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CXCR5$^+$ CD8 T cells displayed higher activation potential despite high PD-1 expression, in tumor-involved lymph nodes from patients with thyroid cancer

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ABSTRACT

Thyroid cancer is one of the malignancies with better clinical outcomes. However, a minority of patients develops an aggressive anaplastic thyroid carcinoma. Development of innovative and multimodal therapeutic strategies is urgently needed. Here, we investigated the role of CXCR5$^+$ CD8 T cells in the peripheral blood, tumor-involved lymph nodes (TILN), and tumor mass of thyroid cancer patients. In peripheral blood mononuclear cells, CXCR5$^+$ cells represented 1.4% ± 0.84% (mean ± s.d.) of total CD8 T cells, while in TILN and in tumor, the frequencies of CXCR5$^+$ CD8 T cells were significantly higher at 27.7% ± 7.8% and 15.5% ± 2.9%, respectively. Compared to CXCR5$^-$ CD8 T cells, CXCR5$^+$ CD8 T cells presented significantly higher PD-1 expression and lower or comparable TIM-3 and CTLA-4 expression. To compare and contrast the functional characteristics of CXCR5$^+$ CD8 T cells and CXCR5$^-$ CD8 T cells, these cells were separated from TILNs and were TCR-stimulated via anti-CD3/CD28. Upon stimulation, CXCR5$^+$ CD8 T cells presented stronger downregulation of CD27, higher expression of proinflammatory cytokines IL-2, IFN-γ, and TNF-α, and higher proliferation capacity than CXCR5$^-$ CD8 T cells. Moreover, CXCR5$^+$ CD8 T cells presented higher expression of cytotoxic molecules Gzm-A, Gzm-B, and perforin. Overall, these results demonstrated that in thyroid cancer patients CXCR5$^+$ CD8 T cells infiltrated the TILNs and the tumors, and were functionally more potent compared to their CXCR5$^-$ counterpart.

1. Introduction

Thyroid cancer is a malignancy with rapidly increasing incidence across all ethnicities and genders [1]. Most patients present localized and differentiated tumors in the thyroid, which are usually well-managed by surgery and radioiodine therapy [2]. However, some patients may present metastasis in the regional lymph nodes and distant sites. These patients tend to respond less well to standard therapy, require further therapeutic interventions, and present higher rates of recurrence and mortality [3]. In addition, a minority of patients develops anaplastic thyroid carcinoma (ATC), which is a poorly differentiated and aggressive form of thyroid cancer with a median survival time of < 5 months [4]. Although clear etiology of ATC is yet to be demonstrated, the fact that ATC is frequently found in proximity with well-differentiated thyroid malignancies seems to suggest that ATC may develop from lower risk thyroid malignancies [5]. Development of multimodal therapies and innovative strategies is required to improve the control of thyroid cancer and manage complications.

CD8 T cell-mediated immunity is essential in preventing cancer initiation and limiting cancer progression. CD8 T cell, under ideal conditions, can eliminate neoplastic cells via the release of granzyme and perforin-containing cytotoxic granules, while propagating inflammation through the secretion of proinflammatory cytokines [6]. Previously, the intratumoral CD8 T cell/Treg ratio inversely correlated with the size of thyroid tumor [7], while the ratio of CD4/CD8 T cells was inversely correlated with tumor stage [8]. These associations suggested a beneficial role of CD8 T cells in cancer. However, CD8 T cells in thyroid cancers displayed many features of exhaustion [9], including elevated expression of checkpoint markers PD-1, CTLA-4, and TIM-3, failure to downregulate CD27, and loss of proliferative and cytotoxic

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potential. The PD-1$^+$ CD8 T cells, which were enriched in tumor-involved lymph nodes, displayed inability to produce perforin, and reduced capacity to produce IL-2 and TNF-α. Overall, these results suggest that CD8 T cells present beneficial roles in thyroid cancer patients, but their functional capacity is reduced due to exhaustion mechanisms.

The function of CXCR5$^+$ CD8 T cell subset is increasingly recognized. This subset is rarely observed in peripheral blood mononuclear cells (PBMCs) but is primarily found in the B cell follicles and T cell zones of lymphoid tissues [10, 11]. In murine LCMV infection and human HIV infection, He et al. showed that CXCR5$^+$ CD8 T cells presented lower PD-1 and TIM-3 expression and higher CD107a, IFN-γ and TNF-α expression, compared to autologous CXCR5$^-$ CD8 T cells [12]. In chronic LCMV-infected mice, Im et al. reported that the PD-1$^+$CXCR5$^+$ CD8 T cells, despite the high PD-1 expression, presented potent proliferative capacity after PD-1/PD-L1 blockade, while PD-1$^+$CXCR5$^-$ CD8 T cells failed to proliferate [11]. Interestingly, recent studies demonstrated that CXCR5$^+$ CD8 T cells could infiltrate pancreatic tumor and tumor-associated lymph nodes in colorectal cancer [13, 14].

In the current study, we investigated the CXCR5$^+$ CD8 T cell subset in PBMCs, tumor-involved lymph nodes (TILNs), and tumors from thyroid cancer patients. We found that the frequency of CXCR5$^+$ CD8 T cells was high in TILNs, moderate in tumors, and low or undetected in PBMCs. Contrary to the findings in chronic virus infections, the CXCR5$^+$ CD8 T cells presented much higher PD-1 expression than CXCR5$^-$ CD8 T cells, and also displayed other exhaustion markers, such as TIM-3 and CTLA-4 expression to varying degrees. However, compared to CXCR5$^-$ CD8 T cells, CXCR5$^+$ CD8 T cells more readily downregulated CD27 upon stimulation, and expressed higher levels of IL-2, IFN-γ, and TNF-α. CXCR5$^+$ CD8 T cells also presented higher proliferative capacity and cytotoxic capacity than CXCR5$^-$ CD8 T cells, characterized by high thyimidine incorporation, and high granzyme (Gzm)-A, Gzm-B, and perforin expression. Overall, this study suggests that despite presenting multiple exhaustion markers, CXCR5$^+$ CD8 T cells are functionally more potent compared to their CXCR5$^-$ CD8 T cell counterpart.

2. Methods

2.1. Patients

PBMCs, TILNs, and tumors were harvested from thyroid cancer patients undergoing surgical neck dissection at the First Affiliated Hospital of Jiamusi University. All patients presented conventional primary papillary thyroid cancer confirmed by histopathology findings and were composed of twelve females and four males between 25 and 57 years of age. Healthy controls were recruited from twelve healthy female volunteers and four healthy male volunteers between 25 and 55 years of age who did not present any form of malignancy, or any other ongoing diseases, including acute or chronic infections, autoimmune diseases, cardiovascular diseases, diabetes, and inflammatory bowel disease. All patients and controls provided written informed consent.

2.2. Sample collection

The institutional review board of First Affiliated Hospital of Jiamusi University approved the collection and use of human samples. Peripheral blood was collected from each patient before surgery and other treatments. PBMCs were harvested via Ficoll (Simga) gradient centrifugation. TILNs and tumors were harvested via surgical resection. TILNs were then separated from tumors as much as possible, and dissected to expose lymphocytes. To increase lymphocyte yield, the interior portions of multiple TILNs were pooled. The tumor samples were then minced into small pieces. Both the TILNs and tumors were digested in sterile Hanks balanced salt solution (HBSS; GIBCO) supplemented with 2 mg/mL Liberase DL and 5 kU/mL DNase I (Roche) for 1 h at 37 °C. Red blood cells were lysed by 5 min incubation at 37 °C in RBC lysis buffer (BioLegend). The cells were then washed in sterile HBSS and rested overnight in RPMI 1640 media supplemented with 10% heat-inactivated FBS, 0.2% penicillin/streptomycin, and 0.2% L-glutamine (GIBCO). Non-adherent lymphocytes were aspirated and adherent tumor cells were removed. Unused PBMCs and TILN lymphocytes were cryopreserved in 90% FBS/10% DMSO (Sigma) in −80 °C.

2.3. Flow cytometry

To quantify CXCR5$^+$ CD8 T cells and examine surface PD-1, TIM-3, and CTLA-4 expression, fresh cells from each tissue type were first incubated with FcX FcR blocker (BioLegend) for 10 min at room temperature, and then incubated with Fixable Aqua Cell Viability Dye (Invitrogen), anti-CD8, anti-CXCR5, and one of anti-PD-1, anti-TIM-3, or anti-CTLA-4 (BioLegend) for 30 min in dark on ice. To examine CD27 expression, sorted CXCR5$^+$ CD8 T cells and CXCR5$^-$ CD8 T cells from TILNs before or after stimulation were incubated with Fixable Aqua Cell Viability Dye and anti-CD27 (BioLegend) for 30 min in dark on ice. Afterwards, cells were washed twice and fixed in 2% formalin.

To sort CXCR5$^+$ CD8 T cells and CXCR5$^-$ CD8 T cells, TILN lymphocytes were thawed, rested overnight, and incubated with anti-CD8 and anti-CXCR5 for 30 min in dark on ice. Cells were then washed twice and sorted in the FACS Aria system. Viability was determined using Trypan Blue counting (Thermo Fisher).

2.4. Stimulation

Sorted CXCR5$^+$ CD8 T cells and CXCR5$^-$ CD8 T cells from TILNs were placed in 96-well round-bottom plates at 2×10^4 live cells per 100 μL media per well, in the presence of Human T Activator beads (Thermo Fisher) at 2 beads per cell. After 24 h, supernatant was removed for the quantification of cytokines by ELISA. Half of cells were collected for anti-CD27 staining and Transcript analysis. The rest of the cells were incubated for a total of 72 h, after which the cells were pulsed with 0.1 μCi radioactive thymidine (Amersham) for 8 h and harvested in a cell harvester, and the thymidine incorporation was examined in a beta-counter.

2.5. Transcript analysis

Expression of gene transcripts was examined using commercial TaqMan Gene Expression (Thermo Fisher) assays following instructions from the manufacturer. Briefly, total RNA was harvested using the TRIzol Reaget (Invitrogen), and the cDNA of the mRNA transcripts was synthesized using SuperScript III revserse transcriptase (Invitrogen). Preassembled primer and probe sets for *IL-2* (Hs00174114_m1), *TNF* (Hs00174128_m1), *IFNG* (Hs00989291_m1), *GZMA* (Hs00989184_m1), *GZMB* (Hs00188051_m1), and *PRF1* (Hs00169473_m1; Thermo Fisher) were used as indicated by the manufacturer. Assays were run on the Real-Time PCR 7500 system (Applied Biosystems).

2.6. ELISA

The Human IL-2, IFN-gamma, and TNF-alpha Quantikine ELISA Kits, Human Granzyme A and Granzyme B DuoSet ELISA systems (R&D Systems), and Human Perforin ELISA Kit (Abcam) were applied according to instructions from the manufacturers.

2.7. Statistical analysis

One-way ANOVA followed by Tukey's post-test, Student's *t*-test, and 2-way ANOVA followed by Sidak's post-test were applied as specified per experiment. Two-tailed P values smaller than 0.05 were considered statistically significant. The making of figures and statistical analyses