Original paper

Histological perspective on the effects of tumor-associated macrophages in the tumor microenvironment surrounding papillary thyroid carcinoma

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Tumor-associated macrophages (TAMs) are one of the most noticeable elements of the tumor microenvironment. The present study investigated the relationships between the density of CD163 immunolabeled M2-like TAMs with other histological properties of the tumor microenvironment and clinipathological features in 90 patients with papillary thyroid carcinomas (PTC). The percentage of TAMs was higher in tumors with significant lymphocytic tumor response ($p = 0.020$), in tumors with a significant degree of stromal tumor response ($p = 0.014$), those with infiltrative tumor borders ($p = 0.029$), in conventional variant papillary carcinoma ($p = 0.032$), and in patients with autoantibodies for thyroid peroxidase ($p = 0.014$). The tumors associated with lymphocytic thyroiditis had lower numbers of TAMs ($p = 0.027$). In conclusion, for the first time, the present study attempts to establish a full assessment of interactions of CD163 expressing M2-like TAMs with the triad of primary tumor- tumor microenvironment- tumor behavior and above all, with markers of autoimmunity. Thus, these alternatively polarized macrophages may act in tumor progression and dissemination according to their various products, which may be ordered by tumor cells or neighboring immune cells. The molecular studies may reveal their roles in various tumors and may improve the therapy strategies targeting TAMs in various malignant tumors, including PTCs.

Key words: tumor associated macrophages, papillary thyroid carcinoma, tumor microenvironment.

Introduction

Papillary thyroid carcinoma (PTC) is the most common malignancy occurring in endocrine organs. Because the incidence of these tumors has been increasing during the last two decades due to diagnostic procedures and because most of these tumors exhibit an indolent clinical course, clinical management of these tumors has become one of the most important subjects in endocrinological oncology [1, 2]. In the context of this issue, the properties of the tumor microenvironment and the prognostic features of the primary tumor have been the themes of several studies [1, 3, 4, 5, 6, 7]. In 1863, Virchow proposed

a theory about relationship between the immune response and cancer for the first time [8]. Beginning in the 1980s, studies about the tumor microenvironment and immune response to tumors have revealed that not only the features of the primary tumor but also tumor-related events may affect the prognosis [7, 9, 10, 11, 12, 13]. On the other hand, immunotherapy models against thyroid carcinoma have been attempted since as early as 1975 [8]. Pre-clinical data about therapies targeting macrophages reveal that ablation or de-polarization of macrophages may have benefits in cancer therapy and may also enhance the effects of chemotherapy or radiotherapy [14, 15].

The tumor microenvironment is primarily composed of fibroblasts, vascular endothelial cells, lymphocytes, neutrophils, mast cells, and macrophages inside or around the tumor. Macrophages are one of the most noticeable elements of the tumor microenvironment, and they are monocytes that transform through two subtypes of macrophages, namely M1 type (classically activated) and M2-like type (M2 type, promoting tumorigenesis, alternatively activated) by the influence of chemoattractants [15, 16, 17]. M2-like macrophages, particularly the M2d phenotype, expressing high levels of transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α), interleukin 10 (IL-10), monocyte colony stimulating factor (M-CSF), vascular endothelial growth factors (VEGFs), matrix metalloproteinases (MMPs), and chemokines – including chemokine (C-C motif), ligand (CCL) 17, and CCL22 – have been defined as tumor-associated macrophages (TAMs) regardless of the argument about their origins (in the bone marrow, yolk sac, or via extramedullary hematopoiesis) [14, 16]. Several reports about TAMs in thyroid carcinomas have revealed that M2-like types of TAMs can be identified by CD68 (also M1 type), CD163, CD206, and CD204 immunoexpression and also that higher numbers or percentages of these cells are associated with higher histological grade (anaplastic carcinoma, poorly differentiated carcinoma), lymph node metastasis, and advanced tumor stage [1, 11, 16, 18, 19]. Recent reports have shown that TAMs promote tumorigenesis by inducing neovascularization and invasion/metastasis and by suppressing antitumor immunity via cell-to-cell interactions [20, 21, 22]. In addition, it has also been reported that TAMs may comprise both M1 and M2-like subtypes of macrophages localized in different parts of the tumor and that the ratio of M2-like to M1 macrophages may predict poorer prognosis in pancreatic cancer [23].

Despite the presence of several previous reports on the effects of TAMs on some of the clinicopathological features in thyroid tumors and the lymphocytic tumor response, for the first time, the present study attempts to establish a full assessment of interactions of CD163 expressing M2-like TAMs with the triad of primary tumor–tumor microenvironment–tumor behavior and, above all, with markers of autoimmunity. In this view, the relationships between the density/number of M2-like TAMs with patterns of tumor border, intrathyroidal dissemination of the tumor (intraglandular dissemination or multifocality), other histological properties of the tumor microenvironment [principally with lymphocytic tumor response (LTR), concomitant lymphocytic thyroiditis (LT), stromal tumor response (STR)], markers of autoimmunity and clinicopathological features (tumor size, extrathyroidal extension, lymph node metastasis) in papillary thyroid carcinoma are investigated via three distinct methods.

Material and methods

Patient selection

The medical reports of patients who presented to the hospital's pathology department between August, 2007 and August, 2014 were reviewed. The study protocol was approved by the local ethics committee (TUTF-BAEK 2014/208). In all, 90 patients who had been diagnosed with PTC and who had undergone total thyroidectomy with central/cervical lymph node dissection were included in the study. Patient data (age at the time of diagnosis, sex, and serum levels of thyroid autoantibodies) and data on the number and size of tumors were obtained from the hospital database. Hematoxylin and eosin-stained (HE) slides of the specimens were re-evaluated by one pathologist (N.C.) The histopathological features were evaluated in the largest tumor focus in cases in which multifocal tumors were present. The clinical features included age at the time of diagnosis (< 45 and ≥ 45 years), sex (male or female), serum levels of thyroglobulin autoantibodies (anti-TG) (positive, negative), and serum levels of thyroid peroxidase autoantibodies (anti-TPO) (positive, negative).

Definitions of clinicopathological criteria

Tumor border (TB), lateral tubular growth (LTG) and intraglandular dissemination (IGD) were interpreted according to Jung *et al*. [24]. Tumors exhibiting irregular spiculated margin or more than 3 penetrating structures including tumor follicles into adjacent thyroid were accepted as infiltrative tumors. Regardless of presence of a fibrous tumor capsule, well-circumscribed tumors without infiltration into surrounding thyroid tissue were defined as smooth bordered tumors. Tumors encircled by a fibrous capsule were regarded as encapsulated. Lateral tubular growth was defined as an elongated tubular follicle (not more than one follicle) extending perpendicular to the invasive tumor border. Intraglandular dissemination was regarded as clusters of tumor follicles mea-

suring less than 1 mm and located at least 500 μm away from the primary tumor. Stromal tumor response (STR) was regarded as the degree of the tumor surrounding/infiltrating fibrosis, and lymphocytic tumor response (LTR) was regarded as the degree of the tumor surrounding/infiltrating lymphocytic infiltration regardless of concomitant lymphocytic thyroiditis in non-neoplastic thyroid. Cases exhibiting no metastatic foci in lymph nodes by evaluation of two serial slides were classified as "lymph node negative".

Clinical features considered for statistical analyses were age at the time of diagnosis (< 45 years and ≥ 45 years) and gender (male and female). Histopathological features considered for statistical analyses were histological variant [conventional variant PTC (CVPTC), follicular variant PTC (FVPTC)], tumor size (≤ 10 mm, > 10 mm), tumor focality (unifocal, multifocal), extrathyroidal extension (ETE) [according to staging system proposed by American Joint Committee on Cancer [25] (absent, present [minimally extension, widely extension]), tumor border (smooth bordered/encapsulated, infiltrative), lateral tubular growth (absent, present), intraglandular dissemination (absent, present), stromal tumor response (absent, present [mild, severe]), lymphocytic tumor response (as three grades according the severity of infiltration and regardless of concomitant LT; absent, present [mild, severe]), tumor surrounding LT (absent, present), lymphovascular invasion (LVI) (absent, present), and lymph node metastasis (LNM) (absent, present).

Immunohistochemistry

Immunostaining for the CD163 antibody was performed with a fully automated immunohistochemistry and *in situ* hybridization (IHC/ISH) staining machine (Ventana BenchMark XT, USA). The sections were deparaffinized in 3 changes of xylene and washed in 96%, 80% and 70% ethyl alcohol. Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide (H_2O_2) for 10 minutes. Antigen retrieval was performed by immersing the slides in Tris-EDTA buffer, pH 9.0 and incubating at 95-97°C in a water bath for 25 minutes. The primary antibody at the indicated dilutions was used for IHC: lyophilized mouse monoclonal antibody for CD163 antigen (NCL-CD163 1 : 200; Novocastra, United Kingdom). *Ultra*View Universal DAB Detection Kit (Ventana) was used. The slides were counterstained with hematoxylin. A single pathologist (N.C.), who was blinded to the clinical assessments of each case, scored the cases by counting the numbers of CD163 positive nontumoral cells and converting these numbers into percentages within the nucleated cells in five independent fields under 400× magnification (with a Nikon Eclipse 80i microscope). Initially, a two-tiered scoring schema for density of TAMs was used. Low density was regarded as ≤ 25% immunostaining of the nucleated nontumoral cells (Fig. 1A, B), whereas high density was 26-100% immunostaining of the nucleated nontumoral cells (Fig. 1C, D) [18].

In addition, a four-tiered scoring schema was used in order to determine whether the increasing density of these cells might effect the behavior of the tumor. This schema was designed by scoring the percentages as follows: score 1: < 25% positive nontumoral nucleated cells in the targeted field, score 2: 26-50% positive nontumoral nucleated cells in the targeted field, score 3: 51-75% positive nontumoral nucleated cells in the targeted field, and score 4: 76-100% positive nontumoral nucleated cells in the targeted field.

Statistical analysis

The statistical analysis was carried out using SPSS v20.0 (IBM Corp, Released 2011, IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY: IBM Corp.). Appropriate chi-square tests (Pearson, Yates, or Fisher) were used to compare possible differences between the percentage of TAMs in tumor tissue and clinicopathological features. The Spearman correlation test was used to examine relationships between the number of TAMs and clinicopathological features. The effects of histopathological and clinicopathological features on density of TAMs were examined using a logistic regression analysis. The odds ratio and the 95% confidence interval of the histopathological and clinicopathological features were calculated. A p value of <0.05 was considered statistically significant.

Results

Clinicopathological features of patients in the study group

The mean age of the patients was 48.6 ±13.2 years. Of the 90 patients, 75 (83.3%) were female and 15 (16.7%) were male. Anti-TG was present in the serum of 15 (25.4%) of 59 patients, whereas anti-TPO was present in the serum of 15 (25.9%) of 58 patients who had preoperative serum autoantibody analysis. Lymph node metastasis was present in 20 (22.2%) of the patients, whereas 70 (77.8%) of the patients exhibited no lymph node metastasis. Among all cases, the histological variant of the tumor was CVPTC in 65 (72.2%) of the patients and FVPTC in 25 (27.8%) of the patients. When the patients were grouped according to tumor size, 60 (66.7%) had microcarcinoma, whereas the tumor size was larger than 10 mm in 30 (33.3%) of the cases. Multifocality of the tumor was present in 55 (61.1%) of the cases, whereas an ETE was present in 29 (32.2%) of the patients. In 46 (51.1%) patients, the pattern of the tumor border was infiltrative. LTG was present in 65 (72.2%) cases, and

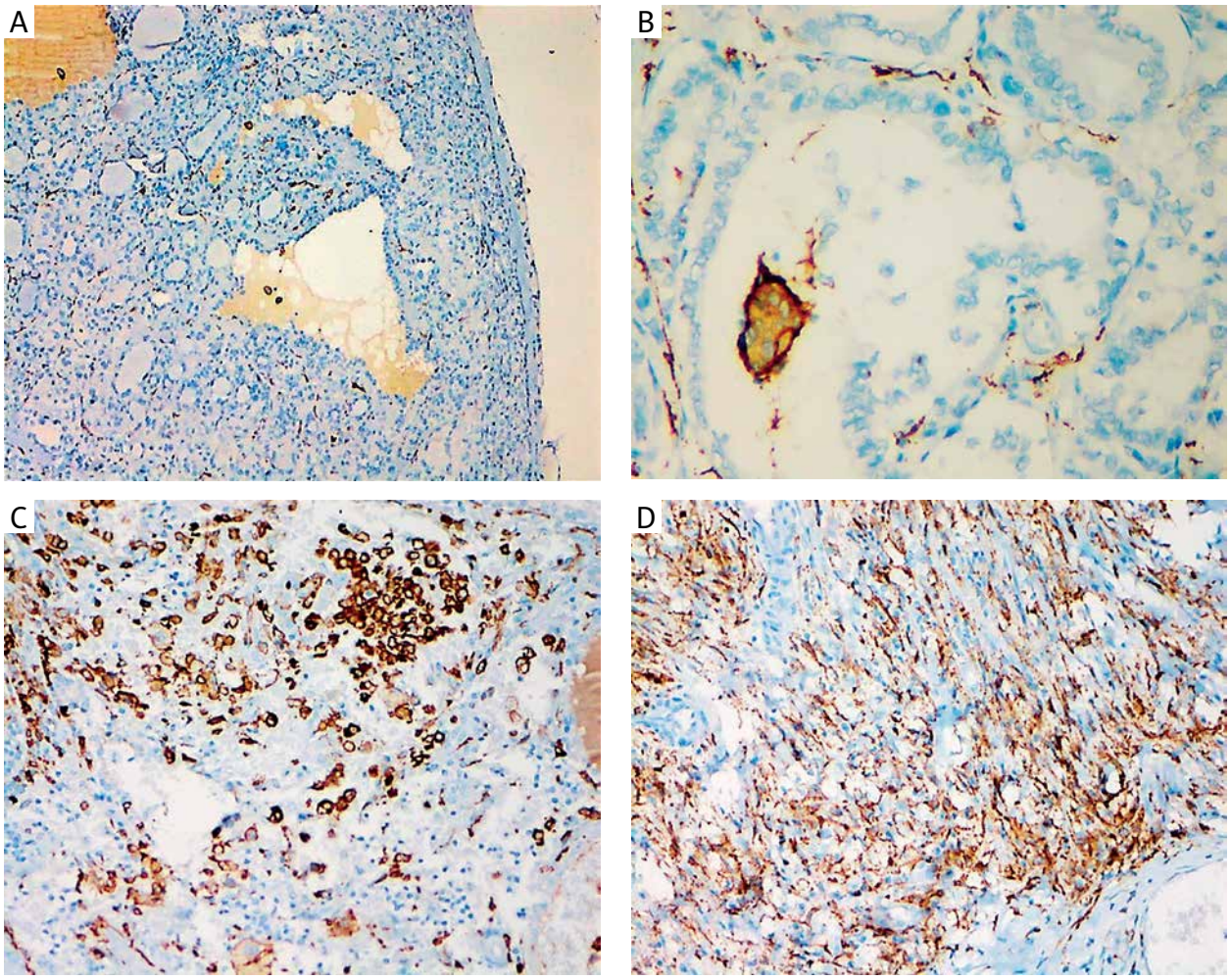

Fig. 1. Low density (A, B) and high density (C, D) of CD163 positive TAMs in PTC (A – immunoperoxidase, original magnification 40×; B-D – immunoperoxidase, original magnification 100×)

the tumor revealed IGD in 28 (31.1%) patients. LTR (mild and significant) was observed in 54 (60.0%) of the cases, STR (mild and significant) was seen in 84 (93.3%) of the cases, and LT accompanied by PTC was seen in 39 (43.3%) of the cases.

Correlations of clinicopathological features and the number of CD163 expressing macrophages in the tumor

The correlations between the density of TAMs and histological and prognostic characteristics were evaluated (Table I). The density of TAMs was significantly associated with the presence and the degree of LTR ($p = 0.033$, correlation coefficient $= 0.255$). We did not observe any significant association between the density of TAMs and other tumor features, such as age, gender, tumor size, TB, LTG, IGD, LVI, tumor focality, ETE, or LNM.

Correlations between the clinicopathological features significantly associated with the density of TAMs and other clinicopathological features were analyzed (Table I). These analyses revealed statistically significant correlations between infiltrative tumor border and the presence of LTG, IGD, ETE, a significant degree of LTR, a significant degree of STR, and conventional variant histology. A significant degree of LTR was statistically correlated with an increasing number of TAMs, infiltrative tumor border, presence of LTG, a significant degree of STR, and absence of LT. A significant degree of STR was correlated with infiltrative tumor border, presence of LTG, ETE, IGD, a significant degree of LTR, and conventional variant histology. The presence of LT was inversely correlated with the presence of LVI and LNM, a significant degree of LTR, and larger tumor size. CVPTC was correlated with infiltrative tumor border, presence of LTG, LVI, ETE, and LNM, and a significant degree of STR.

Comparisons of clinicopathological/histopathological features and the density of CD163 expressing macrophages using a two-tiered scoring schema

As a second step, TAM densities were scored with a two-tiered schema as low or high. On the other hand, STR and LTR were regarded as absent or present, and

Table I. Correlations of clinicopathological features and the number of CD163 expressing macrophages in the tumor

Spearman's rho		Tumor Border	Lateral tubular growth	Intraglandular diseminaton	Lymphocytic tumor response	Stromal tumor response	Lymphocytic thyroiditis	Lymphovascular invasion	Histological variant	Tumor focality	Tumor size	Extrathyroidal extension	Lymph node metastasis
TAMs (number)	Correlation Coefficient	0.158	0.116	0.041	0.225*	0.158	–0.161	0.132	0.050	0.094	–0.183	–0.080	0.145
	Sig. (2-tailed)	0.137	0.276	0.703	0.033	0.136	0.129	0.216	0.642	0.381	0.084	0.455	0.174

serum levels of anti-TG and anti-TPO were included in the comparisons. The density of TAMs was compared with the clinicopathological and histological features and summarized in Table II. According to univariate analysis based on the two-tiered scoring schema, density of TAMs was significantly associated with LTR, TB, and positivity of anti-TPO. The percentage of TAMs was higher in tumors associated with LTR ($p = 0.045$) (Fig. 1A, B), in tumors with infiltrative tumor borders ($p = 0.029$) (Fig. 1C, D), and in patients with autoantibodies for thyroid peroxidase ($p = 0.014$). Multivariate analysis revealed that the presence of LTR in a tumor may be a predictor for TAM density in the tumor ($p = 0.007$, odds ratio: 4.6 (1.5-14.4) (95% confidence interval)).

Comparisons of clinicopathological/ histopathological features and the density of CD163 expressing macrophages by a four-tiered scoring schema

Initially, relationships between the percentages of CD163 positive macrophages with a four-tiered scoring schema in tumor tissue (Fig. 2A-D) and the histological and prognostic features were compared (Table III). There was a statistically significant relationship between intensity of LTR ($p = 0.020$), intensity of STR ($p = 0.014$), presence/absence of concomitant LT ($p = 0.027$), and histological variant ($p = 0.032$). The conventional histological variant seemed to contain a higher percentage of TAMs than the FVPTC (Fig. 3A-D). In terms of LTR, the percentage of TAMs was higher in tumors with significant LTR (Fig. 4A, B), whereas the percentage of TAMs was lower in tumors with a mild degree of LTR and in tumors that were not associated with LTR (Fig. 4C, D). Similar results were also present in the comparisons of STR and the percentage of TAMs. The percentage of TAMs was higher in tumors exhibiting a significant degree of STR (Fig. 4A, B). Tumors associated with LT had lower numbers of TAMs than tumors without LT. The percentage of TAMs was higher in tumors with infiltrative tumor borders than in those with smooth bordered/encapsulated tumors (Fig. 4A-D), and the percentage was also higher in tumors with ETE than in tumors limited to the thyroid. However, the latter findings were not statistically significant, although there was a nearly statistically significant association.

Discussion

Investigations on the tumor microenvironment have introduced the two subtypes of macrophages: M1 macrophages and M2-like macrophages, namely, TAMs. In addition, it is necessary to discover the tumor-associated roles of these cells and the possi-

Table II. Comparisons of clinicopathological features and the density of CD163 expressing macrophages by a two-tiered scoring schema

		Univariate Analysis			Multivariate Analysis		
		Low density <25% n (%)	High density 26-100% n (%)	p	OR (95% CI)	OR (95% CI)	p
Tumor border	Smooth bordered-encapsulated	24 (54.5)	20 (45.5)	0.029	1 (Reference)	1 (Reference)	0.176
	Infiltrative	15 (32.6)	31 (67.4)		2.4 (1.0-5.8)	2.2 (0.6-7.5)	
Lateral tubular growth	Absent	14 (56.0)	11 (44.0)	0.103			
	Present	25 (38.5)	40 (61.5)		–	–	–
Intraglandular dissemination	Absent	29 (46.8)	33 (53.2)	0.227			
	Present	10 (35.7)	18 (64.3)		–	–	–
Lymphocytic tumor response	Absent	20 (55.6)	16 (44.4)	0.045	1 (Reference)	1 (Reference)	0.007
	Present	19 (35.2)	35 (64.8)		2.3 (0.9-5.4)	4.6 (1.5-14.4)	
Stromal tumor response	Absent	2 (33.3)	4 (66.7)	0.473			
	Significant	37 (44.0)	47 (56.0)		–	–	–
Lymphocytic thyroiditis	Absent	25 (49.0)	26 (51.0)	0.151			
	Present	14 (35.9)	25 (64.1)		–	–	–
Lymphovascular invasion	Absent	29 (42.0)	40 (58.0)	0.418			
	Present	10 (47.6)	11 (52.4)		–	–	–
Histological variant	CVPTC	27 (41.5)	38 (58.5)	0.374			
	FVPTC	12 (48.0)	13 (52.0)		–	–	–
Tumor focality	Unifocal	17 (48.6)	18 (51.4)	0.280			
	Multifocal	22 (40.0)	33 (60.0)		–	–	–
Tumor size	≤ 10 mm	24 (40.0)	36 (60.0)	0.249			
	>10 mm	15 (50.0)	15 (50.0)		–	–	–

Table II. Cont.

		Univariate Analysis			Multivariate Analysis	
		Low density <25% n (%)	High density 26-100% n (%)	p	OR (95% CI)	OR (95% CI)	p
Extrathyroidal extension	Absent	28 (45.9)	33 (54.1)	0.315			
	Present	11 (37.9)	18 (62.1)		–	–	–
Lymph node metastasis	Absent	29 (41.4)	41 (58.6)	0.333			
	Present	10 (50.0)	10 (50.0)		–	–	–
Anti-thyroglobulin autoantibody	Absent	20 (45.5)	24 (54.5)	0.165			
	Present	4 (26.7)	11 (73.3)		–	–	–
Anti-thyroid peroxidase autoantibody	Absent	21 (48.8)	22 (51.2)	0.014	1 (Reference)	1 (Reference)	0.143
	Present	2 (13.3)	13 (86.7)		6.2 (1.2-30.8)	3.6 (0.6-20.0)	

OR – odds ratio.

bility of their being candidate targets for treatments in many organ tumors, including thyroid tumors. In this context, we investigated the relationships between CD163 immunolabeled TAMs with features of primary tumor, tumor behavior, thyroid autoimmunity, and properties of the tumor microenvironment by using three distinct scoring schemas. The results of this study can be summarized as follows: i) CD163 expressing TAMs (in numbers and percentages) may have histomorphological interactions with other components of the tumor microenvironment, such as lymphocytic tumor response, stromal tumor response, and concomitant lymphocytic thyroiditis, ii) autoimmunity may have interactions with the density and the function of TAMs in PTC due to the relationships with positivity of anti-TPO, the presence of LTR, and the absence of tumor surrounding lymphocytic thyroiditis, and iii) TAMs exhibit no significant direct relationship between lymph node metastasis and other clinicopathological features of the primary tumor, except the conventional histological variant and local invasiveness of the tumor. However, TAMs may affect tumor behavior in an indirect manner by organizing the tumor microenvironment.

Komohara *et al.* [20] reviewed the published literature about TAMs and reported that tumor cells may introduce monocytes/macrophages from tumor surrounding tissue by producing various chemokines, such as chemokine (C-C motif) ligand (CCL) 2, CCL5, and CCL7, and these macrophages are then prompted to differentiate into M2-like macrophages by various factors produced by tumor cells. Published data about TAMs have indicated their possible roles in cancer progression via neovascularization, invasion/metastasis, and immunosuppression pathways by using their cell-to-cell interactions. Several studies have discovered that TAMs produce some immunosuppressive factors – such as prostaglandin E2 (PGE2) and interleukin 10 (IL-10) – for induction of regulatory T cells and some chemotactic factors, such as CCL17, CCL18, and CCL22, for regulatory T cells [14, 26, 27, 28]. Additionally, it is known that TAMs (particularly M2d subtype) secrete other cytokines, such as TNF-α and TGF-β [14, 16].

Cunha *et al*. [29] reported that inheritance of the G allele in *IL-10*-1082 may support lymphocyte migration as an antitumor response and antitumor autoimmunity, yielding better prognosis in differentiated thyroid carcinoma. On the other hand, Yu *et al*. [30] reported that levels of IL-10 were higher in patients with PTC and multinodular disease than in patients with only multinodular disease, and PTC patients may have a special type of regulatory T cells, resulting in a tumor response that facilitates tumor progression. One of the most recent reports states that IL-10 overexpressing B cells may act as regulatory T cells and may inhibit antigen-specific

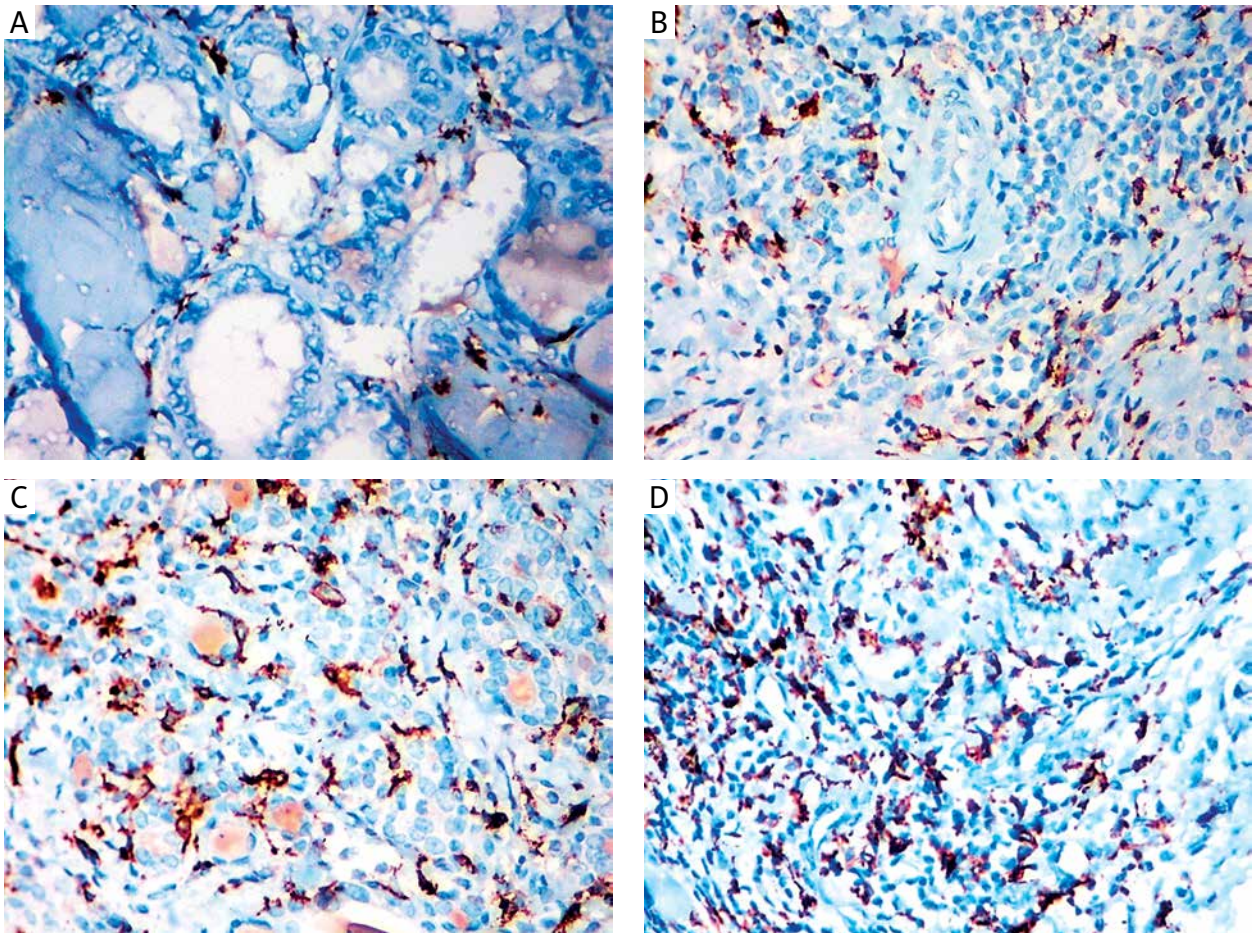

Fig. 2. Density of TAMs according to four-tiered scoring schema. A) score 1, B) score 2, C) score 3, D) score 4 (A-D, immunoperoxidase with CD163, original magnification 200×)

T cell responses [31]. Rotondi *et al*. [32] reported that TNF-α-induced chemokine (C-X-C motif) ligand 8 (CXCL8) secretion might play a role in tumor-related inflammation rather than autoimmunity-related inflammation in the thyroid gland. We have also previously reported that lymphocytic tumor response is a predictive factor for lymph node metastasis in papillary thyroid carcinoma, whereas LT is a protective factor [2]. The results of the present study reveal the statistically significant relationship and correlation between the presence and intensity of LTR and the density of TAMs in tumor tissue. The tumors exhibiting a significant degree of LTR exhibited a higher density of TAMs. Additionally, LTR was a predictor for higher density of TAMs in multivariate analyses. On the other hand, LTR was correlated significantly with infiltrative tumor border, a pattern of lateral tubular growth, and a significant degree of STR, but an inverse correlation was present between LTR and the presence of tumor-surrounding LT. However, as a limitation of the study, neither the immunophenotype of these lymphoid cells nor cytokines secreted by them were evaluated in this study. If the previous conflicting results are taken together with the results of the present study, these results may explain the possible suppression of anti-tumor immunity by TAMs via production of IL-10 and the special type of tumor-associated inflammation induced by the production of TNF-α in these polarized macrophages localized in PTC, as well as the indirect effects of these cells on lymph node metastasis and the invasive nature of the tumor.

Anti-TPO is one of the major autoantibodies in thyroid autoimmune diseases. Initiation of TPO autoimmunity by genetic and environmental factors is followed by several immunological events, including the effects of TNF-α, such as the increase in antigen presentation and increase in anti-TPO autoantibodies via the increase in major histocompatibility complex (MHC) II in thyroid epithelial cells. Finally, clonal B and T cell proliferation occurs regardless of antigen presentation in thyroid tissue. It has also been reported that increasing thyroid stimulating hormone (TSH), which is associated with thyroid carcinogenesis, initiates increased levels of anti-TPO antibodies and also increases TNF-α [8, 33]. Recently, it has been reported that higher levels of anti-TPO antibodies (>1300 IU/ml) are associated with multifocality for PTC [33]. In the present study, positivity of anti-TPO antibodies in the serum of patients was

Table III. Comparisons of clinicopathological features and the density of CD163 expressing macrophages by a four-tiered scoring schema

		< 25% n (%)	26-50% n (%)	51-75% n (%)	76-100% n (%)	Total n (%)	p
Tumor border	Smooth bordered-encapsulated	24 (54.5)	10 (22.7)	7 (15.9)	3 (6.8)	44 (48.9)	0.081
	Infiltrative	15 (32.6)	22 (47.8)	7 (15.2)	2 (4.3)	46 (51.1)	
Lateral tubular growth	Absent	14 (56.0)	6 (24.0)	2 (8.0)	3 (12.0)	25 (27.8)	0.092
	Present	25 (38.5)	26 (40.0)	12 (18.5)	2 (3.1)	65 (72.2)	
Intraglandular dissemination	Absent	29 (46.8)	19 (30.6)	9 (14.5)	5 (8.1)	62 (68.9)	0.232
	Present	10 (35.7)	13 (46.4)	5 (17.9)	0 (0.0)	28 (31.1)	
Lymphocytic tumor response	Absent	20 (55.6)	11 (30.6)	1 (2.8)	4 (11.1)	36 (40.0)	0.020
	Mild	16 (39.0)	16 (39.0)	8 (19.5)	1 (2.4)	41 (45.6)	
	Significant	3 (23.1)	5 (38.5)	5 (38.5)	0 (0.0)	13 (14.4)	
Stromal tumor response	Absent	2 (33.3)	2 (33.3)	0 (0.0)	2 (33.3)	6 (6.7)	0.014
	Mild	17 (56.7)	9 (30.0)	2 (6.7)	2 (6.7)	30 (33.3)	
	Significant	20 (37.0)	21 (38.9)	12 (22.2)	1 (1.9)	54 (60.0)	
Lymphocytic thyroiditis	Absent	25 (49.0)	19 (37.3)	3 (5.9)	4 (7.8)	51 (56.7)	0.027
	Present	14 (35.9)	13 (33.3)	11 (28.2)	1 (2.6)	39 (43.3)	
Lymphovascular invasion	Absent	29 (42.0)	25 (36.2)	10 (14.5)	5 (7.2)	69 (76.7)	0.595
	Present	10 (47.6)	7 (33.3)	4 (19.0)	0 (0.0)	21 (23.3)	
Histological variant	CVPTC	27 (41.5)	25 (38.5)	12 (18.5)	1 (1.5)	65 (72.2)	0.032
	FVPTC	12 (48.2)	7 (28.0)	2 (8.0)	4 (16.0)	25 (27.8)	
Tumor focality	Unifocal	17 (48.6)	11 (31.4)	5 (14.3)	2 (5.7)	35 (38.9)	0.873
	Multifocal	22 (40.0)	21 (38.2)	9 (16.4)	3 (5.5)	55 (61.1)	
Tumor size	≤ 10 mm	24 (40.0)	23 (38.3)	10 (16.7)	3 (5.0)	60 (66.7)	0.778
	>10 mm	15 (50.0)	9 (30.0)	4 (13.3)	2 (6.7)	30 (33.3)	
Extrathyroidal extension	Absent	28 (45.9)	17 (27.9)	11 (18.0)	5 (8.2)	61 (67.8)	0.087
	Present	11 (37.9)	15 (51.7)	3 (10.3)	0 (0.0)	29 (32.2)	
Lymph node metastasis	Absent	29 (41.4)	26 (37.1)	10 (14.3)	5 (7.1)	70 (77.8)	0.524
	Present	10 (50.0)	6 (30.0)	4 (20.0)	0 (0.0)	20 (22.2)	

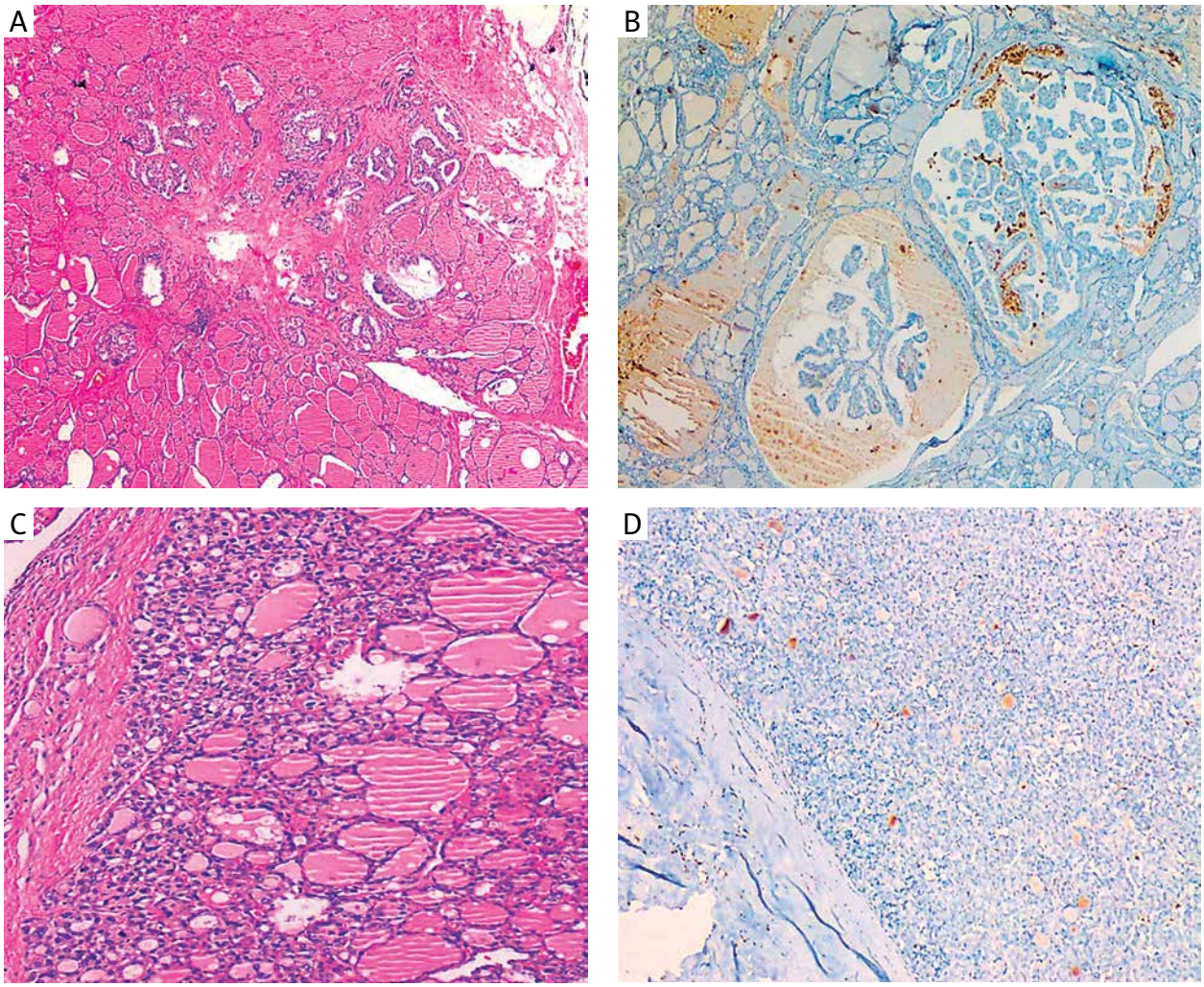

Fig. 3. A, B) Higher density of TAMs in a conventional variant of papillary thyroid carcinoma with infiltrative tumor borders, C, D) lower density of TAMs in an encapsulated follicular variant papillary thyroid carcinoma (A – HE, original magnification 40×; B – immunoperoxidase with CD163, original magnification 100×; C – HE, original magnification 100×; D – immunoperoxidase with CD163, original magnification 100×)

associated with higher density of TAMs, suggesting the possible origin of TNF-α, which causes an increase in anti-TPO antibodies because of the increase in available TPO antigens. Therefore, TAMs may be histological substitutes for high levels of anti-TPO antibodies, and TSH thus may be an indicator of multifocality in patients who have undergone lobectomy. On the other hand, anti-TPO antibodies may be a humoral component of tumor-associated inflammation in PTC, which is induced by TNF-α.

Although Qing *et al.* [1] reported no relationship between the density of CD68 expressing TAMs and LT, which is one aspect of the immune response, our results revealed that the percentage of TAMs was higher in the tumors without surrounding LT. Additionally, the presence of concomitant LT was inversely correlated with the significance of LTR, LVI, LNM, and larger tumor size. These data may support the results of Rotondi *et al*. [32], which indicated different chemokine expression of the tumor-associated lymphocytic response and the autoimmune lymphocytic response. However, the immunophenotype of these lymphoid cells was not evaluated in this study, so these results may be possible signs of the interactions between TAMs and lymphoid cells associated with the immunosuppressive effects of these polarized macrophages. This would support our previous results indicating that the absence of concurrent LT is a predictor for lymph node metastasis in papillary thyroid carcinoma [2]. Thus, these findings may reflect the indirect effects of TAMs for lymph node metastasis in PTC; however, studies including molecular and/or immunohistochemical analyses in a larger series are required.

TGF-β is one of the molecules produced by TAMs [16], and it has antiproliferative effects. It is also an important regulator in epithelial-mesenchymal differentiation and metastasis. In the thyroid, production of this growth factor is regulated by iodine and TSH [8]. Eloy *et al.* [34] reported that the expression of TGF-β in well-circumscribed PTC is lower than in tumors with infiltrative borders. The presence of

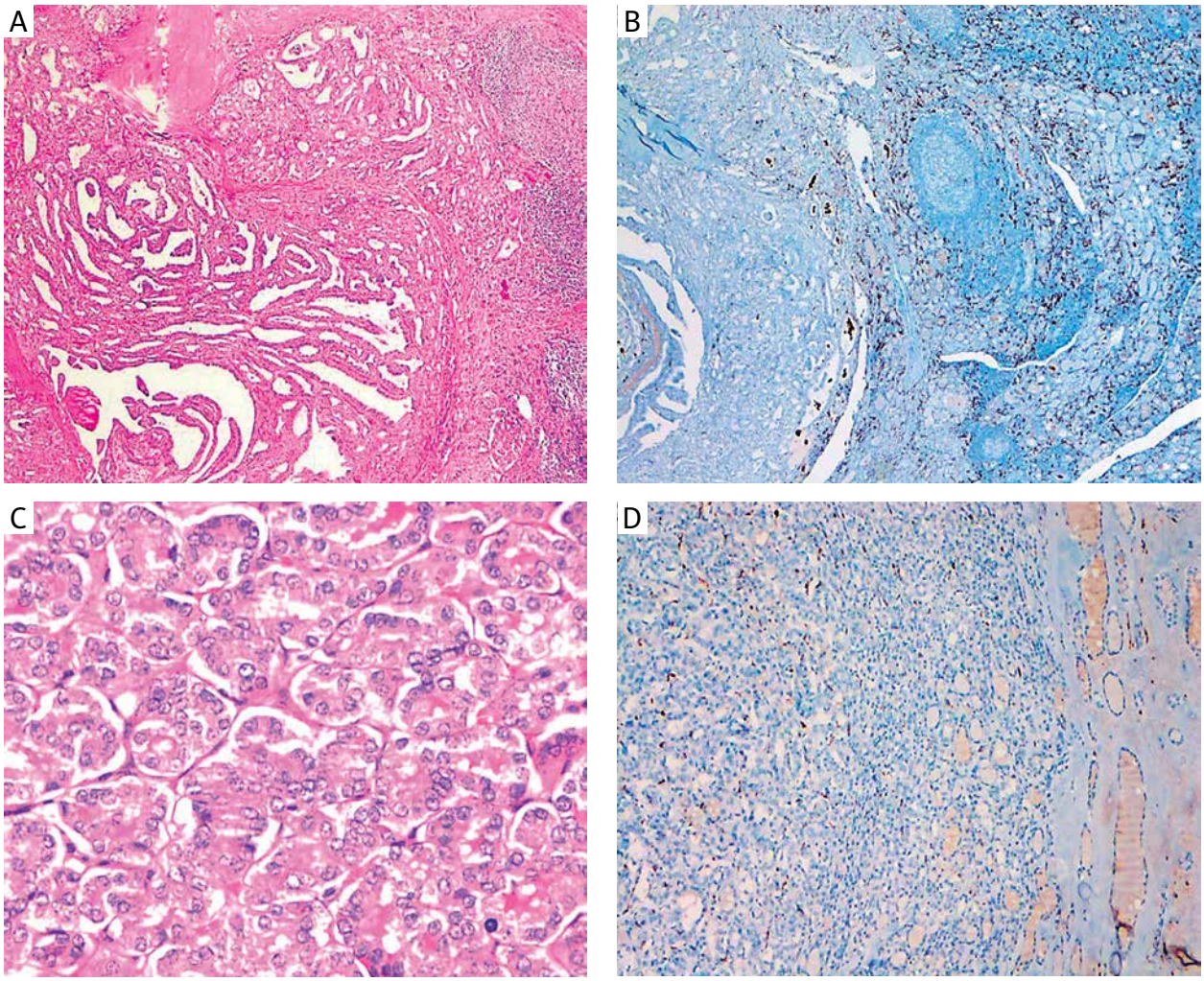

Fig. 4. A, B) Higher density of TAMs in a conventional variant papillary thyroid carcinoma with infiltrative tumor borders associated with significant degree of lymphocytic tumor response and stromal tumor response (A – HE, original magnification 40×; B – immunoperoxidase with CD163, original magnification 40×) and C, D) lower density of TAMs in a follicular variant of papillary thyroid carcinoma without a stromal or lymphocytic tumor response (C – HE, original magnification 200×; D – immunoperoxidase with CD163, original magnification 100×)

a higher density of TAMs in tumors with infiltrative borders and the correlation between the infiltrative tumor border with IGD and ETE in our study may indirectly support the authors' suggestion, which was due to the awareness of the production of TGF-β by TAMs. Therefore, expression of TGF-β by CD163 positive TAMs may indicate the effect of TAMs on the invasiveness of PTCs.

Fibroblasts are one of the major components in the tumor microenvironment. According to the reported data, TAMs may play a role in matrix remodeling in tumors [20]. In the present study, percentages of TAMs in tumors with significant STR were higher than in tumors with a mild degree of STR or tumors without STR, and a significant degree of STR was correlated with infiltrative tumor borders, IGD, ETE, LTR, and CVPTC. These histological features may resemble the highlighted role of TAMs in matrix remodeling and the effects of the stromal tumor reaction on the invasive behavior of PTCs. In the present study, the relationship between vascular invasion and density of TAMs was also examined, but we could not define any association between these two parameters. In our previous study, the percentage of lymph node positive cases was correlated with an increase in the severity of the stromal tumor response, whereas a stromal tumor response was associated with larger tumor size, LVI, and ETE [2]. Thus, TAMs may affect local tumor progression, invasion, and metastasis by producing VEGFs and MMPs, which may play roles in the formation of an appropriate environment, intravasation, and matrix remodeling [26, 27, 28].

The histological variant of PTC influences the behavior of the tumor, as has been proposed in most recent reports [2, 35]. The present study aimed to investigate whether the density of TAMs in the conventional variant and follicular variant of PTC are different. The results of this study indicate that CVPTCs have higher densities of TAMs than FVPTCs, in contrast with previous results indicating that there is no

relationship between histological variant and TAM density [1]. In addition, conventional histology was correlated with infiltrative tumor borders, LTG, LVI, ETE, LNM, and significant STR in our study group, indicating the adverse effect of conventional histology of PTC on tumor prognosis. In addition, variable rates of TAMs in different histological variants and in tumors with different histological grades may indicate that one of the checkpoints for these cells may be the primary tumor cells or that a tumor microenvironment rich in TAMs contributes to aggressive tumor behavior.

Qing *et al*. [1] reported the positive association of TAMs with lymph node metastasis and advanced tumor stage in PTC. In their study, the investigators used CD68 antibodies to evaluate the density of TAMs in tumors. On the other hand, Kim [13] reported that a higher density of TAMs is associated with larger tumor size in lymph node positive patients via CD68 immunohistochemistry. CD68 antibody may label all of the macrophages, including M1 and M2-like subtypes, and M2-like subtypes can be detected by some other markers, such as CD163, CD204, and CD206 [36]. Angell *et al*. [37] reported that an increasing ratio of CD68 positive macrophages to CD163 positive macrophages might have role in immune escape of papillary thyroid carcinoma cells. Jung *et al*. [38] investigated the effect of TAMs on behavior of several types of cancers including thyroid carcinomas. The authors found that density of TAMs has an effect on anaplastic thyroid carcinoma but does not have any effect on well-differentiated thyroid carcinoma. A recent study revealed that CD163 expressing TAMs are associated with tumor cell invasion and lymph node metastasis in PTC due to CXCL16 signaling [15]. In the present stud

y, although CD163 immunohistochemical antibody was used to label TAMs solely, and also the ratio of CD68 positive macrophages to CD163 positive macrophages could not be defined in a relatively small study group, the density of TAMs was not directly associated with lymph node metastasis, ETE, or tumor size. It should be noted that the observations of different studies using different antibodies labeling TAMs – such as CD163 and CD204 – in the tumors with the same histological types have shown that CD163 expressing TAMs could be associated with poorer prognosis, whereas TAMs positive for CD 204 antibody could not. However, opposite results have been reported [36, 39, 40, 41]. The clinicopathological features significantly associated with the density of TAMs – such as infiltrative tumor border, significant STR, absence of tumor surrounding LT, and conventional histology – were significantly correlated with ETE and/or LNM in the study group. Therefore, immunohistochemical expression of different antibodies associated with different clinicopathological features may reflect the multifunctional nature of TAMs. Detection of TAMs by CD68 antibody alone may not provide an accurate description of these cells, and it would be appropriate to evaluate the density of TAMs by using a combination of markers that are specific for M2-like subtype macrophages. In addition, the role of TAMs in LNM or ETE may be mediated by some other properties of the primary tumor or the tumor microenvironment, as has been presented in BRAF mutant papillary thyroid carcinomas [37].

In conclusion, although several previous reports have described the effects of tumor-associated macrophages on some of the clinicopathological features in thyroid tumors and the lymphocytic tumor response, for the first time, despite the presence of some limiting factors, the present study attempts to establish a full assessment of interactions of CD163 expressing M2-like TAMs with the triad of primary tumor–tumor microenvironment–tumor behavior and, above all, with markers of autoimmunity. Thus, these alternatively polarized macrophages may act in tumor progression and dissemination according to their various products, which may be ordered by tumor cells or neighboring immune cells. So, tumor-associated macrophages may be concertmasters connecting between the maestro, or the primary tumor cells, and the orchestra, or the tumor microenvironment. The molecular studies detailing the properties of these special cells may reveal their roles in various tumors and may improve the therapeutic strategies targeting TAMs and prognostic impacts of TAMs in various malignant tumors, including papillary thyroid carcinomas.

The authors declare no conflict of interest.

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