Phenotypical Analysis of Lymphocytes with Suppressive and Regulatory Properties (Tregs) and NK Cells in the Papillary Carcinoma of Thyroid

Foteini Gogali, George Paterakis, George Z. Rassidakis, Gregory Kaltsas, Chrysoula I. Liakou, Panagiotis Gousis, Evangelos Neonakis, Menelaos N. Manoussakis, and Charis Liapi

Departments of Pharmacology (F.G., C.I.L., C.L.) and Pathophysiology (G.K., M.N.M.) and First Department of Pathology (G.Z.R.), Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece; Flow Cytometry Laboratory (G.P.), Department of Immunology, Athens Regional General Hospital G. Gennimatas, Athens, Greece; Department of Endocrine Surgery (P.G.), Metropolitan Hospital, 18547 Athens, Greece; and Department of Surgery (E.N.), Naval Hospital, 11521 Athens, Greece

Context: The immune system seems to play a key role in preventing metastasis and recurrence of thyroid cancer. T regulatory lymphocytes (Tregs) and natural killer (NK) cells play an important role in the dysfunction of the host immune system in cancer patients.

Objective: We investigated thyroid gland infiltration by Tregs and NK cells in patients with papillary thyroid cancer (PTC) and thyroid nodular goiter (TNG). The correlation between the extent of the disease and the lymphocytic infiltration of Tregs and NK cells was examined.

Design, Setting, and Participants: A total of 65 patients with PTC, 25 with TNG, and 50 healthy controls were studied. Blood and tissue samples from 28 patients with PTC and 13 with TNG and blood samples from the healthy controls were analyzed for T4 ($CD3^+CD4^+$), T8 ($CD3^+CD8^+$), NK ($CD3^-CD16^+CD56^+$), and $CD4^+CD25^+CD127^{-/low}$ Tregs by flow cytometry (FC). Tissue samples were also analyzed for $Foxp3^+$ Tregs by immunohistochemistry.

Results: Tregs showed greater infiltration in thyroid tissue of PTC patients compared with patients with TNG ($P < 0.0009$ for FC and $P < 0.0001$ for immunohistochemistry); FC analysis of blood samples showed no difference between the groups. Flow cytometry analysis showed significantly increased NK cells in PTC tissue compared with TNG tissue ($P = 0.037$), whereas blood samples showed no difference. $CD4^+$ and $CD8^+$ T cells did not differ in blood and tissue samples. Increased Tregs tissue infiltration was positively correlated with advanced disease stage ($P < 0.0026$), whereas NK infiltration was negatively correlated ($P < 0.0041$).

Conclusion: Tregs and NK cells may be important regulators of thyroid cancer progression. (***J Clin Endocrinol Metab*** **97: 1474–1482, 2012)**

Thyroid gland carcinoma constitutes approximately 1% of all human malignancies (1–3). Several studies have reported an increased incidence of thyroid cancer (TC) over the last four decades (4, 5) with approximately 10,000 newly diagnosed cases annually in the United States (1, 3). The prevalence of TC is higher in women (five to nine per 100,000) compared with men (two to four per 100,000). Papillary thyroid carcinoma (PTC) accounts for approximately 70% of all thyroid cancers, and although it has a relatively good prognosis, 50% of patients have

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Abbreviations: FC, Flow cytometry; IHC, immunohistochemistry; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; NK, natural killer; PI3K, phosphatidylinositol 3-kinase; PTC, papillary thyroid carcinoma; TC, thyroid cancer; TNG, thyroid nodular goiter; TNM, tumor-lymph nodes-metastasis; Treg, regulatory T cell.

nodal metastasis and 1% distant metastasis at initial diagnosis. Among the various mechanisms involved in cancer development, the dysfunction of the immune system is increasingly being considered (6).

The main function of human immune system is the protection from a diverse range of harmful agents, including tumor cells, while minimizing collateral tissue damage. In a malignant environment, immune system homeostasis and control of self-tolerance are significantly altered. T lymphocytes display extensive diversity in terms of phenotype and function with T helper ($CD4^+$) and cytotoxic ($CD8^+$) lymphocytes being directly involved in cell-mediated tumor destruction, whereas natural killer (NK) cells are important in tumor rejection (7).

Function and activity of these effector T cells are tightly controlled by suppressor, regulatory T cells (Tregs) that inhibit T cell proliferation and maintain tolerance to self-antigens (8, 9) by the secretion of inhibitory cytokines and/or contact-mediated inhibition (10). A malignant tumor environment directs regulatory cells to suppress immune attack, although the mechanism of suppression has not been elucidated yet. The importance of Tregs in human cancer development has only recently been explored in ovarian (11), breast (12), non-small-cell lung (13), and pancreatic cancer (14) and malignant melanoma (15).

Tregs, identified as $CD4^+CD25$ (IL-2 receptor α-chain)$^+$ T cells, specifically express Forkhead box P3, a forkhead/winged-helix transcription factor (16, 17), which is critical for the development and function of these cells (16, 18, 19); mutations in the *FoxP3* gene have been linked to the autoimmune manifestations observed in the Scurfy mouse and humans with immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (20, 21). Transcription factor Foxp3, although reported as the best and most specific marker for the identification of $CD4^+CD25^+$ Tregs, is also expressed in a smaller $Foxp3^+$ T cell population ($Foxp3^+CD25^-$ and $Foxp3^+CD8^+$) (22, 23). Thus, Tregs identification presents various difficulties because the lack of a specific cell surface marker that enables the purification of Tregs can lead to contamination by $CD25^+$ effector T cells. In addition, Foxp3, which is an intracellular marker, requires extensive cell fixation and permeabilization, and its detection by flow cytometry (FC) analysis appears to be inferior to immunohistochemistry (IHC) in Tregs separation. On the contrary, CD127, the IL-7 receptor, as a cell surface marker can be easily assessed by FC in *in vitro* functional studies. CD127 is a protein with a critical role during lymphocyte development, which remains negative/low in $Foxp3^+$ Tregs (24) in comparison with effector and memory T cells (25–27). CD127 expression inversely correlates with Foxp3, and a combination of $CD4^+CD25^+CD127^{-/low}$ results in a highly purified population of Tregs (24, 28) by FC (it is the interaction between Foxp3 and CD127 promoter that reduces the expression of CD127 in Tregs).

Because the lymphocytic infiltration is frequently observed in PTC (29, 30) and may contribute to the tumor process (31), the aim of this study was to identify Tregs, NK cells, and T4 and T8 lymphocytes in patients with PTC and thyroid nodular goiter (TNG), both in thyroid tissue and peripheral blood, and to investigate possible correlations between the extent of the disease and the lymphocytic infiltration of regulatory cells.

Patients and Methods

Patients and healthy blood donors

Sixty-five patients with PTC (53 females and 12 males) and 25 patients with TNG (20 females and five males) undergoing total thyroidectomy were included in the study. All patients were clinically and biochemically euthyroid and had undergone thyroid ultrasound. Peripheral blood and fresh tissue samples were used for the assessment of Tregs. Tissue samples from all patients were analyzed by IHC for Foxp3. Tissue and blood samples from 28 of the 65 patients with PTC and 13 of the 25 patients with TNG were analyzed by FC for $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD16^+CD56^+$, and $CD3^+CD4^+CD25^+CD127^{-/low}$ cells. Blood samples analyzed with FC were compared with blood samples from 50 healthy blood donors.

Blood and tissue samples were obtained with human ethics committee approval and informed consent from patients and healthy blood donors.

Sample preparation

Blood sample preparation

Peripheral blood samples (2 ml) from patients with PTC or TNG at the time of surgery and from healthy blood donors were collected in EDTA-anticoagulated tubes. The blood samples after collection were immediately processed for FC analysis.

Thyroid sample preparation

After thyroidectomy, thyroid tissue was kept immediately on ice till arrival to pathologist. All thyroid samples were prepared by a pathologist who selected regions of the tumor or goiter using macroscopic criteria. The selected sample of thyroid tissue was placed in PBS and was immediately sent to the FC laboratory. The remaining thyroid gland was formalin fixed, sliced horizontally at 4 μm, and totally embedded. Specimens were dehydrated, cut at 4 μm, and stained with hematoxylin and eosin. All slides were reviewed by a pathologist, verifying that cancer and goiter samples were malignant or benign, respectively.

To prepare a cell suspension for FC analysis, thyroid tissue samples were removed from PBS, dissected, and minced into small pieces. Minced thyroid tissue samples were washed in PBS, and the pellet was suspended in PBS and filtered through a 100-μm mesh filter (Filcons 100-98-S; Wipak Medical, Nastola, Finland) ready to be processed for FC analysis.

Methods

Flow cytometric analysis

Flow cytometry was performed by Cytomics FC 500 Beckman Coulter analyzer (Nyon, Switzerland), using directly conjugated monoclonal antibodies (mAb), against the following antigens: CD127 (R34.34), CD3 (UCHT1), CD25 (B1.49.9), CD4 (SFCI 12T4D11), CD8 (B9.11), CD16 (3G8), and CD56 (N901), all from Beckman Coulter. The analysis was performed in whole blood samples and thyroid-derived lymphoid cells collected by mechanical disaggregation. This method was selected due to minimal interference of enzymatic digestion in the expression of surface antigens, especially CD4 and CD25 (32). The mAb were titrated according to manufacturer's instructions. After 15 min incubation at room temperature of fresh cell suspensions or whole blood, with 10 μl labeled antibodies, the erythrocytes were lysed (BD FACS lysing solution; BD Biosciences, San Diego, CA) for 10 min and centrifuged (1800 rpm for 5 min). The supernatant fluid was discarded, and the cells were suspended in 0.5 ml PBS for analysis Two five-color combinations were used per sample: 1) CD27-FITC/CD127-PE/CD3-ECD/CD25-PC5/CD4-PC7 and 2) CD8-FITC/CD4-PE/CD3-ECD/CD16-PC5/ CD56-PC7 (where FITC is fluorescein isothiocyanate, PE is phycoerythrin, ECD is phycoerythrin-Texas red conjugated, PC5 is phycoerythrin-cyanine 5 conjugated, and PC7 is phycoerythrin-cyanine 7 conjugated).

Gating strategy. Selective gating by forward *vs.* side scatter was performed to avoid low forward scatter apoptotic cells. The gated cells were plotted by CD4 *vs.* low side scatter and subsequently coexpressed with CD3 to verify the CD3/CD4 T4 lymphocyte population. To identify subsets of CD4 lymphocytes coexpressing CD25 (Fig. 1, A1–A5) with diminished CD127 expression, we plotted CD127 *vs.* CD25 (33). Tregs were determined as $CD4^+$, $CD25^{bright}$, and $CD127^{-/low}$ lymphocytes, expressed as a percentage within T4 cells (Fig 1, B1–B5). NK cells were determined as $CD3^-CD16^+CD56^+$ expressed as percentage of total lymphocytes. A total of 100,000 events were counted in each sample.

Immunohistochemistry

Foxp3 expression in thyroid tissue sections was analyzed by immunostaining using an antihuman Foxp3 mAb (Abcam, Cambridge, UK). The 4-μm sections were cut from paraffin blocks and captured into slides. Sections were deparaffinized with xylene and rehydrated through a series of graded alcohol solutions. Afterward, antigen retrieval was performed according to the manufacturer's suggestions and endogenous peroxidase (3% H_2O_2) was quenched. Sections were incubated with primary

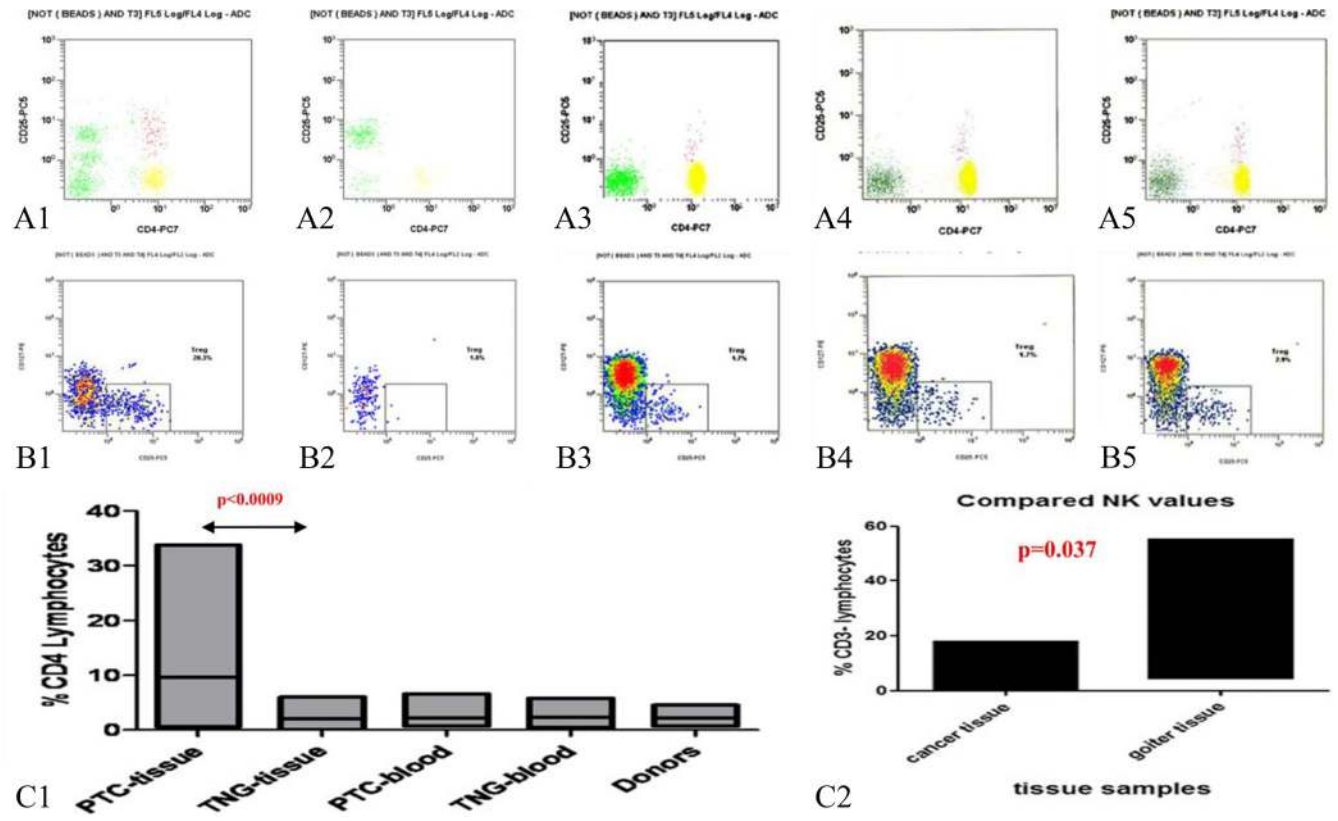

FIG. 1. Treg infiltration of thyroid tissue and peripheral blood from patients with PTC and TNG analyzed by FC. A, $CD4^+CD25^+$ lymphocytes: 1, PTC tissue; 2, TNG tissue; 3, healthy donor's blood; 4, PTC patient's blood; 5, TNG patient's blood. B, $CD4^+CD25^+CD127^{-/low}$ lymphocytes: 1, PTC tissue; 2, TNG tissue; 3, healthy donor's blood; 4, PTC patient's blood; 5, TNG patient's blood. C, Tregs percentage (FC): 1, median value and 25–75th percentile was 7.4 (4.3–11.8) in tissue and 2.1 (1.65–2.42) in blood of PTC patients, 1.4 (0.65–2.9) in tissue and 1.8 (1–2.75) in blood of TNG patients, and 1.85 (1.45–3) in healthy blood donors; 2, NK values as percentage of $CD3^-$ lymphocytes in patients with PTC and TNG tissue samples ($P = 9.937$).

anti-Foxp3 mAb overnight. The next day, slides were washed with PBS with Tween 20, and then immunodetection was performed with biotinylated antimouse secondary antibody, streptavidin-biotin complex, and diaminobenzidine chromogen (LSAB kit; Dako, Copenhagen, Denmark). Finally, slides were counterstained with hematoxylin and mounted. Lymphocyte aggregates were imaged at ×60 magnification and counted manually. Three to seven aggregates were imaged from each sample; 100–1500 lymphocytes were counted in each aggregate depending on lymphocytic infiltration. Histopathological examination and staging of the tumor were performed by experienced pathologists.

Data analysis

Statistical comparisons were carried out with Mann-Whitney *t* test and one-way ANOVA. The correlation between FC and IHC results was assessed by Spearman correlation coefficient (r). Analysis was performed using GraphPad version 5.0b software. Significance level was set at $P < 0.05$.

Results

The median age of the 65 PTC patients was 40 yr (range 17–68), and the median age of the TNG patients was 49 yr (range 17–74). Tumor stage and size, intrathyroidal or lymph node metastasis, and associated disease are shown in Table 1.

FC analysis

T4, T8, CD4/CD8 ratio, and NK cell screening

Blood and tissue samples of patients with PTC and TNG and blood samples of healthy blood donors were analyzed for $CD3^+CD4^+$ (T4), $CD3^+CD8^+$ (T3), and $CD3^-CD16^+CD56^+$ (NK) cells.

TABLE 1. Clinical features of patients with PTC and TNG

	PTC	TNG
No. of patients	65	25
Age (yr)	40	49
Range	17–68	17–74
Sex (female/male)	53/12	20/5
Stage		
I	29	
II	5	
III	14	
IVA	17	
IVB		
Tumor size (cm)	1.3	
Range	0.7–9	
Intrathyroidal metastasis (n)	30+	
Lymph node metastasis (n)	16+	
Related disease (n)		
Hashimoto's thyroiditis	21	
TNG	14	25
TNG plus Hashimoto's thyroiditis	13	2

Mean values of $CD3^+CD4^+$ lymphocytes and $CD3^+CD8^+$ lymphocytes (expressed as percentage of total lymphocytes) in tissues from PTC and TNG patients and in blood from PTC and TNG patients and the healthy blood donors are shown in Table 2; statistical analysis showed no difference for $CD3^+CD4^+$ (T4) and $CD3^+CD8^+$ populations among the groups either in tissue or in blood. Blood ratio of CD4/CD8 was significantly lower in PTC patients (1.87 ± 1.07) compared with TNG patients (2.7 ± 1.0) (*t* test, $P < 0.002$) and with healthy donors (2.3 ± 1.1) (*t* test, $P < 0.03$); no significant difference was noted between TNG patients and healthy donors ($P > 0.27$).

NK cell values in blood from PTC and TNG patients and healthy donors showed no significant difference (Table 2). A significantly greater NK infiltration in PTC tissue samples compared with TNG samples was shown ($P = 0.037$) (Table 2 and Fig. 1C2). We found no statistical difference of NK cell infiltration between PTC patients and patients with PTC in coexistence with Hashimoto's thyroiditis ($P = 0.68$).

Treg screening

$CD3^+CD4^+CD25^{bright}$ lymphocytes analysis

Thyroid tissue samples from 28 PTC and 13 TNG patients were screened by FC for $CD4^+CD25^{bright}$ T cells as well (Fig. 1, A1 and A2). The mean frequency ± SD of $CD3^+CD4^+CD25^{bright}$ T cells in TNG tissue was significantly lower than in PTC tissue [5.1 ± 3.25 *vs.* 8.9 ± 1.89%, respectively ($P < 0.0005$)]. Blood samples from 28 PTC and 13 TNG patients and 50 healthy donors were screened by FC for the presence of $CD3^+CD4^+CD25^{bright}$

TABLE 2. Mean values ± SD of $CD3^+CD4^+$ lymphocytes, $CD3^+CD8^+$ lymphocytes, and $CD3^-CD16^+CD56^+$ cells in PTC and NTG tissues and PTC, TNG, and healthy donors' blood

	$CD3^+CD4^+$ lymph	$CD3^+CD8^+$ lymph	$CD3^-CD16^+$ $CD56^+$ NK
Tissue			
PTC	38.17 ± 14.8[a]	36.3 ± 14.8[c]	5.68 ± 4.6[e]
TNG	32.0 ± 14.0[b]	29.6 ± 16.0[d]	2.7 ± 1.9[f]
Blood			
PTC	57 ± 12.5[a]	32.1 ± 10.5[d]	29.1 ± 18.5[g]
TNG	63.4 ± 11.0[b]	26.4 ± 6.9[e]	23.7 ± 17.0[h]
Healthy donor	62.5 ± 10.0[c]	31.4 ± 11.0[f]	20.0 ± 10.0[i]

Values are expressed as percentage of lymphocytes (mean ± SD). Comparisons were made using Mann-Whitney *t* test.

[a–i] Statistical significance between the groups is as follows: PTC tissue and TNG tissue (T4 *P* value *a*/*b* = 0.23, T8 *P* value *c*/*d* = 0.158, NK *P* value *e*/*f* = 0.037); PTC blood, TNG blood, and healthy donor's blood (T4 *P* values *a*/*b* = 0.122, *a*/*c* = 0.074, *b*/*c* = 0.44; T8 *P* values *d*/*e* = 0.071, *d*/*f* = 0.3, *e*/*f* = 0.55; NK *P* values: *g*/*h* = 0.38, *g*/*i* = 0.15, *h*/*i* = 0.69).

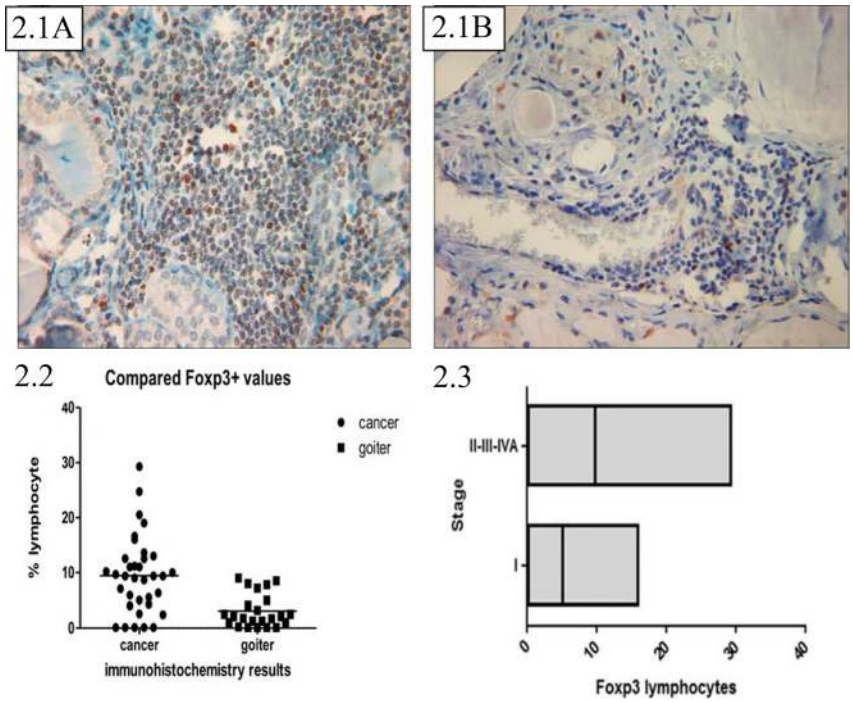

FIG. 2. Treg infiltration of thyroid tissue from patients with PTC and TNG analyzed by IHC. 1, Infiltration of Foxp3 Tregs in PTC (2.1A) and TNG (2.1B) patients, respectively. 2, Comparison of Tregs in cancer and goiter tissue by IHC ($P < 0.0001$). Median range and 25–75th percentile was 9.4 (1.2–12.5) in PTC patients and 1.5 (0.2–2.5) in TNG patients. 3, Expression of Foxp3 lymphocytes in relation to stage I and II, III, and IVA of PTC ($P < 0.0026$). Median range and 25–75th percentile was 3.8 (0.1–8.9) for stage I and 7.9 (5.9–11.8) for advanced stages (II, III, and IVA).

T cells (Fig. 1, A3–A5). In peripheral blood, the mean frequency ± SD of $CD3^+CD4^+$ T cells that were also $CD25^{bright}$ was 10.8 ± 0.5% from normal donors, 9.3 ± 1.2% PTC patients, and 9.7 ± 0.4% in TNG patients. No significant difference was found in blood values among the groups (ANOVA).

$CD3^+CD4^+CD25^+CD127^{-/low}$ lymphocytes analysis

By gating on the $CD4^+C25^{bright}$ T cells, we analyzed for $CD127^{-/low}$ Tregs. The values of T regulatory cells were measured as percentage of the T4 lymphocytes. In Fig. 1, subpopulations of Tregs in thyroid tissue from PTC patients (Fig. 1B1), thyroid tissue from TNG patients (Fig. 1B2), peripheral blood of healthy blood donors (Fig. 1B3), peripheral blood from PTC patients (Fig. 1B4), and peripheral blood from TNG patients (Fig. 1B5) are shown. The median values and the 25–75th percentiles are shown in Fig. 1C1. Tregs were significantly lower in blood samples compared with tissue samples but presented similar levels in peripheral blood of PTC and TNG patients and normal donors (ANOVA). The percentage of Tregs in PTC tissue was greater compared with TNG tissue (*t* test) ($P < 0.0009$) (Fig. 1C1).

Immunohistochemical analysis

$Foxp3^+$ lymphocytes

Immunohistochemical staining of thyroid tissue sections from the 54 of 65 patients with PTC and all TNG patients (25) were used to determine the presence of Foxp3-positive cells in thyroid. Lymphocytic infiltration of Tregs in PTC and TNG tissue samples is presented in Fig. 2.1, A and B). The values of $Foxp3^+$ lymphocytes were measured as a percentage of the total number of lymphocytes present in lymphocytic aggregates. The median value and the 25–75th percentile are shown in Fig. 2.2. Patients with TNG presented fewer Foxp3-positive cells in comparison with patients with PTC (*t* test) ($P < 0.0001$) (Fig. 2.2).

Correlation of Tregs with clinical features and associated disease

Tregs analysis in PTC, either by FC or by IHC, did not show any statistical difference regarding age and sex of the patients ($P > 0.05$). Foxp3 infiltration appears to be greater in patients with lymph nodes metastasis ($P < 0.0067$) and intrathyroidal metastasis ($P < 0.037$) by immunohistochemical investigation.

No significant difference in Tregs infiltration was found in patients with PTC compared with patients with coexistence of Hashimoto's thyroiditis. The results were confirmed by both FC ($P < 0.339$) and IHC ($P < 0.923$) (Fig. 3.2). In patients with PTC associated with Hashimoto's disease, Tregs percentage showed no statistical difference among the tumor-lymph nodes-metastasis (TNM) stages (stage I *vs.* stages II, III, and IVA; $P > 0.3$). No significant difference was shown among patients with PTC and patients with PTC and concomitant TNG by FC ($P = 0.483$) and/or IHC ($P = 0.198$).

$CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD16^+CD56^+$ NK cells, $CD4^+/CD8^+$ ratio, and $Foxp3^+$ Tregs and TNM stage

In relation to tumor stage, there was no significant difference neither in $CD3^+CD4^+$ or in $CD3^+CD8^+$ lymphocytes (FC) between stage I and advanced stages (II, III, and IVA) (*t* test, $P > 0.5$). NK cells (FC) were inversely correlated to PTC stage; NK infiltration was greater in stage I compared with advanced stages ($P < 0.0041$). Means ± SD of NK cells at each TNM stage are shown in Fig. 3.1.

1	Stage I (mean±SD)	Stage II (mean±SD)	Stage III (mean±SD)	Stage IVA (mean±SD)
Tregs (IHC)	5.05±4.7	4.6±3.8	12.90±7.3	8.26±3.9
Tregs (FC)	6.47±3.7	3.9±0.0	8.12±6.8	14.5±9.1
NK cells (FC)	7.94±5.2	3.5±0.0	3.32±2.8	2.72±2.1
T4/T8 ratio (FC)	1.30±0.8	1.3±0.0	1.17±0.3	0.79±0.3

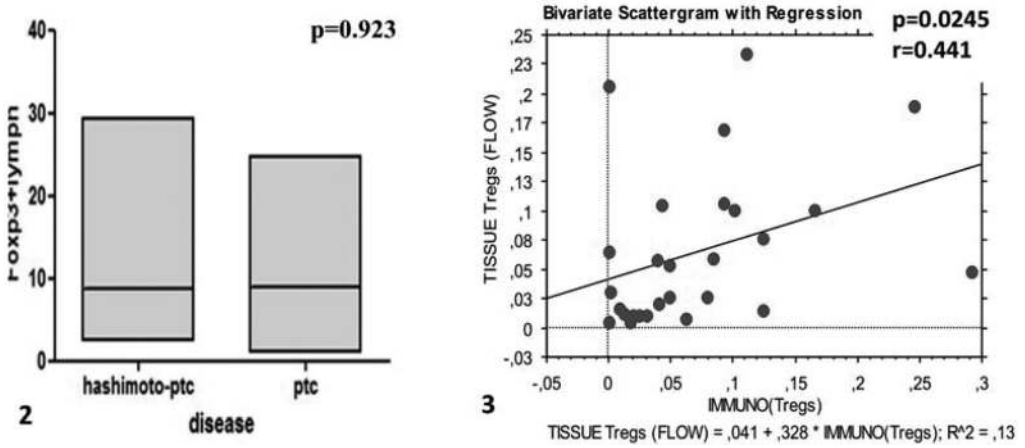

FIG. 3. Treg, NK, and T4/T8 from tissue samples of patients with PTC in correlation with disease tumor stage I. 1, Treg (IHC), NK (FC), and T4/T8 ratio (FC) are expressed as mean ± SD percentage of the total number of lymphocytes. Treg detected by FC are expressed as mean ± SD percentage of T4 lymphocytes. 2, Correlation of $Foxp3^+$ lymphocytes in patients with PTC and PTC and Hashimoto's disease by IHC. 3, FC and IHC Spearman rank correlation for tissue samples of PTC patients (*P* and r values).

$CD4^+/CD8^+$ ratio was greater in primary stage (I) compared with advanced stages (III and IVA) (Fig. 3.1).

Tregs infiltration (mean ± SD) at each TNM stage is shown in Fig. 3.1. Tregs were positively correlated to the stage of the disease, *i.e.* lower infiltration in stage I compared with stages II, III, and IVA by FC ($P < 0.047$) and by IHC ($P < 0.0026$), respectively (Fig. 2.3).

Correlation of FC and IHC for Tregs in tissue samples

For the investigation of the Tregs, two different methods (FC and IHC) were used to identify two different markers, CD127 and Foxp3 (CD127, the receptor of IL-7, is a T cell surface marker, whereas Foxp3 is an intracellular marker). To elucidate whether the results of FC and IHC matched, we used the Spearman rank correlation. Statistical analysis showed that the results of both methods were comparable ($P < 0.0245$; $r = 0.441$) (Fig. 3.3).

Discussion

In the present study, T cell subpopulations, Tregs, T4, T8, and NK cells, are studied in blood and tissue samples of patients with PTC and TNG. We demonstrated increased infiltration of Tregs and NK cells in tissue samples of PTC compared with TNG patients. Tregs infiltration was positively correlated to tumor stage, whereas NK cells were negatively correlated. Although PTC is the most important endocrine malignancy, constituting more than 70% of thyroid cancers, the data on Tregs infiltration in thyroid tissue are limited (34). To our knowledge, no previous study compared blood and tissue samples from TNG and PTC patients. In accordance with French *et al.* (34), our study confirmed the infiltration of PTC tissue with Tregs proportional to disease stage with or without lymph node metastasis (*i.e.* greater stage) or intrathyroidal metastasis (*i.e.* greater tumor load). Because Tregs suppress immune response toward tumors, their increased percentage in advanced stages leads to proliferation of the disease, a fact demonstrated by several investigators. The percentage of Tregs in TNG tissue was significantly lower compared with PTC tissue, and these results are comparable to the results shown by Müller *et al.* (35) between goiter and medullary thyroid carcinoma. The above data suggest that the malignant microenvironment of thyroid carcinoma, contrary to the benign, either activates the already existing Tregs or enhances their thyroid infiltration. The regulatory function of Tregs has been extensively demonstrated in several previous *in vitro* studies. In this context, we have not directly addressed the actual regulatory potential of

the above lymphocyte subset in functional assays. Furthermore, we have no ready explanation on the precise nature of Treg infiltration that occurs in neoplastic thyroid tissues in PTC patients. Recent evidence suggests that $Foxp3^+$ expression by T lymphocytes can be regulated by known signaling pathways. Sauer *et al.* (36) have shown that inibition of phosphatidylinositol 3-kinase (PI3K), Akt, or mammalian target of rapamycin (mTOR) conferred Foxp3 expression and Treg-like gene expression profiles. Similarly, constitutive PI3K/Akt/mTOR activity antagonized Foxp3 induction, suggesting that the PI3K/Akt/mTOR signaling regulates Foxp3 expression (36).

Hashimoto's thyroiditis, a common autoimmune disease, may coexist with PTC. Although immune system activation is important in Hashimoto's thyroiditis, Tregs, which are abundant in inflamed thyroid tissue, do not seem to affect effectively the autoimmune response and the inflammatory evolution (37). In our study, in contrast to French *et al.* (34), no difference in Treg infiltration between PTC and PTC-Hashimoto patients was found, suggesting that the immune response in Hashimoto's thyroiditis was not capable of suppressing Tregs and tumor proliferation. Additionally, there was no correlation between Tregs infiltration in PTC-Hashimoto patients and disease stage; neither did patients with concomitant Hashimoto's disease exhibit a better prognosis. Furthermore, the presence of goiter did not seem to alter the percentage of infiltrating Tregs in PTC patients.

In PTC patients, a mixture of lymphocytes, T4 and T8, including NK cells, has been found, and their presence has been correlated with limited disease recurrence (38). NK cells seem to contribute to tumor development, with important implications in several human malignancies; their reduced number in tumor tissues correlates with poor prognosis and high risk for development of regional and distance metastases (39). Our results, in accordance with Modi *et al.* (38), confirm the presence of NK cells in tissue samples of PTC patients and their participation in tumor development as representatives of the innate immune response and reveal an increased percentage toward TNG. We also demonstrated an inverse correlation of NK cells infiltration with tumor stage, with decreased NK cells infiltration in advanced stages of the disease compared with infiltration in stage I, and no correlation of NK cells in patients with PTC and patients with coexistence of PTC and Hashimoto's disease. This finding highlights the weakness of the innate immune response toward cancer spread in advanced stages. The inverse correlation between Tregs and NK cells with tumor stage confirms the hypothesis that Tregs control NK cells activation and suppresses their immune response (40). According to Ghiringhelli *et al.* (40), the membrane-bound TGF-β of Tregs is responsible for the inhibitory function of Tregs on NK cells cytotoxicity and interferon-γ secretion, resulting in failure of tumor suppression, decreased infiltration, and limited action in advanced stages.

Comparing the percentage of Tregs in blood samples of patients with PTC and TNG and healthy blood donors, except for a slightly increasing tendency in PTC samples, no significant difference was found. This is in contrast to related studies investigating the expression of Tregs in blood samples in ovarian cancer (11), non-small cell lung cancer (13), and even in medullary thyroid cancer (35) in which the percentage of Tregs was significantly higher in the patients compared with healthy blood donors. None of the NK cells populations in peripheral blood presented any differences among the groups.

In our study, T4 and T8 lymphocytes presented similar percentages in tissue and blood of PTC and TNG patients. We demonstrated the inverse correlation between CD4/CD8 ratio and tumor stage, with CD4/CD8 to be double in stage I compared with stage IVA; additionally, CD4/CD8 ratio was lower in blood of PTC patients compared with TNG patients and healthy donors. Our results are in accordance with Shah *et al.* (41), who demonstrated that the ratio of CD4/CD8 correlates with stage and prognosis of the disease, with higher CD4/CD8 ratio related to better prognosis and lower ratio to advanced stages with worse prognosis.

Tregs are part of the suppressor mechanism toward immune response in the tumor microenvironment. Therefore, reducing Tregs function in cancer patients could give an alternative therapeutic pathway. Based on Ghiringelli *et al.* (40), this reduction will lead to stimulation of the innate immunity and increase of NK cells effector functions. Treg-depleting clinical trials have focused on this purpose; administration of pharmaceutical agents and mAb result in reduced numbers of peripheral Tregs (42) and inhibition of their suppressive activity in cancer patients, respectively (43). It is probable that the combination, in addition to other therapeutic approaches (*i.e.* NK cell-based immunotherapy) that focus on the pathways of molecules involved in Tregs trafficking, can offer enhanced antitumor immunity.

In summary, the infiltration of Tregs in tissue of patients with PTC was significantly increased compared with patients with TNG and correlated with disease severity and stage. Tregs and NK cells are characterized from an inversely proportional relation and possess a decisive role in tumor immunity and evolution. Peripheral blood values of Tregs and T4, T8, and NK cells slightly affect PTC. CD4/CD8 ratio could predict disease prognosis. Although current therapy is quite effective for most patients, a considerable number of PTC patients develop distant metas-

tasis and recurrent disease. In these patients, novel tumor immunotherapy targeting Tregs or other suppressive molecules could be considered. Because thyroid epithelial cancers appear to be immunogenic tumors, the development of immunotherapies that enhance immune response against tumor are indispensable.

Acknowledgments

Address all correspondence and requests for reprints to: Charis Liapi, M.D., Ph.D., Endocrinologist, Associate Professor of Pharmacology, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece. E-mail: cliapi@med.uoa.gr.

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