Abstract. T helper 17 (Th17), T cytotoxic 17 (Tc17) and regulatory T (Treg) cells serve important roles in a number of inflammatory and autoimmune diseases. The aim of the present study was to examine the distribution of Th17, Tc17 and Treg cells in patients with differentiated thyroid cancer (DTC) prior to as well as 7, 30 and 90 days following radioactive iodine-131 (131I) therapy, and to elucidate the probable effects of 131I therapy on Th17/Tc17 and Treg/Th17 cells in patients with DTC. A total of 40 patients with DTC (26 female; 14 male) between the ages of 24 and 72 years, as well as 13 age- and sex-matched healthy subjects were included in this study. The number of Th17, Tc17 and Treg cells in the peripheral blood of patients with DTC and of healthy Controls were assessed by flow cytometry. Th17 and Tc17 cells were counted as percentages of the number of CD4+ T cells; Treg cells were counted as a percentage of the number of CD4+T cells. In addition, the serum levels of interleukin (IL)-17, IL-23, IL-10 and transforming growth factor (TGF)-β1 were examined by ELISA. The frequencies of Th17, Tc17 and Treg cells, as well as the serum levels of IL-17, IL-23, IL-10 and TGF-β1 were significantly elevated in patients with DTC compared with healthy Controls, whereas 131I therapy significantly decreased them. In addition, elevated Th17/Tc17 ratio and reduced Treg/Th17 ratio were observed in patients with DTC at day 0, however, these ratios returned to normal levels following 131I therapy for 90 days as compared with healthy Controls. Notably, Th17/Tc17 and Treg/Th17 ratios varied following 131I therapy for 7 and 30 days. In addition, a strong positive correlation between Th17 and Tc17 cells was observed in the healthy Controls and patients with DTC that received 131I treatment for 90 days, whereas a weak positive correlation between Th17 and Treg cell levels was identified in the healthy Controls and no obvious correlation between Th17 and Treg cells was observed in all patients with DTC pre- and post-131I therapy during the entire treatment period. These data suggested a significant involvement of Th17, Tc17 and Treg cells in the pathology of DTC. Restoring the balance of these cells may contribute to the recovery of patients with DTC following 131I therapy.

Introduction

The incidence rate of thyroid tumor has increased over the past 30 years and tends to affect the young (1-3). Although thyroid carcinoma is rare and accounts for approximately 1% among human malignancies, it is the most common malignancy of the endocrine system (4,5). Among thyroid malignancies, differentiated thyroid cancer (DTC) comprises the vast majority, ~90%, and includes papillary and follicular histological types (6). DTC cells have the ability to concentrate iodine and to synthesize and release thyroid hormones. This characteristic enables the administration of radioactive iodine-131 (131I) to treat metastatic diseases and to ablate remnant thyroid tissues following thyroidectomy (7). In accordance with the European Association of Nuclear Medicine guidelines, certain patients with DTC are required to receive 131I therapy after surgery (8).

Immunological mechanisms are vital in the surveillance of malignancy and the control of cancer progression. Immune responses against DTC have long been recognized. T cells are crucial participants in the immune response. Previous studies have revealed that the imbalance between Th helper (Th1 and Th2 cells is a major immunological mechanism in the pathogenesis of DTC (9-11). Three novel T cell subgroups, including Th17 cells [CD4+/interleukin (IL)17+ T] cells, cytotoxic T (Tc17; CD8+/IL17+ T) cells and regulatory T (Treg; CD4+/CD25+/Foxp3+ T) cells have gained increasing attention, as they may serve key roles in the development of infections, allergic reactions, autoimmune diseases and tumorigenesis (12,13). Th17 cells exhibit the characteristics of CD4+ T cells and secrete ILs, including IL-17, IL-21 and IL-22. Differentiation of Th17 cells occurs in the presence of IL-6, IL-21 and tumor growth factor (TGF)-β, and their stability is sustained by IL-1β and IL-23 (14,15). Similar to Th17 cells,
Tc17 cells are characterized by the production of IL-17. Tc17 cells have been implicated in immune-associated diseases and carcinomas (16,17). Treg cells secrete TGF-β and IL-10. The release of IL-10 aids Treg cells to greatly repress host immune responses, mainly by contributing to impaired antitumorimmunity in cancer-bearing subjects (18). In previous reports, the reciprocal relationships between Th17 and either Tc17 or Treg cell numbers in tumors have been emphasized. The imbalance between Th17 and Tc17 or Treg cells has been observed in several types of tumor, such as gastric (19), ovarian (20) and prostate cancer (21). In particular, recent studies have revealed that Th17, Tc17 and Treg cell levels are also abnormal in patients with DTC (11,15,19,22); however, the specific relationships between Th17 cells and Tc17 or Treg cells, as well as the related cytokine secretion, remain unclear. 131I treatment may have an effect on the hematopoietic system of patients with DTC, particularly on the lymphocytes; however, there little is known about T cells in patients with DTC patients following 131I treatment. Therefore, the present study aimed to investigate the effects of 131I on the immune cell subsets, Th17, Tc17 and Treg cells, and the related cytokine production in patients with DTC.

Materials and methods

Patients. The study population included 40 patients with DTC (26 female; 14 male; age, 24-72 years); patient characteristics are provided in Table I. Among them, 29 (72.5%) had papillary carcinomas and 11 (27.5%) had follicular carcinomas. None of the patients had autoimmune diseases, chronic inflammation, allergic disease or other conditions that may affect the tested parameters. All patients with DTC underwent total thyroidectomy. At 4-6 weeks following surgery, patients received a low-iodine diet (<50 µg/day) for 1-2 weeks and subsequently were treated with a single dose of 3.7 GBq (100 mCi) of 131I (Chengdu Gaotong Isotope Cooperation, Chengdu, China) administered orally according to the EANM guidelines (8,12). The Control group comprised 13 age- and sex-matched healthy subjects. This study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China). All patients and Control subjects provided informed written consent prior to enrollment in the study.

Peripheral blood mononuclear cells (PBMCs) and serum preparation. Peripheral blood samples (5 ml) from the study subjects were collected in EDTA tubes following venipuncture. Blood (5 ml) was collected from Control subjects only once, whereas samples were collected from patients with DTC at day 0, as well as 7, 30 and 90 days following 131I treatment. The blood (0.5 ml per subject) was combined with RPMI-1640 medium (5 ml; Sigma-Aldrich; Merck KGaA, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin and incubated at 37°C for 72 h. The supernatant was harvested by centrifugation at 1,400 x g for 12 min at room temperature and stored at -20°C until required (11). Serum for the detection of cytokine levels was obtained by centrifugation at 2,000 x g for 15 min at 4°C and stored at -80°C.

Flow cytometric analysis of Th17, Tc17 and Treg cells. In the present study CD3+CD8+IL17+ T cells were identified as Th17 cells, CD3+CD8+IL17+ T cells as Tc17 cells and CD4+CD25+Foxp3+ T cells as Treg cells. The intracellular cytokine levels were assessed by flow cytometry to identify the cytokine-producing cells, as previously described (13). Briefly, PBMCs (2x10^7) were stimulated with phorbol myristate acetate (25 ng/ml), monensin (1.7 µg/ml) and ionomycin (1 µg/ml; all were purchased from Alexis Biochemicals; Enzo Life Sciences, Inc., Farmingdale, NY, USA). The cells were incubated with anti-CD3-phycocerythrin (PE)-Cy5 (0.6 µg/ml, 15-0038-42, eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and anti-CD8-fluorescein isothiocyanate (FITC) (5 µg/ml, MHCD0801, eBioscience; Thermo Fisher Scientific, Inc.) monoclonal antibodies at room temperature in the dark for 15 min. Following fixation with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) and permeabilization with 1% saponin (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature, cells were stained anti-IL17-PE monoclonal antibody (2.5 µg/ml, 12-7517-42, eBioscience; Thermo Fisher Scientific, Inc.) at 4°C in the dark for 15 min. Similarly, the culture supernatant was incubated with a mixture of anti-CD4-FITC monoclonal antibody (2.5 µg/ml, 11-0048-42, eBioscience; Thermo Fisher Scientific, Inc.) and anti-CD25-allophycocyanin (APC) monoclonal antibody (0.6 µg/ml, 17-0257-42, eBioscience; Thermo Fisher Scientific, Inc.) at 4°C in the dark for 30 min. Following fixation and permeabilization, the cells were stained with anti-Foxp3-PE monoclonal antibody (2.5 µg/ml, 12-4776-42 eBioscience; Thermo Fisher Scientific, Inc.) at 4°C in the dark for 45 min. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed with the BD FACSDiva software (version 6.0, BD Biosciences) (13).

ELISA. Serum was used to determine cytokine levels, including IL-10 (F01360), IL-17 (F01450), IL-23 (F01534) and TGF-β (F02750), with a quantitative sandwich-enzyme immunoassay technique, according to the manufacturer's instructions (Westang; Biotech Co., Ltd., Shanghai, China). All samples were measured in duplicate.

Table I. Clinicopathological characteristics of the 40 patients with differentiated thyroid cancer.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Category</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>≥45</td>
<td>32 (80.0)</td>
</tr>
<tr>
<td></td>
<td>&lt;45</td>
<td>8 (20.0)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>14 (35.0)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>26 (65.0)</td>
</tr>
<tr>
<td>Histology type</td>
<td>Papillary thyroid carcinoma</td>
<td>29 (72.5)</td>
</tr>
<tr>
<td></td>
<td>Follicular thyroid carcinoma</td>
<td>11 (27.5)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>Positive</td>
<td>32 (80.0)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8 (20.0)</td>
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</table>
Statistical analysis. Data are expressed as the mean ± standard deviation and median values. Differences between groups were determined using one-way analysis of the variance, followed by the Tukey’s test. Correlation analysis was evaluated by Pearson’s correlation test. All tests were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Frequencies of Th17 and Tc17 cells in DTC patients pre- and post-131I therapy. The frequency of Th17 and Tc17 cells in the peripheral blood samples from 13 Control subjects and 40 patients with DTC who had total thyroidectomy were analyzed by flow cytometry prior to and following 131I therapy. The percentage of Th17 and Tc17 cells was significantly increased in patients with DTC at day 0 compared with those in the Control group (Fig. 1A-C). At days 7, 30 and 90 following 131I therapy, the percentage of Th17 and Tc17 cells significantly decreased compared with the levels at day 0; in addition, at 90 days following 131I therapy, no significant difference in the percentage of Th17 and Tc17 cells was detected between patients with DTC and healthy subjects (Fig. 1A-C).

Treg cell frequency in patients with DTC pre- and post-131I therapy. At day 0, the percentage of Treg cells was significantly increased in patients with DTC compared with Control group (Fig. 1D and E). At 7, 30 and 90 days following therapy, the percentage of Treg cells was significantly decreased compared with treated patients at day 0; at 90 days post-therapy, no significant difference in the percentage of Treg cells was indicated between patients with DTC and healthy Control subjects (Fig. 1D and E).

Serum concentrations of IL-17, IL-23, IL-10 and TGF-β1 in patients with DTC pre- and post-131I therapy. Serum levels of IL-17, IL-23, IL-10 and TGF-β1 were measured by ELISA. IL-17 and IL-23 levels were significantly increased in patients with DTC at day 0 compared with the levels in the healthy Control patients (Fig. 2A and B), which was consistent with the elevated prevalence of Th17 and Tc17 cells in these patients. Subsequently, at 7, 30 and 90 days following 131I therapy, IL-17 and IL-23 levels were significantly decreased; no significant difference was indicated for the expression levels of IL-17 and IL-23 in patients with DTC 90 days post-therapy compared with healthy subjects (Fig. 2A and B). Similarly, the serum levels of IL-10 and TGF-β1 were significantly increased in patients with DTC at day 0 compared with the Control patients (Fig. 2C and D), which was consistent with the elevated prevalence of Treg cells in these patients. At days 7, 30 and 90 post-therapy, IL-10 and TGF-β1 levels were significantly decreased, and no significant difference was indicated for the levels of IL-10 and TGF-β1 in patients with DTC at 90 days compared with healthy patients (Fig. 2C and D).
Balances between Th17/Tc17 and Treg/Th17 cells pre- and post-\(^{131}\)I therapy. Compared with the Controls, the Th17/Tc17 ratio was significantly elevated in patients with DTC at day 0 (Fig. 3A). Furthermore, this ratio in patients with DTC was reduced following \(^{131}\)I therapy for 7 days. Subsequently, this ratio was unexpectedly increased after \(^{131}\)I therapy for 30 days. Additionally, the Th17/Tc17 ratio was not significantly different from the Control group at 90 days following \(^{131}\)I therapy (Fig. 3A). It is possible that \(^{131}\)I also resulted in radioactive damage to DTC patients. Conversely, the Treg/Th17 ratio was significantly decreased in patients with DTC compared with the Controls, and this ratio in patients with DTC was elevated following \(^{131}\)I therapy for 7 days. Subsequently, at 30 days post-therapy, this ratio was decreased. Furthermore, no significant difference was indicated in the Treg/Th17 ratio between patients with DTC and the healthy Controls following \(^{131}\)I therapy for 90 days (Fig. 3B). In addition, a strong positive correlation between Th17 and Tc17 cell levels was observed in the healthy Controls and in the DTC patients that received \(^{131}\)I treatment for 90 days (Fig. 4A); while a weak positive correlation between Th17 and Treg cell levels was identified in the healthy Controls and no obvious correlation between Th17 and Treg cells was observed in patients with DTC pre- and post-\(^{131}\)I therapy during the entire treatment period (Fig. 4B).

Discussion

A number of previous studies have demonstrated that inflammation orchestrates the tumor microenvironment. For example, inflammatory reactions in the tumor microenvironment were reported to be key components of tumor-associated immunity (23), in which immune cells serve crucial roles. Th17, Tc17 and Treg cells are three representative types of...
immune cells that are important in antitumor immunity and inflammation (19,22,24). Additionally, it was reported that higher levels of Treg cells are associated with poor survival (25). These findings suggested the probable involvement of these three cells in the development of DTC. In the present study, a significant increase in the levels of Th17, Tc17 and Treg cells was observed in patients with DTC; these results were similar to those observed in endometrial carcinoma (13), which suggested that these three effector T cells may serve important roles in the progression of DTC.

Th17 and Tc17 cells have obtained an increasing amount of attention owing to their roles in tumorigenesis; their levels, as well as IL-17 production, are upregulated in the peripheral blood of patients with cancer (19,26,27). Similarly, results from the present study revealed significant increases in the number of Th17 and Tc17 cells in the peripheral blood of patients with DTC. In addition, a strong positive correlation was identified between Th17 and Tc17 cells, and the Th17/Tc17 ratio was significantly elevated in patients with DTC, which suggests that the increase in Th17 cells may exceed that of Tc17 cells. In addition, an elevated proportion of Treg cells, and an imbalance between Treg and Th17 cells in patients with DTC were also observed. Treg cells are known to inhibit host immunity (28); it was reported that higher levels of Treg cells is associated with poor survival (25). Increased Treg frequency may result in tolerance of tumor cells from the host and may aid DTC cell survival and migration. In the present study, the Treg/Th17 ratio may result in tolerance of tumor cells from the host and may aid DTC cell survival and migration. In the present study, the Treg/Th17 ratio was significantly decreased in patients with DTC, and this ratio also fluctuated significantly following treatment with ¹³¹I (34). Results from the present study indicated a significant increase in the number of Th17, Tc17, Treg cells as well as in the production of related cytokines, including IL-17, IL-23, IL-10 and TGF-β1, in patients with DTC following ¹³¹I therapy; these results differed from those reported by Jing et al (33), perhaps owing to the differences in the number and type of inflammatory cells associated with DTC and Graves disease. In addition, the ratio of Th17/Tc17 was significantly elevated in patients with DTC at day 0 and this ratio fluctuated significantly following treatment with ¹³¹I therapy, but it returned to the similar levels detected in healthy Controls 90 days post-¹³¹I therapy. Meanwhile, Treg/Th17 ratio was significantly reduced in patients with DTC at day 0 and this ratio also fluctuated significantly following treatment with ¹³¹I therapy, but it finally returned to the similar levels detected in healthy Controls 90 days post-¹³¹I therapy. These results indicated that ¹³¹I may influence the balance of Th17/Tc17 and Treg/Th17 in patients with DTC.

In conclusion, the numbers of Th17, Tc17 and Treg cells, as well as the levels of related cytokines (IL-17, IL-23, IL-10 and TGF-β1) were significantly increased in patients with DTCs; however, at 90 days following ¹³¹I therapy, the levels decreased and returned to the similar values as detected in healthy Control patients. These data suggested that ¹³¹I may influence the balance of Th17/Tc17 and Treg/Th17 in patients with DTC.
Further studies are required to determine the significance of these findings.

References