regarded as an immune privileged site [13,14], due to its blood brain barrier (BBB), controversially lacking a connection to the lymphatic system and the apparent inability of microglia (the so-called resident brain histiocytes) to induce a T-cell response [15]. However, over the years this principle of CNS immune privilege in glioma patients has been challenged in several ways: 1) in high-grade glioma patients the BBB is leaky; 2) connections exist between cerebrospinal fluid (CSF) and cerebral interstitial fluid (CIF) compartments and cervical lymphatics; 3) microglia can fulfill the role of resident APCs within the CNS; 4) 'professional' APCs, i.e. DCs, are present in the meninges and choroid plexus; 5) active trafficking of immune cells to and from the brain is observed [7].

Glioma antigens are released into the CSF and CIF fluids, and although the brain does not contain specialized lymphatic vessels, there is efficient drainage of CSF and CIF to the deep cervical lymph nodes allowing for initial immune activation. In these lymph nodes glioma antigens encounter cognate B lymphocytes and are processed and presented to circulating naive T-cells by professional APCs such as DCs, leading to immune infiltration of the glioma tissue [7]. It thus becomes increasingly clear that the immune privilege of the CNS in the context of glioma is not defined by the absolute absence and ineffectiveness of immune cells in the CNS, but rather is a consequence of the tight regulation of the immune balance in this system [16]. It is now well established that, beyond this unique immune environment of the brain, glioma cells can actively mediate immune suppression and thereby also influence the effectiveness of immunotherapy [17,18]. Immune suppression and immune evasion is established by glioma cells via multiple mechanisms, such as the down-regulation of HLA class I molecules [19], up-regulation of inhibitory HLA molecules [20–22], and secretion of immunosuppressive factors [23,24]. Moreover, glioma cells directly and indirectly interact with myeloid and lymphoid immune cells. These interactions can result in the inhibition, apoptosis, and anergy of immune cells as well as in their skewing to immature, functionally compromised or immunosuppressive phenotypes [18]. Furthermore, tumour-associated immune cells can in turn promote tumour growth, angiogenesis and invasiveness, and further support immune suppression in the tumour microenvironment [25–27]. The contribution of myeloid cells to immune evasion and tumour progression is achieved by the production of matrix metalloproteases (MMPs), growth factors, cytokines and chemokines, reactive oxygen species and other mediators [28].

The diversity and complexity of CNS tumours has been described as unrivalled by tumours elsewhere in the human body [29]. Systematic histopathological analysis for over more than a century resulted in a World Health Organization (WHO) scheme of CNS tumours that now functions as the global gold standard for classification of these neoplasms [30]. CNS tumours are at the top of the list of 'average number of years of life lost' by cancer [31]. This can be explained by two important facts, i.e. 1) the most frequent primary brain tumour is glioma, and most gliomas show very extensive, diffuse infiltrative growth in the surrounding CNS parenchyma precluding curative therapy [32], and 2) CNS tumours are relatively frequent in children, and many of those are highly aggressive. Gliomas are considered to originate from glial cells or their precursors, and may show astrocytic, oligodendroglial, or ependymal differentiation or a combination thereof. In adult patients, most gliomas are astrocytic, oligodendroglial or mixed oligoastrocytic in nature. A malignancy grade can be assigned to these tumours based on histopathological parameters like marked mitotic activity, a peculiar form of angiogenesis such as 'florid' or even 'glomeruloid' microvascular proliferation, and/or necrosis. The least malignant diffuse glioma is designated as WHO grade II, the most malignant and most frequent astrocytic tumour is glioblastoma (WHO grade IV). The category of anaplastic/WHO grade III diffuse gliomas falls in between these grades. Both low (WHO grade II) and high grade (WHO grade III and IV) diffuse gliomas occur in all age groups, but young adults are more often diagnosed with a diffuse low grade glioma (which can however progress to a high grade glioma), while glioblastomas are relatively frequent in patients over fifty years of age.

Over the last decade insight into the molecular aberrations underlying CNS oncogenesis has increased in a revolutionary way. A combination of morphological and molecular characteristics may soon allow for a much more robust and clinically meaningful 'taxonomy' (typing and grading) of CNS tumours, especially of gliomas. In order to successfully survive and develop into a tumour, malignant cells generally need to sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis [33]. More recently, conceptual progress resulted in the addition of two hallmarks: evasion of immune destruction and reprogramming of energy metabolism. In addition, it was acknowledged that inflammation and genome instability in tumour cells are characteristics facilitating or even enabling these hallmarks [34].Using gene expression data from The Cancer Genome Atlas Project, glioblastomas can now be divided in four subclasses. These four subtypes, classical, neural, proneural and mesenchymal, can be separated according to different patterns of gene expression and aberrations of EGFR, NF1, PDGFRA, and IHD1 [35]. With the growing recognition that immune escape contributes to tumour growth, tumour immunologists have been taking inventory of the significance of immune infiltration for the prognosis of solid tumours. Most notably, Galon and colleagues have shown for Stage I-III colon cancer that memory $CD8^+$ T cell infiltration is more predictive for survival than classic AJCC staging [36,37]. This astonishing finding has led to a world-wide task force with the aim to validate these results in large patient cohorts and to promote the notion that T-cell infiltration rate (the so-called Immunoscore) should be incorporated into a new staging system for colon cancer and possibly also for other solid umours [38,39]. There are indications that also for glioma $CD8^+$ T-cell infiltration rate holds prognostic significance, even in the face of clear immunosuppression in the microenvironment [40].

In this review we will further focus on the infiltration of gliomas by myeloid cells, i.e. glioma-associated microglia and macrophages (GAMs) and myeloid-derived suppressor cells (MDSCs) (Fig. 1). GAMs are considered to represent the most prominent glioma-infiltrating immune cells, constituting up to 30% of all immune cells within the tumour microenvironment [41]. Their presence in the glioma

microenvironment has been reported to correlate with tumour grade and glioblastoma subclass [42–44]. Hence, it may be opportune to consider immunotyping of myeloid-derived immune cells (besides lymphoid cells) in future classifications of CNS tumours.

2. GAMs AND MSDCs in the glioma microenvironment

GAMs are fundamentally involved in the establishment of an immunosuppressive glioma environment. CD68, CD163, CD200, CD204, F4/80, and IBA-1 are generally employed markers to identify GAMs [45–47]. Microglia can be distinguished from macrophages through their low expression of CD45 [47,48]. Although the exact origin of GAMs remains controversial, they are considered to be derived from at least two different lineages. The first group, resident microglia, has been suggested to develop from primitive myeloid precursors in the embryonic yolk sac and to invade the CNS during early embryogenesis [49]. Microglia are resident brain cells which rapidly adopt an actively phagocytic phenotype and release pro-inflammatory factors upon inflammation or injury [50]. Under normal physiological conditions their phenotype is tightly regulated [51]. Although first described as immuno-incompetent, microglia have since been shown to be able to effectively induce innate and adaptive immune responses [52]. In contrast, microglia in the glioma environment have consistently been reported to be impaired in their ability to phagocytose and induce an effective anti-tumour immune response [53,54]. Under certain conditions, these microglial cells can be joined by glioma-associated macrophages (that in contrast to microglia are $CD45^+$ and originate from bone marrow-derived monocyte progenitors) as well as by MDSCs (see Fig. 1) [55,56]. Interestingly, studies in adenocarcinomas of the lung also propose the spleen as an important origin of tumour-associated monocytes [57].

MDSCs represent a heterogeneous population of activated immature myeloid precursors of DCs, macrophages, and granulocytes and can cause remarkable immune suppression via various mechanisms. MDSCs are well known to accumulate in tissues during trauma, infectious disease, and sepsis. Their increased infiltration rates in gliomas and other tumours has been described previously as well [58–60]. In mice a common characteristic of MDSCs is the co-expression of CD11b and GR1 [61]. In humans uniquely identifying markers are unfortunately lacking. MDSCs in the blood of glioma patients have been identified as a $LIN^-HLA\text{-}DR^-CD33^+CD11b^+$subset, but as MDSC populations are remarkably heterogeneous, marker profiles may vary [54,58]. Furthermore, a distinction is made between monocytic MDSCs (in mice: $CD11b^+Ly6G^-Ly6C^{high}$), and polymorphonuclear or granulocytic MDSCs (in mice: $CD11b^+Ly6G^+Ly6C^{low}$) [62]. These two subsets have been suggested to play distinct roles in cancer, infection, and autoimmunity [63–65]. In cancer, granulocytic MDSCs have been shown to regulate antigen-specific T-cell tolerance and non-specific suppression, whereas monocytic MDSCs mediate non-specific suppression and promote tumour angiogenesis [66]. Tumour-associated MDSCs are believed to arise from immature myeloid progenitors through aberrant differentiation which is promoted by factors in the tumour environment [67]. Recruitment of MDSCs to the glioma site is regulated through cytokines

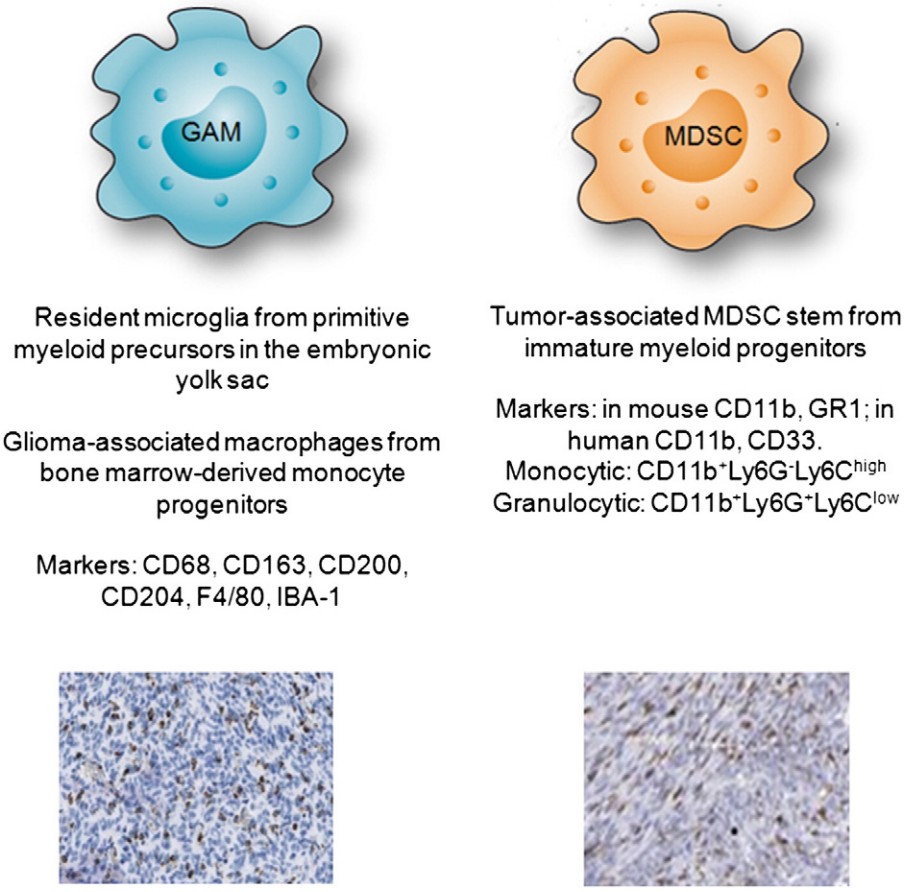

Fig. 1. Glioma-associated microglia and macrophages (GAMs) and myeloid-derived suppressor cells (MDSCs). Schematic representation of the phenotypic and functional characteristics of the two immune suppressive, tumour-associated myeloid subsets and their putative origins. Images are immunostainings of $CD68^+$ cells (left) [246], and $CD11b^+$ cells (right) [247], on glioblastoma tissue sections (www.proteinatlas.org).

and growth factors produced by the tumour. In turn, MDSCs can suppress anti-tumour T-cell responses and promote tumour angiogenesis and invasiveness [67].

3. Interaction between glioma cells and myeloid-derived immune cells

The population of GAMs that is found in glioma is believed to predominantly contribute to the induction of an immunosuppressive glioma microenvironment. They directly and indirectly interact with glioma cells in various ways. First, GAMs are recruited to the tumour via different glioma-derived factors that subsequently contribute to a switch from a tumour-suppressive M1 to a tumour-promoting M2 phenotype (Fig. 2). Acquisition of the M2 phenotype is accompanied by a diminished ability to induce an effective anti-tumour T-cell response. Second, M2 GAMs mediate immune suppression in the glioma microenvironment through secretion of immunosuppressive factors and interaction with other immune cells. Third, the M2 GAMs produce factors that stimulate glioma growth, neovascularization, and invasiveness.

3.1. Recruitment and M2 phenotype polarization of GAMs by glioma cells

To guarantee a constant supply of tumour-associated macrophages, tumours continuously expand bone marrow-derived hematopoietic stem cells by secreting immune modulating factors [68]. A large number of studies has investigated the recruitment of GAMs to the tumour site. This recruitment is mediated through various glioma-derived factors including chemokines, cytokines and matrix proteins [69]. Tumour-derived MCP-1 (CCL2) [27,70,71], GDNF [72], HGF/SF [73], SDF-1 (CXCL12) [74], CSF-1 (M-CSF) [75], TNF [76],VEGF [77,78], and GM-CSF [79] have previously been reported to guide the recruitment and migration of GAMs into the glioma environment. Interestingly, SDF-1 produced by glioma cells is able to recruit GAMs to hypoxic regions in a murine astrocytoma model [74]. Recent studies also indicate that glioma-derived SDF-1 contributes to radiotherapy-induced invasiveness through initiation of macrophage migration and glioma neovascularization [80]. GM-CSF was demonstrated to be involved in GAM recruitment, but also supports the alternative activation of microglia and macrophages into immunosuppressive GAMs [79]. In addition, recent evidence indicates that glioma stem-like cells - the fraction of tumour-initiating glioma cells - play a dominant role in the recruitment of GAMs. Indeed, it has been described that the infiltration of GAMs positively correlates with the density of cancer stem-like cells in gliomas [81]. Moreover, it has been suggested that such glioma stem-like cells recruit circulating monocytes via CSF-1 and the chemokine CCL2 [82], and that this process is more effective than recruitment by regular glioma cells [81]. Recently, it was reported that sphere formation by glioma stem-like cells induced by GAMs in the tumour microenvironment can be blocked by amphotericin B (which was identified by small molecule drug screening) [83]. This may lead to new anti-tumour intervention strategies, preventing the GAM-induced activation of tumour growth. In this context it is of interest that experimental glioma studies revealed that the accumulation of activated microglia occurs early during tumour development, prior to the accumulation of macrophages [84].

As previously alluded to, GAMs can be activated in two distinct ways: a classical M1 activation and an alternative M2 activation (Fig. 2). Classically activated M1 GAMs are stimulated by LPS and IFNγ and are able to effectively promote an anti-tumour immune response [85]. M1 GAMs produce pro-inflammatory mediators, phagocytose tumour cells, present tumour antigens to immune cells, and stimulate them to trigger an antigen-specific T cell response [86]. The transcription factor STAT1 is activated in GAMs upon M1 stimulating factors. As already indicated/discussed above, however, evidence suggests that GAMs in the glioma environment instead possess an alternatively skewed M2 phenotype [87]. The M2 GAM phenotype is induced by various factors such as IL-4, IL-10, IL-13, and TGF-β, and is characterized by an incompetence to induce an anti-tumour T-cell response and by immunosuppressive features [88,89]. In contrast to M1 GAMs, M2 GAMs demonstrate high levels of STAT3, CD163, and CD204 expression [82,87,90]. Especially activation of STAT3 is considered to play a key role in the induction of GAM-mediated immune suppression. However, it is now becoming increasingly clear that various transcriptional and epigenetic mechanisms are involved in the differentiation of M1/M2 macrophages [91].

Tumour cells actively secrete factors that skew the phenotype of GAMs to a M2 phenotype, including expression of GM-CSF [79], CSF-1 [75,92], and S100B [93]. Immunosuppressive factors including IL-4, IL-6, IL-10, MIF, TGF-β, and PGE-2 are secreted by glioma cells, and are also known to drive GAM differentiation towards an M2 phenotype rather than an M1 phenotype [47]. Interestingly, the skewing of GAMs by glioma stem-like cells seems to be mediated through the production of CSF-1, TGF-β1, and macrophage inhibitory cytokine-1 (MIC-1). This

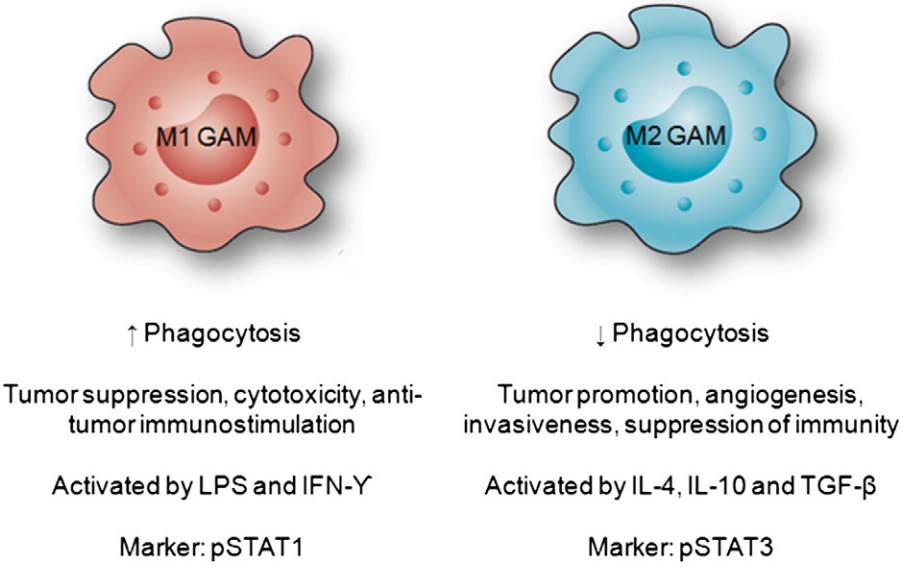

Fig. 2. M1 and M2 GAMs. Schematic representation of the two different GAM subtypes with opposing immunological functions. pSTAT1 and pSTAT3 indicate phosphorylated and activated STAT1 and STAT3, respectively.

could result in STAT3 activation by phosphorylation in GAMs, as well as their secretion of IL-10 and TGF-β. M2-skewed GAMs show an impaired ability to phagocytose tumour cells and induce an antigen specific T-cell response [82]. Besides tumour cells, immune cells in the tumour microenvironment may also actively polarize GAMs towards an M2 phenotype. T_{reg} or B-cells, for example, have been found to stimulate the alternative M2 activation of macrophages *in vitro* [94,95]. Of note, it has been suggested that anti-tumour M1 as well as tumour-promoting M2 GAMs are present in the glioma environment, the balance between these two phenotypes determining the net effect (pro- versus anti-tumour) of the immune response [47].

The response of GAMs to tumour signals can be regulated at a transcriptional as well as post-transcriptional level. It has been suggested that miRNAs regulate hematopoietic stem cell maintenance, macrophage development and alternative activation [96]. For instance, miR-146a was found to control functional heterogeneity of monocyte subsets [97]. miRNAs involved in alternative M2 macrophage activation are up-regulated by tumour-derived signals [96]. On the other hand, tumour-derived immunomodulatory RNAs and proteins can also be collectively transferred through extracellular vesicles (EVs) [96,98,99]. Al-Nedawi et al. noted that the traditionally accepted view of local and distant cell-to-cell communication as primarily mediated by gradients of soluble proteins may need to be complemented by findings that EVs can deliver units of collective information over medium and long-range distances [100]. The role of EVs in glioma and myeloid-derived immune cell cross-talk is further discussed below.

3.2. Immunosuppressive properties of GAMs

The polarization of GAMs towards tumour-promoting M2 GAMs by glioma cells is associated with a range of phenotypic changes leading to the formation of an immunosuppressive microenvironment and the impairment of an effective anti-tumour T-cell response (Fig. 3). It has been demonstrated *in vitro* that GAMs express TLRs and that binding of LPS to TLR-4 on GAMs occurs. However, GAMs failed to subsequently induce T-cell proliferation [53]. This impairment may be caused by the lack of expression of co-stimulatory molecules such as CD40, CD80, and CD86 in GAMs caused by exposure to glioma-derived suppressive factors. Furthermore, these GAMs do not produce pro-inflammatory cytokines such as TNF-α and IL-1 upon stimulation [53]. GAMs contribute to an immunosuppressive glioma environment through the production of a range of immunosuppressive mediators including IL-6 [27], IL-10 [82], and TGF-β1 [82]. IL-10 release has been found to be regulated by STAT3 [101], and mediates a range of immunosuppressive mechanisms. These include amongst others the inhibition of an anti-tumour response by the down-regulation of HLA-DR expression on monocytes [102], and their decreased production of inflammatory cytokines [103].

Immune suppression by GAMs in the glioma environment is also realized through the down-regulation of HLA-DR expression as well as the up-regulation of the inhibitory molecules HLA-G and HLA-E on GAMs, both induced by glioma cells [104,105]. HLA-G is expressed on glioma cells and counteracts NK cell-induced lysis and T-cell activation [18]. Expression of HLA-E on GAMs and glioma cells further enhances immune suppression by mediating resistance to NK cells and CTL cytotoxicity [18]. GAMs show impaired phagocytic competence [53,54]. Effective T-cell activation not only requires the binding of the T-cell receptor (TCR) to MHC, but also co-stimulation, e.g. through the interaction of T-cell CD28 with CD80/CD86 on microglia and macrophages [106]. Binding of CD40 on microglia and macrophages to its ligand on T-cells further promotes T-cell activation [107]. Lack of expression of these co-stimulatory molecules on GAMs, as it is the case in the glioma microenvironment, may therefore result in T-cell anergy [53,108]. Other important mediators of immune suppression in gliomas are FasL and B7-H1 (i.e. PD-L1) expressed on GAMs and glioma cells themselves

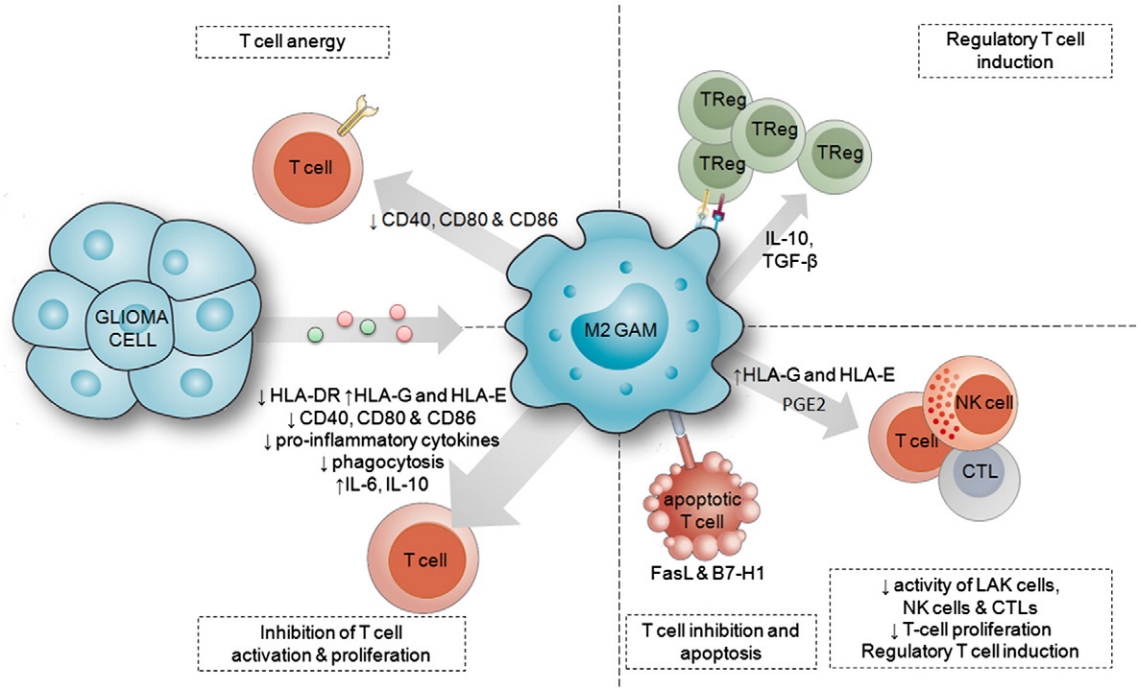

Fig. 3. Immunosuppressive properties of GAMs. Glioma-derived factors skew microglia/macrophages towards a tumour-promoting, immunosuppressive M2 phenotype by the activation of STAT3 by phosphorylation (pSTAT3). Glioma-associated M2 GAMs in turn promote immune suppression via the induction of T-cell anergy and apoptosis, expansion of T_{reg} cells, inhibition of T-cell activation and proliferation and decreasing the activity of LAK cells, NK cells and CTLs. T-cell anergy is established through reduced expression of CD40, CD80 and CD86. Expansion of T_{reg} cells may require direct interaction between MHC II – TCR and CD40 – CD40L as well as secretion of soluble factors such as IL-10 and TGF-β by GAMs. Increased HLA-G, HLA-E and PGE2 expression in GAMs may affect the activity of LAK cells, NK cells and CTLs and further inhibit T-cell proliferation. T-cell apoptosis is mediated through FasL expression on GAMs. In addition, T-cell inhibition is induced by low levels of HLA-DR, co-stimulatory molecules and pro-inflammatory cytokines and low phagocytosis in GAMs as well as increased production of IL-6 and IL-10. Small pink and green circles indicate tumour-derived EVs.

[54,109–111]. Binding of PD-L1 to PD-1 on T-cells results in the down-regulation of TCRs and the induction of T-cell apoptosis. Microglia furthermore account for up to 50% of the FasL expressing cells in gliomas and may thus to a large extent be responsible for the induced apoptosis of lymphocytes in the glioma environment [109].

PGE-2, a small lipid-soluble molecule, is secreted by glioma cells and, amongst other functions, has been reported to provoke the decrease of surface MHC class II expression on microglia and DCs [112]. Interestingly, glioma cells can educate GAMs to up-regulate PGE-2 expression through glioma-derived soluble factors [113]. M2 GAMs may help to establish the immunosuppressive glioma niche by decreasing lymphokine-activated killer (LAK) cell, NK cell, and CTL activity [114], inhibiting T-cell proliferation [78], and inducing a T_{reg} response [78,115]. It has been recently described that microglia are also able to actively induce T_{reg} cells, depending on the local IFNγ levels [116]. At high levels of local IFNγ, as well as low expression of IL-10 and high expression of the co-stimulatory molecules CD86 and CD40 on microglia, potent anti-tumour T-cell responses are induced. However, at low local IFNγ levels, and high IL-10 and low co-stimulatory molecule expression on microglia, the response switches to an induction of T_{reg} cells [116]. As M2 GAMs release high levels of IL-10 [26], and harbor low expression of CD86 and CD40 [53], it is reasonable to consider that M2 GAMs are able to directly induce $FOXP3^+$ T_{reg} cells, and thus even further enhance immune suppression in the glioma environment. The observation that macrophages are able to induce T_{reg} cells strengthens this hypothesis [117].

3.3. Promotion of glioma proliferation and differentiation, angiogenesis, and invasiveness by GAMs

In recent years, there has been an increasing amount of literature on the cross-talk between glioma cells and GAMs leading to enhanced glioma proliferation and invasiveness. An important illustration of this cross-talk is represented by the glioma-induced production of MMPs, especially MMP-2 and MMP-9, by GAMs. This may at least partly account for the degradation of extracellular matrix in parts of the glioma microenvironment and facilitate glioma invasiveness [118]. It has been conclusively shown that glioma-derived factors activate TLR2 (and TLR1/TLR6) on GAMs and thereby trigger the expression of MT1-MMP in a MyD88-dependent fashion [119,120]. Subsequently, MT1-MMP produced by GAMs can activate glioma-derived pro-MMP2 and promote glioma invasiveness [119]. In addition, Bhat et al. demonstrated that GAMs can induce differentiation of proneural glioblastoma cells into radioresistant mesenchymal glioblastoma cells through TNF-α and NF- B translocation [44]. This concept of "GAM abuse" by glioma cells further demonstrates the close interaction between glioma cells and GAMs [119]. MMP-2 is produced by both glioma cells and microglia, and is believed to promote matrix remodeling and to manipulate glioma cells in various other ways [121,122], by affecting glioma cell metabolism, receptor turnover and apoptosis resistance [121]. MMP-9, the second key matrix metalloprotease in glioma progression, is also secreted by microglia and glioma cells [122,123]. The expression of MMP-9 can be triggered by multiple factors derived from GAMs. Stress inducible protein 1 (STI1) secreted from GAMs, for example, has been suggested to increase the activity of MMP-9 in a PrP(C)-independent manner [124].

Multiple lines of evidence suggest that immunosuppressive cytokines expressed by GAMs might also play an important role in the promotion of glioma proliferation and motility. IL-6 [27], IL-10 [26], and TGF-β [125] have all been reported as potent inducers of glioma invasiveness. Beside its effects on immune suppression, TGF-β1 has been demonstrated to enhance MMP-9 expression in glioma stem-like cells and consequently promote their invasiveness [118,125]. Moreover, TGFβ1 can induce up-regulation of VEGF in glioma cells and thus potentially contribute to glioma angiogenesis [126]. In addition, GAMs stimulate glioma proliferation and angiogenesis by various other mechanisms including the production of EGF and, again, VEGF [127,128]. Conversely, glioma cells support microglia recruitment and proliferation by the production of multiple factors, including EGF and HGF/SF [78]. The tight and supportive interaction between glioma cells and myeloid immune cells may thus be regarded as a symbiotic relationship, with a clear benefit for the glioma cells [78].

3.4. MDSCs in glioma

MDSCs are believed to interact with glioma cells, resulting in the inhibition of an effective anti-tumour response (Fig. 4). However, the exact processes of interaction remain to be defined. In a first step, glioma cells are believed to recruit circulating monocytes or other immature myeloid cells, to block their maturation, and to promote their differentiation to MDSCs [18]. Additional evidence from other tumour types has shown that tumour cells can also skew differentiation of immature myeloid cells in hematopoietic organs to MDSCs by releasing EVs [129]. This may also hold true for gliomas, as glioma EVs are able to pass the blood-brain barrier [130]. According to current knowledge, which is mostly based on *in vitro* or murine studies, MDSCs mediate immune suppression and support glioma growth, invasion, and vascularization, as well as the systemic expansion of T_{reg} cells (further discussed below).

3.5. Blockade of myeloid cell maturation and promotion of MDSC differentiation and recruitment

Glioma cells have been found to express multiple factors such as IL-6, IL-10, VEGF, PGE-2, GM-CSF, and TGF-β2 that were previously associated with the expansion of MDSCs [6,54,131–133]. Moreover, IL-6, M-CSF, COX-2/PGE-2, IL-1β, TNF-α, and VEGF were previously shown to stimulate the differentiation of normal human peripheral blood mononuclear cells into MDSCs *in vitro* [134]. As gliomas are known to over-express these factors, it is likely that they also induce the generation of MDSCs *in vivo* [24,135–138]. In addition, it has been described that inhibition of glioma-derived galectin-1 can affect the number of MDSCs present in the tumour [139]. Monocytes of healthy donors gain an MDSC phenotype after co-culture with glioblastoma cells [54]. These monocytes consequently show reduced CD14 levels, increased IL-10, TGF-β, and PD-L1 expression and have a decreased phagocytic potential. Surprisingly, data suggest that a direct interaction between glioma cells and monocytes is needed to optimally achieve a tumour-promoting MDSC phenotype [54]. Furthermore, glucocorticoids also stimulate the activation of an anti-inflammatory MDSC-like monocyte subset [140].

Differentiation and activation of antigen-presenting DCs is an essential step in order to induce an effective immune response. However, evidence also points to tumour-induced suppression of DC maturation and an increased accumulation of immature DCs, as well as macrophage-like cells and MDSCs, resulting in a compromised immune response [133]. Differentiation and activation of DCs is impaired by tumour-derived factors such as IL-6, IL-10, PGE-2, and TGF-β [141]. These factors are also known to be expressed by glioma cells at high levels. Interestingly, glioblastoma-derived IL-6 inhibits the differentiation of $CD34^+$ myeloid precursors into Langerhans cells (another subtype of myeloid-derived APC) and triggers STAT3 activation [142]. We previously demonstrated that STAT3 inhibition can antagonize the suppressive effect of IL-6 on DC differentiation [141]. Remarkably, STAT3 inhibition combined with p38 MAPK inhibition strongly abrogated DC suppression induced by primary glioma supernatants [141]. Importantly, tumour-induced inhibition of DC development has been shown by different groups to result from COX-2 regulated PGE-2 as well as IL-6 and to lead to the alternative development of M2-macrophage- or MDSC-like cells [143–145]. Tumour-derived factors that drive the differentiation of MDSCs can be shed in the tumour microenvironment as individual soluble factors, but also via EVs, including to hematopoietic organs [129]. It was previously reported that EVs can transfer factors that hinder the normal differentiation of myeloid cells and promote the generation of MDSCs as well as their migration to the tumour site [129]. Recruitment of MDSCs to the glioma environment

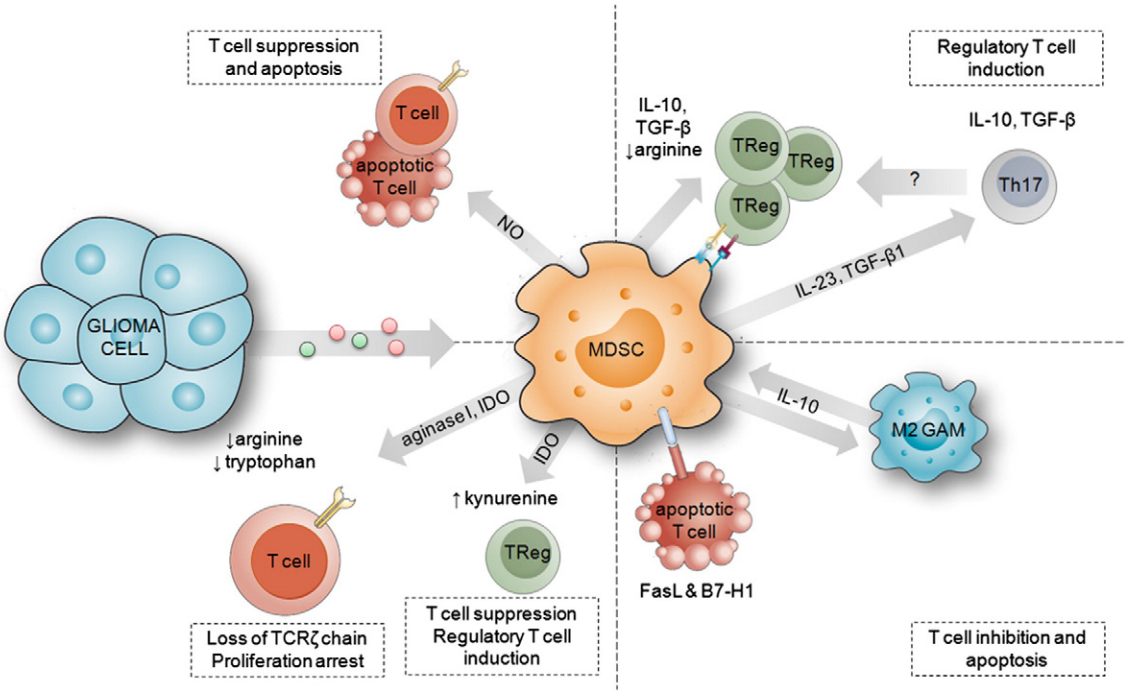

Fig. 4. Current understanding of the immunosuppressive properties of myeloid-derived suppressor cells (MDSCs). Glioma-derived factors block myeloid cell maturation and promote differentiation into MDSCs. MDSCs in turn may contribute to the immunosuppressive glioma environment through induction of T_{reg} cells and T-cell apoptosis, inhibition of T-cell proliferation and loss of the T-cell receptor TCRζ chain. MDSCs in gliomas induce T-cell suppression and apoptosis through the production of oxidative stress. Expansion of T_{reg} cells may require direct interaction between MHC II – TCR and CD40 – CD40L as well as secretion of soluble factors such as IL-10 and TGF-β by MDSCs. MDSCs further recruit T_{h17} cells which may transdifferentiate into T_{reg} cells. Mutual production of IL-10 by MDSCs and GAMs may also enhance the immunosuppressive phenotype of these myeloid cells. Interaction of FasL on MDSC and Fas on T-cells induces T-cell apoptosis. Production of IDO and arginase by MDSCs further results in T-cell suppression and loss of the TCRζ chain through arginine and tryptophan starvation as well as the production of immunosuppressive metabolites. Small pink and green circles indicate tumour-derived EVs.

is mediated by different chemokines and mediators including CCL2 [146], and COX-2-regulated lipid mediators [147].

3.6. Immunosuppressive properties of MDSCs

MDSCs are known to exert remarkable immunosuppressive activities in cancer. MDSCs mediate immune suppression by depleting essential nutrients for lymphocytes, hampering their trafficking and viability, producing reactive nitrogen species and reactive oxygen species (ROS), as well as activating and inducing expansion of T_{reg} cells [67]. Glioma-infiltrating MDSCs in rat models express arginase I, iNOS, indoleamine 2,3-dioxigenase (IDO), and TGF-β [148]. It was revealed that NO production is the primary mechanism of immune suppression in this glioma model [148]. MDSC-derived NO has been described in other tumour types as a potent inducer of T-cell suppression through the induction of oxidative stress. This suppression is mediated by the inhibition of JAK3 and STAT5 activation, a decrease in MHC class II expression as well as T-cell apoptosis [149]. Arginase I contributes to MDSC-induced immune suppression by the depletion of L-arginine which is essential for the growth and differentiation of T-cells. This results in the proliferative arrest of T-cells and a decrease in their expression of the TCR-associated CD3ζ chain [150]. Kohanbash et al. recently proposed that glioma-derived GM-CSF stimulates the up-regulation of IL-4Rα on MDSCs and thereby triggers the IL-13 dependent production of arginase I [131]. Interestingly, arginase I inhibition or supplementation of arginine can relieve T-cell suppression and restore T-cell function in glioblastomas [151]. IDO expression in tumour-associated MDSCs may additionally suppress T-cell activation by catalyzing the degradation of tryptophan into kynurenine and other metabolites. The ensuing tryptophan starvation leads to the inhibition of T-cell activation and proliferation [152]. Furthermore, kynurenine and other immunosuppressive tryptophan catabolites inhibit the induction of an effective T-cell response and promote the differentiation of T_{reg} cells [153,154]. TGFβ represents another important immune-regulatory factor which is produced by glioma-associated MDSCs. MDSC-derived TGFβ, together with IL-10 and a decreased arginine concentration, has been shown to induce the differentiation and expansion of $FOXP3^+T_{reg}$ cells in other tumour types [155,156]. TGF-β, IL-10 and arginase 1 are known to be over-expressed in glioma-associated MDSCs [54,148], and T_{reg} cells and MDSC are both present in the glioma microenvironment [58,157]. This suggests that an MDSC-mediated expansion of $FOXP3^+T_{reg}$ cells may also occur in the glioma microenvironment. Surprisingly, the abrogation of TGFβ signaling has been reported to attract MDSC in mammary carcinomas [158]. Moreover, glioma cells and MDSCs both recruit Th17 cells through cytokine secretion [159]. These Th17 cells subsequently assist MDSCs in generating an immunosuppressive glioma environment by producing IL-10 [159]. As MDSCs have been described to stimulate the trans-differentiation of Th17 cells into $FOXP3^+T_{reg}$ cells via the production of TGFβ and retinoic acid, this process may also occur in gliomas [160].

MDSCs, induced by co-culture with glioma cells, were reported to express increased levels of IL-10 and PD-L1, and to exhibit an impaired phagocytic potential [54]. MDSCs may therefore also induce T-cell apoptosis through binding of PD-L1 on the MDSC surface to PD-1 on T-cells. The impaired ability of MDSCs to phagocytose glioma cells may further result in diminished glioma-derived antigen presentation to T-cells and thus hamper the effective induction of an anti-tumour response. As both glioma-associated MDSCs and GAMs were shown to express high levels of IL-10 and are skewed towards an immunosuppressive, tumour-promoting M2 phenotype by IL-10, it is likely that GAMs and MDSCs in the glioma microenvironment also promote each other's polarization.

3.7. Promotion of glioma proliferation, vascularization, and invasiveness by MDSCs

Despite the great attention given to MDSCs in cancer research during recent years, the role of MDSCs in glioma invasiveness and

vascularization remains to be further elucidated. Besides their remarkable immunosuppressive ability, MDSCs can also exert activities that enhance tumour growth, neovascularization (i.e. angiogenesis and/or vasculogenesis), and invasiveness. Resistance of glioblastoma cells to anti-VEGF treatment has been shown to be associated with infiltration of myeloid cells and macrophages in the tumour tissue [161]. Gene expression analysis suggests that glioma-associated MDSCs enhance vascularization by the expression of TNF, FGF, and MMPs [162]. Glioma-associated MDSCs, moreover, have been reported to express IL-1β, TNF-α, and TGF-β [163]. Membrane-bound TNF-α on breast cancer-associated MDSCs has been proposed to stimulate tumour progression, vascularization, and additional accumulation of MDSCs [164]. Studies analyzing different tumour types revealed that MDSCs directly promote vascularization by the production of MMPs and pro-angiogenic factors as well as by their adoption of endothelial cell-like properties in the tumour environment [165]. Additionally, MDSCs can enhance tumour invasiveness and metastasis by the induction of MMPs and chemo-attractants as well as by the generation of a pre-metastatic niche [165]. However, it remains to be clarified if these properties are also found in glioma-associated MDSCs [118].

4. Extracellular vesicle and platelet-mediated cross-talk between glioma and myeloid cells

The secretome of a cell represents all macromolecules secreted by the cell and contributes notably to cell-cell communication [166]. Secreted molecules can be transported in different manners including the transport in EVs. Glioma cells release large amounts of EVs [167]. EVs are membrane limited vesicles originating from cells and are released in the extracellular space and fluids such as blood, cerebrospinal fluid, urine, saliva, and milk. The term comprises exosomes, shed microvesicles and apoptopic bodies. EVs contain nucleic acids, lipids, and proteins, which they can be delivered through body fluids to local and distant cells [167–170]. Glioma cells produce EVs that carry a diversity of proteins such as cytokines, chemokines, enzymes, signaling protein kinases, oncogenic growth factors and growth factor receptors, integrins, chaperons, transporters, ECM proteins, transcription factors, MMPs, and immunomodulatory molecules [171–178]. Importantly, EVs released into the circulation are potentially capable of hijacking myeloid cells and 'educating' them for pro-tumoural support. Glioma-derived EVs released in the blood contain mRNAs and proteins for most of the GAM and MDSC recruitment factors [167]. In turn, immune cells might communicate with tumour cells in an EV-mediated two-way mode of communication.

An important step in EV transfer is the uptake by the recipient cell. For the internalisation and transfer of EVs and their content, different modes of action have been reported [179,180]. For instance, fusion of melanoma EVs with melanoma cells was shown to be affected by pH, i.e. more EVs fused with the tumour plasma membrane in an acidic environment, involving lipid-lipid interactions and yet to be identified proteins exerting structural functions key for the fusion process [180]. Other studies showed that EVs are taken up by endocytosis and fuse with endosomes. A possible explanation is that the uptake pathway depends on the interaction between surface molecules of EVs and receptors on recipient plasma membranes. Different origins of EVs and different kinds of recipient cells may lead to different interaction and internalisation modes [181]. Christianson et al. recently reported that the uptake of glioblastoma EVs is mediated by heparin sulfate proteoglycan receptors [175]. In addition, uptake of glioblastoma EVs was also reported to be blocked by heparin [182]. However, receptors for the attachment and internalization of glioma EVs expressed on GAMs and MDSCs remain to be identified.

Tumour-derived EVs are believed to induce immune suppression by hampering DC maturation, stimulating the activity of MDSCs and T_{reg} cells, inhibiting T-cells and NK cells and inducing apoptosis of effector T-cells [98,183,184]. Moreover, EVs secreted from myeloid cells in turn may promote the growth of tumour cells [98]. Various other immune cells such as B-cells, DCs, platelets, and macrophages also release EVs which mediate immune-modulatory effects [185]. Although there is increasing evidence for immune-modulatory tumour-derived EVs in cancer, little is known about the role of EVs in the cross-talk between glioma and immune cells and the resulting immunosuppressive effects. However, initial evidence suggests that EV-mediated immune suppression may also occur in gliomas [184]. De Vrij et al. revealed that peripheral blood monocytes from healthy donors adopted a suppressed phenotype with enhanced CD14 and decreased HLA-DR expression after incubation with glioblastoma-derived EVs [186]. Furthermore, glioma-derived EVs have been shown to inhibit T-cell proliferation *in vitro*, implicating EV-associated FasL as an inducer of T-cell apoptosis [187]. In addition, membrane-associated HSP72 on tumour EVs released by other tumour types has been found to induce STAT3 activation in MDSCs [188]. Recent findings imply that the uptake of glioma-derived EVs is dependent on ERK1/2-HSP27 signaling [176]. It remains, however, unclear if glioma EVs can also affect STAT3 activity in GAMs and/or MDSCs. EVs released by MDSCs have recently been reported to be involved in the M2 polarization of macrophages as well as in the chemotactic recruitment of MDSCs in mammary tumours [189]. Interestingly, the protein content of EVs released by MDSCs appears to be dependent on inflammatory factors during MDSC development [190]. In addition, tumour-associated macrophages have been shown to transfer miRNAs via EVs to breast cancer cells, promoting invasion [191].

Increasing evidence suggests an important role of glioma-derived miRNAs on immune regulation and the tumour microenvironment [192]. miRNAs might operate as signaling molecules which are either transported in EVs or as stable argonaute2-miRNA complexes [192]. miRNAs are believed to influence the tumour microenvironment by regulating tumor angiogenesis, immune invasion and tumour-stromal interactions [193]. Interestingly, several miRNAs have been reported to regulate STAT-3 and might thus influence the phenotype of immune cells in the tumour microenvironment [192]. EVs contain a plethora of miRNAs that may function in recipient cells. Katakowski et al. demonstrated that EVs of marrow stromal cells containing excess miR-146 reduced glioma growth in an *in vivo* rat model [194]. Recently, Bronisz et al. reported that the function of glioma cell-derived EVs depends on miR-1 expression [195]. Ectopic expression of miR-1 in glioblastoma cells blocked *in vivo* growth, neovascularization, and invasiveness, and the observed effects were associated with intercellular communication in the microenvironment mediated by EVs. In addition, the authors observed an EV-dependent phenotype defined by glioblastoma invasion, neurosphere growth, and endothelial tube formation, which was mitigated by loading miR-1 into glioblastoma-derived extracellular vesicles. However, further research is warranted on the dynamics of miRNAs transported via EVs [99,167,171,196–198].

In glioma, EVs can also be loaded with tumour-specific oncogenic RNAs and proteins such as EGFRvIII, a constitutively active form of the EFGR receptor that is expressed in a heterogeneous manner [167]. Importantly, EVs released from EGFRvIII-positive glioma cells can transfer EGFRvIII to glioma cells that are lacking EGFRvIII expression [100]. Thus, glioma EVs can potentially mediate the expansion of oncogenic products amongst different tumour cells [100]. It remains to be clarified if this transfer of oncogene activity also occurs from glioma cells to immune cells [199]. Theoretically this could lead to tumour-associated antigen expression in immune suppressive antigen-presenting cells, thereby contributing to T-cell tolerance.

An emerging concept based on EV-mediated interaction between glioma cells and immune cells is that of "organ teaching". Peinado et al. previously formulated this concept for melanomas [200]. A mechanism was presented through which tumour cells via EVs educate bone marrow progenitors and myeloid cells [199,201]. First, tumour-derived factors and EVs may recruit immunosuppressive myeloid cells to the tumour microenvironment in order to locally enhance tumour growth and invasive behavior [199]. Moreover, myeloid cells in the bone

marrow may be educated through tumour-derived EVs resulting in cells that promote tumour growth and invasiveness. Tumour-derived EVs may further stimulate these skewed myeloid cells in the bone marrow to migrate to the primary tumour site as well as to sites of local and distant invasive tumour expansion [199]. Thus, myeloid cells may contribute to the creation of suitable tumour niches, even before tumour cells migrate to these sites [199]. This hypothesis is supported by previous findings which revealed that a variety of tissue-specific EVs are able to alter the phenotype of bone marrow cells and thus might influence their behavior [202,203]. This process was considered to be realized by the direct transfer of EV mRNA which results in a long-term stable change in the transcriptional profile of bone marrow cells [202]. Nevertheless, Li et al. reported effects of glioma EVs on endothelial cells that could not only be explained by the direct transfer of transcripts [198], indicating that EVs can mediate their function through the concerted action of their collective components.

Previously it was demonstrated that glioma-derived EVs are efficiently taken up by blood platelets, thereby transferring RNA and proteins from glioma cells to platelets [204,205]. Platelets isolated from the blood of glioma patients contain tumour-associated RNA such as EGFRvIII [204]. The function of most tumour-associated RNAs and proteins taken up by platelets remains unclear, although Bastida et al. reported that glioma EVs contain tissue factor that can cause aggregation and coagulation of platelets in *in vitro* assays [205]. It was reported that platelets can directly interact with tumour cells [206], and various types of immune cells [207], and that they have a function in tumour growth and immune modulation [208,209].

Evidence suggests that platelets also release EVs that regulate inflammatory gene expression in monocytes [207]. Platelet-derived EVs may therefore represent another modulator of monocyte differentiation. Moreover, platelets have been suggested as a potential source of cancer-specific protein biomarkers [210], which may allow the recruitment of GAMs and MDSCs to sites of tumour neovascularisation [211]. Platelets secrete chemokines and factors such as CCL2, CCL7, and SDF-1, which are known to induce recruitment of macrophages, MDSCs and/or bone marrow-derived precursor cells [208,212]. Studies with luminal breast cancer also propose an important role of platelets in the establishment of a tumour-promoting environment [213]. Also, ovarian carcinoma cells were reported to secrete IL-6, leading to the paraneoplastic accumulation of platelets promoting tumour growth [214]. Of note, MMP-2 activity in human glioma cells is reported to result in the release of significant amounts of IL-6, but also IL-8, IL-10, GM-CSF, TNF-α, angiogenin, VEGF, and PDGF-BB [215], most of which have also been identified in glioma-derived EVs [167]. Furthermore, it was reported that platelets can efficiently release pro-tumoural EVs themselves, thereby supporting tumour metastasis and angiogenesis [216], and possibly allowing for a blood-based distribution network of (tumour-derived) RNA and proteins [208].

Platelets were reported to suppress activated microglia via release of serotonin acting as a radical scavenger [217], and to activate neural stem cells (reported as "oligodendroglial precursor cells") [218]. Although the effect of platelets on glioma stem-like cells is as yet unclear, cytokines released by platelets have the ability to induce proliferation and migration of glioma and endothelial cells *in vitro* [219]. The effects of platelet depletion on glioma growth in orthotopic glioma mouse models *in vivo* remains controversial, with no beneficial effects of platelet loss noted [219]. However, in 153 glioblastoma patients preoperative thrombocytosis (>400 platelets/nl blood) was described as a significant prognostic factor associated with shorter survival [220]. In addition, in 84 glioblastoma patients receiving radiotherapy and concomitant and adjuvant temozolomide a decrease in platelet count from baseline to week 6 significantly correlated with prolonged survival [221]. These findings suggest that platelets exert a pro-tumoural effect in glioma patients. Altogether these results indicate that investigation of the role of glioblastoma-derived EVs alone might not be sufficient, and that the effect of platelet-derived proteins and EVs should be taken into account as well. The role of tumour EVs and platelets on the recruitment of GAMs and MDSCs warrants further investigation (Fig. 5).

5. Dynamics of the glioma cell–myeloid immune cell interaction

As myeloid immune cells can suppress immune responses and furthermore promote tumour progression, it is of interest to address whether these cells are already involved in the early phases of gliomagenesis. Most studies reflect a later developmental tumour stage, at which immune cells are already present and immune suppression is established, and it remains to be investigated to what extent immune cells can also contribute to tumourigenesis. Kennedy et al. studied the dynamics of late and early interaction of tumour and immune cells by employing a murine glioma model. This report described a relatively late occurrence of GAMs in the glioma microenvironment [222]. Furthermore, the authors suggested that early accumulation of T_{reg} cells could cause subsequent activation of M2 GAMs. With regard to the potential efficacy of glioma immunotherapy, the late activation and accumulation of GAMs in the glioma microenvironment may point to a potential immunotherapeutic window preceding GAM-mediated immune suppression. It has been shown *in vitro* that microglia are present in an activated state and possess phagocytic properties after one hour of co-culture with glioma cells [223]. After prolonged contact with glioma cells microglia seem to lose their phagocytic properties. Moreover, it was reported that MDSCs associated with advanced stage disease show stronger suppressive properties than MDSCs occurring early in murine breast cancer models [224]. Down-regulation of transcription of immune response-related genes as well as an up-regulation of tumour-promoting genes in MDSCs was detected at a late time point of tumourigenesis. In this study, FKBP51 - a cis-trans prolylisomerase that binds to the immunosuppressant FK506 and rapamycin - was identified to play an important role in the regulation of the suppressive functions of MDSCs [224].

Another potential inducer of glioma immune suppression is hypoxia. Hypoxia alters the expression of cytokines and co-stimulatory molecules in tumour-associated immune cells [225]. In addition, hypoxia induces changes in tumour cells and vascular cells that can indirectly result in the recruitment of immunosuppressive, tumour-promoting immune cells [225]. Hypoxia has been found to dramatically alter the function of MDSCs in the tumour microenvironment. Indeed, the hypoxia-related transcription factor HIF-1α has been identified to direct the differentiation towards tumour associated macrophages and their switch to non-specific suppressor cells [226]. In addition, MDSC functionality in the tumour microenvironment is believed to be mediated through the up-regulation of arginase I and inducible iNO synthase as well as the down-regulation of NADPH oxidase and reactive oxygen species [226].

6. Consequences for therapy

It was previously shown that tumour cell death triggered by TMZ and RT initiates an immune adjuvant pathway that contributes to the success of cytotoxic treatment. Numerous endogenous danger signals transferred by dying tumor cells to innate immune effectors may account for the immunogenicity of tumour cell death, the exact mechanisms of such transfer in glioblastoma patients remain unclear. Fadul et al examined the phenotype and function of peripheral blood mononuclear cells in 25 glioblastoma patients prior to and 4 weeks after treatment with RT-TMZ, indicating that treatment with RT-TMZ is associated with changes in regulatory and effector peripheral blood mononuclear cells that tilt the balance towards an immune suppressive state [227]. Kioi et al. nicely demonstrated that irradiation induces recruitment of bone marrow-derived cells into the tumors in an HIF-1-dependent manner, restoring the radiation-damaged vasculature by vasculogenesis and thereby allowing the growth of surviving tumour cells [228]. Blocking of the HIF-1-dependent stromal cell-

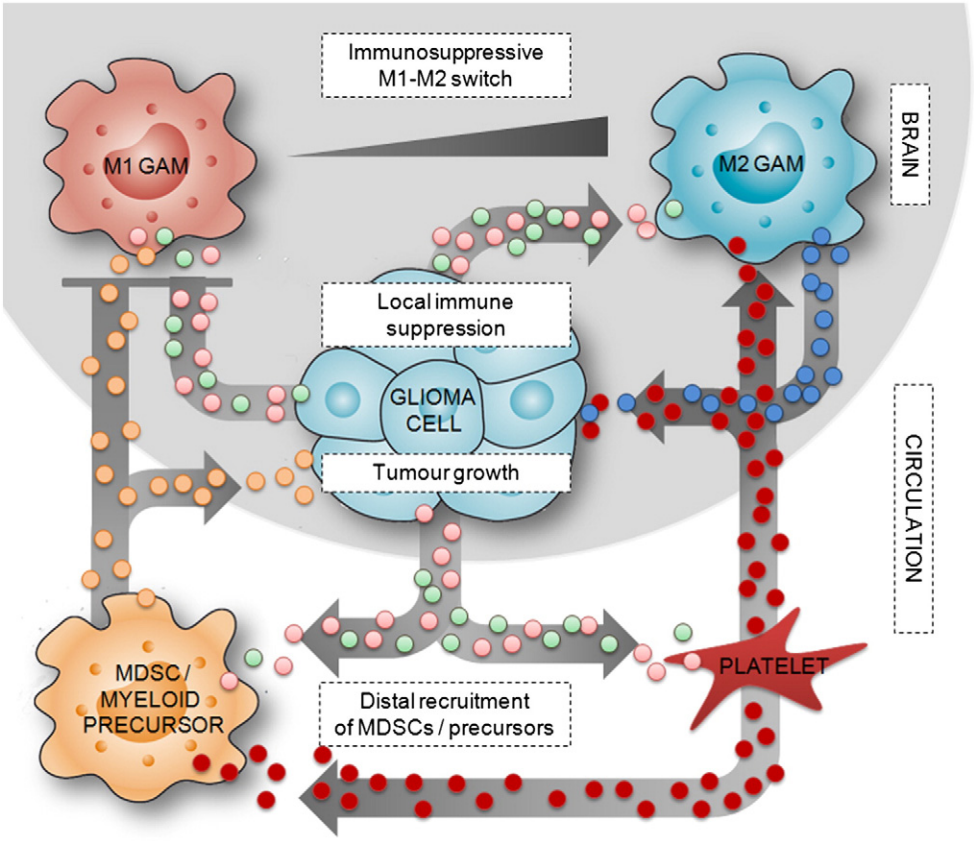

Fig. 5. Schematic representation of the EV-mediated cross-talk hypothesis between tumour cells, platelets, and the immune system. Glioma cells and myeloid-derived immune cells in the brain release individual immunomodulatory proteins as well as EVs (indicated by colored circles) that can collectively transfer immunomodulatory RNA and proteins to local and distal recipient cells, including glioma cells and immune cells. EVs may cause recruitment of myeloid precursor cells and MDSCs and result in an immunosuppressive M1-M2 switch in GAMs. Glioma and immune cell-derived EVs and soluble proteins that reach the circulation can be acquired by circulating blood platelets, and in turn may release EVs and proteins that contribute to immune suppression and tumour growth. The different colored circles indicate EVs with different cells of origin. Pink/green = tumour; red = platelet; yellow = MDSC/myeloid precursor; blue = GAM. Arrows indicate the flows of EVs with stimulating effects whereas blocking bars indicate negative EV regulation.

derived factor-1 (SDF-1) and its receptor, CXCR4, prevented the influx of BMDCs, primarily CD11b + myelomonocytes, and the post-irradiation development of functional tumor vasculature, resulting in abrogation of tumor regrowth. Similar results were found using neutralizing antibodies against CXCR4, or perhaps by using the clinically approved drug AMD3100, a small molecule inhibitor of SDF-1/CXCR4 interactions.

Bevacizumab is an anti-VEGF agent that has been recently added to the treatment options of glioblastoma patients and has demonstrated favourable effects on progression free survival. Although two recently published trials that combined bevacizumab with RT/TMZ show significant prolongation of progression free survival, no effect on overall survival could be confirmed on (non-subclassified) glioblastoma [229–231]. Recently, Gabrusiewicz et al. demonstrated evidence for the accumulation of Tie2-expressing monocytes in brain tumours of mice treated with anti-VEGF therapies in regions with increased tumoral invasion [232]. The monocytes enhanced the invasive properties of glioma cells and secreted high levels of gelatinase enzymatic proteins, and $Tie2^{+}MMP9^{+}$ monocytic cells were consistently detected in the invasive tumor edge upon anti-VEGF therapies. Moreover, they noted the presence of monocytes in surgical glioma specimens taken from tumours that recurred after bevacizumab treatment. These results suggest the presence of a specific myeloid/monocytic subpopulation that plays an important role in the mechanism of escape of malignant gliomas from anti-VEGF therapies and therefore constitutes a new cellular target for combination therapies in patients selected for anti-angiogenesis treatment. Hence, one of the reasons of the suboptimal effects of bevacizumab may be the supportive influence of GAMs on vasculogenesis and on glioma cell invasion in response to anti-angiogenic therapy. Additional effects of bevacizumab on the cross-talk of GAMs and MDSCs remain to be investigated and further research is warranted.

Another drug that is frequently used in glioma patients to suppress oedema formation is dexamethasone, with significant effects on monocytes. Gustafson et al. identified changes in systemic immunity associated with dexamethasone treated patients and identified unique dexamethasone dependent altered monocyte phenotypes [233]. The major population of altered monocytes had a phenotype consistent with the phenotype reported for monocytic MDSC (i.e. $CD14^{+}HLA\text{-}DR^{lo/neg}$). These cells inhibited T cell proliferation, were unable to fully differentiate into mature dendritic cells, were associated with dexamethasone-mediated changes in CCL2 levels, and could be re-created in vitro using tumor supernatants.

Immunotherapeutic approaches to the treatment of glioma have been studied for many decades [7]. However, cross-talk between glioma cells and immune cells and the consequent immunosuppressive glioma environment are likely to antagonize and impair the effects of glioma immunotherapy. In order to develop effective strategies that pass beyond phase I/II clinical trials, immunosuppressive activities and tumour-promoting effects of immune cells have to be overcome [234]. Future approaches combining conventional therapies and immunotherapy of glioma may benefit from the blockade of the development and functionality of immunosuppressive GAMs and MDSCs. A variety of targets have been identified, that can potentially inhibit the differentiation and/or functionality of myeloid-derived cells with immunosuppressive activities. These targets include STAT3, p38 MAPK, COX-2, CCL2, TGF-β1, IL-4, ATP, and CSF-1R [47].

Importantly, STAT3 is a key mediator for alternative polarization of GAMs and MDSCs and may therefore represent a promising therapeutic target [69]. STAT3 inhibition has been reported to activate macrophages in the glioma environment and decrease glioma growth [101]. Moreover, oleanolic and corosolic acids have been found to inhibit STAT3 expression in glioma-associated macrophages and thereby suppress their polarization towards an M2 phenotype [235,236]. Likewise, STAT3 inhibition has been shown to enhance the efficacy of adoptive T-cell transfer therapy in a murine glioma model [237]. We previously reported that combination of STAT3 and p38 MAPK inhibition can overcome glioma-mediated immune suppression which interferes with DC differentiation and activation [141]. In *ex vivo* cultures of metastatic melanoma this combination treatment resulted in an increased ability of DCs to induce a T_{h1} effector response [141]. Hence, STAT3 and/or p38 MAPK inhibition in combination with glioma immunotherapy may represent a promising approach to reverse immune suppression and elicit an effective anti-glioma immune response.

Interestingly, COX-2 inhibition has been shown to delay glioma development by blocking the systemic development of MDSCs as well as their CCL2-mediated recruitment to the glioma microenvironment [146,147]. A similar strategy addressed the inhibition of CCL2 in combination with temozolomide chemotherapy [238]. This approach resulted in a significant increase in survival compared to temozolomide treatment alone in a murine glioma model. CCL2 inhibition was suggested to block GAM and MDSC infiltration into the glioma microenvironment [238]. Surprisingly, M2 associated markers such as CD163, CD204, and CD206 have also been detected on the surface of glioblastoma cells and targeting these markers could hence result in a direct inhibition of these tumour cells as well as of immunosuppressive M2 GAMs [239].

A promising reported approach to overcome the influence of glioma-derived soluble factors on immune cells is the combination of CD137/4-1BB stimulation and p38 MAPK inhibition in addition to tumour-lysate-pulsed DCs [240]. This strategy enabled enhanced immune stimulation in an *in vitro* model for glioma immunotherapy. As an alternative to tumour lysate, Mahaweni et al. recently reported the use of glioma EVs as antigen delivery carriers in DC-based immunotherapy in preclinical glioma mouse models, and reported increased survival of mice after treatment with tumour EV-pulsed DCs as compared to tumour-lysate-pulsed DCs [241]. Stimulatory antibodies against CD137/4-1BB on T-cells induce T-cell proliferation and cytokine production, and enhance survival of $CD8^+$ effector T-cells [242,243], while p38 MAPK inhibition counteracts the secretion of pro-inflammatory cytokines by microglia and glioma cells, resulting in the inhibition of T_{reg} cells and CTL apoptosis and affecting T_{h1} polarization [240,244,245] - and in addition may alleviate DC suppression [141].

As glioma and platelet EVs can possibly play an important role in the cross-talk between myeloid and glioma cells, the inhibition of EV-mediated interactions may potentiate the effect of glioma immunotherapy. Inhibition of highly expressed mediators of membrane trafficking in tumour-derived EVs may impair EV production, and thereby hamper detrimental EV-mediated cross-talk between tumour cells and immune cells [200]. Furthermore, uptake of glioma-derived EVs has been found to be dependent on ERK1/2-HSP27 signaling and may be hampered by modulating the expression of Caveolin-1 and ERK1/2 signaling pathways [176]. Increasing evidence for EV-mediated cross-talk between tumour cells, platelets, and immune system supports the relevance of studying these interactions in gliomas in order to enhance efficacy of glioma immunotherapy.

7. Conclusion and future directions

GAMs and MDSCs play an important role in the formation of an immunosuppressive glioma microenvironment, and likely promote progression of tumour growth in glioma patients. Inhibiting the local and long-distance cross-talk between glioma cells and GAMs and MDSCs may enhance immunotherapeutic efficacy and improve clinical benefit. Mounting evidence points to a role for EVs and platelets in the cross-talk between tumour and myeloid cells in the generation of an immunosuppressive niche. Glioma-myeloid cross-talk thus represents a potential target for therapeutic interference. More research into the underlying mechanisms of this cross-talk is warranted in order to identify and validate molecular targets for more effective next-generation immunotherapeutic modalities.

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