CD8+CD103+PD1+TIM3+ T cells in glioblastoma microenvironment correlate with prognosis.

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Abstract

Glioblastoma (GBM) is the most common and aggressive brain tumor with poor outcome. Immune checkpoint inhibitors (ICIs) have been tested in GBM and, despite disappointing results, the identification of a small subgroup of responders underlies the need to improve our understanding of the tumor microenvironment (TME) immunity. The aim of this study was to determine whether the expression of selected immune checkpoints on tissue-resident memory T cells (Trm) may predict patient outcome. We conducted a single cohort observational study. Tumor samples were collected from 45 patients with histologically confirmed, IDH wild type, GBM (WHO grade IV) and processed to obtain single cell suspensions. Using multiparametric flow cytometry and uni/multivariate analyses, patients were assessed for the correlation of Trm with overall survival (OS) and progression-free survival (PFS). High and low frequency of Trm expressing PD1 and TIM3 was found to be linked to clinical outcome. In fact, low frequency of Trm expressing PD1 or TIM3 or both markers defined subgroups as independent positive prognostic factors for patient survival. On multivariate analysis, low CD8+CD103+PD1+TIM3+ Trm and KPS [?]70 were confirmed to be the most predictive independent factors associated with longer OS (HR [95%CI]: 0.14 [0.04 - 0.52] p¡0.001, 0.39 [0.16 - 0.96] p=0.04, respectively). The CD8+CD103+ Trm subgroups also resulted age-linked predictors for survival in GBM.

Introduction

Glioblastoma (GBM) is the most common and malignant primary tumor of the central nervous system (CNS) in adults[1]. The standard-of-care treatment consists of surgery followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ)[2]. The prognosis is poor, with median overall survival (OS) of approximately 18 months and 2-year survival rate of less than 20%[3].

Therapies of GBM are limited by several factors, including the immunosuppressive tumor microenvironment (TME)[4], which is thought to be the major cause of failure of many clinical trials with immunotherapies. Despite this, in the first clinical trial using nivolumab, about 8% of patients showed responses longer than bevacizumab (11.1 months *vs* 5.3 months)[5].

Several studies indicated that multiple therapies for GBM may lead to substantial changes in the TME[6], whose immune contexture is one of the most important player for tumor progression and response to therapies in many cancer types [7]. TME is a highly complex and dynamic entity that is responsible for defining GBM

as cold tumor[8], dominated by a highly immunosuppressive milieu and dysfunction of T cells[9]. A diverse rate of tumor infiltrating lymphocytes (TILs), including tissue-resident memory T cells (Trm), and the expression of specific stimulatory or inhibitory molecules are determinant factors in defining TME immune reactivity[10]. Accordingly, T cells infiltrating GBM express multiple immune checkpoints, such as PD1 and TIM3, and exhibit impaired function[11].

Solid tumors show enrichment of CD4+ and CD8+ Trm that, upon recruitment into the tissue and in the presence of local inflammatory signals, undergo maturation with CD103 up-regulation[12]. CD8+CD103+ T cells, also defined as CD8+CD103+ Trm, have been shown to be the major anti-tumor effector cells, and their high rates correlates with longer OS in many types of cancer[13]. CD8+CD103+ Trm populate the human brain, playing a key role in immune surveillance[14], and have also been implicated in the response to neo-adjuvant vaccination of GBM patients[15]. However, the role of CD8+CD103+ Trm in GBM needs to be further elucidated.

Elderly GBM patients may benefit from treatments based on *MGMT*status[16], and age-dependent factors have been reported to be critical for GBM prognosis [17]. Noteworthy, the age-related changes of immune function increase at age 65[18]. In GBM, age variable has not been linked to specific immune parameters yet.

Here, we analyzed intratumoral T cells in GBM and we postulate a major role of CD8+CD103+ Trm changes in shaping TME immune contexture. Importantly, such changes hold prognostic significance since low frequency of CD8+CD103+ Trm expressing PD1 and TIM3 predicts better survival.

METHODS AND MATERIALS

Detailed information on study design, reagents and antibodies, biological sample processing, multiparametric flow cytometry analysis of tumour infiltrating lymphocytes and PBMCs, *in vitro* intratumoral lymphocyte expansion, phenotypic characterization and cytokine production, and statistical analysis are described in the Supporting Information.

Results

Study design

This study included 45 newly diagnosed GBM patients (Table 1). Twenty-seven patients (60.0%) had *MGMT* promoter methylation, and twenty-four (53.3%) were males (Figure 1A). Median age at diagnosis was 64 years; median age of young patients ([?]63years) *vs* elderly patients (>63 years) was 57 *vs* 71, respectively. Males and females exhibited equally distributed baseline features, except for no-significant trend towards younger age in males (median age in males *vs* females 61 *vs* 65 years; p =0.62). Median follow-up was 12 months. Predictors of survival were KPS [?]70 and GTR (Table 1). Kaplan-Meier analysis showed that OS differed significantly according to age (young *vs* elderly, 14.7 *vs* 10 months,p= 0.01), KPS ([?]70 vs <70, 14.7 *vs* 7,5 months,p =0.0007), and GTR (GTR *vs* non-GRT, 15 *vs* 9 months,p= 0.004). Patients with methylated *MGMT* promoter had a longer median OS compared to those with unmethylated promoter (14.5*vs* 10.5 months; Table 1), but the difference was not significant. Conversely, PFS resulted significantly changed only in patients grouped by KPS (median [?]70 *vs* <70 group, 8 *vs* 6 months,p= 0.002) and GTR (median GTR *vs* no-GRT, 8 *vs* 5 months, p= 0.002) (Figure 1B, Table 1). These data were confirmed by the univariate analysis, which showed significant correlation with better OS of patients aged [?]63 years, KPS [?]70, and GTR (HR [95%CI]: 0.42 [0.20 - 0.89] p ¡0.001, 0.26 [0.12 - 0.58]p ¡0.001, 0.31 [0.15 - 0.65] p ¡0.001, respectively). At once, better PFS was associated only with KPS [?]70 and GTR (HR [95%CI]: 0.36 [0.18 - 0.74] p =0.01, 0.35 [0.17 - 0.74] p=0.01, respectively). Sex was not significantly correlated with reduced patient HR (Figure 1C, Table S1). Overall, the clinical features of our patients are in line with current literature.

Significance of PD1 and TIM3 expression on CD8+CD103+ Trm

Although the immune infiltrate in GBM have been largely characterized[19], the association of immune checkpoint expression on intratumoral T cell subsets with disease outcome wants to further be exploited.

Therefore, we performed a deep characterization of T cells in tumor samples collected at surgery for lineage, differentiation, memory, activation, and inhibition markers. The frequency of total leukocyte infiltrates, identified as cells highly expressing CD45 levels as opposed to microglial cells characterized by low CD45 expression (Figure S1), was found to be extremely variable among GBM tumors, ranging from 0.01 to 60.3% of whole live cells (median 1.7%) (Figure 2A). As well, high variability of CD45+CD3+ T lymphocytes among patients was found (Figure S2), along with high inter-patient heterogeneous frequencies of CD4+ and CD8+ T cells (CD8+ T cells: 32,4% [7,4% - 74,9%]; CD4+ T cells: 34,6% [0% - 59,1%]) (Figure 2B). A significantly higher frequencies of CD4+ and CD8+ T lymphocytes expressing PD1 with respect to TIM3 or both immune checkpoints were also found (Figure 2C). Similarly to other solid tumors, GBM also recruit CD103+ Trm, which resulted co-expressing the Trm-linked markers CD103 and CD69[14] (Figure S1), herein referred as CD103+ Trm. CD4+CD103+ Trm rate resulted significantly lower to that of CD8+CD103+ Trm (median: 1,8% *vs*7,0%, p =0.0002). In addition, the frequency of CD8+CD103+PD1+ Trm was significantly higher than CD4+CD103+PD1+ Trm (median: 6.3% vs 1.4%, p =0.0002) whereas TIM3 was present at comparable levels on the-two T subpopulations (Figure 2D). The further analysis of the diverse subgroups within CD8+CD103+Trm showed the significant prevalence of cells expressing PD1 with respect to TIM3 and both immune checkpoints (Figure 2E). A deeper assessment of the correlation between the diverse intratumoral T subsets was performed by Spearman's analysis. CD45+ immune cells were found to positively correlate with CD3+ T cells (R=0.58, p ¡0.0001). In turn, CD3+ T cells displayed a strong correlation with CD8+ T cells (R=0.53, p =0.0001), a weak association with CD4+ T cells (R=0.34, p =0.02) and a strong inverse correlation with all CD4+CD103+ Trm subsets. Conversely, strongly positive associations were found between CD8+ and CD8+CD103+ Trm (R=0.46, p =0.001), CD8+CD103+ Trm and CD8+CD103+PD1+ Trm (R=0.98, p <0.0001), CD8+CD103+TIM3+ Trm and CD8+CD103+PD1+TIM3+ Trm (R=0.91, p <0.0001) (Figure 2F). Essentially, CD8+CD103+ Trm were strongly correlated with the presence of CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm subsets (Figure 2G). These data suggest that intratumoral CD45+ immune cells in GBM are positively associated with CD8+CD103+ Trm, and in particular with CD8+CD103+ Trm expressing PD1 and TIM3 rather than CD4+ Trm.

Next, we analyzed the correlation of intratumoral T cells expressing PD1 or TIM3 or both inhibitory markers with prognosis, by categorizing each T cell subset as "high or low" frequency on the cut-off value from the asymptotic distribution of re-scaled rank statistic using the Contal-O'Quigley method. Four out of six CD8+ T subsets were found to be predictors of patient outcome with all CD8+CD103+ Trm subsets resulting the most statistically significant (Figure 3A). Specifically, low CD8+CD103+PD1+ Trm were associated with significantly better OS and PFS (p =0.02 and 0.0031, respectively) and even higher significant was the correlation with better OS and PFS of low CD8+CD103+TIM3+ Trm (p =0.0002 and 0.0033, respectively) and CD8+CD103+PD1+TIM3+ Trm (p <0.0001, both) (Figure 3B). On univariate analysis, low CD8+CD103+PD1+, CD8+CD103+TIM3+, and CD8+CD103+PD1+TIM3+ Trm subsets resulted independent predictors of improved OS (HR [95%CI]: 0.49 [0.35 - 0.92] p ¡0.001, 0.23 [0.09 - 0.54] p ¡0.001, 0.08 [0.02 - 0.25]p =0.04, respectively) and PFS (HR [95%CI]: 0.37 [0.18 - 0.77] p ¡0.001, 0.32 [0.14 - 0.74] p ¡0.001, 0.13 [0.05 - 0.39] p ¡0.001, respectively) (Figure 3C, Table S2). Next, we used a multivariate model to explore the relationship between each CD8+CD103+ Trm subsets and clinical parameters, namely age, sex, MGMT methylation, KPS and GTR, in predicting survival. Better OS was observed with low CD8+CD103+PD1+ Trm (HR [95%CI]: 0.29 [0.12 - 0.74] p =0.01) associated with GTR, KPS [?]70 and age [?]63, with low CD8+CD103+TIM3+ Trm (HR [95%CI]: 0.21 [0.08 - 0.53]p ¡0.001) associated with KPS [?]70 and age [?]63, and lastly with low CD8+CD103+PD1+TIM3+ Trm (HR [95%CI]: 0.14 [0.04 - 0.52]p ¡0.001) in association with KPS [?]70 only (Figure 4A, Table S3). Better PFS resulted associated to low CD8+CD103+PD1+ Trm and GTR and KPS, and low CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm with KPS (Figure S3). Then, high or low CD8+CD103+ Trm subsets were evaluated for predicting OS in GBM patients grouped by KPS and age. Kaplan-Meier plots showed that all three high CD8+CD103+ Trm subsets associated with KPS[?]70 in predicting better OS (Figure 4B). Of note, elderly patients with low CD8+CD103+ Trm subsets had significantly better OS when compared with same age patients distinguished by high immune subsets; in particular low CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm re-

sulted the most significant predictors (p =0.0002) (Figure 4C). In addition, CD8+CD103+ Trm subsets were statistically significant predictors of longer OS for young patients as compared to elderly ones. Confirming the multivariable analysis, young patients with high CD8+CD103+PD1+TIM3+ Trm were absent in the Kaplan-Meier plot (Figure 4C). Low CD8+CD103+ Trm subsets were also found to be predictors of improved PFS in patients with KPS [?]70 and aged [?]63 (Figure S4A, B). Finally, a multivariate analysis including all CD8+ Trm subsets and clinical variables confirmed that low frequency of CD8+CD103+PD1+TIM3+ Trm was associated with KPS[?]70 in predicting better OS (HR [95%CI]: 0.14 [0.04 - 0.52]p ¡0.001) and PFS HR [95%CI]: 0.25 [0.08 - 0.80]p =0.02, respectively (Figure 4D; Table S4).

Inhibition of PD1- TIM3 on CD8+CD103+ T cells enhances their anti-tumor activity

To functionally characterize CD8+CD103+ Trm, we cultured cells from whole tumor samples for 14 days in the presence of IL-2 with or without the antibodies anti-PD1 pembrolizumab and anti-TIM3 sabatolimab, allowing growth and activation of T cells within the co-culture with tumor and antigen presenting cells. Treatment with these antibodies led to an increased capability of CD8+ T cells to produce Granzyme B (GrzB), IFN-γ, and TNF-α, in spite of their number reduction (Figure 4E). Likewise, a reduced percentage of anti-PD1/TIM3-treated CD8+CD103+ T cells was found to enhance GrzB production (Figure 4F), suggesting a key function role of CD8+CD103+ Trm within GBM intratumoral T cells.

Lack of prognostic significance of PD1 and TIM3 expression on T cells of peripheral blood

The frequency of CD3+ as well as of CD4+ and CD8+ T lymphocytes resulted highly variable among patients (Figure S5A, B). Similarly to TME, the frequencies of both CD4+PD1+ and CD8+PD1+ T cells were higher than the relative subsets expressing only TIM3 or both TIM3 and PD1 (Figure 5A). In addition, the Spearman's analysis revealed a stronger positive correlation within CD4+ rather than CD8+ T cell subsets. In particular, CD4+PD1+TIM3+ T cells robustly linked with CD4+PD1+ T cells (R=0.59, p<0.005), CD4+TIM3+ T cells (R=0.74, p<0.0005) and CD4+ T cells (R=0.58, p<0.005). On the other hand, strongly positive association was observed between CD8+PD1+ T cells and CD8+ T cells (R00.71, p <0.0005) as well as CD8+PD1+TIM3+ T cells and CD8+TIM3+ T cells (R=0.54, p<0.005) (Figure 5B). However, Kaplan-Meier analysis demonstrated the absence of prognostic value of high and low frequencies of T cell subsets for both OS and PFS (Figure S6). The further correlation between CD45+-gathered T cell subsets in TME and peripheral blood revealed that the distribution of both CD4+ and CD8+ T cells was largely enriched in tumor samples with respect to paired blood samples, whereas the value of CD4+/CD8+ T cell ratio remained comparable (Figure 5D). Although without prognostic significance, CD8+ T subpopulations expressing PD1 or TIM3 or both markers were found significantly enriched in tumors with respect to blood samples (p <0.001) (Figure 5E).

DISCUSSION

In this study, we found that low frequency of intratumoral CD8+CD103+ Trm expressing PD1 and TIM3 correlate with significantly reduced risk of death in GBM, suggesting a key role of these immune checkpoints in dictating the anti-tumor immune response.

Intratumoral CD8+CD103+ Trm are positively associated with good prognosis in several high-grade tumors and with response to immunotherapy[20]. Nevertheless, in some cases high frequency of CD8+CD103+ Trm associate with poor outcome[21], generating apparently contradictory results that need to be further exploited. Here, we show that CD8+CD103+ Trm abundantly infiltrate GBM, and low frequency of these cells expressing PD1, or TIM3 or both molecules strongly correlates with better patient survival whereas the high frequencies mark poor disease outcome.

The phenotype of intratumoral Trm differs among tumor types, and can be organ and tissue specific[22]. In the brain, Trm are under strict control of immune checkpoint molecules limiting their immune reaction but with preserved functionality upon activation[14]. In general, Trm exert antitumor immunity eliminating transformed cells through the release of GrzB and cytokines to recruit and activate other immune cells [23]. Although CD8+CD103+ Trm are able to recognize their cognate antigen within the TME, they fail to control

tumor growth in the long-term. Likely, the chronic stimulation within the tumor results in increased expression of exhaustion markers, such as PD1 and TIM3, driving immune function downregulation [24]. Of interest, a high density of intratumoral CD8+CD103+ Trm have been also reported to correlate with poor prognosis in some types of cancer[25]. Here, we show that CD8+CD103+ Trm are present at variable rate within GBM tumors, and the rate of Trm subsets expressing PD1 or TIM3 or both molecules predicts prognosis since low and high frequencies of CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm strongly correlate with disease outcome. Specifically, we first found that the amount of intratumoral CD45+ immune cells was positively associated to total CD8+ T cells rather than CD4+ T cells, highlighting that even in a cold tumor like GBM, the prevalence of intratumoral CD8+ T cells could be therapeutically exploited[26]. However, while total intratumoral CD8+ T cells lacked prognostic significance, low frequencies of CD8+CD103+ Trm expressing PD1 or TIM3 or both molecules were to various extents associated with better prognosis, pinpointing these immune checkpoints as determinant for their exhausted function[27]. Low CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm subsets significantly associated with both better PFS and OS, underlying the functional preservation of CD8+CD103+ Trm non-expressing immune checkpoints. In particular, low frequency of CD8+CD103+PD-1+TIM-3+ Trm was found to be the most predictive immune marker of better prognosis, suggesting that the low frequency of such terminally exhausted T cells with reduced long-term survival is the most important determinant for the antitumor immune response[28].

We also showed that the frequencies of PD1- and TIM3-expressing CD8+CD103+ Trm associated to the clinical variable KPS and age in predicting GBM outcome [29]. To our knowledge, this study is the first to correlate intratumoral low frequencies of CD8+CD103+ Trm subsets with high KPS and younger age identifying GBM patients with better prognosis. In particular, low CD8+CD103+PD1+ and CD8+CD103+TIM3+ Trm subsets resulted significant predictors of better survival for patients with both KPS ¡70 and ¿ 70 as well as aged [?]63 and ¿63. It is worth nothing, that low frequency of CD8+CD103+PD1+TIM3+ Trm were found to predict better outcome in patients with KPS ¡70 and ¿ 70 but only aged ¿63 since no young patients in our cohort was not found to have high frequency of the most exhausted CD8+CD103+PD1+TIM3+ Trm. Accordingly, young GBM patients with KPS [?]70 and low frequencies of CD8+CD103+ Trm subsets resulted the group with better clinical outcome. However, while the association between Trm subsets and age is relevant to the challenge of immune changes in the elderly[30], there is not an obvious link of the above immune populations with KPS variable; in this regard, we can speculate on the significance of tumor location in terms of immune infiltrates[31]. Importantly, we found little to no expression of CD103 on peripheral CD8+ T cells in opposition to what was recently reported[32], and the expression of PD-1 and TIM-3 did not show any relevance in predicting disease outcome. Altogether, these data confirm that CD8+CD103+ Trm populate the human brain and may play a master role in immune surveillance under a tight control of inhibitory markers. Consistent with this finding, our study also disclosed that in vitro blocking of PD1 and TIM3 released the capability of intratumoral T cells to produce the effector cytokines IFN-γ and TNF-α, and the cytotoxic factor GrzB. Altogether, our results support the concept that a timely and targeted use of ICIs could reactivate antitumor Trm function.

PD1-based immunotherapies have been tested in a multitude of phase I/II and phase III clinical trials[33]. In line with the finding that high intratumoral TIM3 expression is linked to glioma severity and progression[34], anti-TIM3 therapy is being explored in GBM[35]. However, although to date no obvious clinical benefits of immune checkpoint blockade have been reported in GBM, few patients have shown long-term responses suggesting a therapeutic window for patient-specific treatment. The concept that boosting the immune system properly may lead to stimulate an effective antitumor response in GBM has been recently demonstrated by the phase III trial with DCVax-L vaccine which has met both primary and secondary endpoints with extended patient survival for many months[36]. These evidences meet our finding that the frequency of CD8+CD103+ Trm expressing PD1 and TIM3, evaluated at the surgery, may shape anti-GBM immunity affecting prognosis. Accordingly, these immune biomarkers were found tightly associated to the clinical variables KPS and age in stratifying patients with diverse disease outcome. In addition, as opposed to the peripheral blood whose CD8+ T cell subsets lacked of any prognostic value, the negative impact of PD1 and TIM3 expression

on CD8+CD103+ Trm cells may be reversed by specific immune checkpoint blockade as it occurred in our *in vitro*experiments leading to production of T effector and cytotoxic mediators. Although our study has the limitation that it was conducted in a relatively small cohort, it allowed a deeper understanding of the biology of CD8+CD103+ Trm, identifying them as intratumoral-reactive T cells whose function could be restored by PD1-TIM3 blockade. Accordingly, the adjuvant administration of immune checkpoint inhibitors may represent an optimal therapeutic window for providing favorable clinical outcomes in GBM patients.

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FIGURE LEGEND

Figure 1 . **A**) Schematic Representation of the Study Parameters. GBM cohort consists of 45 patients stratified by sex (female, males), age ([?]63 years, >63 years), and*MGMT* promoter methylation status (methylated promoter=Met; unmethylated MGMT promoter=Unmet); **B**) Kaplan-Meier analysis for OS and PFS in GBM patients stratified by age, sex, *MGMT* promoter methylation, KPS and GTR. p values were calculated by the Log-rank (Mantel-Cox) test; **C**) Forest plot showing hazard ratios with 95% confidence intervals of sample classifiers age, sex and*MGMT* promoter methylation status for OS and PFS.

Figure 2. Lymphocyte composition and immunophenotypic characterization of intratumoral T subsets in GBM patients. Intratumoral T cell subsets were analyzed by flow cytometry on viable CD45+ cells; dots represent single patients. **A**) Distribution of intratumoral CD45+ cell frequencies in the GBM cohort. **B**) Median frequencies of CD4+ and CD8+ T cells with respect to total tumor CD45+ T cells. **C**) PD1 and TIM3 expression on CD4+ and CD8+ T cells gathers the subpopulations CD4+PD1+ T cells, CD4+TIM3+ T cells, CD4+PD1+TIM3+ T cells, CD8+PD1+ T cells, CD8+TIM3+ T cells, CD8+PD1+TIM3+ T cells. **D**) Frequencies of CD103+ Trm subsets assessed within total CD45+ cells; PD1 and TIM3 expression on CD103+ Trm gathers the following subsets: CD4+CD103+PD1+, CD4+CD103+TIM3+, CD4+CD103+PD1+TIM3+, CD8+CD103+PD1+, CD8+CD103+TIM3+, CD8+CD103+PD1+TIM3+. **E**) Frequencies of CD8+CD103+PD1-TIM3-, CD8+CD103+PD1+, CD8+CD103+TIM3+, CD8+CD103+PD1+TIM3+ subsets assessed within total CD8+CD103+ Trm. T cell subsets in A), C), D) and E) were compared using Kruskal-Wallis test and statistically significant differences are indicated with asterisks (**p<0.005, ***p<0.0005). **F**) Heatmap of Spearman's correlations among subpopulations of intratumoral T cells. Red indicates a positive correlation and blue represents a negative correlation; absence of correlation is indicated by white. The values of Spearman's coefficient are reported, while asterisks mark the significance level (*p<0.05, **p<0.005, ***p<0.0005).**G**) Correlation plot of CD8+CD103+ Trm with the subsets expressing PD1, TIM3 or both molecules. Line shows the LOESS fit to the data.

Figure 3. Correlation of intratumoral T cell subsets with OS and PFS patients. A) Plot of Kaplan-Meier analysis summarizing the correlation of PD1- and TIM3-expressing T cell subsets with OS and PFS patients. The analysis was performed to associate low or high percentages of PD1- and TIM3-expressing T lymphocytes, established based on the cut-off of each subset, with OS and PFS; p values were calculated by log-rank (Mantel-Cox) test; brown colors indicate negative correlation of high frequencies of

PD1- and TIM3-expressing T cells with OS and PFS. **B**) Kaplan-Meier curves showing the significant correlations with OS and PFS of low and high frequencies of CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm subsetss.**C**) Univariate regression analysis for effects of T cell subsets over prognosis of patients in terms of OS and PFS; comparisons were performed using log-rank (Mantel–Cox) test and corresponding error bars show 95% CI.

Figure 4 . Identification of the interdependency of low and high CD8+CD103+ Trm subsets with clinical variables in predicting GBM outcome . **A**) HR and p-value of Cox stepwise multivariate regression including CD8+CD103+ Trm subsets and all clinical variables.**B**) Kaplan-Meier plots showing CD8+CD103+ Trm subset prediction of OS in GBM patients stratified by KPS. **C**) Kaplan-Meier analysis of OS displaying CD8+CD103+ Trm subset prediction in GBM patients stratified by age. **D**) Multivariate analysis including all CD8+CD103+ Trm subsets and all clinical variables showing the stronger predictor value of CD8+CD103+PD1+TIM3+ and KPS [?]70 for OS and PFS. **E**) Functional effects of anti-PD1 and anti-TIM3 antibody treatment on expanded T cells from culture of whole tumor cells. Untreated and antiPD1/anti-TIM3-treated CD8+ T cells were evaluated by flow cytometry for GrzB (middle panels), IFN-γ and TNF-α production (right panels). **F**) Untreated and anti-PD1/anti-TIM3-treated CD8+CD103+ Trm were gathered in intratumoral CD8+ T cells (left panels) and assayed for GrzB production (right panels).

Figure 5. Composition and phenotypic characterization of T subsets in blood of GBM patients. Blood lymphocyte populations were analyzed by flow cytometry on viable CD45+ cells. **A**) Scatter plots of median values of T cell subsets in blood were compared using Kruskal-Wallis test. **B**) Heatmap of Spearman's correlations showing binary associations within subpopulations of T cells in blood. Red, positive correlation; blue, negative correlation; white, no correlation (*p<0.05, **p<0.005, ***p<0.0005). **C**) Pairwise comparisons between CD4+ and CD8+ T cells between blood and TME using Wilcoxon matched-pairs signed-rank test. **D**) Pairwise comparisons of TME blood CD4+/CD8+ T cell ratio. **E**) Pairwise comparisons of CD4+PD1+, CD4+TIM3+, CD4+PD1+TIM3+, CD8+PD1+, CD8+TIM3+ and CD8+PD1+TIM3+ T cell subsets between blood and TME using Wilcoxon matched-pairs signed-rank test. Dots represent single patients. Statistically significant differences are indicated with asterisks (**p<0.005, ***p<0.0005).

Table 1. Clinical features of patients enrolled in the study

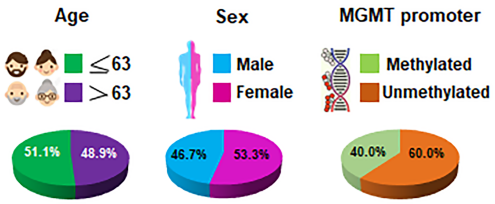
Variable	**All**	**Age**			**Sex**			**MGMT**						
		[?]63	**[?]63**	p	**Male**	**Female**	**p**	**Met**	**Met**	**Unmet**	**Unmet**	**p**	p	**[?]70**
Number (%)	45 (100)	22 (48.9)	23 (51.1)		24 (53.3)	21 (46.7)		27 (60)	27 (60)	18 (40)	18 (40)			34 (74.5
Age at diagnosis median (range)	64 (45-78)	57(45-63)	71 (64-78)	**<0.0001**	61 (45-78)	65 (49-76)	0.62	65 (45-78)	65 (45-78)	63.5 (52-76)	63.5 (52-76)	0.75	0.75	60.5 (45-78)
Sex n (%)				0.55								0.76	0.76	
Male	24 (53)	13 (59)	11 (47.8)					15 (55.5)	15 (55.5)	9 (50)	9 (50)			18 (0.53
Female	21 (47)	9 (41)	12 (52.1)					12 (44.5)	12 (44.5)	9 (50)	9 (50)			16(0.

Tumor site n (%)														
Temporal	19 (42.2)	7 (31.8)	12 (52.1)	0.23	11 (4.8)	8 (38.1)	0.76	11 (40.7)	11 (40.7)	8 (44.4)	8 (44.4)	1	1	14(41
Frontal	15 (33.3)	8 (36.3)	7 (30.4)	0.75	4 (1.6)	11 (5.4)	0.02	10 (37)	10 (37)	5 (27.7)	5 (27.7)	0.74	0.74	12(35
Parietal	8 (17.8)	5 (22.7)	3 (13)	0.45	7 (29.1)	1 (4.7)	**0.05**	4 (14.8)	4 (14.8)	4 (22.2)	4 (22.2)	0.69	0.69	5 (14.7
Occipital	1 (2.2)	1 (4.5)	0 (0)	0.48	1 (4.2)	0 (0)	1	1 (3.7)	1 (3.7)	0 (0)	0 (0)	1	1	1 (2.9)
Multicentric	2 (4.5)	1 (4.5)	1 (4.3)	1	1 (4.2)	1 (4.7)	1	1 (3.7)	1 (3.7)	1 (5.5)	1 (5.5)	1	1	2 (5.9)
KPS median (range)	70 (40-90)	80 (50-909	70 (40-90)	0.05	70 (40-90)	80 (40-90)	0.75	70 (60-90)	70 (60-90)	75 (40-90)	75 (40-90)	0.64	0.64	
[?] 70 (%)	34 (75.5)	20 (90.9)	14 (60.9)	0.73	18 (75)	16 (76.2)	0.9	24 (88.9)	24 (88.9)	10 (55.6)	10 (55.6)	0.007	0.007	
<70 (%)	11 (24.5)	2 (9.1)	9 (39.1)	0.45	6 (25)	5 (23.8)	0.34	3 (11.1)	3 (11.1)	8 (44.4)	8 (44.4)	0.17	0.17	
GTR n (%)				0.51			0.2					0.73	0.73	
Yes	32 (71.1)	17 (77.3)	15 (65.2)		15 (62.5)	17 (81)		20 (74.1)	20 (74.1)	12 (67.7)	12 (67.7)			27 (79.4
No	13 (28.9)	5 (22.7)	8 (34.8)		9(37.5)	4 (19)		7 (25.9)	7 (25.9)	6 (33.3)	6 (33.3)			7 (20.6
Stupp therapy n (%)				1			0.7					0.23	0.23	
[?] 6 cycles	37 (82)	18 (81)	19 (82)		19 (79)	18 (86)		24 (89)	24 (89)	13 (72)	13 (72)			30 (88)
< 6 cycles	8 (18)	4 (19)	4 (18)		5 (21)	3 (14)		3 (11)	3 (11)	5 (28)	5 (28)			4 (12)
IDH mutation n (%)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)		0 (0)	0 (0)	0 (0)	0 (0)			0 (0)
MGMT n (%)				1			0.76							

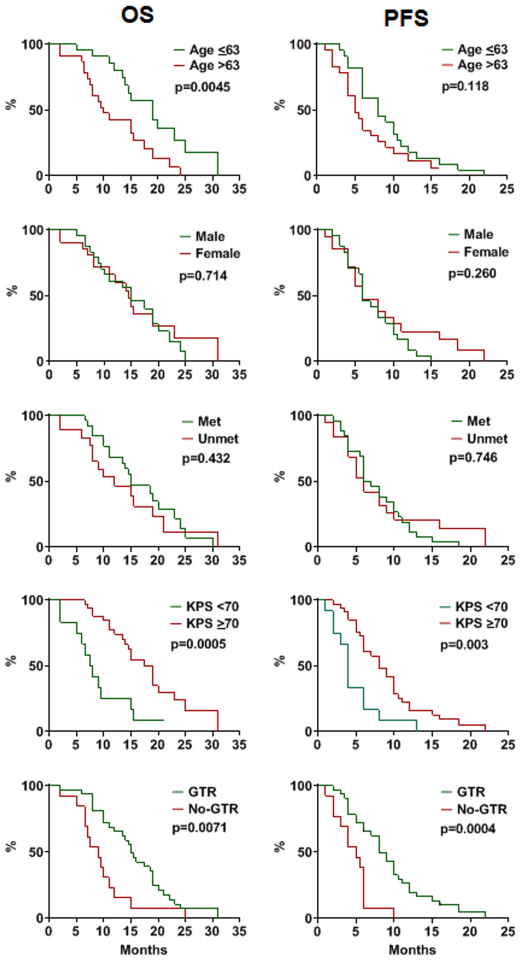
Met	27 (60)	13 (59.1)	19 (82)		15 (62.5)	12 (57.1)									24 (70.6
Unmet	18 (40)	9 (40.9)	4 (18)		9 (37.5)	9 (42.9)									10 (29.4
OS (months) median (range)	12 (2 - 31)	14.7 (5-31)	10 (2-24)	**0.01**	12.7 (5-25)	12 (2 - 30)	0.8	14.5 (6.5-30)	14.5 (6.5-30)	10.5 (2-31)	10.5 (2-31)	0.1	0.1		14.7 (7-31)
PFS (months) median (range)	6 (1 - 22)	8 (3 - 22)	5 (1 – 16)	**0.02**	6 (2 - 15)	6 (1-22)	0.9	6 (2 - 18.5)	6 (2 - 18.5)	6 (1 - 22)	6 (1 - 22)	0.7	0.7		8 (2 - 22)

Statistical tests: Mann Whitney U test for continuous variables (Age, KPS), Fischer's exact test for categorical variables (Sex, GTR, Chemotherapy, MGMT status, Tumor site). MGMT, O6-methylguanine-DNA-methyltransferase; KPS, Karnofsky Performance Status; GRT, Gross Total Resection; IDH, isocitrate dehydrogenase; OS, overall survival; PFS, progression free survival.

A



B



C