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Th9 cells promote antitumor immunity via IL-9 and IL-21 and demonstrate atypical cytokine expression in breast cancer

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ABSTRACT

Breast cancer is a major cause of cancer-related death in women. Antitumor T cell responses play critical therapeutic roles, including direct cytotoxicity mediated by $CD8^+$ T cells and immunomodulatory roles mediated by $CD4^+$ T cells. The IL-9-expressing Th9 cells are recently found to present antitumor immunity in melanoma and lung adenocarcinoma. In this study, we found that IL-9 expression in the serum and in circulating $CD4^+$ T cells were significantly upregulated in breast cancer patients compared to healthy controls. The IL-9-expressing Th9 cells were enriched in the $CCR4^-CCR6^-CXCR3^-$ subset. Upon TCR stimulation, this subset also presented potent IL-10 and IL-21 expression in addition to IL-9 expression. $CCR4^-CCR6^-CXCR3^-$ $CD4^+$ T cells also assisted in the killing of autologous tumor cells by $CD8^+$ T cells, but did not initiate cytotoxicity by themselves. This enhancement in $CD8^+$ T cell-mediated cytotoxicity was dependent on IL-9 as well as on IL-21. Interestingly, the tumor-infiltrating Th9 cells presented comparable IL-9, reduced IL-10, and elevated IL-21 expression compared with their counterparts in the peripheral blood. Together, these results demonstrated that IL-9-expressing Th9 cells were upregulated in breast cancer patients and potentially possessed antitumor roles by enhancing $CD8^+$ T cell-mediated cytotoxicity.

1. Introduction

Breast cancer is the most prevalent cancer in women and a major cause of cancer-related death [1]. Antitumor T cell responses play critical therapeutic roles in breast cancer. $CD8^+$ T cells can mediate the elimination of malignant tumor cells via the release of cytotoxic molecules, while $CD4^+$ T cells can modulate the immune response through the secretion of various sets cytokines. Infiltration of tumor mass by $CD8^+$ T cells and proinflammatory $CD4^+$ T cells predict better prognosis in breast cancer patients [2,3].

The $CD4^+$ T cells can be distinguished into several T helper (Th) subsets with distinctive gene transcription and cytokine secretion profile. These include most commonly the IFN-γ-expressing Th1 cells, IL-4-, IL-5-, and IL-13-expressing Th2 cells, IL-17-expressing Th17 cells, naturally occurring $CD25^+Foxp3^+$ regulatory T (nTreg) cells, and induced IL-10- and TGF-β-expressing Treg (iTreg) cells. The IL-9-expressing Th9 cells are a recently described subset with potential antitumor roles [4]. Aside from IL-9, Th9 cells are also shown to express IL-10 and IL-21 under various stimulation conditions, and can induce epithelial cells to express CCL20 [5]. Particularly in melanoma, Th9 cells demonstrate potent antitumor activity. In peripheral blood and the skin of melanoma patients, the frequency of Th9 cells was significantly reduced [6]. A genetic polymorphism in the IL-9 gene was associated with elevated melanoma risk [7]. Furthermore, adoptive transfer of Th9 cells could significantly reduce the tumor mass in murine melanoma models as well as lung adenocarcinoma models [4,6,8]. Currently, it is thought that Th9 cells mediated tumor regression via IL-9 and IL-21, which augmented existing antitumor immunity [9]. Also, CCL20 expression in tumor cells could attract CCR6-expressing leukocytes to infiltrate the solid tumors [10]. Together, these lines of evidence indicated that Th9 cells presented an antitumor role in multiple tissues.

We designed the following study to examine the role of Th9 cells in breast cancer patients. First, we examined the frequency of circulating Th9 cells. The functional characteristics and their action toward autologous $CD8^+$ T cells were then investigated. Finally, we examined the frequency and cytokine expression of tumor-infiltrating Th9 cells.

2. Materials and methods

2.1. Participants and samples

TNM stage III breast cancer patients were diagnosed using clinical

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Table 1
Demographic and clinical information of patients and controls.

	Patient	Control
N	12	12
Age, y (median, range)	51 (30–65)	53 (32–65)
Sex (% female)	100	100
ER-positive (N, %)	12 (100)	N/A
PR-positive (N, %)	9 (75)	
HER2-positive (N %)	3 (25)	N/A

evaluations including physical examination, chest X-ray, mammography, ultrasound examination, breast MRI, blood tests, and core biopsy at Shengli Oilfield Central Hospital. Age- and BMI-matched healthy volunteers who were not presenting any evidence of inflammatory diseases and not taking any medication were recruited as healthy controls. Detailed demographic and clinical information is presented in Table 1. Written informed consent was obtained from all participants. The ethics committee of Shengli Oilfield Central Hospital approved this study.

Peripheral blood samples were harvested shortly after confirmation of breast cancer diagnosis and before treatment. The ACK lysis buffer (Thermo Fisher) was applied according to the manufacturer's instructions, if necessary. The peripheral blood mononuclear cells (PBMCs) were then collected via standard Ficoll-Paque centrifugation method. Resected tumors were minced into 1 to 2 mm pieces and incubated with 1 cubated with hyaluronidase (Stemcell) at 37 °C for 16 h in a shaking water bath. The resulting cell slurry was further separated into single cells by passing through a 70-μm filter, and the cells were separated into two fractions. In one fraction, the lymphocytes were collected by Ficoll-Paque centrifugation. In the other fraction, the cells resuspended briefly in trypsin-EDTA, washed with PBS supplemented with 2%, BSA resuspended in 5 mg/mL dispase (Stemcell) and 1 mg/mL DNase (Sigma-Aldrich), and washed again for fibroblast removal using the Anti-Fibroblast MicroBeads (Miltenyi). The remaining tumor cells were then pelleted and used in experiments.

2.2. Cell isolation

Whole $CD4^+$ and $CD8^+$ T cells were isolated using the Human CD4 and CD8 T cell Enrichment kits (Stemcell), respectively. For the isolation of $CXCR3^+$, $CCR4^+$, and $CCR6^+$ $CD4^+$ T cell subsets, the isolated $CD4^+$ T cells were first stained with the anti-human PE-CXCR3, PE-CCR4, or PE-CCR6 monoclonal antibodies (BioLegend) in separate experiments, and PE-positive cells then selected in a BD FACSAria instrument while PE-negative cells were discarded. For the isolation of $CCR4^-CCR6^-CXCR3^-$ $CD4^+$ T cells, all three PE-conjugated antibodies were added in one pool and the cells were sorted in FACSAria. PE-negative cells were selected while PE-positive cells were discarded.

2.3. T cell stimulation

For TCR stimulation, Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher) was used according to manufacturer's instructions. For PMA and ionomycin (P/I) stimulation, 50 ng/mL PMA and 1 μg/mL ionomycin (Invivogen) was added.

2.4. Quantitative PCR

Total mRNA was harvested from whole $CD4^+$ T cells or $CD4^+$ T cell subsets using the RNeasy Mini kit (Qiagen). The cDNA was then produced using the QuantiTect Reverse Transcription Kit (Qiagen). The primer/probe sets used were Hs00174125_m1 for IL-9, Hs00961622_m1 for IL-10, and Hs00222327_m1 for IL-21, as selected for TaqMan gene expression assays (Thermo Fisher). Quantification was conducted using the SYBR Green real-time PCR system (Thermo Fisher).

2.5. ELISA

Commercial kits, including IFN gamma (sensitivity < 2 pg/mL, range 25.6 to 1000 pg/mL), IL-4 (sensitivity 2 pg/mL, range 10 to 1300 pg/mL), IL-10 (sensitivity < 1 pg/mL, range 7.8 to 500 pg/mL), IL-17A (sensitivity 5 pg/mL, range 31.25 to 2000 pg/mL), IL-21 (sensitivity 20 pg/mL, range 78 to 5000 pg/mL), and IL-9 Human ELISA kit (sensitivity 0.5 pg/mL, range 3.1 to 200 pg/mL; Invitrogen) were used for ELISA.

2.6. Chromium-release assay

The freshly isolated tumor cells were labelled incubated with 300 μCi of ^{51}Cr per 10^6 cells for 2 h at 37 °C, and then washed with PBS plus 2% BSA. The tumor cells were then plated in triplicates at 1×10^4 cells/mL. Effector $CD8^+$ T cells were added accordingly at various effector/target ratios. For experiments with $CCR4^-CCR6^-CXCR3^-$ $CD4^+$ T cells, $CCR4^-CCR6^-CXCR3^-$ $CD4^+$ T cells were added at 1:1 ratio with $CD8^+$ T cells. Neutralizing antibodies included MH9A3 for IL-9, JES3-9D7 for IL-10 (BioLegend), and MT216G/21.3 m for IL-21 (MabTech). Plates were incubated at 37 °C for 6 h. Supernatant was harvested and the ^{51}Cr level was counted with a gamma counter. For maximum killing, cells were incubated in 1% NP40. For spontaneous release, no T cell was added. The specific lysis was calculated as 100% × [(^{51}Cr release in experiment) − (spontaneous release)]/[(maximum release) − (spontaneous release)].

2.7. Statistical analysis

All statistical tests were conducted by Prism 5 software (GraphPad). Comparisons between two groups used the Mann-Whitney test. Comparisons between paired multiple groups used the Friedman test followed by Dunn's multiple comparisons. Two-way comparisons between paired groups at repeated-measures were performed using two-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Breast cancer patients presented higher levels of IL-9 expression in the peripheral blood and the circulating $CD4^+$ T cells

We first harvested the peripheral blood samples from breast cancer patients and healthy control individuals who were matched with the breast cancer patients in age and BMI. The level of IL-9 in the serum was detected by ELISA. In healthy control individuals, IL-9 was only detected in one out of twelve individuals, while in breast cancer patients, six out of twelve subjects presented positive IL-9 in the serum (Fig. 1). The IL-9 production by circulating $CD4^+$ T cells was subsequently investigated. First, we incubated PBMCs in the presence of anti-CD3/CD28 (TCR) or PMA and ionomycine (P/I) stimulation for 12 h, and found that the IL-9 mRNA transcription was significantly higher in $CD4^+$ T cells from breast cancer patients than from healthy controls (Fig. 1B). Next, we examined the secreted IL-9 protein in the supernatant, and found that the IL-9 protein level was significantly higher in breast cancer patients than in healthy controls (Fig. 1C). Subsequently, we purified $CD4^+$ T cells from PBMCs, which were TCR- or P/I-stimulated for 12 h. The $CD4^+$ T cells from breast cancer patients secreted significantly higher IL-9 than the $CD4^+$ T cells from healthy controls (Fig. 1D).

3.2. IL-9-expressing Th9 cells were enriched in the $CCR4^-CCR6^-CXCR3^-$ subset

Having observed an upregulation in IL-9 expression in $CD4^+$ T cells