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Correlation between $CD4^+CD25^+T_{reg}$ Cells and CCR4 in Nasopharyngeal Carcinoma

Yan-xin REN[1]
Jun SUI[1]
Xin SONG[1]
Gee WAN WONG[3]
Jing MA[2]
Hong YAO[3]
Marie Chia-mi LIN[3]
Xiao-jiang LI[2]

[1] Immunotherapy Center, The Third Affiliated Hospital, Kunming Medical College, Kunming 650118, Yunnan Province, China.
[2] Head and Neck Surgery, The Third Affiliated Hospital, Kunming Medical College, Kunming 650118, Yunnan Province, China.
[3] Institute of Molecular Biology, Department of Chemistry, Open Laboratory of Chemical Biology, The University of Hong Kong, Hong Kong, China.

Correspondence to: Xiao-jiang LI
E-mail: xiaojiangle@yahoo.com.cn
Tel: 86-871-8185656 ext.2180
Fax: 86-871-8181942

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E-mail: editor@cocronline.org
Tel (Fax): 86-22-2352 2919

OBJECTIVE $CD4^+CD25^+$ T regulatory (T_{reg}) cells are a population of T cells which suppress an overactive immune system. CCR4 is a chemokine receptor involved in the recruitment of lymphocytes. Nasopharyngeal carcinoma (NPC) is resistant to immunosurveillance, owing to the increased number of tumor-infiltrating T_{reg} cells which are recruited to the tumor by CCR4.

METHODS The percentage of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in tissue or peripheral blood (PB) lymphocytes of patients with untreated NPC or normal subjects was analysed by flow cytometry.

RESULTS In both tissue and PB lymphocytes, the percentage of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells was significantly elevated in patients with NPC in comparison with that in the normal tissue of controls. Furthermore, in the patients with NPC, a higher percentage of $CD4^+CD25^+$ T_{reg} cells was found in the tumor-infiltrating (TI) lymphocyte population than in the PB population. In the NPC patient group, a general trend towards an increased percentage of TI T_{reg} cells was found in the patients with advanced stage NPC. The number of $CD4^+CD25^+$ T_{reg} cells was positively related to the number of $CCR4^+$ cells in the tumor and in the PB of the patients with NPC, while the number of $CD4^+CD25^+$ T_{reg} cells was negatively related to the number of $CD4^+CD25^-$ T cells.

CONCLUSION Immunosuppression was observed in NPC, especially at the tumor sites. $CD4^+CD25^+$ T_{reg} cells may suppress $CD4^+CD25^-$ T cells. CCR4 may have an important role in the recruitment of $CD4^+CD25^+$ T_{reg} cells to tumor sites, thus causing resistance to immunosurveillance.

KEY WORDS: nasopharyngeal carcinoma, $CD4^+CD25^+$ T_{reg} cells, CCR4, flow cytometry, tumor-infiltrating lymphocytes.

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck malignancy with high incidence in Southeast Asia, Africa, western Canada and Alaska. In China, NPC has a high prevalence in Guangdong, Guangxi, Hunan, Jiangxi, Fujian, and Yunnan provinces. For instance, the incidence of NPC in Guangdong province is 30–50 per 100,000 annually[1].

$CD4^+CD25^+$ regulatory T cells are defined as a subgroup of regulatory T (T_{reg}) cells associated with autoimmune disorders. Transferring $CD4^+CD25^+$ T_{reg} cells into BALB/c athymic nude (nu/nu) mice can spontaneously induce the development of histologically and serologically evident autoimmune diseases[2]. Constantly reconstituting the $CD4^+CD25^+$ T_{reg} cell population within a limited period after transfer of

$CD4^+CD25^+$ T_{reg} cells might prevent those autoimmune diseases[2]. $CD4^+CD25^+$ T_{reg} cells may suppress antigen presenting cells or cytotoxic T lymphocytes through cellular interaction or secretion of cytokines.

It has been found that in most malignancies, especially in early-stage malignancies, immunosuppression is restricted to the tumor sites. The ratio of $CD4^+$ to $CD8^+$ is the most common measure of immune function, but it does not correlate well with tumor stages, tumor volume or survival rate of patients. Therefore, estimating the immune strength of patients with cancer using the $CD4^+/CD8^+$ ratio does not provide a reliable prognosis[3]. For some human cancers, the antigen recognized by T cells is autologous, indicating that tumor immunity is also autoimmunity[4]. $CD4^+CD25^+$ T_{reg} cells recognize antigen in a way similar to that of traditional T cells, but the former are activated more easily by autoantigen owing to their higher affinity to autologous tissue[5]. Therefore, it has been hypothesized that immunosuppression in tumor microenvironments is associated with the activation of $CD4^+CD25^+$ T_{reg} cells.

Chemokines are a group of small, structurally related cytokines which might induce directional migration of different lymphocyte populations, and play important roles in the immune response to infection and tumor. Chemokines are classified into four main subfamilies based on the arrangements of their N-terminal conserved cysteine residues: CXC (α-chemokine), CC (β-chemokine), C (γ-chemokine, just the second and fourth cysteine residue), CX3C (δ-chemokine)[6]. CC chemokine receptor 4 (CCR4) is a member of the CC chemokine receptor family. In gastric adenocarcinoma, elevated levels of CCL22 and CCL17 (both ligands of CCR4), and an increased number of $CD4^+CD25^+$ T_{reg} cells, can be found[7]. This leads to the hypothesis that tumor cells secrete CCL22 and CCL17 which activate and attract $CD4^+CD25^+$ T_{reg} cells through the CCR4 pathway, and hence these ligands stimulate the $CD4^+CD25^+$ T_{reg} cells which accumulate in tumor sites and suppress immunity. We analyzed the number of $CD4^+CD25^+$ T_{reg} cells and the levels of CCR4 in venous blood and in tumor biopsy specimens taken from patients with primary NPC before treatment and from healthy donors using flow cytometry.

Materials and Methods

Sample collection

Heparinized venous blood (35 cases) and tumor biopsy specimens (25 cases) taken from patients with primary NPC at the time of first admittance to The Third Affiliated Hospital of Kunming Medical College were collected. None of the patients had undergone radiotherapy or chemotherapy. Patients with known diseases of their immune systems or taking medicine which might affect their immune system were excluded. Clinical stages were determined according to the UICC/AJCC 1997 classification. For the control group, 12 healthy people, who had been admitted to the hospital for rhinobyon, headache, nasal mucus and nasal bleeding, were recruited and underwent a pathological examination to exclude the presence of NPC. All samples of nasopharyngeal tissue were obtained with the guidance of a nasopharyngeal fiberscope. To avoid the risk of impairing the results of diagnosis of surgically excised specimens only a small piece of each specimen (about 5 mm in diameter) was provided for this study. All samples were stored at −70°C, and the remaining part of each specimen was collected for pathological examination. Full consent was obtained from all subjects before collection of samples.

Antibody

FITC-conjugated anti-human CD4, phycoerythrin (PE)-conjugated anti-human CD25, CCR4, PC5-conjugated anti-human CD45, PE-conjugated anti-human IgG immunoglobulin and FITC-conjugated anti-human IgG immunoglobulin as control were purchased from Beckman Coulter. An EPICS-XL (Beckman Coulter) flow cytometer was used.

Flow cytometry

A single-cell suspension of specimens was obtained, which was digested with collagenase for 2 h at 37°C. The suspended cells of peripheral blood (PB) were immunostained with fluorochrome-conjugated anti-human antibodies for 25 min at 4°C. The PB was dissolved by Q-prep auto hemolysis equipment. Samples were analyzed by flow cytometry, and the frequencies of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells present in lymphocytes of different subsets were calculated; the data obtained were analyzed with EXPO32.

Statistical analysis

All data were analyzed using SPSS 11.5 software; intergroup differences were tested with an independent samples t test; and the correlation among variables was assessed with linear models. A P value < 0.05 was considered significant.

Results

Increased number of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in NPC specimens

Flow cytometric analysis of the specimens (n=25) and normal tissues of the controls (n=12) indicated that clusters of $CD4^+CD25^+$ T_{reg} cells infiltrated into tumor sites, but were not found in the normal tissue of controls or in the PB of the patients with NPC (data not shown). The percentage of tumor-infiltrating (TI) $CD4^+CD25^+$ T_{reg} cells of typical NPC is 31.2% and only 4.1% in the normal tissues of controls (Fig. 1A,B). The percentage of TI $CD4^+CD25^+$ T_{reg} cells in the NPC specimens was $(16.19 \pm 7.78)\%$, which was higher than the $(3.57 \pm 1.25)\%$ found in the normal tissues of controls ($P < 0.05$).

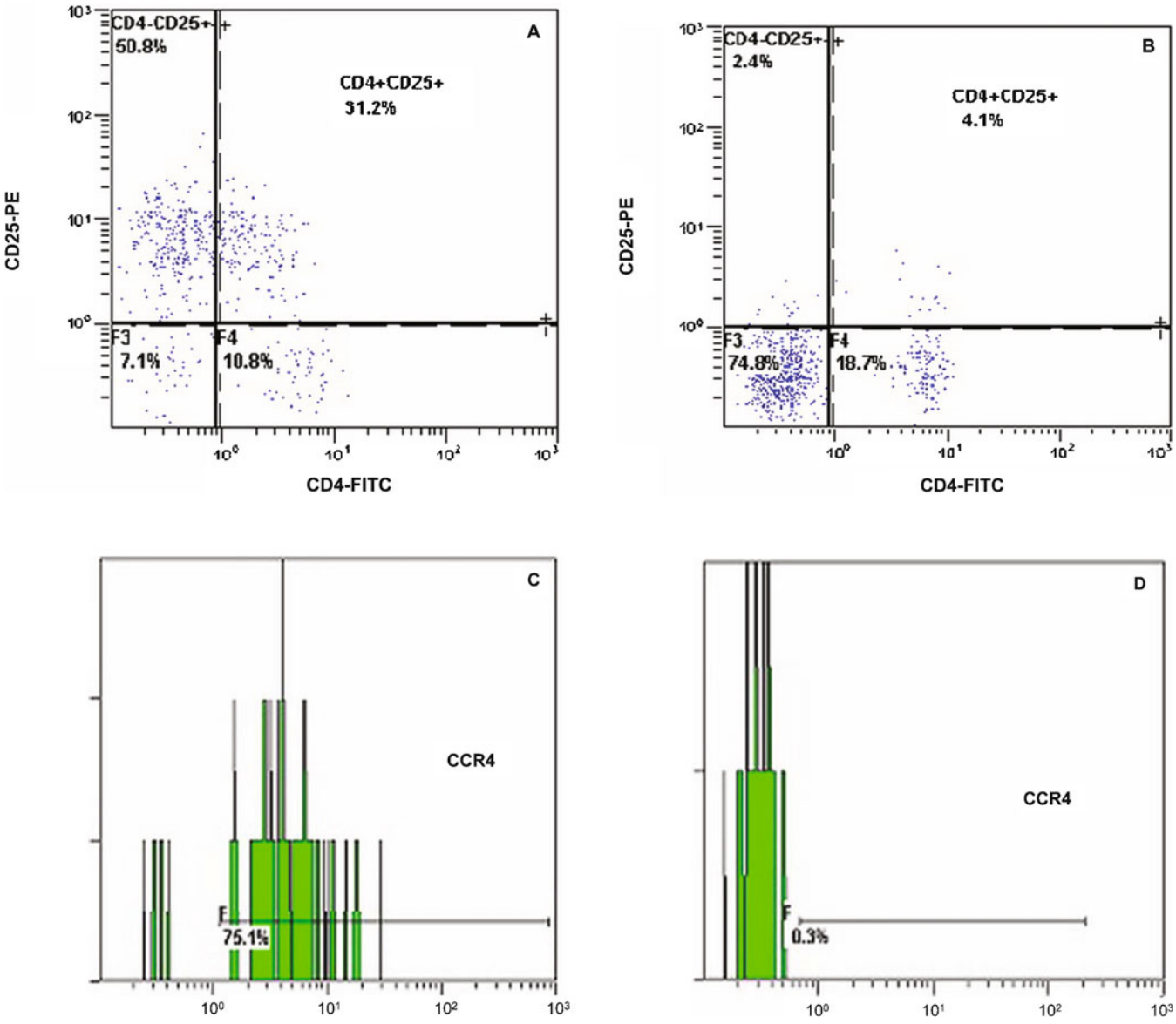


Fig.1. Percentages of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in tissue of patients with nasopharyngeal carcinoma (NPC) and controls. The percentage of $CD4^+CD25^+T_{reg}$ tumor-infiltrating (TI) cells in patients with NPC was 31.2% (A), higher than the 4.1% of the normal tissue of controls (B). The percentage of $CCR4^+$ TI cells of the patients with NPC was 75.1% (C) while that in the normal tissue of controls was 0.3% (D).

Analysis of the same typical NPC specimens showed that the percentage of $CCR4^+$ cells was 75.1% and that in the controls was 0.3% (Fig.1C, D). Consistently, the levels of $CCR4^+$ cells in the NPC specimens were also significantly elevated to $(47.09 \pm 29.77)\%$, which was higher than the $(6.1 \pm 1.12)\%$ found in controls ($P < 0.05$).

Increased number of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in the peripheral blood of patients with NPC

The percentages of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in blood samples were higher in patients with NPC than in controls, but the difference was not significant. The percentage of $CD4^+CD25^+$ T_{reg} cells in the PB of typical patients with NPC is 5.6% and only 3.5% in the PB of controls (Fig.2A,B). The percentage of $CD4^+CD25^+$ T_{reg} cells in the NCP PB was $(5.43 \pm 3.32)\%$, which was higher than the $(3.24 \pm 1.34)\%$ of the controls ($P < 0.05$).

In this study, we also found that the percentage of $CCR4^+$ cells in the PB of the same typical patients with NPC was 20.2% while it was 5.0% in the controls (Fig.2C, D), and the percentage of $CCR4^+$ cells in NPC PB was $(11.22 \pm 7.41)\%$, which was higher than the $(2.43 \pm 1.89)\%$ in control PB ($P < 0.05$).

Increased percentage of $CD4^+CD25^+$ Treg cells in the patients with advanced stages of NPC

The patients were classified into two groups according to the UICC/AJCC 1997 classification: an early-stage group (stage I + II) and an advanced-stage group (stage III + IV). The differences in the percentage of $CD4^+CD25^+$ T_{reg} cells

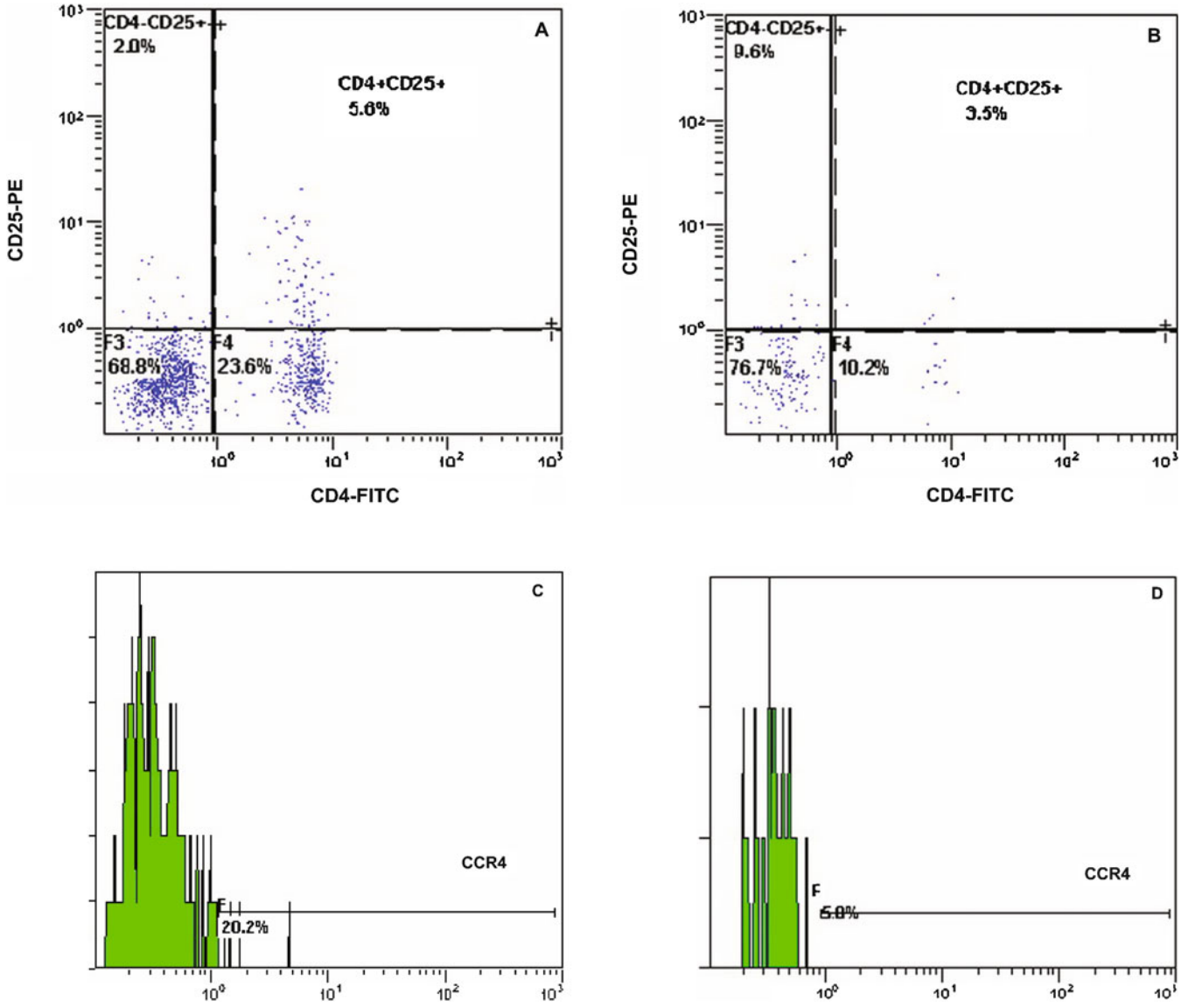


Fig.2. Percentages of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in the peripheral blood (PB) of patients with nasopharyngeal carcinoma (NPC) and controls. The percentage of $CD4^+CD25^+$ T_{reg} cells in the PB of the patients with NPC was 5.6% (A) while that in the normal tissue of controls was 3.5% (B). The percentage of $CCR4^+$ cells in the PB of the patients with NPC was 20.2% (C) and that in the normal tissue of controls was 5.0% (D).

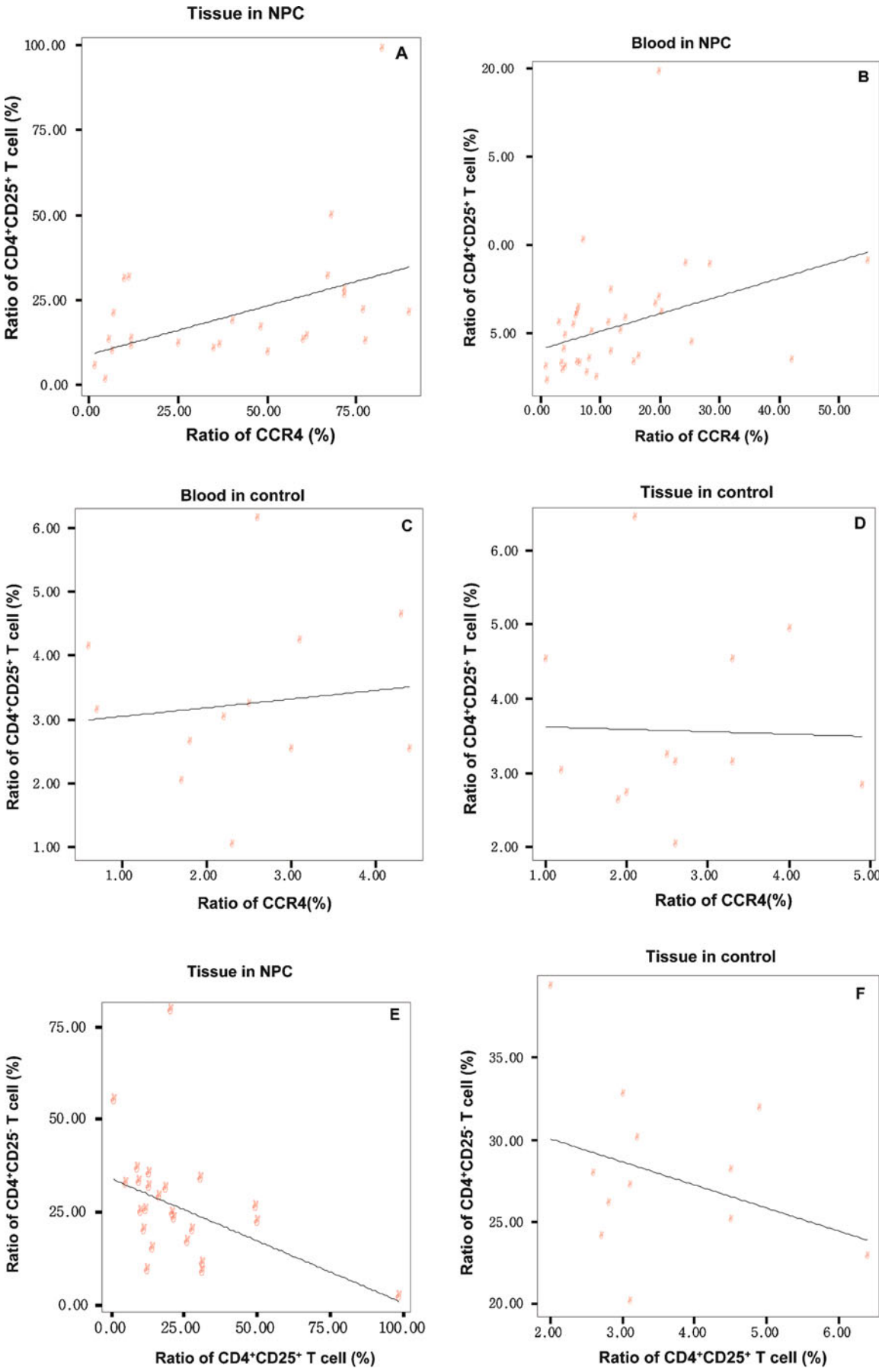
and $CCR4^+$ cells between the two groups were analyzed. The percentage of $CD4^+CD25^+$ T_{reg} cells in the specimens of the patients with NPC at stage III+IV was higher than that at stage I + II [$(23.76 \pm 12.29)\%$ *vs.* $(12.70 \pm 5.76)\%$, $P < 0.05$)], whereas the difference in $CCR4^+$ cells between the two groups was not statistically significant [$(40.25 \pm 25.42)\%$ *vs.* $(48.90 \pm 35.11)\%$, $P > 0.05$]. In PB, the difference in the number of $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells between the two groups was not significant ($P > 0.05$).

Correlations of $CD4^+CD25^+$ Treg cells with $CCR4^+$ cells and $CD4^+CD25^-$ T cells in NPC

To clarify the relationship between $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in NPC, the correlation between the two was analyzed. There was a positive correlation between $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in the NPC specimens and in PB (correlation coefficient 0.485, 0.357, respectively, $P < 0.05$, Fig.3A, B), while there was no correlation between the two in the normal tissues of controls ($P > 0.05$, Fig.3C, D). A negative correlation was found between $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells in the NPC specimens ($r = -0.444$, $P < 0.05$, Fig.3E), but this was not observed in the normal tissues of controls (Fig.3F).

Discussion

Patients with NPC have benefited from radiotherapy and chemotherapy, but more recent studies have examined the role of host immune responses in head and neck


Tissue in NPC
A
Ratio of CD4+CD25+ T cell (%)
Ratio of CCR4 (%)
Blood in NPC
B
Ratio of CD4+CD25+ T cell (%)
Ratio of CCR4 (%)
Blood in control
C
Ratio of CD4+CD25+ T cell (%)
Ratio of CCR4(%)
Tissue in control
D
Ratio of CD4+CD25+ T cell (%)
Ratio of CCR4(%)
Tissue in NPC
E
Ratio of CD4+CD25- T cell (%)
Ratio of CD4+CD25+ T cell (%)
Tissue in control
F
Ratio of CD4+CD25- T cell (%)
Ratio of CD4+CD25+ T cell (%)

carcinoma progression, and suggested that T lymphocytes may play a role in controlling tumor growth[8]. T lymphocytes are composed of two subgroups: suppressor T cells (Ts) $CD8^+$ and helper T cells (Th) $CD4^+$, which interact with each other balancing the immune function. $CD4^+$ T cells regulate the immunity and help B lymphocytes to secrete antibodies, while $CD8^+$ T cells mediate killing of target cells. The percentage of $CD4^+$/$CD8^+$ T cells in PB is the most widely used index of immunity in clinical practice. However, some studies have suggested that there is no good correlation between the percentage of $CD4^+/CD8^+$ T cells and the stage, tumor burden and survival of tumor patients. However, $CD4^+$ T cell and $CD8^+$ T-cell populations are composed of subpopulations with different specific functions. Moreover, since the distribution of different T-cell populations in tumor sites differs from that in PB, it is expected that immunosuppression may occur in tumor sites but not in other parts of the body. Therefore, the $CD4^+/CD8^+$ ratio is not a good index of the status of the immune system.

At tumor sites, lymphocytes infiltrate into primary or secondary tumors and play an important role in killing tumor cells and in reducing the potential for metastasis. Therefore, the quantity and functioning of TI lymphocytes represents the intensity and level of host-anti-tumor immunity. However, it has been reported that the quantity of $CD4^+CD25^+$ T_{reg} cells increases in tumor sites and in the PB of patients with ovarian carcinoma, lung cancer, breast cancer, pancreatic cancer and colon carcinoma[9–13]. $CD4^+CD25^+$ T_{reg} cells act as an immune system regulator by direct cell contact and cytokine secretion[14,15].

In our study, the data showed that the percentage of $CD4^+CD25^+$ T_{reg} cells in NPC specimens was much higher than that in PB or in the normal tissue of controls. We also found that there was a negative correlation between $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells in the NPC specimens. Yang et al.[12] reported that $CD4^+CD25^+$ T_{reg} cells could suppress not only the proliferation of $CD4^+CD25^-$ T cells but also interferon γ and interleukin-4 secretion by $CD4^+CD25^-$ T cells. Experimental depletion of $CD4^+CD25^+$ T_{reg} cells in mice with tumors improves immune-mediated tumor clearance and enhances the response to immune-based therapy. The quantity of $CD4^+CD25^+$ T_{reg} cells is greater than that of $CD4^+CD25^-$ T cells in gastric adenocarcinoma; the close connection indicates that $CD4^+CD25^+$ T_{reg} cells suppress $CD4^+CD25^-$T cells by direct cellular contact[16]. Therefore, there is a possibility that $CD4^+CD25^+$ T_{reg} cells promote tumor growth by suppressing $CD4^+CD25^-$ T cells. This needs further investigation with a larger number of samples.

The percentage of $CD4^+CD25^+$ T_{reg} cells in the patients with NPC at stage III+IV was higher than that at stage I + II, which is consistent with the results of the study on gastric and esophageal carcinomas[17], but in contrast to a recent report by Lau et al.[18] The main reason for this discrepancy is that the percentage of $CD4^+CD25^+$ T_{reg} cells in the study of Lau et al. was that in PB, whereas we analysed both tumor sites and PB. Thus our study suggests that the percentage of $CD4^+CD25^+$ T_{reg} cells in tumor sites is more appropriate for evaluating host immunity.

Recruitment of T cells to a particular site is a multistage process involving secretion of chemokine by tumor and rolling T cells[19]. A limited number of $CD4^+CD25^+$ T_{reg} cells are found in normal tissues, but there are no data on their role in normal tissue[20]. In this experiment, we found that the percentage of $CCR4^+$ cells in TI and PB lymphocytes was significantly higher in patients with NPC than in controls, and the percentage of $CCR4^+$ cells was positively related to that of $CD4^+CD25^+$ T_{reg} cells in both specimens and PB of the patients with NPC. The hypothesis that malignant tumors can attract $CD4^+CD25^+$ T_{reg} cells through CCR4 is logical. It has been reported that the levels of the CCR4 ligand, CCL22, are elevated in gastric adenocarcinoma[16], ovarian carcinoma[21] and Hodgkin's disease[22], and the CCL22 level is related to the prognosis of patients with cancer, and only in lymph nodes infiltrated by tumor cells would $CD4^+CD25^+$ T_{reg} cells suppress immunity[23]. On the other hand, little CCR4 is expressed by T cells in lymph nodes free of tumor cell infiltration such as in Hodgkin's disease[24]. Another experiment proved that CCR4 was highly expressed on $CD4^+CD25^+$ T_{reg} cells[25]. Therefore, it is logical to suggest that effective T cells can recognize tumor antigen and play an important role in anti-tumor activity by means of antigen-dependent-cell-mediated cytotoxicity at an early stage. On the other hand, in order to survive, the tumor would produce copious amounts of CCR4 ligands, CCL17 and CCL22, to attract $CD4^+CD25^+$ T_{reg} cells to suppress immunity in tumor sites.

Investigators also reported that mature dendritic cells (DCs) could secrete CCL22 and CCL17 to mediate the migration of T lymphocytes to tumor sites[26]. There is competition among T lymphocytes, and CCR4 expressed on $CD4^+CD25^+$ T_{reg} cells would give them an advantage, enabling the $CD4^+CD25^+$ T_{reg} cells to suppress the proliferation and function of effective T cells[27,28]. We suggest that DCs can present tumor antigens to effective T cells for their anti-tumor effects; meanwhile DCs also produce chemokines to attract $CD4^+CD25^+$ T_{reg} cells to tumor sites. Therefore, CCR4 plays an important role in recruitment and suppression of $CD4^+CD25^+$ T_{reg} cells at tumor sites.

Fig.3. Correlations of $CD4^+CD25^+$ T_{reg} cells with $CCR4^+$ cells and $CD4^+CD25^-$ T cells in nasopharyngeal carcinoma (NPC). In both (A) and (B), there was positive correlation between $CD4^+CD25^+T_{reg}$ cells and $CCR4^+$ in tissue-infiltrating (TI) cells and peripheral blood (PB) of patients with NPC (correlation coefficient 0.485; 0.357, respectively, $P<0.05$); in both (C) and (D), there was no correlation between $CD4^+CD25^+$ T_{reg} cells and $CCR4^+$ cells in the normal tissue and PB of the controls ($P>0.05$). (E) A negative correlation was found between $CD4^+$ $CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells in TI cells of patients with NPC (correlation coefficient -0.444, $P<0.05$). (F) No correlation was found between $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ T cells in the normal tissue of controls.

$CD4^+CD25^+$ T_{reg} cells facilitate the growth of malignant tumors, so suppression and deletion of $CD4^+CD25^+$ T_{reg} cells will strengthen the host anti-tumor response. Shimizu et al.[29] transferred a splenic cell suspension depleted of $CD4^+CD25^+$ T_{reg} cells to BALB/c athymic nude mice, before implantation with BALB/c-derived radiation leukemia cells. The tumors of the majority ($> 90\%$) of the mice were significantly suppressed and the mice survived for a longer term (> 60 days) than control mice treated with normal rat IgG (< 40 days). Sutmuller et al.[30] eliminated $CD25^+$ T cells by anti-murine CD25 antibody in C57BL/6 female mice inoculated with a B16BL6 melanoma cell line. They found that depletion of $CD25^+$ T cells enhanced tumor rejection, but this rejection was dependent on $CD4^+$ T cells and $CD8^+$ T cells. However, the anti-CD25 antibody could also reduce the anti-tumor effect of $CD8^+$ T cells and natural killer cells. Cytokine-induced killer cells depleted of $CD4^+CD25^+$ T_{reg} cells include more $CD8^+$ and $CD3^+CD56^+$cells than those with $CD4^+CD25^+$ T_{reg} cells, and they can kill tumor cells more effectively[31]. Ishida et al.[24] showed that a chimeric anti-CCR4 monoclonal antibody could deplete $CCR4^+$ cells and inhibit the migration of $CD4^+CD25^+$ T_{reg} cells in vitro, and, therefore, they proposed that a therapeutic strategy with an anti-CCR4 monoclonal antibody combined with tumor-specific cytotoxicity T lymphocyte was worth examining. $CD4^+CD25^+$ T_{reg} cells have been extensively studied and an understanding of their role has advanced, therefore, a safer and more efficient way of treating NPC will be discovered.

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Conflict of interest statement

No potential conflicts of interest were disclosed.

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