

T cytotoxic 17 cells suppress the inflammatory reaction by regulating chemokines in colonic cancer

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Abstract

Objective: The aim of this study was to detect the level of T cytotoxic 17 (Tc17) cells in patients with colonic cancer and explore the relationship between Tc17 cells and colonic cancer cells.

Material and methods: The numbers of Tc17 cells in both colon cancer tissue and co-culture system with SW620 were evaluated by FACS sorting. The expression of the CCL2, CXCL12, G-CSF, M-CSF and GM-CSF in SW620 was detected by qPCR after being co-cultured with Tc17 cells.

Results: Compared to the normal colon tissue, the number of Tc17 cells was significantly increased in the cancer tissue. And this number was remarkably increased after being co-cultured with SW620. Co-cultured Tc17 cells could also enhance the expression of CCL2, CXCL12 and GM-CSF.

Conclusions: Colonic cancer cells can recruit Tc17 cells to cancer tissue. These recruited Tc17 cells can promote the expression of inflammatory suppression chemokines in order to induce the immune suppression in cancer development.

Key words: colon cancer, Tc17 cells, chemokines, inflammatory suppression.

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Introduction

Colon carcinoma as one of the common malignant cancers that cause human death in the world [1], is a kind of complicated disease closely related to such factors as age, life-style, living surroundings and immune status [2, 3]. To find an efficiency treatment method, physicians and scientists focus on the intrinsic mechanism in colonic cancer development, especially the immune-related responses in cancer formation. According to cancer-associated clinical researches, a variety of immune cells play a role in attenuating anti-cancer effect by inducing and sustaining the immunosuppression state.

Recently, interleukin (IL)-17 cells are detected in colonic tumor tissues and noticed to participate in tumor immune response [4]. Interleukin 17 cells are produced in both CD4+ and CD8+ T cells, also known as T helper 17 cells (Th17) [5] and T cytotoxic 17 cells (Tc17 cells) [6], respectively. It has been reported that the over-expressed IL-17 tumor cells are able to induce angiogenesis by stimulating the release of IL-8 [7] and promote tumor growth by increasing the expression of vascular endothelial growth factor (VEGF) [8]. In addition to Th17 cells, many other factors, such as IL-23, can induce Tc17 cells aggregation, but lead to various cytotoxic effects [9-12]. However, the exact effect of Tc17 cells in colonic cancer development

is still unclear. This study aimed to investigate the biological function of Tc17 cells, and explore the immunological mechanism in the colon cancer development process.

Material and methods

Samples

Twenty-nine normal colon tissues and 31 colonic cancer tissues were collected from the First Affiliated Hospital of Zhengzhou University. For experiments *in vitro*, each group had 6 samples and experiments were repeated three times. This study was conducted in accordance with the declaration of Helsinki. This study was conducted upon the approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from all participants.

Preparation of human peripheral blood mononuclear cell (PBMC)

All the procedures were under sterile condition. Peripheral blood from volunteers was extracted and then treated with red blood cell lysis buffer followed by filtration through a nylon membrane (200 hole/25.4 mm) in or-

der to prepare single cell suspension. After centrifuged at a speed of 1500 rpm for 5 min, supernatant was aspirated and the cells were rinsed twice in PBS. Finally, the cell pellets were resuspended with RPMI 1640 culture medium (Gibco, Grand Island, NY, USA) to form mononuclear lymphocyte suspension.

Human peripheral blood T lymphocytes and Tc17 cell sorted by magnetic beads

According to the sorting method reported previously [13, 14], PBMC were divided into two groups with the same cell numbers in each 4 ml of PBS. Cells were centrifuged at 1500 rpm for 5 min. After removing the supernatant, cell pellets were resuspended in 100 μ l of PBS and incubated with 10 μ l of anti-CD8 antibody (Abcam, ab4055) for 15 min at room temperature. After the incubation, 5 μ l immunomagnetic beads were added in and incubated for another 10 minutes. Add PBS with the final volume of 2.5 ml. The tube was set in the magnetic field without cover for 5 min before cell suspension was poured-out and rinsed with PBS. This step was repeated three times. Finally, separated cells were resuspended with culture medium and counted. According to their own surface markers, the numbers of each cell portion were counted individually. By using the same sorting methods, human Tc17 cells were separated from lymphocytes with other Tc17 specific antibodies, including CD8 and IL-17.

Co-culture of colon cancer cell lines and Tc17 cells

Human colon cancer cell line SW620 was purchased from Shanghai cell bank, Chinese Academy of Sciences. Colon cancer cells (SW620) and Tc17 cells were mixed with a 1 proportion ratio and incubated in 5% CO₂ for 42h. Then, 1 μ g/ml phorbol myristate acetate, 50 μ g/ml ionomycin and 0.7 μ l/ml Golgistop (BD Biosciences, New Jersey, USA) were added to the medium to incubate in for another 6 h at 37°C, 5% CO₂.

Flow cytometry analysis

Co-cultured cells were washed by PBS and resuspended in 100 μ l of PBS for antibody incubation. In all the incubation procedures, light exposure needed to be avoided. FITC-CD3 (0.5 mg/ml) antibody and CD8-APC (0.2 mg/ml) antibody (eBioscience, San Diego, CA, USA) were added to cells suspension and incubated at room temperature for 20 minutes. 100 μ l of Fix/Perm A (Invitrogen, Carlsbad, USA) were added in and incubated for another 15 min at room temperature. Next, cell suspension was centrifuged at 1500 rpm, and then resuspended in 100 μ l of Fix/Perm B (Invitrogen, Carlsbad, USA). Interleukin

17-PE (0.2 mg/ml) antibody (eBioscience, San Diego, CA, USA) was added in and incubated for 1 h at 4°C. Finally, after washing by PBS, cells were resuspended with 500 μ l of PBS and sorted by flow cytometer (FACSCalibur, Becton Dickinson, USA).

Fluorescent quantitation polymerase chain reaction (qPCR)

Total RNA (gRNA) of SW620 was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). Then gRNA was reversed to cDNA (Thermo Scientific, Rockford, IL, USA). Fluorescent quantitation PCR (qPCR) reactions were designed with the following protocol: 50°C, 2 min 95°C, 10 min 95°C, 15 s 60°C, 1 min repeat step 3 and 4. 95°C, 15 s 60°C, 30 s 95°C, 15 s.

Statistical analysis

All the data were analyzed by SPSS17.0 (SPSS Inc, Chicago, IL, USA) for normality test. *T*-test was used to find out the differences between each two groups. The significant difference was defined as $p < 0.05$.

Results

Tc17 cells were recruited in colon cancer tissue

Twenty-nine normal colon tissues and 31 tumor tissues were collected from the healthy donors and the patients with colonic cancer, respectively. The population of Tc17 cells in both cancer and normal tissues were analyzed by FACS sorting. In 29 normal colon tissues, the average percentage of Tc17 cells was 2.83%; while in 31 colon cancer tissues, the average percentage was 8.91% (Fig. 1). *T*-test analyses revealed statistical differences between normal and colon cancer group ($p < 0.05$).

Tc17 cell proportions were elevated in co-culture with human colon cancer cell line SW620 cells

According to the above result, Tc17 cells could also be stimulated in the cancer microenvironment. To test this speculation, Tc17 cells which were collected from human peripheral blood were co-cultured with human colon cancer cell line SW620. And then the population of Tc17 cells was investigated under these conditions. The FACS sorting result showed that Tc17 cells accounted for 5.74% in lymphocytes group and account for 11.8% in the co-culture group. This increased proportion was confirmed as a statistical difference between by *t*-test ($p < 0.01$) (Fig. 2).

Chemokines expressions were raised in co-culture system

Tc17 cells have been shown that they could promote the anti-inflammatory chemokines expression and induce

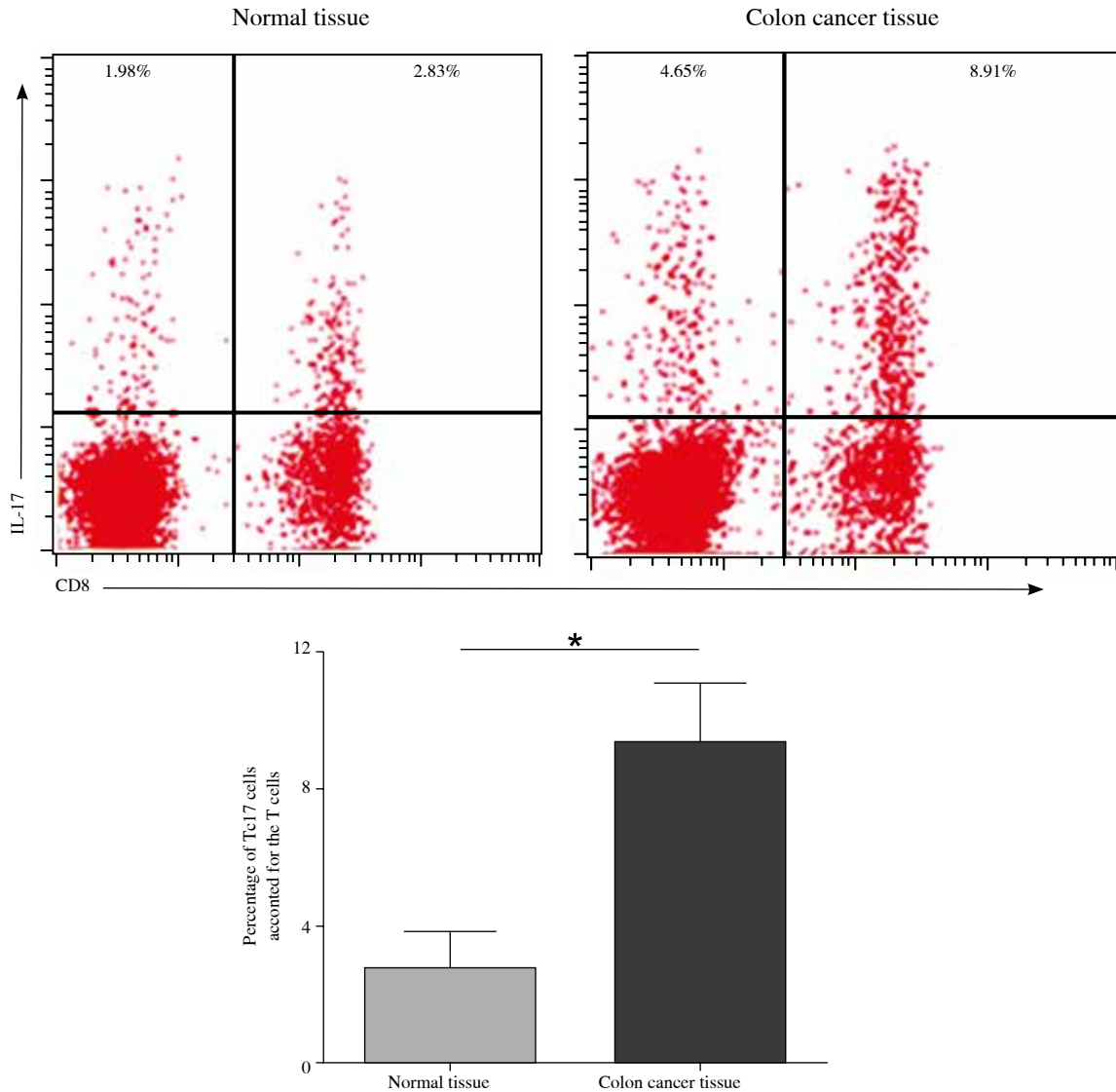


Fig. 1. Tc17 cell proportion was elevated in colon cancer tissue ($n > 6$, $*p < 0.05$, repeated three times)

the immunosuppressive status in gastric cancer cells and breast cancer cells [15, 16]. Upregulated CCL2, CXCL12, G-CSF, M-CSF and GM-CSF which are all known as anti-inflammatory chemokines were also reported in colon cancer cells [17]. To study whether Tc17 cells affect the chemokine expression in colonic cancer cells, we analyzed the chemokine expression in the SW620 cells with or without Tc17 cell co-culture stimulation by the qPCR test. The data showed that all the CCL2, CXCL12 and GM-CSF were significantly upregulated in the co-culture group, compared to the group without Tc17 cells (Fig. 3A, B and E); however, although the expression of G-CSF was also elevated in the co-culture group, this increase does not show a statistical difference ($p > 0.05$, Fig. 3C). Meanwhile, M-CSF showed no change

es between two groups (Fig. 3D). These results supported the hypothesis that Tc17 cells could promote the expression of anti-inflammatory chemokines including CCL2, CXCL12 and GM-CSF in colonic cancer tissue.

Discussion

It has been reported that the population of Tc17 cells was surprisingly increased in hepatocellular carcinoma [6], and played a role of an immunosuppression agonist by promoting immune inhibitory cells [15]. However, rare data were reported about the function of Tc17 cells in colonic cancer. The aim of this study is to investigate the effect of Tc17 cells in colonic cancer development and immunosuppression.

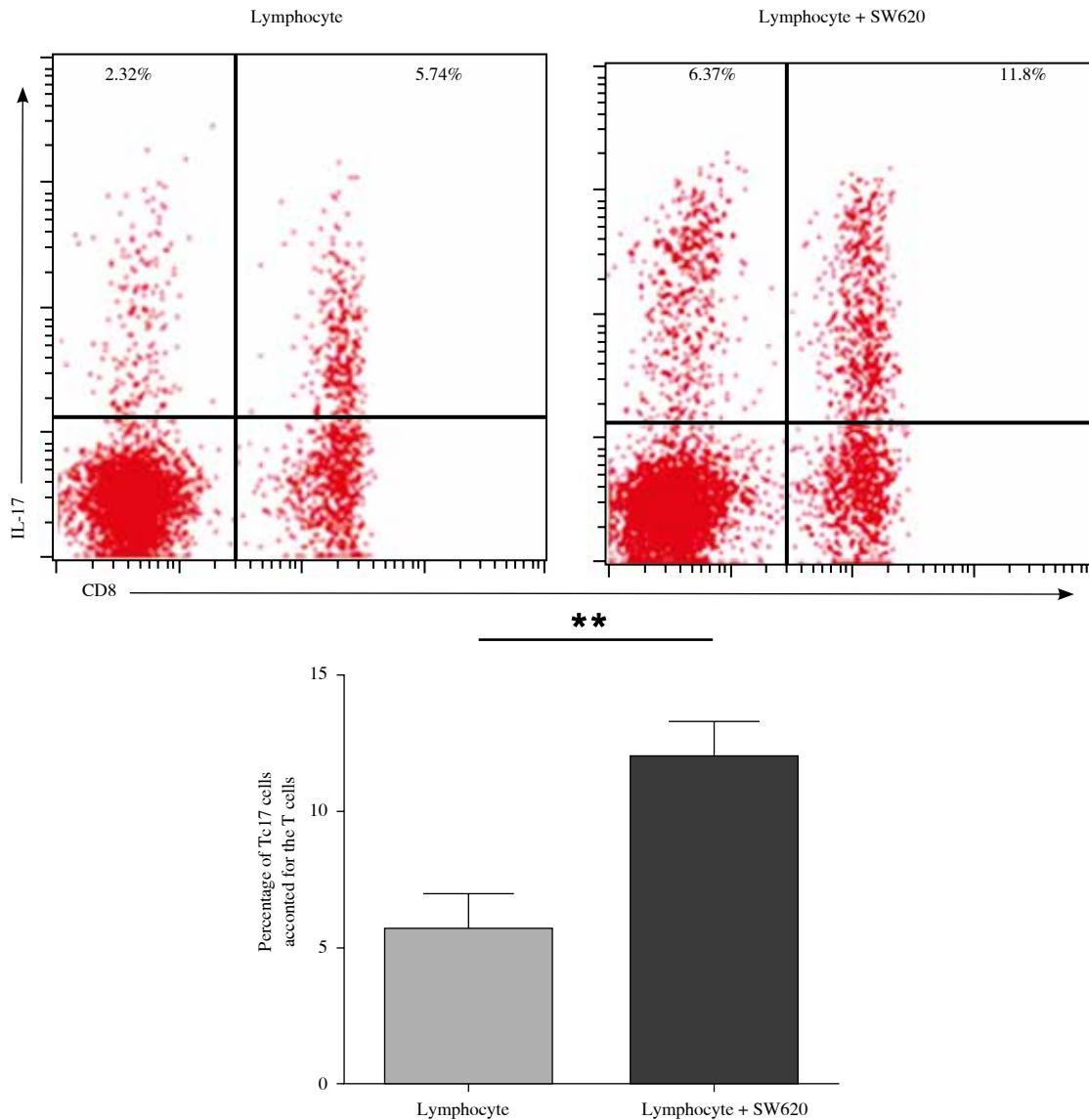


Fig. 2. Tc17 cell proportion was elevated in the co-cultured system with SW620 cell line ($n = 6$, $**p < 0.01$, repeated three times)

We firstly detected the Tc17 cells in the tumor tissues of colon cancer tissues. Consistently with previous findings, the results showed that Tc17 cells had a higher proportion in colon cancer tissues than normal colon tissues [15]. It suggested that Tc17 cells were obviously recruited in colon tumor.

However, it still not clarified whether this increased number of Tc17 cells was due to the upregulated proliferation or the recruited Tc17 cells from another location. To explain the mechanism of increased Tc17 cells number in colonic cancer microenvironment, a co-culture system composed with Tc17 cells and human colon cancer cell lines SW620 was performed *in vitro*. The FACS sorting

revealed that the proportion of Tc17 cells was similarly increased in the co-culture system with SW620. This result implied that the total number of Tc17 cells was affected by the stimulation of SW620 cells, indicating that the proliferation rate of Tc17 cells was changed in the colonic cancer microenvironment.

The increased Tc17 cells might lead to the immunosuppression status of local colonic cancer tissue by inducing more immunosuppressive chemokines [16]. To determine whether Tc17 cells induce the expression of immunosuppressive chemokines in SW620 cells or not, the mRNA level of CCL2, CXCL12, G-CSF, M-CSF and GM-CSF were tested in co-cultured SW620 cells. Simi-

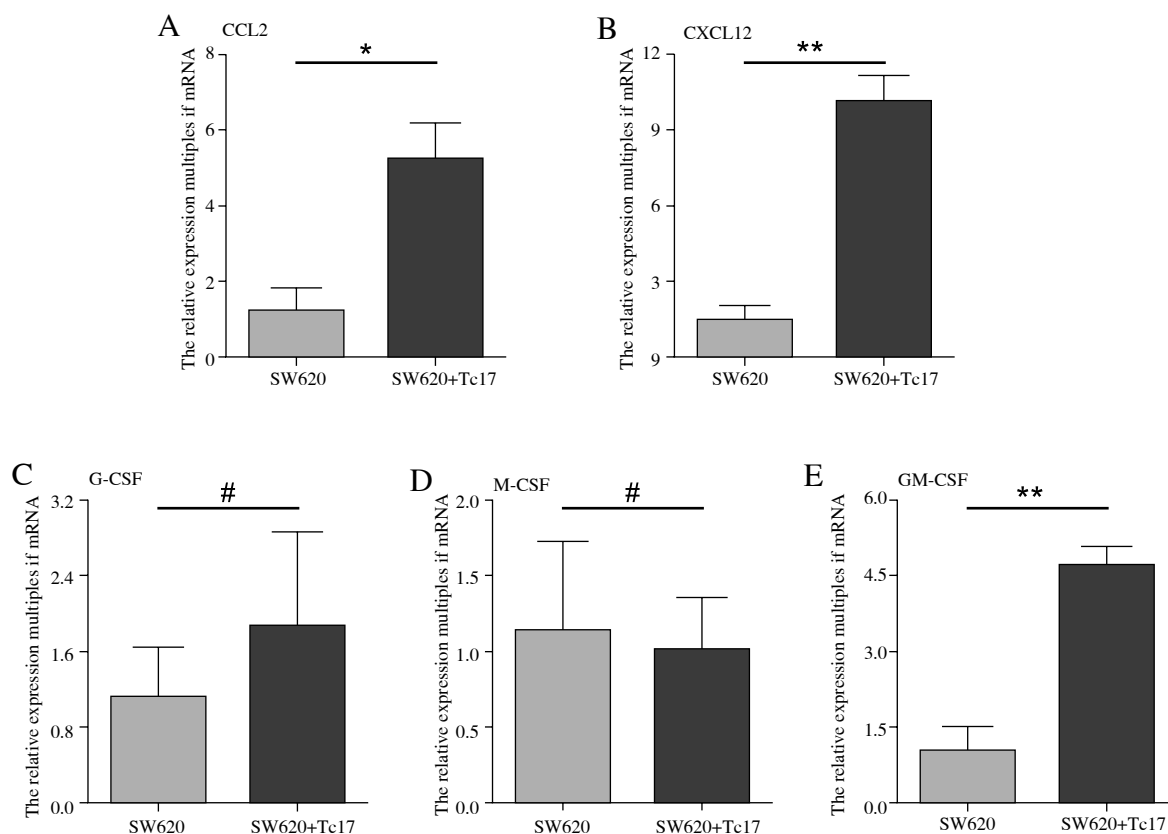


Fig. 3. The expression of anti-inflammatory chemokines were upregulated in SW620 with Tc17 cells co-culture ($n = 6$, $*p < 0.05$, $**p < 0.01$, $\#p > 0.05$, repeated three times)

larly with previous studies about CCL2 [14, 18], CXCL12 [19] and GM-CSF [20], our results demonstrated higher CCL2, CXCL12 and GM-CSF expressions in SW620 cells. Therefore, the result suggested that Tc17 cells could induce the immunosuppression status by promoting the expression of immunosuppression chemokines.

In summary, our results demonstrated that Tc17 cells were increased in colonic cancer tissues. The upregulated expression of immunosuppressive chemokines was noticed in a Tc17 and SW620 cell co-cultured system. Herein our research provided a new connection between colon cancer and immune reactions, and presented a new mechanism of immunosuppression in cancer development.

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