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CXCR5$^+$CD8$^+$ T cells infiltrate the colorectal tumors and nearby lymph nodes, and are associated with enhanced IgG response in B cells

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ABSTRACT

Colorectal cancer is the third most prevalent cancer type worldwide and contributes to a significant percentage of cancer-related mortality. Recent studies have shown that the CXCR5$^+$CD8$^+$ T cells present more potent proinflammatory function than CXCR5$^-$CD8$^+$ T cells in chronic virus infections and in follicular lymphoma, but the role of CXCR5$^+$CD8$^+$ T cells in colorectal cancer is yet unclear. In this study, we demonstrated that CXCR5$^+$CD8$^+$ T cells were very rare in peripheral blood mononuclear cells from healthy and colorectal cancer individuals, but were significantly enriched in resected tumors and tumor-associated lymph nodes. Compared to CXCR5$^-$CD8$^+$ T cells, the CXCR5$^+$CD8$^+$ T cells demonstrated significantly higher Bcl-6 expression and lower Blimp1 expression, suggesting that CXCR5$^+$CD8$^+$ T cells might represent a memory CD8$^+$ T cell subset. CXCR5$^+$CD8$^+$ T cells also enhanced the IgG expression by autologous B cells. Under *ex vivo* condition, the CXCR5$^+$CD8$^+$ T cells demonstrated lower degranulation, TNFα expression and IFNγ expression than CXCR5$^-$CD8$^+$ T cells. However, after PMA + ionomycin stimulation, the degranulation and TNFα expression by CXCR5$^+$CD8$^+$ T cells were significantly elevated to a level comparable with CXCR5$^-$CD8$^+$ T cells, whereas the IFNγ expression by PMA + ionomycin-stimulated CXCR5$^+$CD8$^+$ T cells were significantly higher than that by CXCR5$^-$CD8$^+$ T cells. Following long-term TCR-stimulation, CXCR5$^+$CD8$^+$ T cells demonstrated significantly more potent proliferation capacity and higher IFNγ expression than CXCR5$^-$CD8$^+$ T cells. TCR-stimulated CXCR5$^+$CD8$^+$ T cells also showed a gradual downregulation in CXCR5 expression. We further found that TCR-stimulated CXCR5$^+$CD8$^+$ T cells demonstrated higher granzyme B production and induced more specific lysis of autologous tumor cells than CXCR5$^-$CD8$^+$ T cells. Together, these data demonstrate that CXCR5$^+$CD8$^+$ T cells represent a significant CD8$^+$ T cell subset in colorectal tumors and have the potential to contribute to antitumor immunity, but their specific roles require further studies *in vivo*.

1. Introduction

CD8$^+$ T cells represent one of the most important cell types in antitumor immunity [1]. The canonical CD8$^+$ T cell can be activated by peptide antigens expressed by MHC class I molecules on the target cell surface, together with signal transduction from costimulatory molecules such as CD28 and IFNγ. When activated, CD8$^+$ T cells can release perforin and granzymes at the junction space between the T cell and the target cell, which causes the disruption of target cell membrane and the activation of apoptosis in the target cell. Due to this cytotoxic function, it is thought that CD8$^+$ T cells infiltrated inside the tumor microenvironment might be able to directly eliminate tumor cells. Indeed, in many human cancers, higher levels of tumor-infiltrating CD8$^+$ T cells were correlated with better clinical outcomes [2–4]. Moreover, immunotherapeutic strategies utilizing activated and/or genetically engineered CD8$^+$ T cells are showing increasing promises in treating established cancers [5,6]. On the other hand, it is known that the malignant cells and non-malignant cells in the tumor interact to create an immunosuppressive microenvironment that can reduce the number and efficacy of tumor-infiltrating CD8$^+$ T cells [7]. This intratumoral microenvironment is enriched with immune checkpoint molecules, such as PD-1/PD-L1, inhibitory cytokines, such as IL-10 and TGF-β, and CTLA-4-expressing regulatory T cells [8–11]. Therefore, strategies to improve CD8$^+$ T cell frequency and efficacy in cancers are urgently needed.

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In recent years, a subset of $CD8^+$ T cells with high CXCR5 expression is discovered in human lymphoid tissues [12,13]. CXCR5 is a chemokine receptor expressed at high level on $CD19^+$ B cells, as well as dendritic cells and T cell subsets [14]. Its ligand, CXCL13, is mainly expressed by follicular dendritic cells and stromal cells in the germinal center B cell follicles. $CXCR5^+CD4^+$ follicular helper T (Tfh) cells are considered the main B helper cell and have critical roles in the generation and maintenance of humoral immunity [15]. The role of CXCR5 on $CD8^+$ T cells, however, is less clear. It was shown that $CXCR5^+CD8^+$ T cells may infiltrate the B cell follicles and assist the Tfh cells in mediating antibody responses [12,16]. Interestingly, a recent study demonstrated that $CXCR5^+CD8^+$ T cells in chronic lymphocytic choriomeningitis virus (LCMV)-infected mice demonstrated fewer features of exhaustion than $CXCR5^-CD8^+$ T cells [13]. Compared to $CXCR5^-CD8^+$ T cells, $CXCR5^+CD8^+$ T cells expressed significantly lower levels of inhibitory molecules Tim-3 and PD-1 but higher levels of proinflammatory cytokines TNFα and IFNγ. Upon adoptive transfer, mice receiving $CXCR5^+CD8^+$ T cells presented significantly lower viral load than mice receiving $CXCR5^-CD8^+$ T cells. Similar proinflammatory features were also observed in $CXCR5^+CD8^+$ T cells harvested from human tonsillar tissues and follicular lymphoma tumors, where a high level of TNFα and IFNγ secretion was observed in $CXCR5^+CD8^+$ T cells but not $CXCR5^-CD8^+$ T cells [17].

Colorectal cancer represents one of the predominant causes of cancer-related mortality in the world [18]. High number and frequency of tumor-infiltrating $CD8^+$ T effector cells have been associated with improved clinical outcomes in colorectal cancer patients [2,19]. However, it was also found that patients with colorectal cancer presented immunodysregulation with exhaustion and apoptosis of tumor-resident $CD4^+$ and $CD8^+$ T cells [20]. The existence and frequency of $CXCR5^+CD8^+$ T cells in colorectal cancer patients is unclear. Moreover, whether $CXCR5^+CD8^+$ T cells could contribute to antitumor immunity in colorectal cancer remains unknown. Therefore, we examined $CXCR5^+CD8^+$ T cells in colorectal cancer patients in this study.

2. Methods

2.1. Study population

Recruitment of colorectal cancer patients and age- and sex-matched healthy volunteers was performed at Changhai Hospital. A total of 23 patients (45–70 years of age), who presented pTNM stages IIA, IIB or IIC based on the 7th edition of the TNM classification system, underwent surgical removal of colorectal cancer [21]. Cell frequency, B cell help function, transcription factor expression, and IFNγ/TNAα expression of $CXCR5^+CD8^+$ T cells were examined in the first batch of 18 patients, while the Granzyme B (GzB) production and cytotoxicity were examined in the second batch of 5 patients. The first and second batches were recruited under the same standard with no significant differences in demographic and clinical characteristics. Peripheral blood was drawn at the time of diagnosis for colorectal cancer patients or at the time of recruitment for healthy volunteers. No patient received neoadjuvant therapy at the time of sample collection. No metastasis was detected in the patient cohort. In addition, no patient or healthy control presented cardiovascular disease, chronic hepatitis B or hepatitis C infection, diabetes, ongoing acute infections, or other malignancies. Written informed consent was received from every participant. Changhai Hospital Ethics Review Board approved this study.

2.2. Samples

Peripheral blood mononuclear cells (PBMCs) were harvested from blood by standard Ficoll centrifugation method. Immediately following surgical resection, the gross resected sample was sterilized, with the intestinal wall and fatty tissue removed, and was dissected by an experienced surgeon into the tumor mass and associated lymph nodes. The tumor was cut into tiny fragments and put into an enzymatic digestion solution with 3000 U/mL DNase, 10 mg/mL collagenase and 10 mg/mL hyaluronidase (all purchased from Sigma Aldrich) for overnight incubation with continuous rotation. The single cell suspension were then obtained by pushing the digestion product through a 70-μm sized strainer, and were layered on top of Ficoll for centrifugation to obtain tumor-infiltrating mononuclear cells. The associated lymph nodes were disrupted into small fragments with continuous washing with cold HBSS. Cell clumps were broken up by pipetting, and the cell suspension was pushed through a strainer. Mononuclear cells were harvested by Ficoll centrifugation.

2.3. Flow cytometry

For the detection of $CXCR5^+CD8^+$ T cell frequency, freshly isolated PBMCs, tumor-infiltrating lymphocytes or tumor-associated lymph node-infiltrating lymphocytes were incubated with Aqua Dead Cell stain (Invitrogen) and anti-human CD3, CD4, CD8 and CXCR5 for 30 min in 4 °C. For intracellular cytokine or transcription factor detection, after surface staining, cells were treated with Cytofix/Cytoperm buffer set or Transcription Factor buffer set (both from BD Pharmingen) according to the manufacturer's instructions, respectively, and were stained with anti-human TNFα, IFNγ and/or Bcl-6. For CD107a staining, the anti-human CD107a antibody was added 6 h prior to cell harvest. All antibodies were purchased from BioLegend.

2.4. Cell subset isolation

Tumor-infiltrating $CXCR5^+CD8^+$ and $CXCR5^-CD8^+$ T cells were isolated using the Human $CD8^+$ T cell Enrichment kit, followed by PE-conjugated anti-human CXCR5 staining and Human PE Positive Selection kit, according to manufacturer's protocols. $CD19^+$ B cells were isolated from freeze-thawed PBMCs using the Human B cell Enrichment kit. All kits were purchased from Stemcell.

2.5. ELISA

The Human IgA, IgG and IgM ELISA kits (Abcam) were used to examine B cell antibody secretion, following the manufacturer's instructions. The human granzyme B ELISA was performed using Legend Max kit with pre-coated plates (BioLegend) following manufacturer's instructions.

2.6. mRNA analysis

The total RNA was extracted from isolated $CXCR5^+CD8^+$ and $CXCR5^-CD8^+$ T cells using TRIzol reagent (Invitrogen). The mRNA was then converted into cDNA by Taqman Reverse transcription kit and was quantified using the SYBR-Green system (Applied Biosystems). Relative expressions of transcription factors were calculated using the $2^{-\Delta CT}$ method.

2.7. ^{51}Cr release assay

2×10^5 primary tumor cells from each patient were labeled with 50 μL of 1 mCi/mL ^{51}Cr for 45 min in 37 °C. The cells were then washed, and resuspended at 2×10^4 cells/mL. 50 μL of ^{51}Cr labeled tumor cells were added to a 96-well round-botton plate and effector cells were added at 0.3/1, 1/1 and 3/1 E/T ratios. All wells were topped with complete medium and were incubated for 16 h before radioactivity measurement. 1 M HCl solution was added to total release (TR) control and culture medium only was added to spontaneous release (SR) control. Percentage specific lysis was calculated as 100×(experiment - SR)/(TR-SR).

2.8. Statistics

Data were analyzed in GraphPad Prism. Data in Figs. 1b, 1c and 2