Comparison of Tumor Immune Environment Between Newly Diagnosed and Recurrent Glioblastoma in Matched Patients

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Research Article

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Abstract

**Purpose:** Glioblastoma (GBM) is the most lethal primary brain tumor in adult patients. The disease progression, response to chemotherapy and radiotherapy at initial diagnosis, and prognosis are profoundly associated with the tumor microenvironment (TME), especially the features of tumor-infiltrating immune cells (TII). Recurrent GBM is even more challenging to manage. Differences in the immune environment between newly diagnosed and recurrent GBM and an association with tumor prognosis are not well defined.

**Methods:** To address this knowledge gap, we analyzed the clinical data and tissue specimens from 24 GBM patients (13 at initial diagnosis and 11 at recurrence). The expression levels of multiple immunobiological markers in patients’ GBM at initial diagnosis *versus* at recurrence were compared, including five patients with both specimens available (paired). The distribution patterns of TII were evaluated in both the intratumoral and perivascular regions.

**Results:** We found that tumors from recurrent GBM have significantly more tumor-infiltrating lymphocytes (TILs) and macrophages and higher PD-L1 expression than tumors at primary diagnosis and benign brain specimens from epilepsy surgery. The pattern changes of the TILs and macrophages of the five paired specimens were consistent with the unpaired patients, while the CD8 to CD4 ratio remained constant from diagnosis to recurrence in the paired tissues. The levels of TILs and macrophages at initial diagnosis did not correlate with OS. TILs and macrophages were increased in recurrent tumors both in intratumoral and perivascular areas, with higher distribution levels in intratumoral than perivascular regions. Higher CD4 or CD8 infiltration at recurrence was associated with worse prognosis, respectively.

**Conclusion:** Our study elucidated that TIL and TAM tend to accumulate in perivascular region and are more abundant in recurrent GBM than newly diagnosed GBM.

Introduction

Glioblastoma (GBM) is the most common and aggressive malignant brain tumor in adults with limited therapeutic options and a grim prognosis. Despite great efforts to study this disease in the past few decades, GBM is still the leading mortality in brain cancer with an 80% mortality rate within one year after diagnosis. According to a recently published systematic review including 56,626 patients in 438 studies globally from 1979 to 2017, the median overall survival of GBM patients after the adoption of radiation therapy (RT) and temozolomide (TMZ) as standard in 2005 improved only from 12.5 (range 2.3-28) months to 15.6 (range 3.8-29.6) months with only 5.8% (range 0.01–29.1%) of patients living to 5 years [1].

Tumor recurrence is almost inevitable in GBM, and a considerable portion of patients will suffer second and third-time recurrences [2]. Unfortunately, recurrent GBM is much more resistant to radio- and chemotherapy, and fewer than 30% of patients are eligible for a second surgery [3]. There is no established standard care for recurrent GBM. Besides bevacizumab +/- RT, treating recurrent GBM largely
relies on our knowledge of the primary GBM, even though evidence is emerging showing substantial differences in pathological features and the tumor microenvironment between initial and recurrent GBM [2, 4]. Therefore, studies on the unique features and the potential treatment targets are urgently needed for recurrent GBM.

In the past decade, the rapid development of immune therapy, especially the discovery of immune checkpoint proteins and the application of immune checkpoint blockade antibodies, has ignited our enthusiasm to battle cancer. Targeting immune checkpoint proteins has revolutionized treating melanoma, lung cancer, renal cell carcinoma, and urothelial carcinoma [5]. However, clinic trials using immunotherapy for GBM, including recurrent GBM, are much less exciting. So far, most of the clinical trials evaluating the antibodies targeting PD-1/PD-L1 axis combining with either standard treatment or other targeted treatment have not been able to provide substantially improved results in newly diagnosed or recurrent GBM patients [6].

The unique heavily immunosuppressive tumor microenvironment (TME), as well as the patterns of immune cells infiltrating GBM are the major factors that contribute to the resistance to the standard and immune therapy in GBM. Tumor-infiltrating lymphocyte (TIL) and macrophages are two populations that play a central role in TME of GBM. Increased numbers of CD8+ cytotoxic T cells, as well as the general CD3+ T cell infiltration are associated with prolonged overall survival of GBM patients [7]. However, the role of CD4+ T cells is more controversial due to the diverse functions of its subgroups, including CD4+ T helpers, regulatory T cells (Treg), CD4+CD56+ immunosuppressive T cells [8, 9]. Some studies show that CD4+ T cells are the predominant T cell constituents in primary GBM, which strongly correlate with tumor angiogenesis as well as poor prognosis [10]. As the predominant infiltrating immune cells in tumor tissue, macrophages are also suggested to have a prognostic and predictive role in GBM. GBM recruits tumor-associated macrophages (TAM) to the TME and promotes their polarization into an immunosuppressive phenotype with high expression of PD-L1 and production of pro-tumor cytokines, which dampens the anti-tumor effect of TIL and facilitates a protumorigenic function [11]. In addition to the density of TILs and macrophages, their distribution patterns are also closely related to the clinical outcome of GBM patients [9, 12].

Although many studies have explored the features of infiltrating immune cells in newly diagnosed GBM, few have focused on recurrent GBM and the comparison between these two, in particular using the paired primary-recurrence samples from the same patients. In the present study, we investigated and compared the densities and the distribution patterns of CD4+ T cells, CD8+ T cells and macrophages, as well as programmed death ligand-1 (PD-L1) expression between tissue samples from newly diagnosed and recurrent GBM tumor specimens. Additionally, we also studied paired tumor samples obtained from five patients at their initial diagnosis and disease recurrence. Our data deliver crucial insight into the features of the TME in recurrent GBM, to help design new immune therapy to improve the treatment outcome of recurrent GBM.

**Materials And Methods**
Study population

The formalin-fixed, paraffin-embedded GBM sections were taken from 26 patients treated at the University of Nebraska Medical Center (UNMC) with archived tissues at Department of Pathology and Microbiology under the approval of the Institutional Review Board (IRB). Among them, 13 samples were from GBM patients at initial diagnosis before treatment. Eleven samples were at recurrence after chemotherapy and RT. There are 5 paired tumor samples collected both from initial diagnosis and recurrence. Additionally, two non-lesional epilepsy patients without brain tumors also were included as controls. The study population included 15 females and 11 males with age ranging from 30 to 84 years old at the time of diagnosis. The H&E staining slides were reviewed independently by two neuropathologists at UNMC, blinded to each other and to the patients’ clinical information. We aimed to study the numbers as well as the location of tumor-infiltrating immune cells in tumor specimens at initial diagnosis and at recurrence.

Immunohistochemistry

Serial unstained slides were cut from the tumor blocks and immunohistochemistry (IHC) staining was performed using the BenchMark Ultra IHC/ISH system (Roche, Basel, Switzerland). The unstained slides were stained with anti-CD4 (SP35) rabbit monoclonal primary antibody, anti-CD8 (SP57) rabbit monoclonal primary antibody, anti-CD68 (KP-1) mouse monoclonal primary antibody, and anti-PD-L1 (SP263) monoclonal primary antibody. All primary antibodies were ordered from Ventana Medical Systems (Arizona, U.S.). Human tonsillar tissues were stained simultaneously as positive controls.

Quantification Of Immunostainings

After staining for all the markers, slides were scanned with a Ventana iScan HT slide scanner at 40x magnification and analyzed using Definiens Tissue Studio (Munich, Germany).

Images were imported into the Definiens Tissue Studio image analysis software in Tagged Image File Format (TIFF). Firstly, several areas of interest were selected to train the algorithm of the software. After adjusting the counterstain threshold and IHC threshold, the nuclei and IHC markers were recognized by the Definiens, with the consistency confirmed by a trained researcher. Then the slides were quantified by the trained algorithm of the software.

The distribution of immune cells in the intratumoral region was studied by counting the number of cells in intratumoral areas in each high-power field (HPF; 40x objective and 10x eyepieces). The distribution of immune cells in the perivascular region was studied by counting the number of cells in perivascular spaces divided by the number of vessels in each HPF. The mean counts of immune cells were obtained from at least 10 different HPFs of each section.
To compare the distribution of the immune cells in intratumoral and perivascular areas, we used the scoring system as described in Yang's and Yeung's studies [13, 14]. Briefly, the infiltration of immune cells in intratumoral and perivascular regions in each section was scored as follows: for intratumoral cell infiltration, 0 = 0 to 5 cells per 10 HPF; 1 = 5 to 20 cells per 10 HPF; 2 = 20 to 100 cells per 10 HPF; 3 = more than 100 cells per 10 HPF; for perivascular cell infiltration, 0 = less than 1 cell per vessel; 1 = 2 to 5 cells per vessel; 2 = 5 to 20 cells per vessel; 3 = more than 20 cells per vessel. At least 10 HPF with more than 20 vessels were identified for each section.

Statistical analysis

Results of the immunostaining features were presented as an absolute number (%) or median (range). The comparisons of these features between newly diagnosed and recurrent GBM were carried out by SASS using Wilcoxon test. Overall survival of patients with newly diagnosed GBM was defined as the time from the date of the initial surgery until the death of any cause or until the last follow-up date. Survival after recurrence (SR) was defined as the date of surgical resection for the recurrence until death from any cause or until the last follow-up date. The associations of survival with immunostaining features at diagnosis or at recurrence were performed using the univariate Cox proportional hazard model.

For each immune marker, the percentage of positively stained cells over all nucleated cells were calculated for each specimen. Data were shown as Mean ± standard error of the mean. Unpaired t-tests were performed to compare between newly diagnosed and recurrent GBM using the Prism Software (GraphPad, USA). Paired t-tests were used to compare the infiltrating immune cell numbers/markers from specimens derived from the 5 patients with paired tissue samples at their initial diagnosis and recurrence. The association of survival with CD4+ TIL, CD8+ TIL, CD4+/CD8+, CD68, and PD-L1 expression was explored using a linear regression model. For all statistical analyses, results were considered statistically significant with a (2-tailed) p-value threshold of 0.05.

Results

Demographics and clinical features

Thirteen tissue specimens from newly diagnosed GBM, 11 from recurrence and 2 benign control specimens from patients with epilepsy were analyzed in this study. Among them, there were 10 paired samples from 5 patients at both initial diagnosis and at recurrence. The demographic and clinical features of all patients (n=26) are summarized in Table 1. The median age of all GBM patients at diagnosis was 59.25 (range 30.56-84.36). Among them, 57.7% were females, and all the patients in this study were Caucasian. No statistically significant difference in gender and median age was found between patients at initial diagnosis and patients at recurrence. Gross total resection (GTR) was performed in 53.8% at initial diagnosis and 81.8% at recurrence. Radiotherapy was administered in 84.6% at initial diagnosis and in 91% at recurrence, both as standard adjuvant treatment after initial diagnosis.
with 6000 cGy as the median dose. Seventy-seven percent of patients at initial diagnosis and 100% of recurrent patients received standard chemotherapy (TMZ). The median OS after the initial diagnosis was 19.7 months, and 96.2% of patients died during follow-up. The median length of survival after date of surgery for recurrence (SR) was 12.77 months (Table 1).

The impact of clinical features in the prognosis of newly diagnosed and recurrent GBM were evaluated using univariate survival analysis. KPS ≤ 70 is an adverse prognostic factor for OS with HR (Hazard Ratio) of 9.6 (95% CI 1.065-86.874) when compared to KPS of > 70. GTR showed a trend towards improved OS compared with biopsy only. Gender and age had no significant association with OS (Table 2). None of these were associated with survival after recurrence (Table 3).

**Immune cell infiltration is greater at recurrence than in newly diagnosed GBM**

To explore the immune cell infiltration features in GBM patients, we analyzed TILs and macrophages as well as PD-L1 expression in tumor tissues from newly diagnosed and recurrent GBM patients. Additionally, we used two brain tissue samples from patients undergoing non-lesional epilepsy surgery as control. Compared to GBM tissue at primary diagnosis, the recurrent tumors showed significantly higher levels of infiltration of CD4+ T cells, CD8+ T cells, and CD68+ macrophages, whereas there was no significant difference in the CD8 to CD4 ratio observed between the two groups (Fig. 1A, B, C, D). Moreover, we found the expression level of PD-L1 greatly increased in recurrent GBM compared with tumors at initial diagnosis (Fig. 1E). The density of infiltrating TILs in epilepsy patients is numerically similar compared to recurrent GBM and higher than GBM at initial diagnosis but GBMs have much more CD68+ macrophages than epilepsy specimen. On average, about one third of all cells in GBM tissue are CD68+ macrophage in recurrent GBM specimen (Fig. 1D). However, no statistically significant difference was found, likely due to the insufficient number of patients in the epilepsy group. There was no significant correlation between OS and the infiltration of CD4+ T cells, CD8+ T cells, CD68+ macrophage, as well as the CD8/CD4 ratio and PD-L1 expression level for patients at initial diagnosis (Fig. 2A, B, C, D, E).

Intra-tumoral heterogeneity is one of the most prominent features of GBM, which is responsible for the different responses to treatment as well as the tumor relapse after the treatment [15]. Tumor samples from different patients may carry even greater heterogeneity caused by the phenotypic and genetic differences of tumor cells between individual patients. Therefore, we identified and selected five patients who had tumor samples from both the initial diagnosis and recurrence (paired samples) and studied the changes of infiltrating immune cells between the paired samples.

Consistent with the unmatched patients, the number of CD4+, CD8+ TIL as well as CD68+ macrophage were robustly higher at recurrence. The expression of PD-L1 also increased in recurrent GBM compared with matched samples at initial diagnosis (Fig. 3). No significant differences were observed in the CD8/CD4 ratio between the two groups. Notably, one patient with a remarkably increased CD8/CD4 ratio in the recurrent tumor had the highest overall survival (2411 days) among all GBM patients included in this study. This patient, a 73-year-old man, was first diagnosed with GBM in February 2008, with two
recurrences in August 2011 and July 2013, and passed away in September 2014. The CD8/CD4 ratios of the tumor tissue samples collected at his first diagnosis and twice recurrence were 0.65, 2.64 and 2.31, respectively, with quite drastic increase at recurrence.

**Increased densities of infiltrating immune cells in recurrent GBM with CD4+ TIL predominant in the perivascular region**

To learn more about the distribution patterns of tumor-infiltrating immune cells in GBM, we further looked at their infiltrations at perivascular and intratumoral regions separately. There were significantly increased densities of CD4+ TIL, CD8+ TIL, macrophage, and PD-L1 positive cells in recurrent GBM compared with GBM samples at initial diagnosis in intratumoral regions (Fig. 4B). Interestingly, the densities of CD4+ TIL and CD8+ TIL infiltration in the perivascular regions were also robustly increased in recurrent GBM tumor tissues than in initially diagnosed GBM, while there was no difference in the distributions of macrophage and PD-L1+ cells in perivascular spaces between initially diagnosed and recurrent specimens (Fig. 4B). The infiltration of CD4 TIL was almost twice denser than CD8 TIL in initially diagnosed GBM tumors across the entire tumor area. The predominance of CD4+ over CD8+ TIL was also seen in recurrent GBM, with the difference being more significant in the perivascular region (Fig. 4B).

In order to compare the immune cell infiltration levels between the intratumoral region and perivascular region, we used a 0-3 scoring system to evaluate the cells infiltration pattern (Fig. 4C). There were significant higher number of TIL in the intratumoral regions than in perivascular areas at both initial diagnosis and recurrence. The distribution of macrophage and PD-L1+ cells were also dramatically higher in the intratumoral regions than perivascular areas at either initial diagnosis or recurrence (Fig. 4A, C).

We further look into the pattern of TILs in recurrent GBM parenchymal tissues and their correlations with the prognosis after tumor recurrence, ie, the event-free survival (EFS) which is defined as the time interval from the recurrence when the tissues were obtained to the next recurrence or death, whichever came first. Interestingly, both the infiltrating CD4 and CD8 T cell densities predicted worse EFS with trend toward significance in recurrent specimens and significantly when all specimens were combined (Table 4). No significant correlations to EFS of CD8 to CD4 ratio (CD8/CD4), macrophage infiltration (CD68) or PD-L1 expression levels in recurrent only or combined tumor tissues were identified.

**Discussions**

Several studies have reported the correlation between tumor-infiltrating immune cells and the outcome of GBM patients, suggesting an important role of tumor-infiltrating immune cells in disease progression. However, some of the results have been quite conflicting. CD8 cytotoxic T lymphocytes (CTLs) are considered to be directly involved in anti-tumor cytotoxic responses and play a critical role in fighting against cancer, while the roles of CD4+ T cells are more complicated. On one side, CD4 T helper cells, activated by an antigen-presenting cell (APC), are crucial for priming CD8 CTLs, thus assisting and optimizing the immune response against the tumor. On the other hand, CD4+ Treg cells are well recognized immunosuppressive cells and facilitate the tumor growth through dampening the function of
APC and through their interaction with other T cells, *e.g.*, inhibiting the activities of effector T cells and natural killer (NK) cells. Many studies have agreed that elevated CD3+ total T cells and CD8+ T cells infiltrating into tumor were associated with extended overall survival of GBM patients [7, 16, 17] but controversial results also exist regarding the correlation between CD4+ T cells infiltration and patient's survival [7]. Some have reported that CD8 T cells were predominant TIL in primarily diagnosed GBM patients [12, 18], while others have shown that CD4+ TIL were more frequent [10]. One study including 90 primarily diagnosed GBM patients has shown that both CD4 and CD8 TIL levels were negatively associated with the GBM patients' outcome [12]. Our study is not able to show a significant correlation between the tumor-infiltrating levels of CD4+T cells, CD8+ T cells or CD4+/CD8+ ratio at primary diagnosis and OS of GBM patients but a trend of negative correlation coefficients seen in Fig. 2, most likely due to the limited number of patients with insufficient statistical power. However, by examining the immune cell infiltrating patterns in intratumoral parenchyma and perivascular regions, respectively, we are able to show that CD4+ TIL is predominant over CD8+ TIL in intratumoral and perivascular regions, and in both primary and recurrent GBM tumor tissues. Although not statistically significant, we have observed a trend showing that TILs, both CD4+ and CD8+, are more likely to accumulate in the perivascular area than the intratumor region, indicating the suppressive immune microenvironment of tumor parenchyma on TIL migration/survival.

In GBM, most of studies on the infiltrating immune cells have been focused on primarily diagnosed tumors. Very limited information is available when tumor recurs. Tumor relapse/recurrence is, however, almost inevitable for GBM patients. A study included 38 primary and 12 recurrent GBM patients failed to show any difference of CD3 TIL, CD8 TIL, Treg, and macrophage infiltration values between primarily diagnosed and recurrent GBM [19]. With purposely identifying and including the patients with both tissue samples at primary GBM diagnosis and at recurrence to reduce inter-specimen heterogeneity, we have confirmed that the densities of CD4+ TIL, CD8+ TIL, and CD68+ macrophage were significantly higher in recurrent than primarily diagnosed GBM, which is consistent with the results from a Japanese study showing the elevated expression of CD3 and CD8 in secondary resected tumor tissues than initially resected tissues [20]. However, the increase of TIL in recurrent tumor does not correlate with a prolonged survival. In contrast, recurrent GBM is much more resistant to chemotherapy and radiotherapy than primarily diagnosed GBM, leading to extremely limited treatment strategies and dismal outcomes [21]. Our data supported this finding that increased CD4 or CD8 T cell infiltration in recurrent GBM tumor predicted worse EFS after recurrence (Table 4). One of the potential reasons is the dysfunction of T lymphocytes caused by the hostile and immunosuppressive TME in GBM. The activation and function of CD8+ T cells are influenced by numerous of environmental factors. The cytokines secreted by tumor cells, myeloid-derived suppressor cell (MDSC), and TAM could all affect the trafficking, localization, differentiation, and activation of T cells, resulting in exhaustion and other forms of dysfunction of T cells [22]. A study that analyzed the TIL in both primarily diagnosed and recurrent GBM demonstrated significant exhaustion of CD8+ TIL as defined by increased expression of PD-1, CD39, Tim-3, CD45RO, and HLA-DR [2]. Our study also showed a distinct more CD68+ macrophage infiltration and PD-L1 expression in recurrent GBM than newly diagnosed tumors, which indicated a potential more
immunosuppressive environment in recurrent GBM since TAMs in GBM have been known to be immunosuppressive. Of note, the CD68+ macrophages are the dominant immune cell type as demonstrated by our and other groups particularly in recurrent tumor comprising of, on average, one third of the cells in tumor with some specimen being more than 50%. It remains unknown whether the increased expression level of PD-L1 in recurrent GBM, although still very low, may prone the tumor to be more responsive to anti-PD1/PD-L1 immunotherapeutics with baseline expression levels of PD-L1 in primarily diagnosed GBM known to be very low and its expression level overall is a negative predicting factor for prognosis [23, 24].

Our study also showed an interesting finding that the CD8/CD4 ratios remained stable between the primarily diagnosed GBM and recurrent GBM. CD8+/CD4+ TIL ratio has long been recognized as a measure of relevant immune activity in the tumor environment and was reported to have a positive correlation with prognosis in some cancers [25, 26]. Although the significance of such association is still controversial in glioma, with the considerable low number of TIL and the relatively "cold" immune environment in glioma, accumulating evidence supported CD8+/CD4+ ratio as a sign of tumor regression and showed the reverse relationship between CD8+/CD4+ ratio and the malignant grade in glioma [18, 27]. This prompted us to study the CD8+/CD4+ ratio in primarily diagnosed and recurrent GBM context. Despite the significant increase of CD8+ TIL in recurrent GBM, there was also remarkable recruitment of CD4+ TIL in recurrent GBM compared with primarily diagnosed GBM, which ended with an unchanged CD8/CD4 ratio between these two, indicating a similar hostile immune environment of recurrent GBM as in the primarily diagnosed GBM. The question is raised whether increased TIL infiltration is a nature of the recurrent disease or a consequence of prior chemo- and/or RT, with the median time interval from initial diagnosis to tumor recurrence being 23.9 months in the recurrent tumor cohort of our study which is approximately one year after completing the adjuvant chemotherapy. Multiple clinical trials testing anti-PD1/PD-L1 immunotherapy, including CheckMate-143 testing nivolumab in recurrent GBM patients have failed to show benefit. Future strategy in treating recurrent GBM, based on our results from this study, could be incorporating an immune cell modulator to convert the hostile environment such as a drug that can polarize TAM to anti-cancer phenotype, combining with anti-PD1/PD-L1 immunotherapeutics, which may take advantage of the increased immune cell infiltration in recurrent tumor although most likely those newly infiltrated cells are immediately immunosuppressive.

Collectively, by analyzing and comparing the infiltrating immune cells in tissues from newly diagnosed and recurrent GBM samples, we found that TIL and TAM are more abundant in recurrent GBM with a predominantly perivascular accumulation pattern, particularly for CD4+ TIL. Our study may contribute to deliver novel insights into the TME of recurrent GBM and provide valuable information for designing future combinatory therapies by selecting novel drugs for patients with recurrent GBM.

Declarations

Compliance with Ethical Standards
Funding

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Conceptualization: Chi Zhang (UNMC); Methodology: Chi Zhang (UNMC), Fei Wang; Formal analysis, investigation: Fei Wang; Resources: Dominick J. DiMaio, Michele R. Aizenberg, Nan Zhao; Data curation: Fei Wang, Sahara J. Cathcart, Jie Chen; Writing—original draft preparation: Fei Wang, Nan Zhao; Writing—review and editing: Fei Wang, Jie Chen, Nicole A. Shonka, Chi Lin, Chi Zhang; Funding acquisition: Chi Zhang (UNMC). All authors have read and agreed to the published version of the manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data Availability statement

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Tables

Due to technical limitations, tables 1 to 3 are only available as a download in the Supplemental Files section.
Figures

Figure 1

**Immune cell infiltration in GBM at initial diagnosis versus recurrence.** Dot plots depicting CD4 (A), CD8 (B), CD8/CD4 ratio (C), CD68 (D), and PD-L1 (E) positive cell number per 1k cells in GBM group at initial diagnosis (n=13), recurrent GBM group (n=11) and epilepsy group (n=2). P values are defined as * < 0.05; ** < 0.01; and ***< 0.001. ns: not significant (P ≥ 0.05).

Figure 2

**Correlation of the immune cell infiltration / PD-L1 expression levels with overall survival in GBM patients at initial diagnosis.**

The correlations of the density of CD4+ T cells (A), CD8+ T cells (B), CD8/CD4 ratio (C), CD68+ macrophage (D), and PD-L1 expression levels (E) in GBM at initial diagnosis with OS were analyzed using simple linear regression.

Figure 3

**Comparison of immune cell infiltrating densities at initial diagnosis and recurrence from the same patients.**

Densities of CD4+, CD8+, CD68+, PD-L1+ cells and CD8/CD4 ratio were calculated in tumor tissues obtained at the initial diagnosis and recurrence of the five GBM patients with paired tissue samples. Paired t-tests were used to determine the significances. P values are defined as * < 0.05 and ** < 0.01. ns: not significant (P ≥ 0.05).

Figure 4

**Infiltration patterns of immune cells in TME of GBM at initial diagnosis and at recurrence.**

(A): Selected IHC staining images of perivascular regions in tumor tissues obtained at the initial diagnosis and at recurrence of GBM patients with paired tissues at both times. Immunostainings of CD4,
CD8, CD68 and PD-L1 markers were illustrated at low magnification (left, 200×, scale bar represents 50 μm) and high magnification (right, 400×, scale bar represents 20 μm). (B): Comparisons of the densities of CD4, CD8, CD68, and PD-L1 positive cells per HPF (40× objective and 10× eyepiece) in intratumoral and perivascular areas between initially diagnosed (n=13) and recurrent GBM (n=11). A mean of 10 randomly selected HPFs in tumor parenchymal regions were used for intratumoral counting, while a mean of 10 randomly selected HPFs with at least one vessel per HPF were used for perivascular counting. (C): Comparisons of the infiltration scores of CD4, CD8, CD68, and PD-L1 positive cells between intratumoral and perivascular regions at initial diagnosis (n=13) and at recurrence GBM (n=11). Bars represent mean ± SEM. *: p < 0.05; **: p < 0.01; ***: p < 0.001; ****: p < 0.0001. Data were analyzed by unpaired Student's t tests.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.png
- Table2.png
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