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REVIEW ARTICLE

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The roles of glioma-associated macrophages/microglia and potential targets for anti-glioma therapy

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ABSTRACT

Glioblastoma (GBM) is the central nervous system tumor with the most aggressive behavior, and no definitive therapy has yet been found. The tumor microenvironment of GBM is immunosuppressive and is considered a 'cold tumor' with low lymphocytic infiltration, but is characterized by a high proportion of glioma-associated macrophages/microglia (GAMs). GAMs promote tumor growth and also affect treatment resistance in GBM. In this review, we describe the origin and classification of GAMs in humans and describe the mechanisms of their activation and the cell-cell interactions between tumor cells and GAMs. We also describe the history of GAM detection methods, especially immunohistochemistry, and discusses the merits and limitations of these techniques. In addition, we summarized chemotactic factors for GAMs and the therapies targeting these factors. Recent single-cell RNA analysis and spatial analysis add new insights to our previous knowledge of GAMs. Based on these studies, GBM therapies targeting GAMs are expected to be further developed.

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KEYWORDS
Tumor-associated macrophage (TAM); microglia; brain tumor; glioblastoma

1. Introduction

The progression and acquisition of resistance to treatment in malignant tumors are intricately linked not only to genetic alterations within the tumor cells but also to a pro-tumor microenvironment. Glioblastoma (GBM), the most aggressive of all brain tumors, comprises a complex milieu of tumor and non-tumor cells, including numerous immune cells and vascular endothelial cells [1]. The tumor microenvironment (TME) of GBM is immunologically characterized as a 'cold tumor' due to its low lymphocytic infiltration, the absence of cancer-associated fibroblasts, and the distinct morphology of vascular endothelial cells that contribute to the blood-brain barrier (BBB) [2, 3]. Owing to these attributes, efficacious evidence-based treatments for GBM are exceedingly scarce, and the prognosis remains dismal. It is established that a relatively high proportion of macrophages/microglia exists within the TME of GBM [3, 4], highlighting their significance in the pathologic analysis and therapeutic advancement of this condition. This review elucidates the role of glioma-associated macrophages/microglia (GAMs) in brain tumors, predominantly GBM. It encompasses their origins, the evolution of research to date, and their potential as therapeutic targets, considering recent scholarly works. While numerous reviews on GAM exist [5–8], their content often amalgamates human and animal model studies. Given the notable disparities in the phenotypes and roles between human and murine macrophages [9], it is imperative to evaluate them based on independent criteria. Consequently, this review predominantly concentrates on GAM in human samples.

2. The origin of macrophages/microglia in human brain

Tissue-resident macrophages in the central nervous system are categorized into microglia, perivascular, meningeal, and choroid-plexus macrophages, according to their distribution [10]. These cells are derived from the yolk sac except for some choroid-plexus macrophages, and migrate to the brain during embryonic development and sustain themselves through prolonged self-renewal [10]. They differ from circulating monocytes and may alter under

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pathological conditions such as tumors or inflammation. Under physiological conditions, microglia are pivotal in maintaining brain homeostasis and the innate immune defense against pathogens [11]. Furthermore, they are crucial for brain development, influencing neurons and glia, and contributing to synaptic maturation and brain circuitry [12]. However, within the TME of GBM, microglia also foster tumor progression by supporting tumor growth [13].

3. GAM detection in human glioma

Historically, the IgGEAC assay, which employs enzyme-treated tumor samples, was utilized to detect and quantify GAM infiltration in human GBM. In this assay, IgG-coated erythrocytes bind to cells expressing Fc and C3 receptors when incubated with tumor homogenates. It has been reported that GAMs constitute approximately 45% of the cellular content in GBM [14]. In addition, lectin histochemistry, a method of binding lectins to specific sugar chains and observing cellular distribution, has been used [15]. However, this technique tends to overestimate GAM infiltration as lectins also bind non-specifically to vascular endothelial cells apart from GAMs.

Subsequently, immunohistochemistry (IHC) has been adopted for the identification of specific proteins and cells within samples. Roggendorf et al. proposed classifying GAMs into four categories based on macrophage markers such as CD68, MHC class II, and CD11c [16]. This classification retains relevance as activated microglia at tumor margins are flat with elongated processes, whereas macrophages in the tumor core are relatively rounded with fewer processes (Figure 1). Recent advancements in intravital two-photon microscopy have further elucidated the morphological distinctions between macrophages and microglia in GBM tumors [17].

IHC techniques now permit the precise identification of macrophages within tumor tissues. CD68 and Iba-1 have been widely used as pan-macrophage/microglia markers, whereas scavenger receptors such as CD163 and CD204 are known to react to specific subpopulations of macrophage/microglia. A study investigating the GAM subpopulation using these markers indicated that high ratio of CD163-positivity in GAM correlates with poor prognosis in GBM patients [18]. When comparing GBM with normal brain, low-grade glioma, and high-grade glioma, an incremental increase in CD163-expressing GAM infiltration with rising malignancy grades suggests a tumor-promoting role for CD163-expressing GAM (Figure 2). It is noteworthy that the utility of each marker varies by organ and species; for instance, CD206 serves as a functional protumor macrophage marker in non-brain organs (e.g. breast cancer) but is seldom detected in human GBM. Conversely, CD206 is a practical macrophage marker in murine glioma. Other valuable markers include Iba1 and CD68 for pan-macrophage identification, and MHC class I/II and CD86 for inflammatory phenotype of GAM. Various markers for microglia have been proposed [6, 19–21], and TMEM119 in combination with CD11b and CD45 (high CD11b and low CD45 in microglia, high CD11b and high CD45 in non-microglia) are considered relatively useful, but their merits are controversial and no specific marker has yet been found.

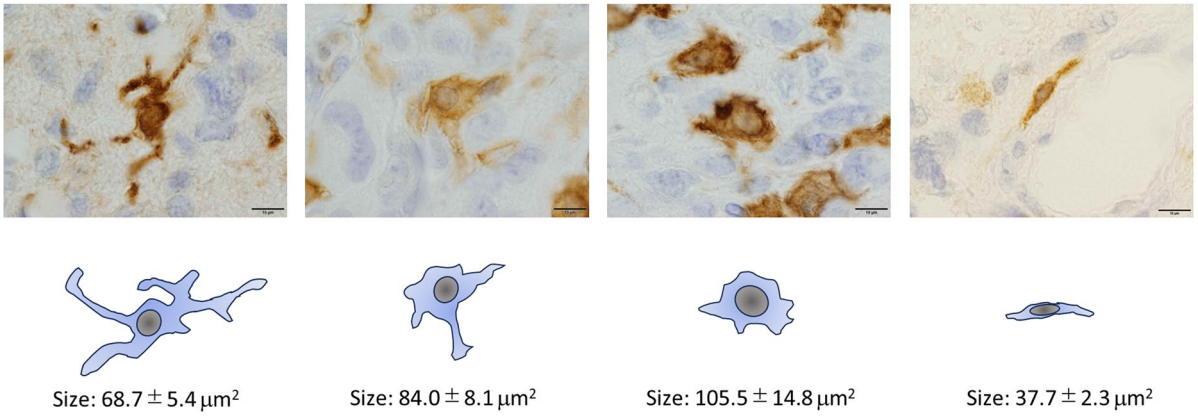
4. Chemotactic factors for GAM infiltration

MCP-1/CCL2 is one of the most renowned chemotactic factors associated with monocyte infiltration. The seminal study suggesting a correlation between GBM and macrophages was conducted by Yoshimura et al. who demonstrated that GBM cell lines produce monocyte/macrophage migration factors and identified MCP-1/CCL2 [22]. Subsequent analyses of GAM in human GBM revealed that higher MCP-1 expression correlates with more frequent GAM infiltration, which also contributes to angiogenesis [23–26]. Recent in silico studies have indicated that immune checkpoint genes such as PD-L1 and TIM3 are upregulated in cases with elevated MCP-1 expression, which is significantly associated with a poorer clinical trajectory and higher WHO grades in glioma [27].

M-CSF, also known as colony stimulating factor 1, is a pivotal factor for the survival, proliferation, and differentiation of myeloid cells. M-CSFR, or colony stimulating factor 1 receptor, functions not only as a receptor in myeloid cells but also as a proto-oncogene in various tumor types [28]. Co-expression of M-CSF and M-CSFR in GBM cell lines and surgical specimens has been observed and is posited to contribute to GBM growth *via* autocrine and paracrine mechanisms [29]. M-CSF expression correlates positively with higher glioma grades and is more closely associated with the ratio of CD163 positivity in GAM than with the density of GAM [18]. Consequently, M-CSF and M-CSFR signaling are considered crucial in GBM progression, and inhibition of M-CSFR is viewed as a promising therapeutic approach against GBM, as detailed in another section of this article.

Vascular endothelial growth factor A (VEGF-A) plays a critical role in GBM angiogenesis; it suppresses antitumor immunity by inhibiting dendritic cell maturation and fostering regulatory T cell

A



B

Human GAM markers

Ramified Microglia	Avtivated Microglia	Macrophages	Perivascular cells
Iba1 (+++)	Iba1 (+++)	Iba1 (+++)	Iba1 (+++)
CD68 (+++)	CD68 (+++)	CD68 (+〜+++)	CD68 (+++)
CD163 (+)	CD163 (+++)	CD163 (+++)	CD163 (+)
CD169 (-)	CD169 (-)	CD169 (-)	CD169 (-)
CD204 (+)	CD204 (+++)	CD204 (+++)	CD204 (+)
CD206 (±)	CD206 (±)	CD206 (±)	CD206 (±)

Mouse GAM markers

Ramified Microglia	Avtivated Microglia	Macrophages	Perivascular cells
Iba1 (+++)	Iba1 (+++)	Iba1 (+++)	Iba1 (+++)
CD68 (+++)	CD68 (+++)	CD68 (+〜+++)	CD68 (+++)
CD163 (+)	CD163 (+)	CD163 (±)	CD163 (±)
CD204 (+)	CD204 (+++)	CD204 (+++)	CD204 (+)
CD206 (++)	CD206 (+++)	CD206 (+++)	CD206 (++)
F4/80 (±)	F4/80 (+〜++)	F4/80 (+〜++)	F4/80 (±)

Figure 1. (A) Classification of GAMs in GBM proposed by Roggendorf et al. [16]. Ramified microglia exist mainly at tumor margins and has flat cell body with elongated processes. Activated microglia and Macrophages exist in the tumor core. The former has relatively rounded cell body with several short processes and the latter has rounded cell body with fewer processes. Perivascular cells, which has small and flat cell body, can found around vascular lumen. (B) Tables representing the degree of expression of GAM markers in human and mouse.

proliferation [30, 31]. VEGF-A is also known to exhibit chemotactic properties toward macrophages [26]. Bevacizumab, an anti-VEGF antibody, has been reported to normalize vascular structures, reduce tumor vascular density, and enhance tumor oxygenation [32]. Bevacizumab is extensively utilized in the management of recurrent high-grade gliomas, noted for extending progression-free survival, though it does not necessarily impact overall survival [33, 34]. The presence of increased VEGF-A in hypoxic areas influences GAM chemotaxis within these regions [35]. A reduction in GAM infiltration and an increase in T-cell infiltration have been noted in GBM cases treated with bevacizumab [36]. Given the impact of VEGF-A on GAM, the efficacy of bevacizumab is anticipated to improve when used in conjunction with other therapeutic modalities in the future.

5. The phenotypic activation of GAM in human glioma

Macrophages/microglia typically function to remove dead cells and foreign substances. However, GAM exhibits an atypical activated state. They facilitate GBM growth by expressing and releasing a variety of angiogenic factors, growth factors, and immunosuppressive factors, driven by the invasion and activation

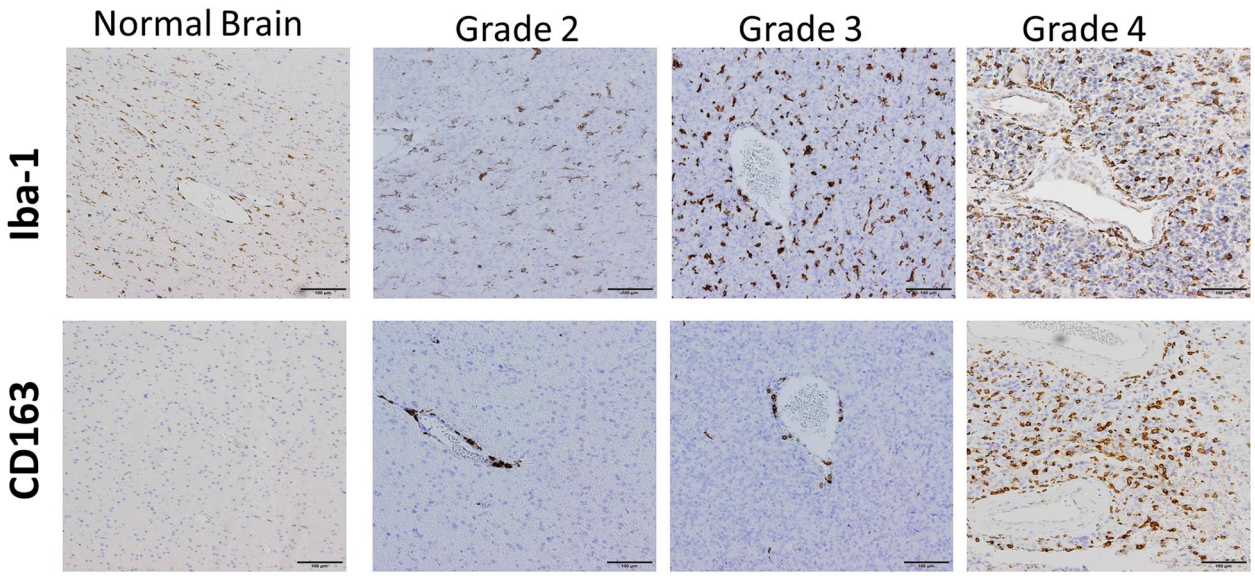


Figure 2. Expression of macrophage markers by WHO grade of human gliomas. Iba-1 is a pan-macrophage/microglia marker and CD163 is one of macrophage-specific antigen related to protumor activation. The infiltration of GAM increases according to WHO grade, whereas significant elevation was observed in the density of CD163-positive GAM.

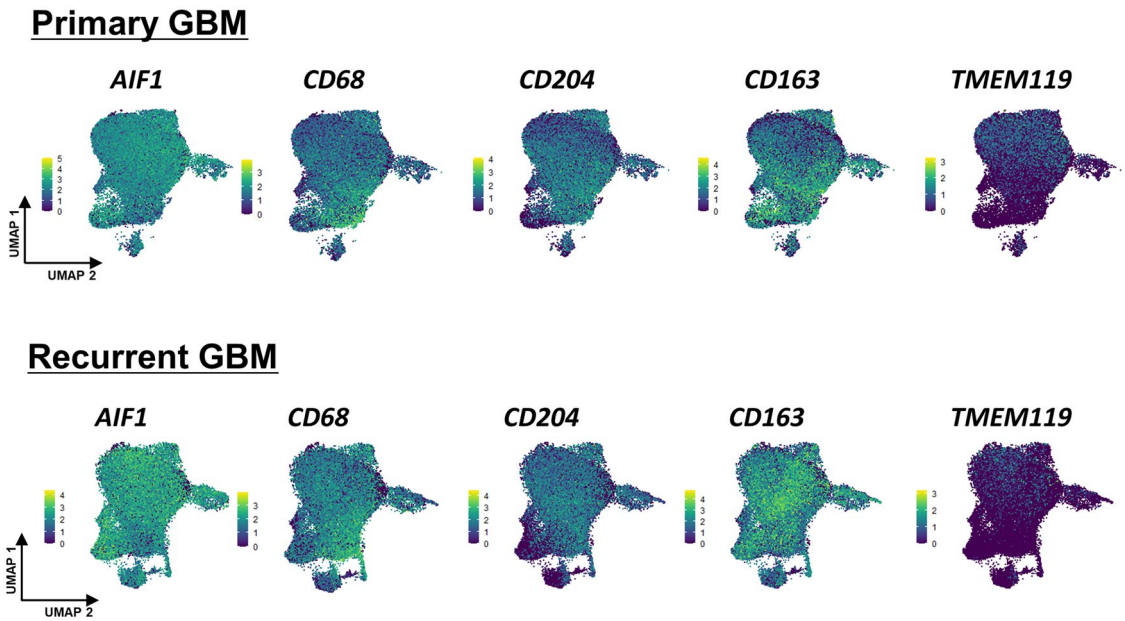


Figure 3. The UMAP plots of the expression of the indicated genes in the myeloid lineage cells (Iba-1 high expressing) in primary ($n = 51{,}035$ cells) or recurrent ($n = 38{,}662$ cells) glioblastoma tissues.

in cancer tissues under the influence of diverse factors produced by GBM cells. The activation state of monocyte-derived macrophages has been bifurcated into two primary categories: those stimulated by TLR ligands or IFN-γ produced by Th1 cells, which induce inflammation (classical activation, M1-polarization), and those stimulated by IL-4 or IL-13 produced by Th2 cells, which suppress inflammation (alternative activation, M2-polarization). However, distinguishing clearly between M1 and M2 phenotypes is challenging, as macrophages exist in various stages of activation. The M1/M2 paradigm, initially proposed for mouse bone marrow-derived macrophages, does not readily translate to human macrophages. Consequently, the use of 'M1-like' and 'M2-like' designations is recommended wherever possible [37]. Predominantly, M2-like macrophages/microglia are engaged in angiogenesis and immunosuppression, and GAMs in GBM are generally considered to be 'M2-like'. Recent single-cell RNA-sequence analyses have suggested that macrophages within the TME can be categorized into tissue-resident and monocyte-derived groups rather than M1 and M2 [38,39] (Figure 3). Thus, the binary M1/M2 classification appears inadequate for GAM studies. Given that CD163 and CD204 do not mark

TREM119-expressing microglia, these markers may be suitable for identifying monocyte-derived GAMs in GBM.

6. Cell-to-cell interaction between GAM and GBM cell

Research on cell-to-cell interactions between GBM cell and GAM has expanded over the past decade. Our previous studies indicated that patients with higher levels of CD163 in GAM exhibited increased tumor cell proliferation rates and poorer prognoses [18, 40]. *In vitro* experiments have shown that direct co-culture of M2-like macrophages with GBM cells promotes cell growth *via* STAT3 activation, underscoring the significance of direct interactions between GBM cells and GAMs in human GBM. GAM secreted several growth factors include IL-1β, IL-10, and M-CSF [4, 41]. The interaction between membrane type M-CSF on GBM cells and M-CSFR on GAMs is showed to trigger potent pro-tumor activation of TAMs [40]. Membrane type M-CSF induces a more robust activation of M-CSFR compared to its soluble form, although the specific mechanisms remain elusive [40]. Furthermore, a significant number of oligodendrocyte and GAM are located at the interface between GBM and normal brain regions, where cytokines produced by these cells are known to promote stemness in glioblastoma cells [42]. GAM-derived factors significantly influence the proliferation of oligodendrocyte progenitor cells (OPCs), subsequently enhancing tumor growth and chemoresistance in GBM. Factors such as FGF-1 and EGF from OPCs, along with HB-EGF and IL-1β from GAMs, are implicated in creating a niche at the tumor border that influences the stemness of GBM cells. Therefore, GAM exerts a profound impact on tumor growth in GBM, and further investigations into their functionality are anticipated.

7. GAM is a promising target for anti-glioma therapy

Macrophages infiltrating the TME exhibit pro-tumor functions by promoting tumor cell growth, resistance to chemotherapy and radiotherapy, metastasis, invasion, neovascularization, and immune suppression. Consequently, inhibiting macrophage chemotaxis and their differentiation into a pro-tumor phenotype is considered a promising approach for various types of tumors [43]. Inhibition of M-CSF, a prevalent GAM migration factor, has been identified as a significant therapeutic target for GBM in numerous studies. Mouse experiments have demonstrated that inhibition of M-CSFR extends overall survival and diminishes tumor volume by reducing M2-like GAMs within the tumors. However, tumor recurrence was observed in over half of the mice, indicating a recovery in M-CSFR sensitivity over time [44, 45]. Moreover, the anti-tumor effect was enhanced when M-CSFR inhibition was combined with radiation therapy [46]. Nonsteroidal anti-inflammatory drugs have been shown to elicit anti-glioma immune responses by preventing the production of prostaglandin E_2 from myeloid cells [47]. Inhibition of the glycolytic pathway in GBM cells through a lactate dehydrogenase inhibitor also blocks the extracellular signal-regulated kinase pathway, thereby preventing the activation of CCL2 and CCL7 and the subsequent recruitment of GAMs to the TME [48]. Since GBM is characterized as a 'cold tumor' with sparse lymphocytic presence, one novel therapeutic strategy involves alleviating the immunosuppressive state within the tumor to attract T cells or to activate them. Pant et al. reported that the co-inhibition of CCR2 and CCR5 in transplanted GBM mice decreased infiltration of myeloid-derived suppressor cells into the tumor and increased CD8-positive T cells, thereby enhancing the effectiveness of immune checkpoint inhibition therapy [49]. It has also been reported that inhibiting the JAK/STAT pathway and the vascular endothelial growth factor receptor (VEGFR) is effective in transforming 'cold tumors' into 'hot tumors' [4, 50]. Immune checkpoint inhibitors, previously deemed less effective in GBM, may warrant reevaluation of their utility when used in conjunction with these therapies. Recently, anti-CD47 antibodies have gained attention as a novel immunotherapeutic option because they enhance the phagocytic activity of tumor cells by GAMs [51]. Although the efficacy of these new therapies has been proven in animal models, their effectiveness in human clinical settings remains insufficient. It is important to acknowledge that macrophages, unlike other immune cells, exhibit significant differences between humans and mice in their clinical applications.

8. Findings from recent GAM research

Recent studies employing single-cell RNA sequencing analysis in cancers of various organs have demonstrated that macrophages within tumors segregate into distinct clusters from those of tissue-derived and monocyte-derived origins, with monocyte-derived macrophages exhibiting more pronounced pro-tumor activities [38, 39]. This characterization of monocyte-derived macrophages aligns with what was previously identified as M2-like GAMs, with markers such as CD163 and CD204 being expressed on monocyte-derived

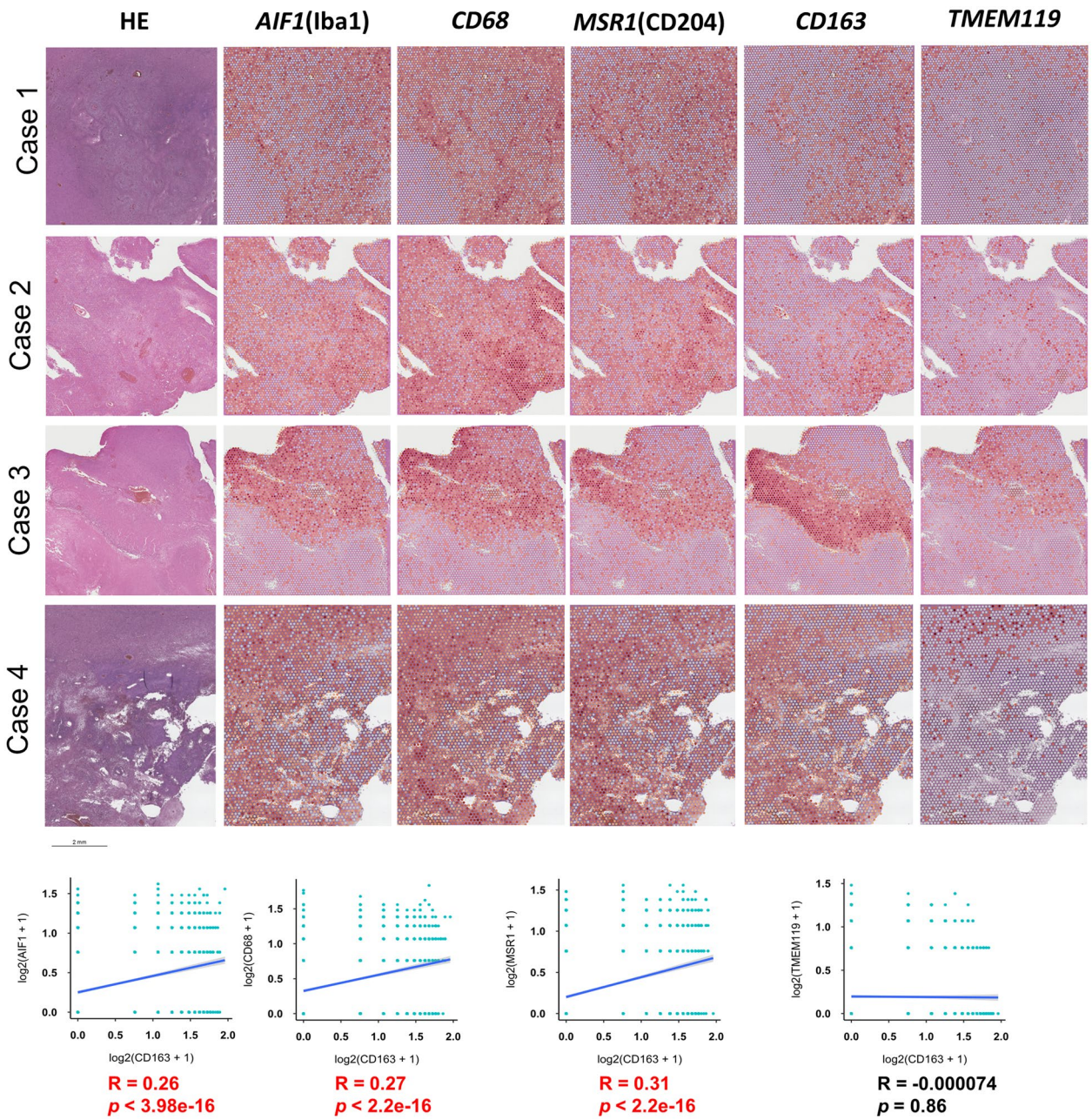


Figure 4. Spatial transcriptomics (10× Genomics Visium) of human GBM showing the distribution of each gene expression. TMEM119 is microglial marker and the others are macrophage markers. The distribution of CD163 correlates with AIF-1(Iba-1), CD68, MSR1 (CD204), but does not correlate with TMEM119.

macrophages in glioblastomas. Single-cell RNA analysis of the GBM public dataset al.so indicated that CD163 and CD204 expression distinctly clusters with tissue-resident microglia, corroborating these prior findings (Figure 3). Spatial analysis, which allows for the examination of gene expression while preserving the morphology of tumor tissues, proves invaluable for deciphering the tumor microenvironment in GBM. Data from 10× Genomics Visium underscored that CD163 and CD204 do not coincide with the microglial marker TMEM119 in human GBM (Figure 4). Iba-1(AIF1) was expressed both in TMEM119-positive resident microglia and TMEM119-negative monocyte-derived macrophages, and Iba-1(AIF1) is considered to be a marker for myeloid lineage cells. Although Iba-1(AIF1) at first described as a marker for microglia in previous study [52], recent studies described that Iba-1(AIF1) expression was seen in macrophages and other myeloid lineage cells such as dendritic cells [38, 39].

Recently, changes in the TME composition in recurrent GBM has also focused. In such cases, an increased proportion of GAMs within the tumor significantly influences tumor growth and the development of therapeutic resistance. These increased levels of GAMs did not correlate with other genomic abnormalities nor were they observed in IDH-mutant astrocytoma [53]. Moreover, radiotherapy for recurrent GBM is known to convert tissue-resident

microglia to monocyte-derived macrophages in the TME [46], suggesting that GAM-targeted therapies might also aid in preventing the recurrence of GBM. On the other hand, these studies on GAMs still have little affinity with the genetic diagnosis of GBM. The relationship between genetic mutations and the tumor microenvironment is an issue to be investigated in the future.

9. Macrophages/microglia in brain tumors other than GBM

The effect of GAMs has been documented in brain tumors other than GBM. Diffuse midline glioma (DMG) exhibits even less lymphocytic infiltration and lacks BBB disruption compared to GBM, thereby limiting the efficacy of immunotherapies. Nonetheless, VEGFR inhibitors have shown partial effectiveness in DMG [54, 55]. In primary central nervous system lymphoma (PCNSL), while no direct correlation was found between GAM infiltration and prognosis, enhanced Stat3 activation in lymphoma cells was observed in cases with an abundance of CD163-positive macrophages/microglia within the tumor tissue [56]. In meningiomas, CD163 expression has been linked to prognosis [57, 58]. Additionally, certain highly invasive and treatment-resistant neuroendocrine tumors, such as pituitary neuroendocrine tumors (PitNETs), display elevated levels of CD163-positive GAMs and M-CSF, indicating the potential effectiveness of M-CSFR inhibitors [59].

10. Conclusion

GAMs constitute a significant portion of the TME in GBM and play a crucial role in promoting tumor growth and progression. The dynamics of cell-cell interactions between GAMs and tumor cells are progressively being elucidated, and their functions and roles are expected to be further clarified through the new technologies such as single-cell RNA and spatial analysis. Intervening in the immune microenvironment of GBM, including targeting GAMs, is useful for development of novel therapeutic strategies for GBM.

Disclosure statement

No potential conflict of interest was reported by the authors.

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