


Analysis of human glioma-associated co-inhibitory immune checkpoints in glioma microenvironment and peripheral blood

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Abstract

One biomarker for a better therapeutic effect of immune checkpoint inhibitors is high expression of checkpoint in tumor microenvironment. The purpose of this study is to investigate the expression of immune checkpoints in human glioma microenvironment and peripheral blood mononuclear cells. First, single-cell suspension from 20 fresh high-grade glioma (HGG) specimens were obtained, and analyzed for lymphocyte composition, then six co-inhibitory immune checkpoints were analyzed at the same time. Second, 36 PBMC specimens isolated from HGG blood samples were analyzed for the same items. In GME, there were four distinct subtypes of cells, among them, immune cells accounted for an average of 51.3%. The myeloid cell population (CD11b⁺) was the most common immune cell identified, accounting for 36.14% on average; the remaining were most CD3⁺CD4⁺ and CD3⁺/CD8⁻/CD4⁻ T lymphocytes. In these cells, we detected the expression of BTLA, LAG3, Tim-3, CTLA-4, and VISTA on varying degrees. While in PBMCs, the result showed that when compared with healthy volunteers, the proportion of NK cells decreased significantly in HGG samples ($p < 0.01$). Moreover, the expression of BTLA, LAG3, and Tim-3 in CD45⁺ immune cells in PBMC was more remarkable in glioma samples. In conclusion, the CD11b⁺ myeloid cells were the predominant immune cells in GME. Moreover, some immune checkpoints displayed a more remarkable expression on the immune cells in GME. And the profile of checkpoint expression in PBMC was partially consistent with that in GME.

Keywords

high-grade gliomas, tumor microenvironment, myeloid cell, immune checkpoint

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Introduction

Glioblastoma (GBM) is the most aggressive malignant primary brain tumor in adults, and has an invariably terminal prognosis and a median survival time of only 15 months.^{1–4} Despite recent advances in surgery, radiation therapy and chemotherapy, treatment options for GBM remain limited. Novel treatment strategies are urgently needed. Immunotherapy plays a prominent role in some malignant tumor, but it needs to be clarified in GBM whose main therapy is still based on the STUPP protocol by now.

Immune checkpoint modulator is the most popular star in cancer immunotherapies and has produced dramatic changes in the treatment paradigms of some advanced cancers,^{5–7} it also provides prospect for the treatment of GBM.

So far, several immune checkpoint inhibitors (ICIs) have been widely investigated. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and Programmed Death 1 (PD-1) are the two best-studied ICIs and in certain tumors they do manifest compelling clinical effectiveness, however most patients inevitably develop adaptive resistance and the overall efficiency remain unsatisfactory. It has been reported that the durable objective response rate following anti-PD-1 therapy is 31–44% in advanced melanoma,^{5,8,9} 19–20% in NSCLC^{6,10,11} and 22–25% in RCC.⁷ In GBM, PD-1/PD-L1 inhibitors are currently the most widely researched ICIs and more than 30 clinical trials are under way to explore their clinical utility. However, the result of phase III clinical trial on anti-PD-1 antibody (Checkmate-143) did not meet their primary end point.¹² One of the reasons maybe associated with the low PD-L1/PD-1 expression in glioma microenvironment (GME), which is a biomarker for predicting treatment efficacy. An analysis of *PDCO1* (codes for PD-1) expression in the GBM/normal brain samples from the TCGA and REMBRANDT data sets showed that there was no significant difference between GBM and normal brain samples.¹³ For rational application of ICIs, it is important to analyze the expression profile of immune checkpoints in GME.

In addition to PD-1/PD-L1 and CTLA-4, recent studies have identified several new immune checkpoint targets, like lymphocyte activation gene-3 (LAG3), T cell immunoglobulin and mucin-domain containing-3 (Tim-3), V-domain Ig suppressor of T cell activation (VISTA), and B- and T- lymphocyte attenuator (BTLA). In our study, we analyzed the immune cell composition and co-inhibitory immune checkpoints expression profile in newly diagnosed high-grade glioma (HGG) microenvironment and the change of immune cell proportion and the expression of checkpoints in PBMCs. The purpose of these works is to provide instructions for the future application of ICIs in newly diagnosed HGG.

Methods

Obtaining human glioma specimens and preparation of single-cell suspensions

This study is fundamental research based on clinical data, and it belonged to prospective cohort study. According to the method for sample size estimation ($n = \frac{(Z_{\alpha} + Z_{\beta})^2 * 2\sigma^2}{\delta^2}$, $\alpha = 0.05$, $\beta = 0.8$), 15 samples were needed in each group. Fresh surgical glioma specimens from 20 patients with untreated, newly diagnosed primary supratentorial HGG were collected at the time of surgery and processed immediately from Jan 2018 to Jan 2020. Using an intraoperative image guidance system based on preoperative Gd-enhanced MR imaging, samples were taken from enhancing tumor and overlying “normal” cortex. The midline or bilateral glioma was excluded. All specimens from enhancing areas were histopathological confirmed as HGGs (World Health Organization Grade III/IV), the diagnosis was based on 2016 WHO classification and the details of clinical characteristics were presented in Table 1. Then, in order to remove obvious hematoma, the fresh surgical glioma specimens were minced and washed repeatedly with PBS. The resulting slurry was subjected to partial enzyme digestion (Miltenyi Biotec, brain tumor dissociation kit) and passed through 70 μm nylon mesh. These single-cell suspensions were used directly for flow cytometry. In addition to glioma specimens, six normal brain samples from patients undergoing fistula surgery were collected and worked as control group.

Isolation of PBMCs

Blood specimens from 36 pre-treatment primary HGG patients were collected in the mean time. Human peripheral blood mononuclear cells (PBMCs) were isolated using sequential Ficoll and Percoll density gradient centrifugations (Ficoll-Paque Plus, Amersham Biosciences) as described previously.¹⁴ Cells at the interface were harvested, washed once in PBS, and used immediately for flow cytometry analysis. In addition, another 36 blood samples from healthy volunteers were collected and worked as control group.

Flow cytometry

Single-cell suspensions from operative specimens were washed once in PBS, then resuspended in PBS, and counted on a hemocytometer with trypan blue staining. These single cells were divided into 10^6 -cell aliquots and were washed again in PBS for flow cytometry, then they were resuspended in 100 μL of PBS with 1% human AB serum, and incubated at room temperature for 10 min for

Table 1. Clinical characteristics of the study cohort in glioma microenvironment analysis.

	Age	Gender	WHO grade ^a	IDHmut	MGMTmet	I p /19q loh	TERTmut	H3K27M
1	56	M	IV GBM					
2	48	F	IV GBM					
3	47	F	III AA					
4	44	M	III AA					
5	66	F	III AA					
6	53	F	IV GBM					
7	55	F	IV GBM					
8	54	M	III AO					
9	51	F	IV GBM					
10	64	M	IV GBM					
11	67	M	IV GBM					
12	64	M	III AA					
13	48	F	IV GBM					
14	35	M	IV GBM					
15	40	M	IV GBM					
16	37	M	III AA					
17	54	F	IV GBM					
18	54	M	IV GBM					
19	46	F	III AO					
20	69	F	IV GBM					

AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma.

^aPer to the revised World Health Organization 2016 classification., anaplastic astrocytoma; AO, anaplastic oligodendroglioma

Fc-receptor blocking. Thereafter, 10 μ L of antihuman CD45-AmCyan (clone: HI30), CD11b-APC-Cy7 (clone: ICRF44), CD56-APC (clone: B159), CD3-PE-Cy7 (clone: SK7), CD4-PerCP-Cy5-5 (clone: SK3) and CD8-FITC (clone: RPA-T8) were mixed together and added to each sample, then VISTA-PE (clone: MIH65), CD-223-PE (LAG3, clone: T47-530), CD366-PE (Tim-3, clone: 7D3), CD272-PE (BTLA, clone: J168-540), CD152-PE (CTLA-4, clone: BNI3), and CD279-PE (PD-1, clone: BNI3) were added to the samples, respectively; similar staining was performed with isotype-matched control antibodies. All of the antibodies were purchased from BD Pharmingen. These samples were incubated at room temperature in the dark for 15 min. Cells were washed in PBS again and resuspended in 100 μ L of PBS, an additional 300 μ L PBS was added to each sample, and the samples were immediately read on a flow cytometer. An analysis was performed using Flow-Jo software (TreeStar, Inc.).

In addition to surgical HGG specimens, the normal brain tissue, the PBMCs from HGG patients and healthy volunteers were all analyzed by Flow Cytometry with the same methods mentioned above.

Statistical analysis

Data are expressed as the mean, median, quartile, range, and standard deviation for continuous variables and counts (percentages) for categorical variables. Comparison

between groups was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. All analyses were performed using SPSS software (version 21). All statistical tests were 2-sided, and $p < 0.05$ was considered statistically significant.

Ethics statement

Blood and tumor tissue was collected from brain tumor patients who were operated at blinded for peer review and signed our Clinical Specimen Bank acquisition consent form. The study was approved by the Ethics Committees of blinded for peer review. This study has been performed in accordance with the principles of the Declaration of Helsinki (1964) as revised in Tokyo (1975) Venice (1983), Hong Kong (1989), Somerset West (1996), and Edinburgh (2000). The study was also based on the following ethical and formal considerations: (1) Informed consent of the subject. (2) Declaration of Helsinki. (3) Laws and regulations in the China and Beijing.

Results

The cell composition in GME

Fresh surgical glioma specimens were obtained from 20 patients harboring HGGs. First flow cytometric analysis of CD45/CD11b/CD56/CD3/CD4/CD8 expression patterns

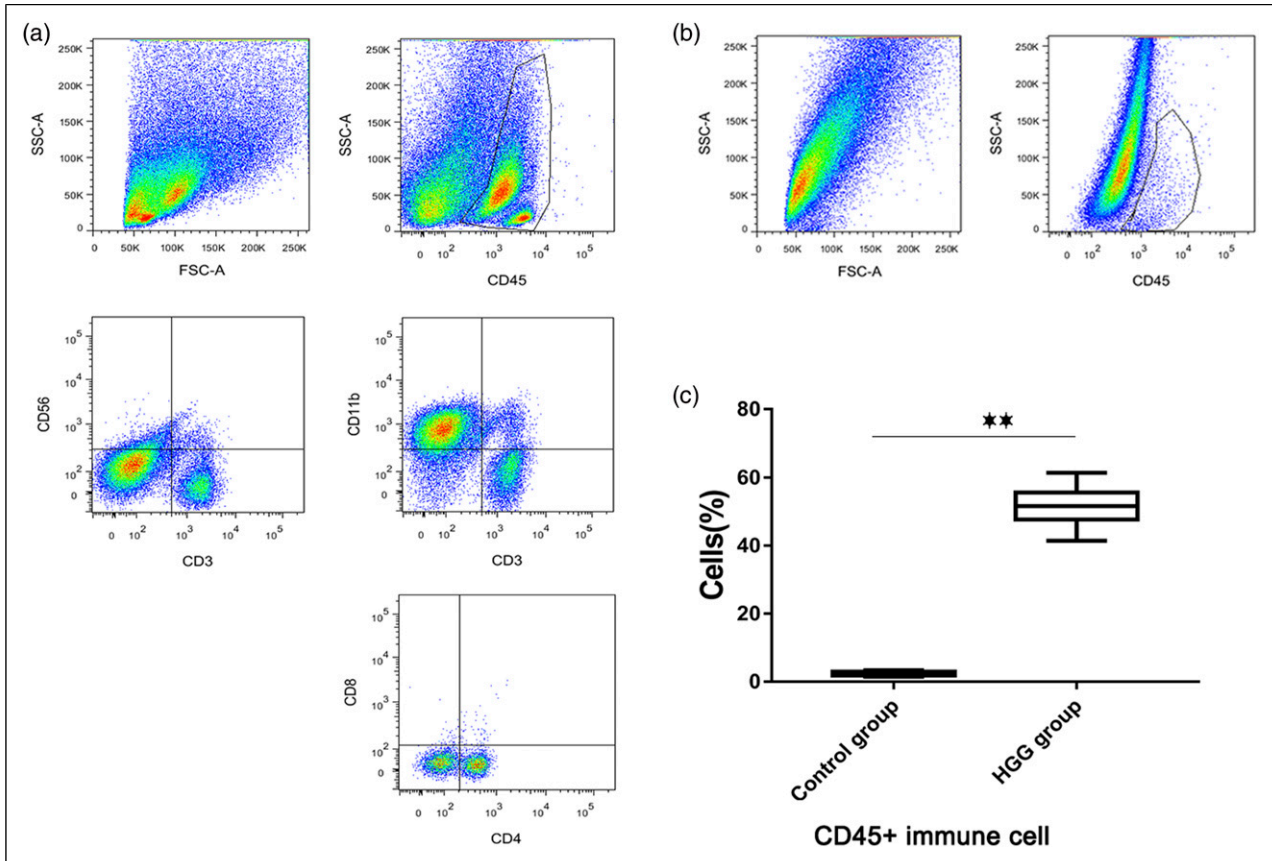


Figure 1. Analysis of the immune cell composition in glioma microenvironment. (a) analysis of CD45/CD11b/CD56/CD3/CD4/CD8 expression in fresh HGG specimens, the result showed that there were four distinct subtypes of cells in GME, it included CD45⁻ non-immune cells, CD45⁺/CD3⁻/CD11b⁺ myeloid cells, CD45⁺/CD3⁺/CD4⁺ T lymphocytes, and CD45⁺/CD3⁺/CD8⁻/CD4⁻ T lymphocytes. Among them, immune cells accounted for an average of 51.3%. There were scarcely any CD45⁺/CD3⁺/CD8⁺ T lymphocytes and CD3⁻CD56⁺ NK cells in HGGs immune microenvironment. (b) single-cell suspension from normal brain samples were analyzed as the control group; the result showed that there were only two distinct subtypes of cells including CD45⁻ non-immune cells and few CD45⁺ immune cells. The CD45⁺ immune cells in these specimens accounted for an average of 2.35%. (c) There was a significant difference in the infiltration of immune cells between the glioma specimen and normal brain specimen. (** $p < 0.01$).

in fresh HGG specimens revealed four distinct subtypes of cells in GME, it included CD45⁻ non-immune cells, CD45⁺/CD3⁻/CD11b⁺ myeloid cells, CD45⁺/CD3⁺/CD4⁺ T lymphocytes and CD45⁺/CD3⁺/CD8⁻/CD4⁻ T lymphocytes. Among them, immune cells accounted for an average of 51.3%. There were scarcely any CD45⁺/CD3⁺/CD8⁺ T lymphocytes and CD3⁻CD56⁺ NK cells in HGGs immune microenvironment (Figure 1(a)).

In these immune cell subtypes, the myeloid cell population (CD45⁺/CD3⁻/CD11b⁺) was the most common immune cell identified (mean (\pm SD) 36.14 \pm 5.01% of viable cells, range 29–43%); collectively these myeloid cells were called glioma-associated myeloids cells (GAMs), while CD45⁺/CD3⁺/CD4⁺ T lymphocytes (mean (\pm SD) 4.9 \pm 1.85% of viable cells, range 3.3–7.9%) and CD45⁺/CD3⁺/CD8⁻/CD4⁻ T lymphocytes (mean (\pm SD) 3.3 \pm 0.89% of viable cells, range 2.3–4.5%) were less common (Figure 1(a)).

In our study, single-cell suspension from normal brain samples were analyzed as control group, flow cytometric analysis of these specimens showed that in normal brain, there were only two distinct subtypes of cells including CD45⁻ non-immune cells and sparse CD45⁺ immune cells. The CD45⁺ immune cells in these specimens accounted for an average of 2.35% (Figure 1(b)), and there was significant difference in the infiltration of immune cells between the glioma specimen and normal brain specimen ($p < 0.001$) (Figure 1(c)).

Immune checkpoints profile in GME

Next, we analyzed the expression profile of immune checkpoints in glioma infiltrating immune cells. First, we analyzed their expression on myeloid cells, and the analyzed checkpoints included BTLA, LAG3, Tim-3, PD-1,

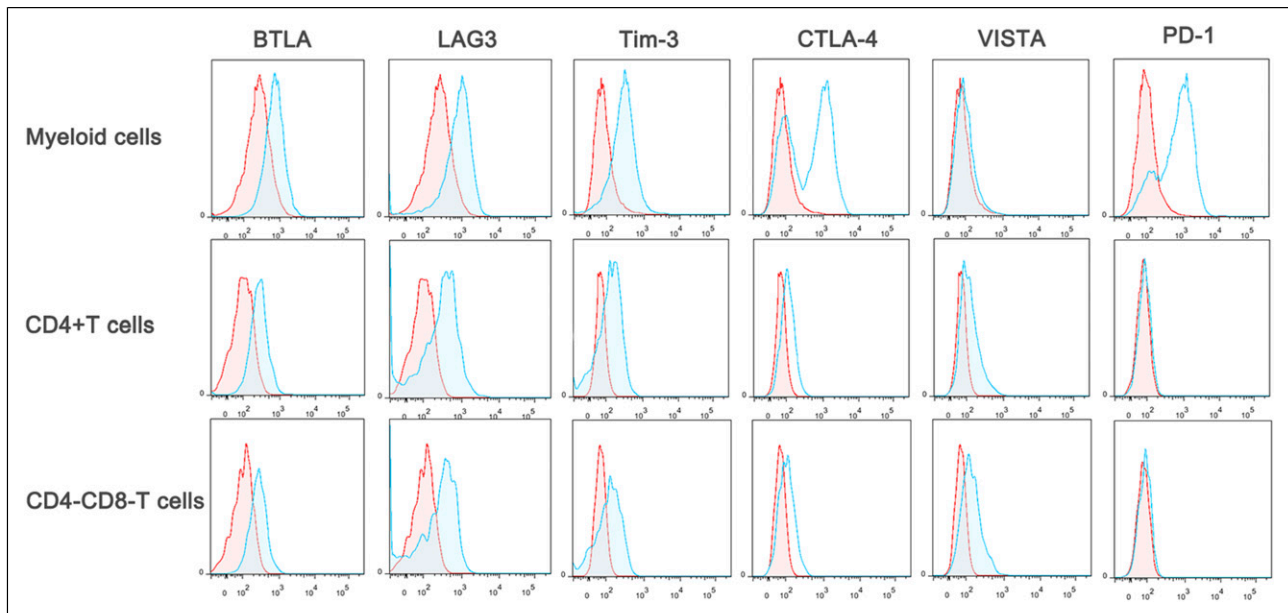


Figure 2. Analysis of the expression profile of immune checkpoints in high-grade glioma infiltrating immune cells. The analyzed immune cells include CD11B⁺ myeloid cells, CD4⁺ T, and CD8⁺ T cells, and the analyzed immune checkpoints included BTLA, LAG3, Tim-3, PD-1, CTLA-4, and VISTA.

Table 2. Expression of immune checkpoints in glioma microenvironment.

Immune checkpoints	TAM		CD4 ⁺ CD8 ⁻		CD4 ⁻ CD8 ⁻	
	Positive specimens number	Proportion of positive cells mean (± SD)	Positive specimens number	Proportion of positive cells mean (± SD)	Positive specimens number	Proportion of positive cells mean (± SD)
LAG3	15	14.88 ± 11.46	13	22.86 ± 22.61	13	23.2 ± 22.23
Tim-3	13	9.33 ± 7.2	4	1.58 (0.76–4.14)	4	2.97 (2.12–4.54)
BTLA	16	6.86 ± 3.54	17	10.22 ± 5.72	15	11.00 ± 12.45
PD-1	3	0.83 ^a (0.83–1.92)	1	0.43 ± 0.15	1	0.27 ± 0.27
VISTA	0	0.40 ± 0.44	18	26.43 ± 19.42	12	23.67 ± 17.29
CTLA-4	3	1.21 (0.43–2.15)	13	7.5 ± 6.34	13	8.59 ± 7.30

^aWhen the data does not confirm to a normal distribution, the result is represented as median and quartiles.

CTLA-4, and VISTA. The result showed that in infiltrating myeloid cells, the expression of LAG3, Tim-3 and BTLA were obviously higher than other checkpoints (when the number of positive cells were more than 5%, we defined the expression of immune checkpoint as positive), while the expression of PD-1, VISTA, and CTLA-4 were quite few (Figure 2). And the details were presented in Table 2. Then we analyzed the immune checkpoints expressed on T lymphocytes in GME. In infiltrating CD3⁺CD4⁺ and CD3⁺CD8⁻/CD4⁻ T lymphocytes, the expression of LAG3, BTLA, VISTA, and CTLA-4 were obviously higher than Tim-3 and PD-1, and the details were also presented in Table 2.

In conclusion, LAG3 and BTLA were the most widely expressed co-inhibitory immune checkpoints in GME

and expressed on both infiltrating myeloid cells and T lymphocytes. In addition, Tim-3, PD-1, VISTA, and CTLA-4 were also expressed in varying degrees in different immune cell subtypes and specimens. The expression of these immune checkpoints may result in the exhaustion of tumor infiltrating immune cells.

PBMCs can partially reflect the expression of immune checkpoints in GME

In our study, we also analyzed the expression of immune checkpoints in HGG patients' PBMCs. The main purpose was to observe the consistency of PBMCs and GME in immune checkpoints expression.

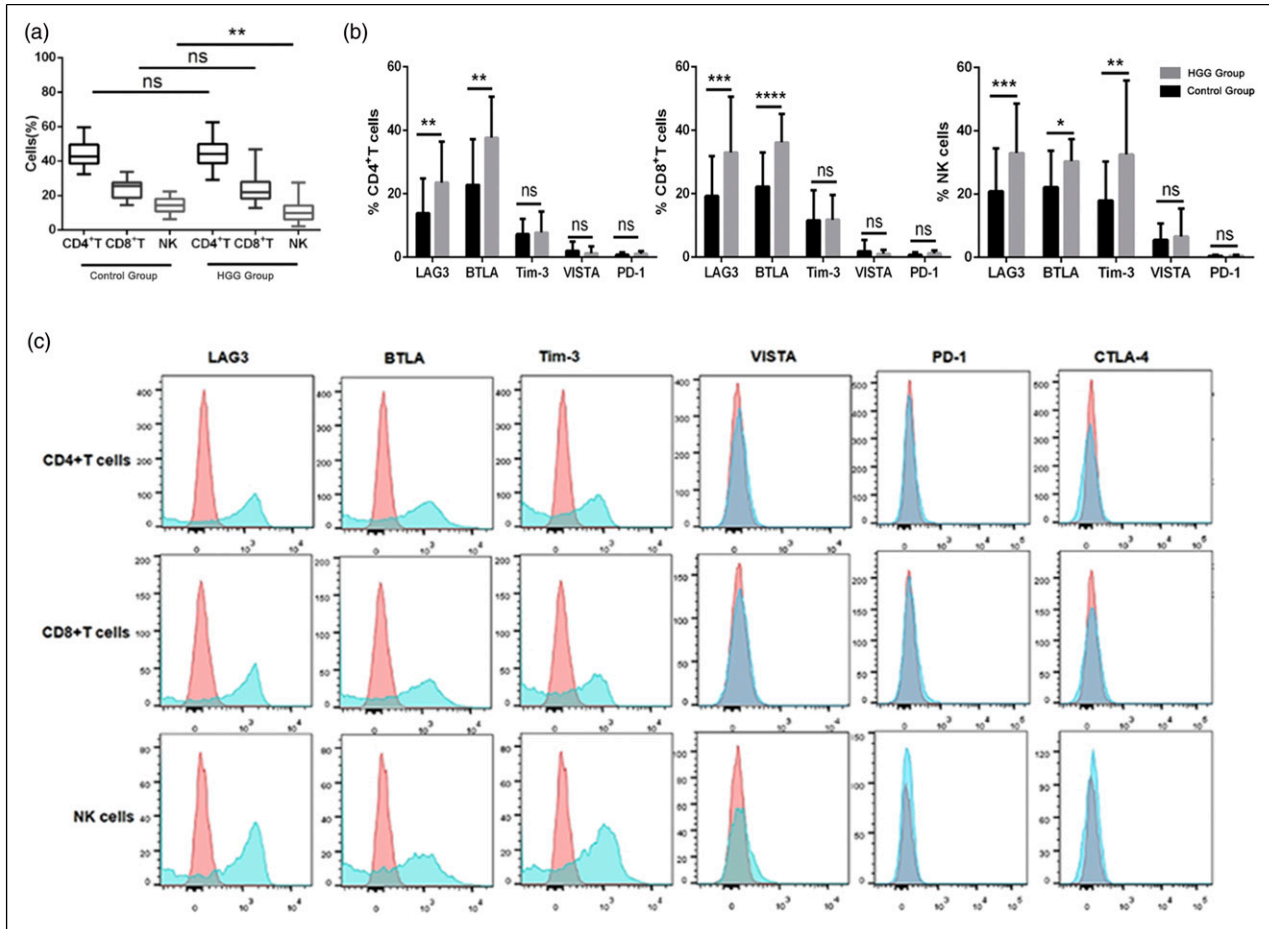


Figure 3. Analysis of the change of immune cell composition and the expression profile of immune checkpoints in high-grade glioma patients' PBMC. (a) The proportion of NK cells decreased significantly in HGG patients, and there was significant difference between the two groups. However, there was no difference in the proportion of CD4⁺ and CD8⁺ T lymphocytes between the two groups. (b and c) The analyzed immune checkpoints included BTLA, LAG3, Tim-3, PD-1, CTLA-4, and VISTA. The expression of BTLA, LAG3, and Tim-3 were more remarkable when compared with healthy volunteers; and we did not detect the remarkable expression of TIGIT, CTLA-4, and PD-1 in both groups. (* $p < 0.05$, ** $p < 0.01$).

Similarly, we first analyzed the changes of lymphocytes composition in PBMC. The results showed that when compared with healthy volunteers, the proportion of NK cells ($(14.45 \pm 4.062)\%$ in healthy volunteers) decreased significantly in HGG patients ($(10.52 \pm 5.691)\%$), and there was significant difference between the two groups ($p < 0.01$). However, there was no difference in the proportion of CD4⁺ and CD8⁺ T lymphocytes between the two groups (Figure 3(a)).

Then, we analyzed the immune checkpoints expressed in PBMCs, the result showed that the expression of BTLA, LAG3, and Tim-3 were more remarkable when compared with healthy volunteers (Figure 3(b) and (c)); and we didn't detect the remarkable expression of TIGIT, CTLA-4, and PD-1 in both groups (Figure 3(b) and (c)). After that, we analyzed the expression in different lymphocyte subgroups, and the detail information was presented in Table 3.

Discussion

Studies have shown that tumor development and progression are influenced by tumor microenvironment (TME) and controlled by the host immune system. Therefore, lymphocyte composition and immune system biomarkers in TME are important for evaluations of tumor prognoses and treatment response. Different from other tumors, the lymphocyte composition of GME is characterized by a more intense myeloid cells (including macrophage and microglia) infiltrate. These myeloid cells account for up to 30–50% of the total tumor cell mass in human GBM.¹⁵ In our study, the CD11b⁺ myeloid cells accounted for 36.14% in our HGG specimens, which is consistent with previous studies. So these cells are regarded as potential therapeutic targets in glioma immunotherapy.¹⁶ However, most studies by now revealed that they mainly

Table 3. Expression of immune checkpoints in PBMC.

Immune checkpoints	CD4 ⁺			CD8 ⁺			NK		
	HGG mean (± SD)	Volunteers mean (± SD)	p	HGG mean (± SD)	Volunteers mean (± SD)	p	HGG mean (± SD)	Volunteers mean (± SD)	p
LAG3	23.56 ± 12.99	14.1 ± 10.57	p < 0.01	33 ± 17.56	19.26 ± 12.66	p < 0.001	33.03 ± 15.65	20.88 ± 13.59	p < 0.001
Tim-3	7.825 ± 6.527	7.254 ± 4.862	p > 0.05	11.77 ± 7.857	11.59 ± 9.556	p > 0.05	32.59 ± 23.35	17.93 ± 12.05	p < 0.01
BTLA	37.71 ± 12.94	23.53 ± 11.14	p < 0.01	36.23 ± 9.003	22.09 ± 10.32	p < 0.0001	30.42 ± 7.004	25.68 ± 8.572	p < 0.05
PD-1	1.061 ± 0.794	0.7528 ± 0.7538	p > 0.05	1.156 ± 0.9458	0.7464 ± 0.8012	p > 0.05	0.5417 ± 0.2951	0.4031 ± 0.3576	p > 0.05
VISTA	1.244 ± 2.152	2.036 ± 2.914	p > 0.05	1.022 ± 1.296	1.837 ± 3.577	p > 0.05	6.558 ± 8.856	5.465 ± 5.234	p > 0.05

played a role in suppressing antitumor immune response in GME. So it raised the intriguing potential of reeducating these cells to act as anti-glioma effector cells and to reduce tumor burden. In addition to myeloid cells, as many as 8.2% of the cells in HGG specimens were tumor infiltrating T lymphocytes in our study, they were mainly $CD3^+/CD4^+$ and $CD3^+/CD8^-/CD4^-$ T lymphocytes and also played an important role in glioma immunity.

In the interaction between glioma and these infiltrated immune cells, co-inhibitory immune checkpoints have profound effects on their function, especially in inducing the exhaustion of these cells and the tumor immune escape. Therefore, in our study, we detected the expression profile of co-inhibitory immune checkpoints including BTLA, LAG3, Tim-3, PD-1, CTLA-4, and VISTA in these immune cells. Some of them have been widely studied in glioma immunity and been adopted to glioma clinical trials, while some still lack information and need more researches.

By now, CTLA-4 and PD-1/PD-L1 are the two best-studied immune checkpoints and are regarded as the first tier of co-inhibitory checkpoint molecules that are primarily responsible for maintaining self-tolerance, while other molecules are regarded as the second tier that have distinct and more specific roles in regulating the immune response.¹⁷

There have accumulated some experiences in the application of CTLA-4 and PD-1/PD-L1 antibodies in GBM. On the whole, CTLA-4 antibody is not widely used in GBM clinical trials because it plays a role in the earlier phase of T cell activation and causes an extensive impact on the immune network.¹⁸ PD-1/PD-L1 inhibitors are currently the most widely researched ICIs in GBM as a result of their safety and effectiveness in other tumors. Currently, more than 30 clinical trials have been performed.^{19,20} However, the response rate in overall patients is far from satisfactory and the extended survival is variable.²¹ In Topalian SL's review,⁸ they provided a systematic summary on biomarkers associated with the therapeutic efficacy of ICIs based on the existing tumor treatment experiences. In these biomarkers, intratumoral lymphoid infiltration and intratumoral checkpoint expression upregulation played an important role in predicting efficacy. However, an analysis of *PDCO1* (codes for PD-1) expression in the GBM/normal brain samples from the TCGA and REMBRANDT data sets showed that there was no significant difference between GBM and normal brain samples.¹³ In our study, the result showed that the expression of PD-1 is low in GME and there were only three samples with PD-1 positive. Therefore, exploring additional immune checkpoint molecules is a hot research topic, recent studies have identified several new immune checkpoint targets like LAG3²² and TIM-3.²³ They were described as the second-tier of co-inhibitory molecules and

had different lymphoid, anatomical and functional specifications. The investigations about these molecules have generated promising results in preclinical studies and/or clinical trials.

In our HGG specimens, we detected the expression of BTLA, VISTA, LAG3, and Tim-3 in GME. The results showed that they expressed in different type of lymphocytes. LAG3, Tim-3 and BTLA were detected on GAM and they were also detected in other tumor-associated myeloid cells in previous studies.²⁴ By now, there have been some clinical trials targeting on LAG3 and Tim-3 for the treatment of GBM.²⁴ BTLA is identified as another newly identified inhibitory receptor that belongs to CD28 superfamily,²⁵ there is no clinical trial opened for BTLA. But Junshi Biosciences announced that the world's first anti-BTLA antibody, TAB004/JS004, has been approved for clinical trial by FDA and is expected to be used in clinical trials soon.

LAG3, BTLA, VISTA, and CTLA-4 were detected on $CD3^+$ T cells. VISTA, whose immunoglobulin variable domain homology with PD-1,²⁵ was initially shown to inhibit T cell activation. Humanized anti-VISTA antibody has been adopted into clinical trials for advanced solid malignancies. However, there were little information on its expression and function in glioma patients. In our study, we detected its expression on T lymphocytes in GME.

On the whole, our result showed a widely expressed of LAG3 and BTLA in all immune cells in GME. All these provided a rationale for initiation of clinical trials of anti-LAG3/BTLA antibody in glioma. This is also the original purpose of our study. By analysis the expression of intratumoral checkpoint expression, our study provided direct human evidence for launching clinical trials to establish safety and efficacy of ICIs therapies in combination with the current standard of care in the primary HGG.

Conclusion

In addition to HGG specimens, we also detected the expression of immune checkpoints in HGG patients' PBMCs, the result showed that LAG3, Tim-3 and BTLA expressed more in PBMCs than healthy volunteers'. This indicated that the expression of immune checkpoints in PBMCs was able to partially reflect the condition in GME. However, the expression of immune checkpoints in GME is dynamic with the application of antitumor therapies, so it needs more data to determine if it can reflect the dynamic changes of checkpoints expression.

While there were some limitation in our study that the HGG glioma specimens and PBMC specimens were not obtained from the same group of patients and the dynamic changes of immune checkpoints expression in GME and PBMC were not explored in our study.

In conclusion, our study demonstrated the lymphocytic composition of HGG specimens, and analyzed the expression of immune checkpoints in GME and PBMC, the result showed that the immunity microenvironment and immune checkpoints expression in GME were quite complex. Expression of immune checkpoints in different HGG specimens varied from different samples, this may be associated with the HGG heterogeneity. The expression of checkpoints in PBMCs is partially consistent with that in GME; however, if it can work as an indicator to monitor the change in GME, it needs more work.

Author Contributions

Shaoping Shen and Qiyang Wu conducted the whole experiment and wrote the paper. Jialin Liu acquired the data. Liangliang Wu assisted in data analysis. Rong Zhang and Yasushi Uemura analyzed and interpreted the data and revised the paper. Xinguang Yu, Ling Chen, and Tianyi Liu conceived and designed the project.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. The authors have declared that no conflict of interest exists. Neither the entire paper nor any part of its content has been published or has been accepted elsewhere. It is not being submitted to any other journal. All authors of this paper have read and approved the final version submitted. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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Ethics approval

Ethical approval for this study was obtained from Ethics Committee of Chinese PLA General Hospital, and the approval number/ID is S2018-089-02.

Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

Not applicable.

Data accessibility

The data that support the findings of this study are available from the corresponding author [tianyiliu08@hotmail.com] upon reasonable request

Dear Editor:

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