

Infiltration of a mixture of different immune cells may be related to molecular profile of differentiated thyroid cancer

Dear Editor

Pathologists have long recognized that some tumors are densely infiltrated by cells of both innate and adaptive arms of the immune system, thereby reflecting inflammatory conditions arising in non-neoplastic tissues (Dvorak 1986, Hanahan & Weinberg 2011). Such infiltration may reflect an effort of the immune system attempting to eradicate neoplastic cells. Conversely, there is an increasing amount of evidences for antitumor responses, resulting from a pressure on the tumor to evade immune destruction (Hanahan & Weinberg 2011). Tumor evolution and consequent patient outcomes may depend on this complex interaction between immune system and tumor cells.

The immune responses against differentiated thyroid carcinomas (DTC) have also been extensively studied (Modi *et al.* 2003). CD4+T cells are central to the successful orchestration of this immune response. Naive CD4+T cells differentiate into one of at least four functionally distinct fates, such as Th1, Th2, Th17, and regulatory T cells (Tregs). In general, Tregs are identified as FOXP3+ lymphocytes and are thought to contribute to tumor-specific T-cell tolerance (Zhou & Levitsky 2007). Th17 cells were recently described but, despite their important role in host protection against infectious pathogens and in the pathogenesis of various inflammatory and autoimmune diseases, their prevalence in human cancer is still under investigation (Su *et al.* 2010).

Antitumor immune response is thought to be related to tumor antigenicity. In fact, a correlation between the protein expression profile and immune cell infiltration in DTC was observed (Bruland *et al.* 2009). Some researchers have suggested that MUC1, NIS (SLC5A5), ATM, PTEN, and CD56 (NCAM1) expression might indicate tumor differentiation and tumor progression, demonstrating tumor antigenicity (Larson *et al.* 2007, El Demellawy *et al.* 2008, Morari *et al.* 2010). However, whether these markers are really associated with DTC immune response is still not clear.

In order to examine the putative association between tumor molecular profile and pattern of infiltration of tumor-infiltrating lymphocytes (TIL), tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSCs), we investigated 398 patients whose tissue samples were maintained in the tissue bank of the institution. Thyroid carcinoma was diagnosed in 266 patients: 253 with papillary thyroid carcinomas (PTC) including 153 classical form, 80 PTC follicular variants, 20 PTC tall cell variants, and 13 with follicular carcinomas (FC): seven minimally invasive and six frankly invasive. We also obtained 132 normal or benign thyroid tissues, including 18 normal tissues, 58 nodular goiters, and 56 follicular adenomas (FA).

Patients' clinical information was obtained from their files. Aggressiveness at diagnosis was ascertained using the TNM (tumour, node, metastasis) and stage classification system for DTC. Patients underwent periodic total body scans, serum TSH, and thyroglobulin (Tg) measurements according to a standard protocol for a period of 12–298 (43.50 ± 33.29) months, Mo=21 months. Patients presenting high nonstimulated serum Tg levels (>2 ng/dl and, more recently, Tg >1 ng/dl using a sensitive assay) were submitted to a thorough image search. We defined tumors as persistent/recurrent and/or presenting long distance metastasis, according to the aforementioned parameters.

Formalin-fixed paraffin-embedded tissues from all 398 cases were reviewed for diagnostic confirmation and used to build a tissue microarray (TMA, Beecher Instruments, Silver Springs, MD, USA) for immunohistochemical (IHC) analysis. We obtained four tumor tissue cores from each case. Two spots were chosen from representative areas of the lesion based on the greater leukocyte infiltration while two other spots were chosen from areas free of leukocyte infiltration. The IHC markers were divided into two groups: a) immune cell markers and b) tumor markers. Immune cell markers included TIL and TAM subsets, such as CD3, CD4, CD8, CD20, and CD68, always evaluated in intraepithelial infiltrating cells located within cancer

cell nests, as showed in Fig. 1, panel I. Tumor markers included CD56, NIS, MUC1, PTEN, ATM, and B7H1 (CD274). IHC procedure was held as described previously running positive and negative controls in the same batch of reactions (Morari et al. 2010).

Infiltration of Th17 and MDSCs was assessed by immunofluorescence. CD4, IL17, CD33, and CD11b expressions were analyzed as described previously (Bertelli et al. 2006). The secondary antibodies were FITC or a rhodamine conjugate. The images were obtained using a confocal laser microscopy (LSM510; Zeiss, New York, NY, USA). Analysis and documentation of results were performed using a Leica FW 4500 B microscope.

The slides were scored independently by two pathologists (J Vassallo and F A Soares), both blinded to tumor features. Cells were considered positive for IHC markers when a clear-cut brown staining was observed in the typical cellular localization. Th17 was defined as those cells positive for immunofluorescence markers when a clear CD4+ and IL17+ co-localization was observed in the typical cellular localization, as shown in Fig. 1, panel II. Likewise, MDSCs were defined as those cells positive for immunofluorescence markers when a clear CD33+ and CD11b+ colocalization was observed in the typical cellular localization, as shown in Fig. 1, panel III. An individual evaluation of immune cell markers was completed for each tissue spot, estimating the number of positive cells per TMA spot considering an approximate area of 0.79 mm². The cases were grouped into categories for statistical analysis: 0 (no positive cell), 1+ (up to ten positive cells per spot), and 2+ (ten or more positive cells per spot).

Statistical analysis was carried out using the Winstat Software (Cambridge, MA, USA). Nonparametric analysis was performed using the χ^2 or Fisher's exact test as indicated. A multivariate logistic regression model was applied using tumor type as a dependent variable, protein expression, and clinical risk factors, including gender and age as explicative variables. Mann-Whitney *U* tests were used to compare continuous or arranged measures between two groups whose variable did not present Gaussian distribution; Kruskal-Wallis test was used to compare three or more groups whose variable did not present Gaussian distribution. The *T*-test or ANOVA test was used for variables that presented Gaussian distribution, comparing two, three, or more groups respectively. All tests were conducted at a 0.05 significance level.

As expected, most (83.66%) of our patients were females. Individuals with benign (49.29 ± 15.18 years old) and malignant (43.66 ± 15.91 years old) thyroid

lesions presented similar ages at diagnosis. DTC patients were classified according to the pathological TNM (pTNM) staging system (Shaha 2007) as pTNM I (157 cases), II (28 cases), III (40 cases), and IV (41 cases). Ninety tumors were classified as encapsulated and 176 as nonencapsulated; 118 tumors were multifocal and 148 unifocal; 108 presented extrathyroidal invasion, whereas 158 tumors did not. Forty-eight patients (18.04%, including ten dead-of-disease) presented recurrence, whereas 185 evolved free of disease (69.55%). Thirty-three patients who could not be classified into one of these two groups were further excluded from any analysis involving outcome.

Macrophages were not found in normal thyroid tissue. TAM was more frequent in malignant (82.11%) than in benign tissues (33.91%; $P < 0.0001$). As depicted in Table 1, TAM was more frequent in tumors expressing MUC1 ($P < 0.0001$), ATM ($P < 0.0001$), and B7H1 ($P = 0.0035$).

None of the normal thyroid tissues were CD3+, only one nodular goiter, and four follicular adenomas. Conversely, CD3+ infiltration occurred in 59.17% of malignant tissues ($P < 0.0001$), at similar rates in PTC and follicular thyroid carcinomas tissues ($P = 0.8596$). CD3+ was more frequent in FTC than in FA cases ($P < 0.0001$), and in follicular variant of papillary thyroid carcinomas than in FA cases ($P < 0.0001$), helping to characterize follicular-patterned lesions, but it did not differentiate FVPTC from FC ($P = 0.5309$). Infiltration of CD3+ lymphocytes also correlated with MUC1, CD4+, CD8+, CD20+, and TAM (Table 1).

None of the normal thyroid tissues was CD4+. Only two nodular goiters (3.44%) and one FA (1.78%) displayed CD4+ lymphocytes, in contrast to 41 out of the malignant samples (15.41%), hence distinguishing benign (2.63%) and malignant nodules (15.41%; $P = 0.0010$). CD4+ lymphocyte infiltration was more frequent in tumors positive for B7H1, MUC1, ATM, and CD56, presenting TAM, CD3+, CD8+, and CD20+ lymphocytes (Table 1).

Malignant thyroid tissues showed CD8+ lymphocytes more frequently (46.12%) than goiter samples (37.5%) and FA (37.74%). However, 17 normal thyroid tissues presented evident CD8+ lymphocyte infiltration and one sample displayed only a few scattered cells spread throughout the tissue. Intense CD8+ lymphocytic infiltration occurred more frequently in malignant (23.29%) than in benign (3.53%) thyroid tissues ($P < 0.0001$). CD8+ lymphocyte infiltration was more frequent in tumors expressing MUC1, ATM, PTEN, NIS, CD56, B7H1, and showing TAM, CD3+, CD4+, and CD20+ lymphocytes (Table 1).

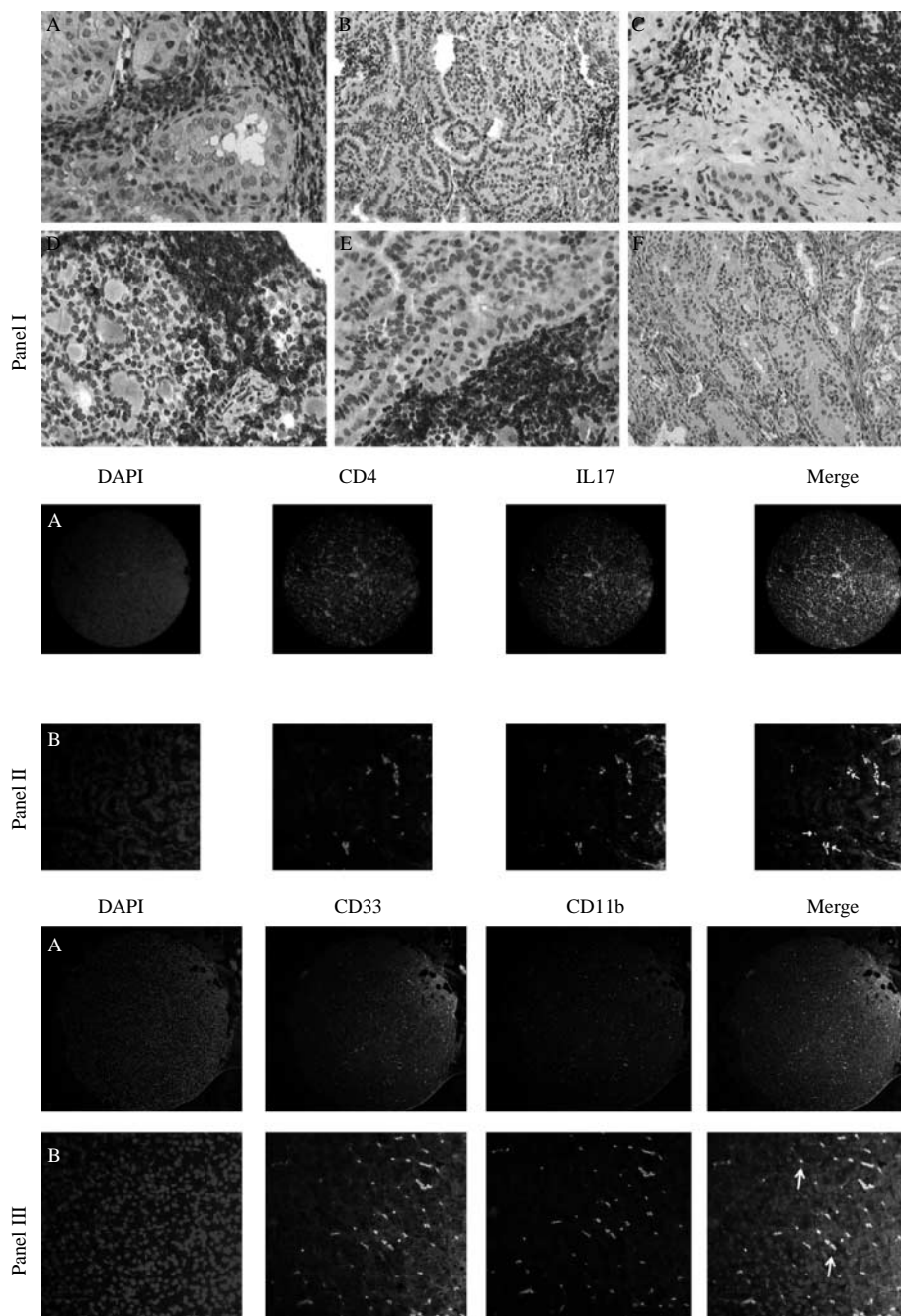


Figure 1 (Panel I) Immune cells in papillary thyroid carcinomas. All images correspond to immunohistochemical reactivity for each of the markers, as evidenced by brown staining in the membrane of lymphocytes. (A) Infiltration of CD3+ lymphocytes (400 \times), which is a pan-T marker. (B) Infiltration of CD8+T lymphocytes. CD8+ (cytotoxic T) lymphocytes are found as a diffuse infiltration between the papillae. (C) Infiltration of CD4+ (helper T) lymphocytes infiltrating PTC lesion (400 \times). (D and E) Infiltration of CD20+ lymphocytes (B lymphocytes). (F) Infiltration of CD68+ cells (macrophages at 50 \times). (Panel II) CD4+/IL17+ lymphocytes infiltrate PTC demonstrated by immunofluorescence colocalization of CD4+ and IL17+ markers. (Panel A) TMA images for CD4+/IL17+ cells in PTC (magnification: 50 \times). (Panel B) PTC with infiltration of CD4+/IL17+ lymphocytes (magnification: 400 \times). White arrows indicate CD4+/IL17+ (Th17) lymphocytes. The nuclei of PTC cells were characterized by their typical appearance and were easily distinguished from infiltrating lymphocytes. CD4+/IL17+ lymphocytes were found as single cells or multicellular clusters within the tumor. (Panel III) CD33+/CD11b+ cells infiltrate PTC demonstrated by immunofluorescence colocalization of CD33+ and CD11b+ markers. (Panel A) TMA images for CD33+/CD11b+ cells in PTC (magnification: 50 \times). (Panel B) PTC with infiltration of CD33+/CD11b+ cells (magnification: 400 \times). White arrows indicate CD33+/CD11b+ myeloid-derived suppressor cells (MDSCs). CD33+/CD11b+ cells were found as diffuse or multicellular clusters scattered within the tumor.

Table 1 Relationship between macrophages and the immune phenotype of inflammatory cells

Immune cells	Tumor markers										Immune cell markers				
	MUC1	ATM	PTEN	NIS	B7-H1	CD56	FOXP3+	MDSCs	Th17	CD20+	CD8+	CD4+	CD3+		
Macrophages	<0.0001	<0.0001 ^a	NS	NS	<0.0001	NS	<0.0001	NS	NS	0.0039	0.0012	0.0338	<0.0001		
CD3+	<0.0001	NS	0.0834	NS	NS	NS	<0.0001	0.0071	NS	0.0001	0.0012	0.0001			
CD4+	<0.0001	0.0013	NS	NS	0.04942	0.0058	<0.0001	0.03153	NS	<0.0001	<0.0001				
CD8+	0.0009	<0.0001	<0.0001	<0.0001	0.0005	0.017	<0.0001	0.00249	0.0144 ^a	<0.0001	<0.0001				
CD20+	<0.0001	<0.0001	NS	0.0029	0.0032	0.0284	<0.0001	0.0181	NS						
Th17	NS	0.0079 ^a	NS	NS	NS	0.0283	NS	<0.0001							
MDSCs	NS	NS	NS	0.02747	0.03256	NS	0.04414								
FOXP3+	0.0044	<0.0001	NS	0.0203	0.0092	0.0005									

NS, nonsignificant.

^aComparison using 'less than ten cells' vs 'more than ten cells' categories.

None of the normal thyroid tissues or the FA examined and only two nodular goiters displayed CD20+ lymphocytic infiltration in contrast to 46 (23.11%) of malignant tissues ($P < 0.0001$). CD20+ lymphocytes were more frequent in PTC (27.33%) than in FTC (5.26%; $P = 0.0024$). CD20+ was more frequent in tumors expressing MUC1, ATM, NIS, CD56; TAM, CD3+, CD4+, and CD8+ lymphocytes infiltration, as shown in Table 1.

CD4+/IL17+ lymphocytic infiltration was more frequent in malignant tissues (98.22%) than in nonmalignant tissues (87.23%; $P = 0.0004$). CD4+/IL17+ lymphocyte infiltration was more frequent in tumors expressing ATM, CD56, and CD8+ lymphocytes as shown in Table 1. Figure 1, panel II, shows a representative immunohistochemistry of CD4+/IL17+ lymphocytic infiltration in PTC tissue.

None of the normal thyroid tissues, goiters, FA, or FC presented FOXP3+ lymphocytes. Only 14.5% PTC were FOXP3+. FOXP3+ lymphocytes were more frequent in tumors expressing MUC1, ATM, NIS, CD56, and B7H1 and in infiltration of macrophages, CD3+, CD4+, CD8+, and CD20+ lymphocytes (Table 1).

We further investigated the association between infiltration of MDSCs and the molecular profile of our DTC cases. Indeed, tumors positive for B7H1 were frequently enriched with MDSCs ($P = 0.0455$). Infiltration of MDSCs was accompanied by infiltration of CD3+ ($P = 0.0284$) and CD8+ ($P = 0.0050$) lymphocytes. Tumors that presented MDSCs were frequently positive for NIS ($P = 0.0439$).

In summary, our data demonstrated that immune cells infiltrate malignant tissues more often than benign lesions, suggesting an immune reaction of the organism against transformed cells. In addition, immune cell infiltration was closely associated with the IHC profile of the DTC specimens examined.

Some proteins involved in the pathogenesis of DTC might account for part of the immune reaction as differentiation antigens, as their expression is significantly associated with the inflammation process. MUC1, NIS, and CD56 were found as common markers of antitumor immune response. There are evidences that these markers may act as autoantigens (Moore et al. 2004, El Demellawy et al. 2009).

We also found PTEN and ATM expression associated with immune cell infiltration, supporting the idea that these proteins might enhance tumor skills to induce an immune response (Noh et al. 2009). Endorsing this assumption, it has been demonstrated that loss of ATM is associated with immune resistance (Gomez et al. 2008). Also, B7H1 is classically engaged

in immune evasion. In fact, cancer cell expression of costimulatory molecule B7-H1 has been implicated as a potent inhibitor of T-cell-mediated antitumoral immunity (Thompson *et al.* 2005). However, association between B7H1 expression in tumor cells and infiltration of different immune cells may rise the idea that these immune cells are not functionally activated. More studies are warranted to figure this question.

In conclusion, we observed an infiltration of a mixture of different immune cells in DTC. The fact that these immune cells are correlated with each other suggests that a complex immune network may be elicited by the cancerous thyroid follicular cell. Also, we demonstrated that the immune response may be influenced by the status of DTC tumor antigenicity. In addition, immune cell infiltration profile helped to characterize specific tumor histotypes.

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Declaration of interest

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Author contribution statement

L L Cunha contributed in conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. E C Morari and A C T Guihen contributed in collection and assembly of data and final approval of manuscript. D Razolli, R Gerhard, and S Nonogaki contributed in data analysis and interpretation, histopathological analysis, and final approval of manuscript. F A Soares and J Vassallo contributed histopathological analysis and final approval of manuscript. L S Ward contributed in conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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