Comparison of the tumor immune microenvironment between glioblastoma and metastatic brain tumors.

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Research Article

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Abstract

Background: Brain metastases (BrM), which commonly arise in patients with melanoma, breast cancer and lung cancer, are associated with a poor clinical prognosis. In this context, the tumor microenvironment (TME) plays an important role since it either promotes or inhibits tumor progression. Our previous studies have characterized the immunosuppressive microenvironment of glioblastoma (GBM). The aim of this study is to compare the immune profiles of BrM and GBM in order to identify potential differences that may be exploited in their differential treatment.

Methods: Tumor and/or blood samples were taken from 20 BrM patients and 19 GBM patients. Multi-parametric flow cytometry was used to evaluate myeloid and lymphoid cells, as well as the expression of immune checkpoints in the TME and blood. The Mann-Whitney test and t-test were utilized for statistical analysis.

Results: High frequencies of myeloid cells dominate both the BrM and GBM landscapes, but there is a higher presence of tumor-associated macrophages in GBM, while BrM are characterized by a significant presence of tumor-infiltrating lymphocytes. Exhaustion markers are highly expressed in all T cells from both primary and metastatic brain tumors. The cell cycle analysis of a BrM and a GBM revealed proliferating tumor cells and blood-derived macrophages, but quiescent lymphocytes and resident microglial cells. As previously demonstrated for GBM, macrophages sorted from a lung BrM exhibited a strong immunosuppressive activity. Finally, a significant expansion of some myeloid cell subsets was observed in the blood of both GBM and BrM patients.

Conclusions: Our results define the main characteristics of the immune profile of BrM and GBM, which are distinguished by different levels of immunosuppressive myeloid cells and lymphocytes devoid of effector function. Understanding the role of the different cells in establishing the metastatic setting is critical to improving the therapeutic efficacy of new targeted immunotherapy strategies.

Background

Brain metastases (BrM) are the most frequent intracranial tumors: the incidence of newly diagnosed BrM is three to ten times that of newly diagnosed primary malignant brain tumors, considered between 9% and 17% according to various studies, although the exact percentage is thought to be higher (1–3). It is hypothesized that this incidence is increasing due to improved cancer survival, an aging of the population, increased awareness of the disease and better diagnostic tests. Lung cancer is considered the most common source of BrM (39–56%), followed by breast cancer (13–30%), melanoma (8–11%), renal cell cancer (2–6%) and colorectal cancer (6–9%). The primary tumor may not be known (2–14%). Breast cancer predominates in women, whereas lung cancer is the most frequent source of BrM in the male population (1–4).

At the onset of neurological symptoms, 50–70% of BrM appear to be prolific on an MRI scan, and melanoma has the highest propensity of all systemic malignant tumors to metastasize to the brain.
Surgery is the preferred treatment for BrM, especially for single lesions, followed by radiation therapy; these treatments are frequently combined. BrM are generally well demarcated from the surrounding brain parenchyma, although infiltrative growth patterns have been observed and described (5). Sodium fluorescein- or 5-aminolevulinic acid (5-ALA)-induced fluorescence may be used to improve the surgical strategy of tumor removal (6–8) and maximize the extent of resection, especially in eloquent areas (9).

We recently demonstrated that 5-ALA makes it possible to safely maximize the extent of resection (9–11), given that 5-ALA-associated fluorescence behaviour of BrM was correlated with the rate of local recurrences, local progression-free survival and overall survival. These results led to the hypothesis that protoporphyrin IX (PpIX) fluorescence of cerebral metastases may be an intrinsic factor related to a more benign character of BrM and of the primary tumor. In contrast, the loss of PpIX fluorescence may indicate a more aggressive BrM behaviour (12). 5-ALA for the visualization of high-grade gliomas has been in use since 2007, and over the years, this intraoperative technique has been proven to increase progression-free and overall survival (13). In this respect, we demonstrated that the sampling of glioblastoma (GBM) specimens from the central or marginal areas based on PpIX emission also identifies bone marrow-derived macrophages (BMDM) endowed with an immune suppressive activity that increases from the marginal to the central region (14). In fact, the immune cell infiltrate of GBM is characterized by a high proportion of myeloid cells, the bulk of which are BMDM, and a paucity of T cells with the morphology and markers of exhausted cells (15).

Given the importance of the immune cell composition in the tumor microenvironment (TME), it is vital to understand its characteristics in order to identify new targets that may enhance antitumor activity. In this study, we compared the immune landscape of GBM with that of the most prevalent BrM, focusing on tumor-associated macrophages and immune suppression mechanisms.

**Methods**

**Patient characteristics**

BrM patients in this study were consecutively recruited at the Department of Neurosurgery in Padova (from 2016 to 2019) and Florence University Hospitals (from 2021 to 2022) in Italy. GBM patients were recruited in Padova between 2016 and 2017 and in Florence in 2021. All participant characteristics are detailed in Table 1. Briefly, 19 first-surgery GBM patients and 20 BrM patients were enrolled, and freshly resected tumor samples and peripheral blood were collected. Thirteen and 19 tumor specimens were obtained from BrM and GBM patients, respectively, together with 17 and 16 blood samples drawn immediately before induction of anesthesia. In addition, peripheral blood was collected from 18 healthy donors (HD).

All the experiments were approved by the ethics committees of the Veneto Institute of Oncology–IRCCS of Padova, Italy (MDSC_SNC 2016/13), and the Padova and Florence University Hospitals (NOL_NCH
All patients gave their written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

**Tumor specimen processing**

After resection, tumor specimens were immediately transferred to MACS® Tissue Storage Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored at 4°C until processing. Sample digestion was performed as reported previously (14). For the cell sorting experiment, the single-cell suspension was subjected to immunomagnetic bead-based separation using human CD33 MicroBeads (Miltenyi Biotec) according to the manufacturer’s instructions, to isolate CD33\textsuperscript{high} cells.

**Phenotypic analysis**

Fresh peripheral blood samples were stained with fluorescently-labeled monoclonal antibodies (mAb) as described in (16) to identify different circulating myeloid populations. In detail, four distinct subsets of myeloid-derived suppressor cells (MDSC), polymorphonuclear cells (PMN) and monocytes were identified according to the following antibody panel: anti-CD11b Alexa Fluor 700 (BD Biosciences, Becton Dickinson, Franklin Lakes, NJ, USA), anti-CD14 APC-H7 (BD Biosciences), anti-CD15 V450 (BD Biosciences), anti-CD33 PE-Cy7 (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), anti-IL4Rα PE (R&D Systems, Minneapolis, MN, USA), anti-lineage cocktail 1 (Lin 1) FITC (BD Biosciences) and anti-HLA-DR APC (BD Biosciences). Fluorescence minus one (FMO) negative controls were prepared for HLA-DR and IL4Rα. The overall staining procedure was standardized as reported previously (16).

Tumor specimens were stained with different antibody cocktails for the analysis of the immune infiltrate. To this end, single-cell suspensions were stained with LIVE/DEAD™ Fixable Aqua (Life Technologies, Thermo Fisher Scientific), anti-CD45 BV421 (BD Biosciences), anti-CD33 APC (BD Biosciences), anti-CD3 APC-H7 (BD Biosciences), anti-CD49d PE (BioLegend, San Diego, CA, USA), anti-CD3 PE-Cy7 (Beckman Coulter, Indianapolis, Indiana, USA) or anti-CD3 APC-H7 (BD Biosciences), anti-CD8 APC-H7 (BD Biosciences), anti-CD4 BV785 (BioLegend), anti-lymphocyte-activation gene 3 (LAG-3) FITC (AdipoGen, San Diego, CA, USA), anti-programmed cell death protein 1 (PD-1) PE (Miltenyi Biotec) and anti-T cell immunoreceptor with Ig and ITIM domains (Tigit) PE-Cy7 (BioLegend). FMO tubes were prepared as negative controls for PD-1, LAG-3 and Tigit.

Multicolor flow cytometry analysis was performed on an LSRII flow cytometer (BD Biosciences), and data analysis was carried out with FlowJo™ software (BD Biosciences).

**Immunosuppressive activity assay**

The immune suppressive activity of CD33\textsuperscript{high} myeloid cells sorted from a lung BrM was evaluated by determining the proliferation of allogenic CellTrace™-labeled peripheral blood mononuclear cells (PBMC) isolated from HD buffy coats as described previously (14). After four days at 37°C, 5% CO\textsubscript{2}, T cell proliferation was assessed by staining cells with anti-CD3 PE-Cy7 mAb (Beckman Coulter) and
calculating the absolute number of proliferating T cells using Trucount™ tubes (BD Biosciences). All data were normalized by assuming that the proliferation of T cells alone was 100%.

**Evaluation of cell subset proliferation by BrdU incorporation**

Single-cell tumor suspensions were stained with the BD Pharmingen™ FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer’s instructions in order to characterize the cell cycle. Briefly, cells were incubated with bromodeoxyuridine (BrdU) for one hour and, after incubation, stained with LIVE/DEAD™ Fixable Aqua (Life Technologies, Thermo Fisher Scientific), anti-CD45 BV421 (BD Biosciences), anti-HLA-DR APC (BD Biosciences) and anti-CD49d PE (BioLegend), as previously described. Cells were then fixed and permeabilized with BD Cytofix/Cytoperem Buffer and BD Cytoperm Permeabilization Buffer Plus, both provided in the kit, and treated with DNase to expose the incorporated BrdU. At the end, cells were stained with an anti-BrdU FITC mAb, to evaluate its incorporation, and with 7-aminoactinomycin D (7-AAD), to stain the total DNA content for cell cycle analysis. The LSRII flow cytometer (BD Biosciences) was used to acquire data and the FlowJo™ software (BD Biosciences) was used for the analysis.

**Statistical analysis**

SigmaPlot software (Systat Software Inc., San Jose, CA, USA) was used for data statistical analysis. The Mann-Whitney test and Student’s t-test were performed, with $P$ values $< 0.05$ considered statistically significant. All tests were two-sided, and for the sake of brevity, the lack of significance was not reported.

**Results**

**Analysis of the myeloid infiltrating cells in the tumor microenvironment of BrM and primary GBM**

Twenty patients with BrM and 19 patients with primary GBM undergoing their first surgery were enrolled in this study. BrM derived from different primary tumors, including melanoma ($n = 6$), lung ($n = 8$), bladder ($n = 1$), ovary ($n = 1$) and breast cancer ($n = 4$) (Fig. 1a). When 5-ALA-assisted surgery was used for tumor resection, all tissue specimens collected were derived from the bright PpIX fluorescent area; in all the other cases, specimens were obtained from the central tumor region. Table 1 summarizes all of the participants’ characteristics.

Using multi-parametric flow cytometry, we investigated the myeloid and T cell infiltrate present in the TME; an example of the gating strategy used is shown in Fig. 1b. Both BrM and GBM had a broad CD45$^+$ leukocyte infiltrate, with a range between 5.3% and 63.9% among live cells (median 19.6%) for BrM and 14.4% and 72.9% for GBM (median 36.4%), as shown in Fig. 1c. In both cases, the bulk of the leukocyte infiltrate was composed of CD33$^{\text{high}}$ macrophages, with a higher proportion in GBM (median 36.6% vs. 73.85% in BrM and GBM, respectively, $P \leq 0.001$). We then dissected the contribution of resident vs. blood-derived macrophages in the CD33$^{\text{high}}$ cells as reported previously (14) and identified BMDM as
CD45+/CD33high/HLA-DR+/CD49d+ cells and microglia (MG) as CD45+/CD33high/HLA-DR+/CD49d− (Fig. 1b), and observed a higher frequency of BMDM in GBM compared to BrM (Fig. 1c, median 16.3% in BrM vs. 47.3% in GBM, P = 0.003). As previously reported, a significantly higher presence of BMDM compared to MG was observed in GBM, and the same also held true for BrM, although the presence of blood-derived macrophages was significantly higher in GBM than BrM.

We then analyzed the myeloid infiltrate of BrM according to their primary site for breast (n = 3), melanoma (n = 5) and lung BrM (n = 3) and compared them to GBM. We found no significant difference in terms of the frequency of leukocytes, but a trend toward an increase of CD45+ cells in GBM (Fig. 1d). Instead, significant differences emerged by analyzing the myeloid populations, with a higher presence of CD33high cells in GBM compared to breast, melanoma and lung BrM. Regarding BMDM, their presence was significantly higher in GBM compared to melanoma BrM (Fig. 1d, P = 0.0222). No significant differences were observed for the presence of PMN, with the exception of breast BrM, in which we found a higher presence compared to GBM (Fig. 1d, P = 0.0343).

Overall, these results suggest that BrM are characterized by a consistent myeloid infiltrate, mainly composed of BMDM and PMN, with a pattern similar to that of GBM, albeit at a lower level.

**Analysis of the T cell infiltrate and the expression of exhaustion markers**

Together with the myeloid populations, we also analyzed the presence of T cells in the immune infiltrate, which is an important component of the TME of BrM, given their potential role as antitumor effectors (17–19).

Results highlighted a significant presence of tumor-infiltrating CD3+ cells in BrM as compared to GBM, both in the CD8+ and in the CD4+ subsets, reinforcing the notion that GBM is a cold tumor (Fig. 2a). We then analyzed the expression of the PD-1 (Fig. 2b), LAG-3 (Fig. 2c) and Tigit (Fig. 2d) immune checkpoints and compared it to that of T cells present in the GBM microenvironment. A high frequency of PD-1+ T cells was present in the TME of both BrM and GBM, with no significant differences (Fig. 2b). The percentage of LAG-3+ (Fig. 2c) and Tigit+ (Fig. 2d) cells was lower than that of PD-1+ T cells, with a tendency for a higher presence in BrM, suggesting a lack of T cell functional response in these tumors.

When we independently analyzed melanoma BrM (n = 4), which have a peculiar immune infiltrate, we observed a significantly higher infiltration of lymphocytes, evaluated by morphological SSC-A parameters combined with CD45 expression, and by the T cell markers CD3, CD4 and CD8 (Fig. 3a). In terms of immune checkpoint expression, PD-1 expression on T cells was high in both melanoma BrM and GBM (Fig. 3b), while LAG-3 expression was reduced (Fig. 3c), as previously mentioned (Fig. 2c).

**Analysis of the myeloid populations in the peripheral blood of BrM and GBM patients**
It has been clearly demonstrated by us and by others that cancer patients have an altered myelopoiesis (20) and that an expansion of MDSC is found in glioma patients, the level of which is a component of a prognostic model for GBM patients (21). We thus analyzed the levels of several myeloid populations, including four different MDSC subsets, monocytes (evaluated as CD14+ cells) and PMN (evaluated as CD15+ cells), in the peripheral blood of BrM and GBM patients and compared them with a group of HD matched for sex and age. The four MDSC subsets were identified as CD14+/IL4Rα+ (MDSC1), CD15+/IL4Rα+ (MDSC2), Lin−/HLA-DR−/CD11b+/CD33+ (MDSC3) and CD14+/HLA-DR− (MDSC4).

Intriguingly, we found that the presence of both types of brain tumors was associated with elevated levels of circulating myeloid cells, including PMN, monocytes and MDSC1, 2 and 4 (Fig. 4) compared to HD. In contrast, a significant drop in the level of MDSC3 was observed in BrM. However, there were no remarkable differences between BrM and GBM for any of the myeloid subsets.

When we separated BrM on the basis of primary tumors, from breast cancer (n = 4), melanoma (n = 5) and lung cancer (n = 6), there were no significant differences between BrM from different sites or between them and GBM (Fig. 5).

**Macrophages from a lung BrM are endowed with immune suppressive potential**

Previous studies from our laboratory demonstrated that macrophages from GBM samples possess an immune suppressive activity that depends not only on their ontogeny but also on the tumor context, since their immunosuppressive potential increases as they migrate to the center of the lesion (14). Therefore, we sought to determine whether macrophages also exert an immune suppressive role in BrM.

To that end, after enriching CD33high cells to 88% of live cells in a lung metastasis, we tested their immune suppressive potential towards the proliferation of activated T cells. As shown in Fig. 6, macrophages exerted a strong suppression on T cell proliferation, as evidenced by the quantitative reduction in T cell proliferation (Fig. 6a, upper part, orange plot) and the higher CellTrace™ fluorescence intensity (Fig. 6a, bottom part, orange plot), but also by the down-regulation of CD3 expression on T cells (Fig. 6b).

**Evaluation of cell subset proliferation in the brain TME**

We sought to determine if cell subsets present in the TME maintain the ability to proliferate *ex vivo*, immediately after resection and without any additional stimulation. To achieve this, BrdU incorporation was conducted in cell suspensions from a GBM and a lung metastasis, and the cell cycle was analyzed after 1 hour in both BMDM and MG macrophages, lymphocytes and tumor cells (evaluated as CD45− cells). As expected, the main cell subset that entered the active phases of the cell cycle (i.e., the S and G2-M phases) was represented by tumor cells, particularly in GBM, and to a lesser extent in the lung BrM, while the proliferation observed in lymphocytes and MG cells was negligible. Notably, BMDM also
exhibited a significant fraction entering the cell cycle and were mainly present in the G$_2$/M phase in both GBM and lung BrM (Fig. 7). To the best of our knowledge, this is the first evidence demonstrating that BMDM proliferate in the TME; these data also reinforce the notion that blood-derived and resident macrophages differ not only in terms of immune suppressive ability but also in terms of proliferative ability. Collectively, our data indicate that, in the TME, suppressive BMDM have the ability to proliferate, whereas lymphocytes with a hallmark of dysfunctional activity are in a resting state.

**Discussion**

Cancer cells that metastasize to the brain need to adapt to a very peculiar microenvironment that is radically different from the site of origin. The colonization of the tumor cells at a distant site also includes the induction of immune evasion mechanisms in the TME, such as the infiltration of immune cells with tumor-promoting activity. Our previous studies on primary brain tumors identified a typical immune landscape mainly composed of immune suppressive macrophages both in meningiomas and in GBM (14, 22). Another recurrent characteristic of the TME in primary brain tumors is the scarcity of lymphocytes, especially in GBM, which is a feature that defines a typical cold microenvironment.

The findings in this study highlight that a sustained recruitment of blood-derived macrophages occurs even in the most common BrM and that such cells possess immune suppressive ability, as seen in GBM (14). This supports the hypothesis that the recruitment of myeloid cells in the brain parenchyma of a growing tumor is not only a defining trait, but it also allows the tumor to avoid its immune-mediated destruction. Remarkably, the T cell infiltrate in the TME of BrM is significantly higher than that of GBM, suggesting that the priming of the antitumor T cell response is higher for tumors of extracranial origin, in line with the high mutational burden of melanoma and lung cancer. Other factors could explain the higher frequency of T cells in BrM from extracranial tumors, such as the release of chemokines and the accentuation of molecular pathways expanding T cell trafficking and extravasation, like an increased expression of cellular adhesion molecules on endothelial cells (23).

However, T lymphocytes that are present in brain tumors show high levels of exhaustion markers, irrespective of the tumor's origin. Thus, despite a higher presence of T cells in tumors of extracranial origin, it appears that their activity is hampered, as evidenced by their lack of proliferation (Fig. 7), the high expression of PD-1, LAG-3, and Tigit on their surface (Figs. 2 and 3), and the concomitant immunosuppressive activity mediated by BMDM (Fig. 6).

The presence of significant numbers of leukocytes in brain tumors also highlights the lack of a functioning blood-brain barrier (BBB), a fact that is also emphasized by the contrast enhancement pattern of these tumors in MRI (24). Although it is well known that the BBB is not homogeneously disrupted in brain tumors (25), the significant presence of leukocytes in these tumors argues in favor of the administration of drugs capable of activating an antitumor immune response. In line with this consideration, the presence of a TME with a strong immune suppressive trait should lead to the careful planning of immune interventions for brain tumors, where a combination of treatments that stimulate the
immune system and block the TME's main suppressive activities represents a new opportunity for the
treatment of both primary and secondary tumors. To fully exploit this opportunity, a rational targeted
approach that exploits the immune landscape and the functional and metabolic connections that sustain
tumor growth is required. In this regard, we have recently demonstrated that by inhibiting heme
oxygense-1 (HO-1), a key metabolic enzyme of iron metabolism in BMDM, the immune suppressive
activity of tolerogenic cells is alleviated and T cell proliferation is restored (26). However, while the activity
of immune suppressive cells appears to be a valuable target for undermining tumor growth, the presence
of T cells in the TME should be preserved to restore their antitumor potential. In order to advance new
clinical studies, it would be prudent to investigate the possibility of tailoring novel strategies that
selectively target key points controlling the suppressed TME without affecting T cells. In this regard, new
drug-loaded nanosystems that selectively target tumor and tumor-promoting cells without penetrating T
cells could be considered as a novel and effective approach in cancer therapy (27). Therefore, a detailed
knowledge of the brain TME and immune evasion patterns is essential for the design of successful
treatment strategies.

As regards the limitations of our study, metastatic brain biopsies are rare and it is difficult to collect
freshly resected specimens in sufficient quantities for functional studies. In addition, our study does not
have a large enough sample size to conduct all the comparisons between the different tumor types in all
the different experiments. Another issue concerns the prior treatments of metastatic patients, as opposed
to GBM that are, instead, treatment-naive. Therefore, future efforts should incorporate the present findings
by addressing the impact of prior treatments on the composition of the TME, a factor we did not consider
for this study. Nevertheless, we have previously demonstrated that, despite therapy, the TME of relapsing
GBM maintains a similar infiltration pattern as the primary tumor (15, 28). This suggests that, regardless
of the patient's therapy, the recruitment of blood-derived cells with immune suppressive activity may also
be a hallmark of BrM.

**Conclusions**

Our analysis reveals that, regardless of its origin, the presence of a brain tumor sculpts the
microenvironment towards an immunosuppressed state, in which blood-derived cells with
immunosuppressive activity and proliferative potential play a prominent role. Given the important role
these cells play in sustaining tumor growth, it is crucial to understand the immunological component of
the TME at both the tumor and systemic levels for the identification of targets and the development of
effective therapeutic approaches.

**Abbreviations**

5-ALA: 5-aminolevulinic acid

7-AAD: 7-aminoactinomycin D
Declarations

Ethics approval and consent to participate

All the experiments were approved by the ethics committees of the Veneto Institute of Oncology–IRCCS of Padova, Italy (MDSC_SNC 2016/13) and the Padova and Florence University Hospitals (NOL_NCH 1536/19). All patients gave their written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Availability of data and materials

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Conceptualization, SuM; methodology, SuM; formal analysis, BM; investigation, BM, MGR, AT, SaM, GB, LP; resources, CB, ADP; data curation, BM, MGR, SaM; writing, BM, CB, SuM; supervision, SuM; project administration, SuM; funding acquisition, SuM. All authors have read and agreed to the published version of the manuscript.

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References


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**Figures**
**Figure 1**

**Analysis of the myeloid infiltrate in the TME of BrM and GBM.** (a) Schematic representation of the localization of primary BrM tumors analyzed in the study. In the upper left, a representative surgical view under white and blue light after 5-ALA administration to a BrM patient. Figure created with biorender.com. (b) Representative flow cytometry gating strategy for the identification of the different myeloid subsets in tumor samples. After morphological evaluation and the exclusion of doublets and dead cells, leukocytes...
were identified on the basis of their CD45 expression. Macrophages and PMN were further discriminated in the CD45$^+$ gate based on their differential CD33 expression, with PMN identified as CD33$^{\text{dim}}$ cells and macrophages as CD33$^{\text{high}}$ cells. Finally, BMDM and MG were further differentiated in the CD33$^{\text{high}}$ subset based on the combined expression of CD49d and HLA-DR, with BMDM distinguished as CD49d$^+$/HLA-DR$^+$ or CD33$^{\text{high}}$/HLA-DR$^+$ and MG as CD49d$^-$/HLA-DR$^+$ or CD33$^{\text{high}}$/HLA-DR$^{\text{dim}}$, respectively. **(c)** Analysis of the frequency of the leukocyte subsets in the TME of BrM and GBM. CD45$^+$ cells were calculated among live cells, while CD33$^{\text{high}}$, BMDM, MG and PMN were gated among CD45$^+$ leukocytes. Violin plots show the frequency distribution and median of tumor-infiltrating leukocytes in the whole BrM (blue plots) and GBM (red plots) (n = 14 for BrM, except for PMN [n = 13]; n = 18 for GBM). Comparison by Mann-Whitney test. * < .05; ** < .01; *** ≤ .001. **(d)** Analysis of the leukocyte infiltrate in the TME of BrM according to their primary tumor site. Bars show the mean ± standard error (SE) of the frequency of tumor-infiltrating leukocytes in BrM from breast cancer (pink bars, n = 3), melanoma (grey bars, n = 5), lung cancer (white bars, n = 3) and GBM (red bars, n = 18). Each black dot represents a sample. Comparison by t-test. * < .05; ** < .01; *** ≤ .001.
**Figure 2**

**T cell infiltrate and exhaustion markers in the TME of BrM and GBM.** CD3⁺, CD8⁺ and CD4⁺ cells were selected among CD45⁺ leukocytes. CD8⁺ cells were gated as CD3⁺/CD8⁺ or CD3⁺/CD4⁻ cells, while CD4⁺ cells were identified as CD3⁺/CD8⁻ or CD3⁺/CD4⁺ cells. Blue and red plots refer to BrM and GBM, respectively. **(a)** Analysis of the frequency of T cell subsets in the TME of the entire BrM and GBM. Violin plots illustrate the distribution and the median of the frequency of tumor-infiltrating leukocytes in the
entire BrM and GBM (n = 9 for BrM; n = 16 for GBM). Comparison by Mann-Whitney test. ** < .01; *** < .001. (b) Percentage of PD-1$^+$ cells in the total CD3$^+$ population and in the CD8$^+$ and CD4$^+$ T cell subsets (n = 9 for BrM; n = 16 for GBM). Each dot represents a sample. (c) Percentage of LAG-3$^+$ cells in the total CD3$^+$ population and in the CD8$^+$ and CD4$^+$ T cell subsets (n = 5 for BrM; n = 16 for GBM). Each dot represents a sample. Comparison by t-test. * < .05. (d) Percentage of Tigit$^+$ cells in the total CD3$^+$ population and in the CD8$^+$ and CD4$^+$ T cell subsets (n = 5 for BrM; n = 3 for GBM). Each dot represents a sample.
T cell infiltrate and exhaustion markers in the TME of melanoma BrM and GBM. Lymphocytes were identified using morphological parameters in the CD45+ cell fraction. CD3+, CD8+ and CD4+ cells were gated among CD45+ leukocytes. CD8+ cells were selected as CD3+/CD8+ or CD3+/CD4- cells, while CD4+ cells were identified as CD3+/CD8- or CD3+/CD4+ cells. Grey and red plots refer to melanoma BrM and GBM, respectively. (a) Analysis of the frequency of lymphocyte subsets in the TME of BrM from
melanoma and GBM. Bars show the mean ± SE of the frequency of tumor-infiltrating leukocytes in BrM from melanoma and GBM (n = 5 for melanoma BrM and n = 18 for GBM for total lymphocytes; n = 4 for melanoma BrM and n = 15 for GBM for CD3\(^+\), CD8\(^+\) and CD4\(^+\) subsets). Each dot represents a sample. Comparison by t-test. * < .05; ** < .01; *** ≤ .001. (b) Percentage of PD-1\(^+\) cells in the total CD3\(^+\) population and in the single CD8\(^+\) and CD4\(^+\) subsets (n = 4 for BrM; n = 16 for GBM). Each dot represents a sample. (c) Percentage of LAG-3\(^+\) cells in the total CD3\(^+\) population and in the single CD8\(^+\) and CD4\(^+\) subsets (n = 3 for BrM; n = 16 for GBM). Each dot represents a sample. Comparison by t-test. ** ≤ .01.
Figure 4

Analysis of circulating myeloid populations in the peripheral blood of BrM and GBM patients. PMN, monocytes and MDSC1-4 were evaluated in the peripheral blood of BrM (blue plots) and GBM (red plots) and compared to a set of HD (light blue plots). Violin plots illustrate the distribution and the median of the frequency of (a) PMN (n = 18 for BrM; n = 16 for GBM; n = 18 for HD), (b) monocytes (n = 19 for BrM; n = 16 for GBM; n = 18 for HD), (c) MDSC1 (n = 19 for BrM; n = 16 for GBM; n = 18 for HD), (d) MDSC2 (n
= 19 for BrM; n = 16 for GBM; n = 18 for HD), (e) MDSC3 (n = 18 for BrM; n = 16 for GBM; n = 18 for HD) and (f) MDSC4 (n = 19 for BrM; n = 15 for GBM; n = 18 for HD) in the peripheral blood of BrM and GBM patients. Comparison by Mann-Whitney test. ** < .01; *** ≤ .001.

Figure 5
Analysis of circulating myeloid cells in the peripheral blood of BrM. Bars show the mean ± SE of the frequency of PMN (a), monocytes (b) and MDSC1-4 (c-f) in BrM from breast cancer (pink bars), melanoma (grey bars), lung cancer (white bars), GBM (red bars) and in a set of HD (light blue bars) (n = 4 for breast BrM, except for PMN and MDSC3 [n = 3], n = 5 for melanoma BrM, n = 6 for lung BrM, n = 16 for GBM, except for PMN [n = 15], and n = 18 for HD). Each dot represents a sample. Comparison by Mann-Whitney test. * < .05; ** < .01; *** ≤ .001.
Immune suppressive activity of macrophages in the TME of a lung BrM. CD33\textsuperscript{high} cells were isolated from a single-cell suspension of a lung BrM using anti-CD33 immunomagnetic beads and cultured for four days with αCD3- and αCD28 mAb-stimulated PBMC. (a) Proliferation of T cells cultured in the presence of CD33\textsuperscript{high} cells (orange plots) in a 1:1 ratio. The bars in the upper section represent the value of quantitative T cell proliferation normalized assuming the proliferation of T cells alone (green bar) to be 100%. The lower section depicts CellTrace histograms of stimulated T cells alone (green plot) or in the presence of sorted CD33\textsuperscript{high} cells (orange plot). (b) Dot plots of stimulated T cells alone (upper section) or in the presence of sorted CD33\textsuperscript{high} cells (lower section).
Analysis of the cell proliferation in the TME of a lung BrM and a GBM. Cell cycle analysis of the main cell subsets present in the TME of a representative lung BrM (upper section) and GBM (lower section). Cell suspensions from dissociated specimens were pulse-labeled with BrdU and then counterstained with 7-AAD for flow cytometry analysis. For each subset, the different cell cycle phases were discriminated by plotting BrdU (Y axis, biexponential scale) vs. 7-AAD (X axis, linear scale) and color-coding them as...
follows: blue square for $G_0$-$G_1$; green square for $G_2$-$M$; orange square for S. The numbers in each box refer to the percentage of cells present in the corresponding cell cycle phase.