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Tim-3 expression predicts the abnormal innate immune status and poor prognosis of glioma patients

Xueen Li[a], Bo Wang[b], Lintao Gu[c], Jie Zhang[b], Xiaoyan Li[b], Lifen Gao[b], Chunhong Ma[b], Xiaohong Liang[b,*], Xingang Li[a,**]

[a] Department of Neurosurgery, Qilu Hospital, Shandong University, Jinan 250012, PR China
[b] Key Laboratory for Experimental Teratology of Ministry of Education, Department of Immunology, Shandong University School of Medicine, Jinan, Shandong 250012, PR China
[c] Department of Otolaryngological, Qianfo Mountain Hospital, Jinan, Shandong 250014, PR China

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ABSTRACT

Malignant glioma, the most common and devastating primary brain tumor, has serious effects on human health with high risk of recurrence and short survival periods. Recently, the exploitation of immunological mechanisms shed new lights for developing novel therapeutic strategies for glioma pathogenesis. Tim-3, a member of T cell immunoglobulin and mucin domain family, has been involved in multiple diseases, including tumor, by regulating the viability and function of immunocytes. In the present study, we detected Tim-3 expression on peripheral innate immunocytes from glioma patients and analyzed their correlation with clinical indices. We found that the number of $CD3^-CD56^+$ NK cells decreased in glioma patients. Compared with healthy controls, glioma patients had higher Tim-3 expression on peripheral $CD3^-CD56^+$ NK cells and $CD14^+$ monocytes. $Tim\text{-}3^+$ NK cells had decreased capability of IFN-r secretion, while $Tim\text{-}3^+$ monocytes showed a M2-like phenotype. Importantly, Tim-3 level on both NK cells and monocytes positively correlated with the ratio of $Ki\text{-}67^+$ tumor cells. Moreover, patients with high percentage of $Tim\text{-}3^+$ monocytes showed high risk of recurrence or death. Our present work gives new insights into the innate immune mechanisms in glioma and might provide new evidences for the clinical practice of Tim-3-based immunotherapy in glioma.

1. Introduction

Malignant glioma, or glioblastoma multiforme, is the most common and devastating primary brain tumor, affecting children and young adults [1]. Despite the development of multimodal interventions, including maximal, safe resection and combination of radiotherapy with temozolomide chemotherapy, the clinical outcome of malignant glioma remains extremely poor because the tumor recurs in virtually all patients. The cumulative 1-year survival rate of glioma patients is < 30% and the mortality rate of patients within 3 years is up to 90% [2].Therefore, it is urgent to develop novel, efficient therapeutic strategies based on a deeper understanding of the molecular and cellular mechanisms of glioma pathogenesis.

Although the central nervous system (CNS) was originally considered to be an immune-privileged site, more recent studies disclosed that it should more accurately be viewed as an immunologically specialized site [3]. Like the periphery, the CNS is also capable of coordinating robust immune responses with the innate and adaptive immune systems. Also, accumulating data are beginning to clarify that a variety of immune cells and molecules underpin anti-glioma immune responses [4]. However, tumor-mediated immunosuppression occurs to most patients with glioma and both innate and adaptive immunity are profoundly inhibited. Up to dates, a lot of mechanisms are proved to participate in glioma-induced suppression, including immunosuppressive cytokines and catabolites, immune checkpoint molecules, and suppressive immune cells, etc. [5], which constitute the complicated immune environment. Therefore, to further exploit the immune molecules and cells involved in this network would undoubtedly illuminate novel approaches to glioma immunotherapy.

Tim-3, a member of T cell immunoglobulin and mucin domain family, initially identified as a specific marker of differentiated IFN-γ-producing T cells, has been proved to be a key immune checkpoint molecule of T-cell-mediated responses [6–8]. Further studies also uncovered the abundant expression of Tim-3 on $CD8^+$ T cells, monocytes/macrophages, mast cells, NK cells and dendritic cells [9–11]. Tim-3 on different types of immune cells showed versatile and complicated

* Correspondence to: X. Liang, 44 Wenhua Xi Road, Jinan, Shandong 250012, PR China.
** Correspondence to: X. Li, 107 Wenhua Xi Road, Jinan, Shandong 250012, PR China.
E-mail addresses: liangxiaohong@sdu.edu.cn (X. Liang), lixg@sdu.edu.cn (X. Li).

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functions and were involved in the multiple physiological or pathological milieu [12]. Aberrant expression pattern of Tim-3 participated in the pathogenesis of various kinds of diseases (e.g. tumor, viral infection, diabetes, atherosclerosis, and autoimmune diseases) by affecting the intensity and duration of innate and/or adaptive immune response [13,14]. These important findings support the fact that Tim-3 is now becoming a remarkable candidate in immune checkpoint therapy [15].

The role of Tim-3 in nervous system diseases was also reported. Zhang et al. [16] disclosed that the accumulation of Tim-3$^+$ cells positively correlated with disease progression of experimental autoimmune neuritis (EAN), and Tim-3 was mostly expressed on T cells during the early phase and on CD68$^+$ macrophages at the peak or during recovery phase of EAN. Our previous studies showed that increased Tim-3 expression was detected in peripheral blood monocytes (PBMCs) of ischemic stroke patients and correlated with abnormal lipid levels [17]. In addition, we also detected the altered Tim-3 expression on peripheral CD8$^+$ T cells in intracerebral hemorrhage (ICH) patients, which closely correlated with the inflammatory response, the disease severity and the outcome of ICH patients [18]. More recently, it was reported that glioma patients had increased Tim-3 level on peripheral CD4$^+$ and CD8$^+$ T cells, which was correlated with tumor grade, serum TNF-a level and the Karnofsky Performance Status score of the glioma patients [19,20]. These observations strongly suggest that Tim-3 might be involved in the pathogenesis of glioma. However, till now, it still remains unknown about the effects of Tim-3 on innate immune status of patients with glioma, which might be more easily induced especially in the CNS [21]. In the present study, we examined Tim-3 expression on peripheral innate immune cells (NK, NKT, and CD14$^+$ monocytes) and analyzed their correlation with clinical indices of glioma patients. Our results demonstrated that Tim-3 expression on peripheral CD3$^-$CD56$^+$ NK cells and CD14$^+$ monocytes in glioma patients were significantly higher than those in healthy donors. Tim-3$^+$ NK cells had decreased capability of IFN-r secretion and Tim-3$^+$ monocytes showed M2-like phenotype. Importantly, Tim-3 level on both NK cells and monocytes positively correlated with the ratio of Ki-67$^+$ tumor cells. Moreover, patients with high percentage of Tim-3$^+$ monocytes showed high risk of recurrence or death. Our present work gives new insights into the innate immune mechanisms in glioma and might provide new evidences for the clinical practice of Tim-3-based immunotherapy in glioma.

2. Materials and methods

2.1. Patients

A total of 25 glioma patients were enrolled at the Department of Neurosurgery, Qilu Hospital, Shandong University, China, from January 2013 to June 2014. All these patients were confirmed with primary glioma by histological analysis, received surgical resection and were available for follow-up visit. Patients with preoperative anticancer treatment or other synchronous malignancy had been excluded from our study. Follow-up data were collected through on-site interview or telephone calling. The latest follow-up date was July 2016.

The control group consisted of 17 healthy volunteers from Medical Examination Center of Qilu hospital. The study was approved by the medical ethics committee of Shandong University, and informed consent was acquired from each subject.

2.2. Blood sampling and flow cytometry analysis

Blood samples were routinely taken from the antecubital vein of normal controls and glioma patients. Flow cytometry was used to determine the ratio of innate immune cells in peripheral blood and Tim-3 expression on these immunocytes. One hundred microliters of whole blood was incubated at 4 °C in a dark room with monoclonal antibodies, FITC-conjugated anti-CD56 (Serotec, Oxford, UK) and FITC-conjugated anti-CD14 (Biolegend, San Diego, USA), PE-conjugated anti-Tim-3 (clone: RMT3-23; R&D, Minneapolis, MN), APC-conjugated anti-CD206 (Biolegend, San Diego, USA), FITC-conjugated anti-IFN-γ (Miltenyi Biotec, Shanghai, China). Thirty minutes later, stained blood samples were subjected to RBC lysis using FACS lysis solution (BD Biosciences, San Jose, CA). Then, cell suspension was washed twice with phosphate buffer solution (PBS) and was detected using a BD flow cytometer (BD Biosciences, San Jose, CA), and finally the data were analyzed.

2.3. Immunohistochemistry staining of Ki-67

Tumor tissues were collected from glioma patients who underwent surgical resection as mentioned above. Immunohistochemistry was performed according to standard protocols using anti-Ki-67 (ab15580; Abcam) antibodies. The ratio of Ki-67 stained cells was independently counted by three pathologists and the final result was the average from ten fields of ~1000 cells from each tumor section.

2.4. Statistical analysis

All data were analyzed using the GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Comparison between groups was analyzed by using the student's *t*-test and Mann-Whitney nonparametric *U* test. *P* values were considered significant at $P < 0.05$.

3. Results

3.1. Percentage of peripheral NK cells decreased in glioma patients

To delineate the innate immune status in glioma patients, we firstly detected the percentage of the major innate immune cells in peripheral blood. As shown in Fig. 1, compared that of healthy donors, peripheral CD3$^-$CD56$^+$ NK percentage was significantly decreased in patients with glioma (Fig. 1A), which was consistent with the report that both NK cell number and function were shaped by solid tumor microenvironment [22]. However, there were no obvious differences in the percentage of CD3$^+$CD56$^+$ NKT cells (Fig. 1B) or CD14$^+$ monocytes (Fig. 1C) between glioma patients and healthy donors.

3.2. Tim-3$^+$ NK cells with low IFN-γ production were increased in glioma patients

Considering the important roles of Tim-3 in regulating innate immune status, we then detected the Tim-3 expression on peripheral NK cells, NKT cells and monocytes in both glioma patients and healthy donors. According to the results from flow cytometry, there was high level of Tim-3 expression on NK cells (the positive rate is about 60% in healthy donors, shown in Fig. 2A) and moderate expression on NKT cells (the positive rate is about 20% in healthy donors, shown in Fig. 2B), which was consistent with the literature [10]. In glioma patients, the positive percentage of Tim-3 on NK cells was further upregulated compared with that of healthy donors (Fig. 2A), while there was no significant difference in Tim-3 expression on NKT cells between these two groups (Fig. 2B). Next, we further observed the IFN-γ production on Tim-3$^+$ and Tim-3$^-$ NK cells in glioma patients. As shown in Fig. 2C, the ability of IFN-γ production by Tim-3$^+$ NK cells was significantly lower than that by Tim-3$^-$ NK cells in glioma patients. Collectively, these results showed that patients with glioma had the increased percentage of Tim-3$^+$ NK cells with low capacity of IFN-γ production.

3.3. Glioma patients had more Tim-3$^+$ CD14$^+$ cells with M2-like phenotype

CD14$^+$ monocyte is also the important player in deciding the host innate immune status and Tim-3 is involved in regulating its activation and function [23]. Our flow cytometry data showed that the positive