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Phenotypic and Functional Characterization of Lymphocytes in Autoimmune Thyroiditis and in Papillary Carcinoma

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Abstract. *Background: Infiltrates of lymphocytes are found in both autoimmune thyroid disease and papillary cancer and are responsible for thyroid destruction in autoimmune disease. Their role in neoplastic transformation is not yet clear. Materials and Methods: Phenotypic studies and the capacity to undergo apoptosis were assessed on peripheral and gland infiltrating lymphocytes from patients with autoimmune thyroiditis and papillary carcinoma. Results: Peripheral lymphocytes in these patients belong to the same phenotype as the infiltrating lymphocytes. A mixed immune response Tc2 and Tc1 is present in thyroid glands of patients with papillary tumors and the capacity to undergo apoptosis in peripheral lymphocytes from both groups of patients increases. Conclusion: We suggest that a switch from a Th1 (Tc1) in autoimmune thyroid disease to a Th2 or mixed response in papillary carcinoma patients in peripheral blood may help the early diagnosis of thyroid cancer and could be used in autoimmune thyroid disease patient follow-up.*

Autoimmune thyroid disease comprises a spectrum of conditions that includes many clinical forms involving thyreocyte destruction (1). From a clinical point of view, thyroiditis often determines the presence of nodules which on FNAB can show cytological alterations such as anisonucleosis that can be of dubious diagnosis. This can lead to the wrong management of patients before surgery.

In Hashimoto thyroiditis (HT), infiltrating lymphocytes, which are of Th1 type in HT, are responsible for the apoptotic dysregulation through the secretion of IFNγ, which is known to up-regulate the expression of caspases 8 and 2 responsible for cell apoptosis (2, 3). By contrast, in Graves disease (GD), thyroid infiltrates are mainly Th2 type lymphocytes and their cytokines (such as IL4 and IL5) prevent apoptosis by increasing Bcl2 expression on thyreocytes (4). Many cytokines and chemokines have been shown to play a part in the destructive process, but the first steps towards autoimmunity (5) and a consequent link between dysregulation in the immune response and cellular transformation have not been elucidated. Many thyroid tumors are infiltrated by lymphocytes and other inflammatory cells. Whether these infiltrates happen before or after the neoplastic transformation is not clear (6, 7). One theory (8) adapts well to the thyroid model in that cells that are targets of autoimmune destruction may have to adapt to life by de-repressing tumor suppressor genes or oncogenes (9). This will signal danger to the immune system, which will in turn continue to maintain the autoimmune process. The present study is an analysis of the phenotypic differences and behaviour of lymphocytes in patients with thyroiditis and papillary cancer.

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Key Words: Tc1, Tc2, papillary carcinoma, autoimmune thyroiditis, thyroid nodule.

Materials and Methods

Patients and healthy subjects. Initially all patients from the Department of Surgery of the "La Sapienza" University of Rome, Italy, were informed of our study and blood samples were sent to the laboratories of the Department of Experimental Medicine without any clinical information about the patients. After diagnosis, only patients with chronic thyroiditis and/or thyroid cancer were included in the study. There were a total of 30 patients with autoimmune thyroiditis (27 women and 3 men, aged from 30 to 72) and 10 patients (7 women and 3 men, aged from 39 to 69) with papillary carcinoma. A group of 15 healthy subjects (5 men and 10 women, aged from 25 to 55) were selected as a control group. One of the diagnostic criteria for autoimmune thyroiditis was the concentration of thyroperoxidase auto-antibodies in serum (Ab TPO). Patients with thyroiditis showed Ab TPO>600 U/ml, while 50% of patients with cancer had Ab-TPO> 100 U/ml. Abs TPO

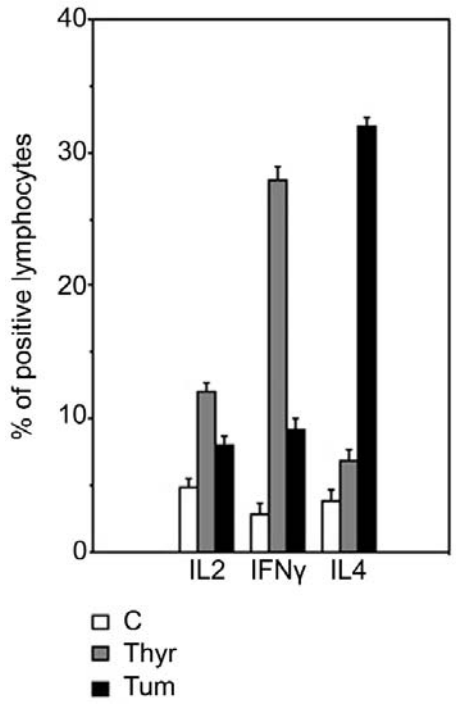

Figure 1. *Cytoplasmic IL2, IFNγ and IL4 expression in peripheral lymphocytes of healthy donors (□), thyroiditis patients (■) and tumor patients (■). Lymphocytes were cultured for 18 h at 37˚C, 5% CO_2 in the presence of 16 mM PMA, monensin and ionomycin and then labelled with monoclonal antibodies anti-IL2, anti-IFNγ and anti-IL4, as described in Materials and Methods. Data represent the mean of all determinations in each group of subjects ±SD.*

were not detectable in healthy subjects. Fine-needle aspiration or excision of the gland confirmed the suspected diagnosis in all patients. All patients with papillary cancer underwent total thyroidectomy. Five patients with multinodular goiter and 10 patients with suspected uninodular lesion of the thyroid underwent surgery. Patients with Graves disease were excluded from the study. None of the patients and healthy subjects was affected by other autoimmune or infectious diseases or were under steroid treatment.

Culture of lymphocytes. Lymphocytes were separated from 20 ml of peripheral blood by centrifugation over a Ficoll/Hypaque gradient (30 min, 1600 rpm) and washed with RPMI 1640 medium (GIBCO BRL, Paisley, UK). Isolated lymphocytes were cultured in RPMI medium without serum for 24 h, 48 h and 72 h at 37˚C, 5% CO_2. Where required, 10% untreated autologous serum was added to the culture medium.

Apoptosis measurement. Two different methods were used to measure the levels of apoptosis: cytofluorimetric evaluation of the hypodiploid peak after propidium iodide (PI) staining and by the binding of FITC-conjugated Annexin V (Annexin V kit, Bender MedSystems Diagnostics GmbH, Wien, Austria). Before the apoptotic assay, cells were divided into two groups: one group ($1x10^6$/ml) was fixed in 70% ethanol for 1 h, and then stained with PI (50mg/ml PBS) (Sigma) (10) for the evaluation of apoptosis. The second group of cells from the same cultures was labelled with

Table I. *Phenotypic characterization of peripheral lymphocytes from healthy donors, patients with thyroiditis and tumor patients.*

	IL2		IFNγ		IL4	
	CD4+%	CD8+%	CD4+%	CD8+%	CD4+%	CD8+%
Healthy	5.0±2	/	3.0±2	1.5±1	4.2±1	/
Thyroiditis	6.8±3	5.3±2	18.2±2	9.8±3	5.2±2	2.3±2
Tumors	4.6±2	3.4±3	6.8±3	2.5±2	5.5±3	26±4

Lymphocytes were double-labelled with either anti-CD4 or anti-CD8 and antibodies anti-IL2, IFNγ and IL4, as described in Materials and Methods. Results are expressed as percentage of IL2, IFNγ and IL4 expressing lymphocytes among the $CD4^+$ and CD^+ subsets. Data are the mean of all determinations in each group of subjects ± standard deviation.

the different monoclonal antibodies for the phenotypic studies and for Annexin V. Apoptotic cells stain with Annexin V. For intracellular cytokine determinations, lymphocytes were incubated for a further 18 h in the presence of specific inducers.

Immunolabelling and flow cytometric analysis. Lymphocytes were immunolabelled at time 0 and at the reported incubation times both with antibodies against surface receptors (CD2, CD3, CD4, CD8, CD56, CD19, CD95, CD25) and antibodies against intracellular cytokines (IL2, IL4, IFNγ) (11). Immunolabelling was carried out in the same way for both intracellular and surface antibodies (12). As negative controls, all assays included lymphocytes from the same patient that were immunolabelled with an irrelevant antibody (anti IgG1 isotypic control). Fluorescence intensity was analysed with an Epics XL-MCL cytometer (Coulter Electronics, Hialeah, FL, USA), as previously reported.

Immunocytochemistry and scanning confocal microscope analysis. Three micron (μ) sections of all resected samples were used for standard histology. Furthermore 5 μ sections from the same paraffin-embedded blocks were rehydrated and stained with the same FITC-conjugated monoclonal antibodies used for peripheral lymphocytes. One section from each block was immunolabelled with a FITC-conjugated irrelevant antibody (anti-IgG) and used as a negative control. Nuclei were stained with PI (10 mg/ml) in PBS. Fluorescence was analyzed by Zeiss laser scanning microscope (LSM 510, Oberkochen, Germany).

FITC was excited at 488 nm and laser power was set at 1 milliwatt. Images were collected at 512 x 512 pixels.

Statistical analysis. Results were expressed as mean±standard deviation and the differences between groups of data were assessed by Student's *t*-test and analysis of variance. The level of significance was set at $p<0.05$.

Results

Phenotypic characterization of lymphocytes from peripheral blood. The number of CD4+ and CD8+ lymphocytes was always in

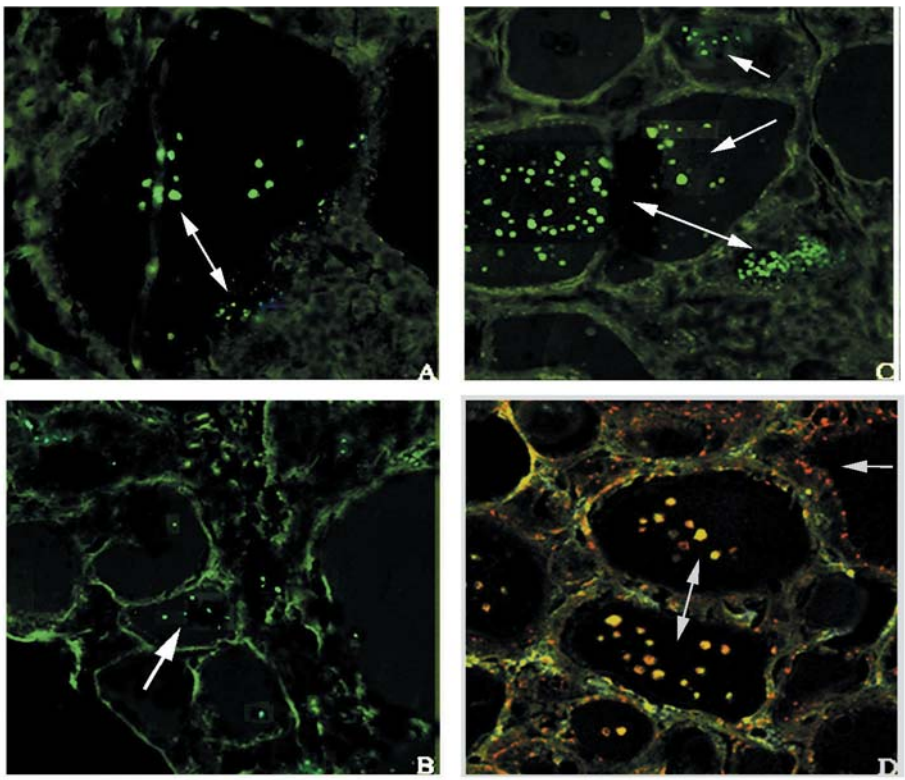

Figure 2. *Confocal laser microscopy of thyroiditis sections. Representative experiment. Panel A: lymphocytic infiltrates in follicles (arrow) and in interstitial spaces labelled with FITC conjugated-anti-CD2 antibody (green). Panel B: lymphocytic infiltrates (arrow) in follicles and in interstitial spaces labelled with FITC conjugated anti-IL2-antibody (green). Panel C: lymphocytic infiltrates (arrow) in follicles and in interstitial spaces co-immunolabelled with FITC conjugated anti-IFNγ (green). Panel D: lymphocytic infiltrates (arrow) in follicles and in interstitial spaces co-immunolabelled with FITC conjugated anti-IFNγ (green) and PE conjugated anti-CD8 (red). Overlapping signals are yellow and orange.*

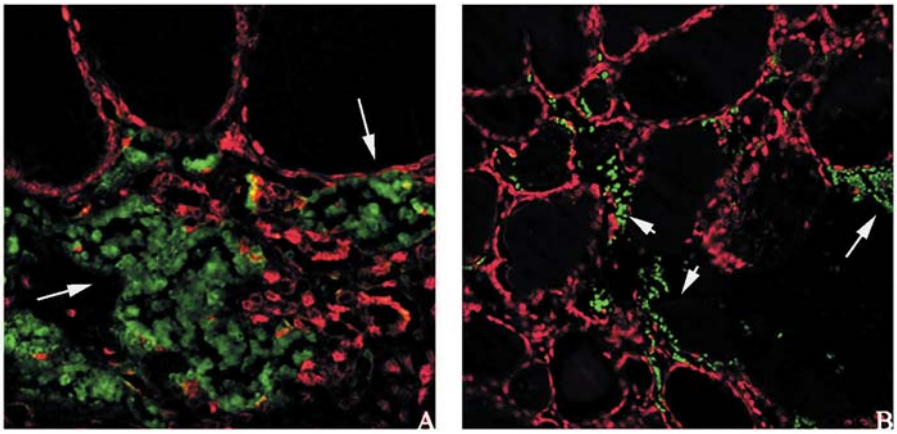

Figure 3. *Confocal microscopy of papillary tumor sections. Representative experiment. Panel A: infiltrating lymphocytes in follicles immunolabelled with FITC conjugated anti-IL4 (green). Thyroid cells and infiltrating inflammatory cells are colored with propidium iodide (red). Panel B: infiltrating lymphocytes in follicles immunolabelled with FITC conjugated anti-IL2 (green). Red colour (PI staining) highlights the follicles and the papillae.*

the normal ranges (respectively 40-53% and 22-36%) in the three groups of subjects studied. In order to detect cytoplasmic IL2, IL4 and IFNγ, from both CD4+ and CD8+ subsets, lymphocytes were double-labelled with monoclonal antibodies.

As shown in Figure 1, the positive rate for cytoplasmic IL2, IFNγ and IL4 was different in thyroiditis and tumor patients. IL2 expression was significantly higher in thyroiditis patients than in tumor patients ($p<0.01$) and the positive rate for

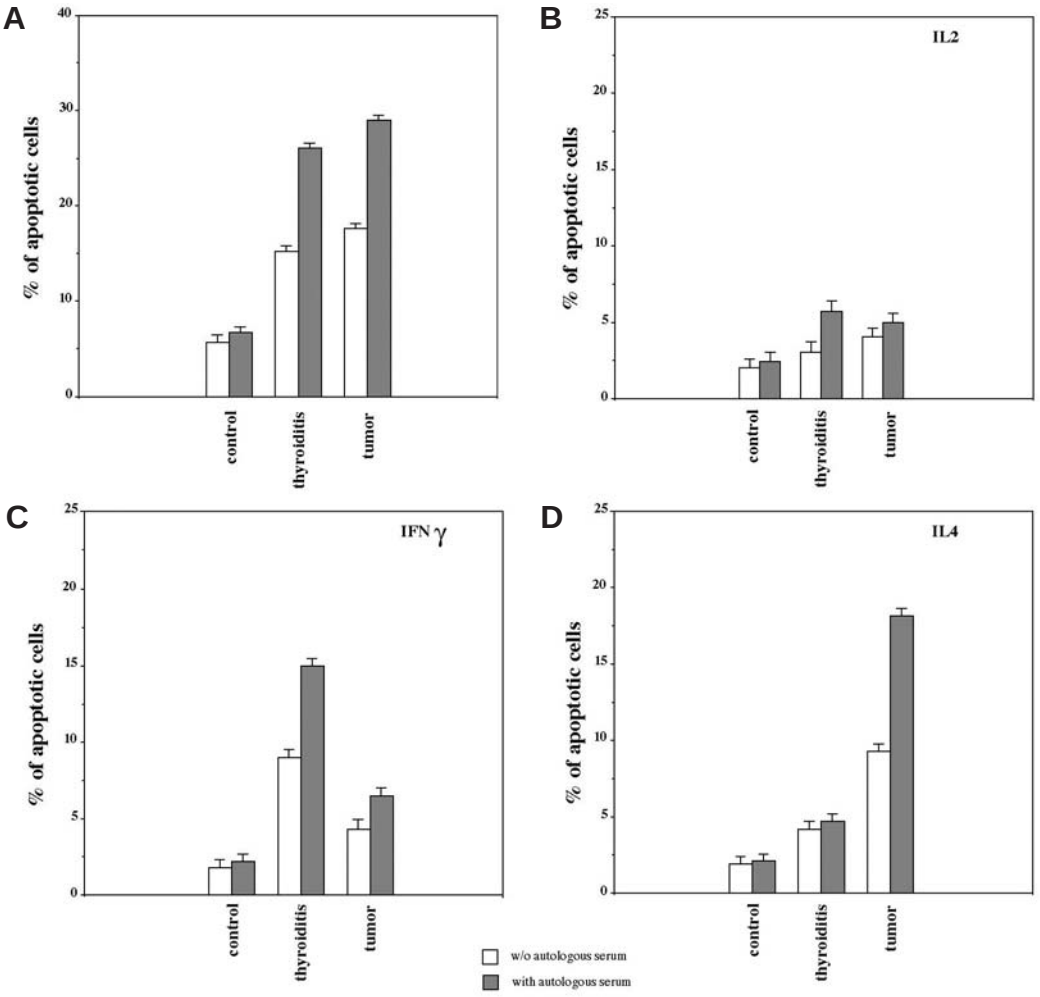

Figure 4. *Apoptosis of peripheral lymphocytes. Healthy donors (control) thyroiditis patients and tumor patients after 72 h of culture at 37˚C, 5% CO_2 without (□) or with (■) autologous serum. Panel A: apoptosis of total lymphocytes, panel B: apoptosis of IL2-positive lymphocytes, panel C: apoptosis of IFNγ-positive lymphocytes, panel D: apoptosis of IL4-positive lymphocytes. Apoptosis was determined by the binding of FITC-conjugated Annexin V and by cytofluorimetric evaluation of the hypodiploid peak after PI staining as described in Materials and Methods. Data represent the mean of all determinations in each group of subjects ±SD.*

IFNγ was dramatically higher in thyroiditis patients than in tumor patients ($p<0.0001$). Cytoplasmic IL4 was five times higher ($p<0.00001$) in tumor patients than in all the other groups studied, meaning that in the tumor patients there was a mixed population of lymphocytes producing both types of cytokines: IL2 and IL4. As shown in Table I, most of the IL4-expressing lymphocytes in tumor patients belonged to the CD8+ subpopulation, meaning that the predominant subset in this pathology was Tc2 and not Th2. On the other hand, CD8+ lymphocytes expressed IFNγ in thyroiditis patients.

Phenotypic characterization of lymphocytes from surgical samples. In order to confirm whether the same T lymphocyte subsets were found in thyroid infiltrates from the patients who had undergone surgery, we performed immunocytochemistry with the same antibodies used for peripheral lymphocytes and confocal microscope analysis. This technique was chosen in order to have a general *in situ* control about the cytokine expression. In order to show that the infiltrating cells were effectively T lymphocytes, we immunolabelled each section with FITC-conjugated anti-CD2. Figure 2A shows one representative microscopic field of a section from a patient with thyroiditis labelled with anti-CD2. Scattered lymphocytes expressed IL2 (Figure 2B) and a higher percentage of them expressed IFNγ (Figure 2C). Double fluorescence with both fluorochromes green-IFNγ and red-CD8+ (Figure 2D), showed that most of the infiltrating lymphocytes belonged to the Tc1 subset. In the samples from patients with thyroiditis IL4 (not shown) fluorescence was absent. Papillary tumors infiltrating

lymphocytes were positive for CD2 (Figure 3A) and for IL4 (Figure 3B), meaning that Th2 type lymphocytes were predominant over the Th1 type. Healthy areas from lobes not affected by either thyroiditis or papillary cancer were used as negative controls and labelled each time with the same antibodies described (not shown). This type of analysis does not allow a reliable quantification of negative or positive cells, our samples were always compared with the same sections stained with hematoxylin-eosin.

Apoptosis of lymphocytes. Because of the differences in the phenotype and in order to study the behavior of the lymphocyte subsets in patients with thyroiditis and with cancer, we analyzed lymphocyte apoptosis in peripheral lymphocytes from the three groups of subjects after different times of culture in the presence of (or without) autologous serum. Figure 4 Panel A shows the *in vitro* increased apoptosis in peripheral lymphocytes from thyroiditis ($p<0.0001$) and tumor patients ($p<0.0005$) compared with T cells from healthy donors. The addition of autologous serum increased the phenomenon in tumor and thyroiditis patients ($p<0.0001$), while it did not affect apoptosis in lymphocytes from healthy controls ($p=0.2$). Panels B, C and D show the apoptosis of lymphocytes expressing IL2, IFNγ and IL4, respectively. It is clear from the histograms that apoptosis increases in lymphocytes cultured with autologous serum and that a much higher percentage of Th1 cells undergo apoptosis in thyroiditis patients, while the highest number of apoptotic cells from tumor patients express IL4.

Discussion

It is known that Th1 lymphocytes infiltrate thyroid glands in autoimmune thyroiditis; we have shown that peripheral lymphocytes in patients with autoimmune thyroiditis are phenotypically identical to the gland-infiltrating lymphocytes. It has been shown that Th1 cytokines exert thyroid autoimmunity by CD95-mediated apoptosis. We have found that a high number of both peripheral and gland infiltrating lymphocytes that produce IFNγ belong to the CD8+ subset. This means that destruction of thyreocytes may happen in two ways: Fas- mediated apoptosis and CD8 cytotoxic activity. In tumors with lymphocyte infiltrates the local and systemic immune response is mixed. Cytokines of both types are expressed by lymphocytes: CD4+ lymphocytes express IL2 while CD8+ lymphocytes express IL4.

The presence of a mixed immune response, Th1/Tc2 in both glands and peripheral blood of papillary cancer patients may be interpreted as follows: thyroids affected by autoimmune thyroiditis are infiltrated by Th1 (Tc1) type lymphocytes which start and maintain an autoimmune process that is sometimes very mild and clinically silent. Chronic inflammation and local concentration of cytokines would have significant consequences: attempted replacement of thyreocytes causes increased cell turnover, which may contribute to neoplastic transformation and switch the immune response from Th1/Tc1 to Th2/Tc2. The rate of *in vitro* apoptosis of peripheral lymphocytes seems to have worked as a diagnostic factor in our study. Th2 cells are able to undergo apoptosis only in the presence of Th1 cells that express high levels of Fas L. Unpublished data obtained in our laboratory show a high expression of soluble Fas in the serum of the above patients. The high expression of CD95 on lymphocytes, soluble Fas and cytokines may explain the increased *in vitro* lymphocyte apoptosis (13). Most of the lymphocytes of both pathologies undergoing *in vitro* apoptosis after treatment with autologous serum belonged to the (CD45RO+CD8+) memory cytotoxic subset. Alongside their cytotoxic activity, they are able to induce helper cells (CD4+) to differentiate into Th1 or Th2 (14). Recent studies suggest a potential role for Tc1 and Tc2 subsets in tumor regression and immunotherapy (15); furthermore, it has been shown that Tc1 cells are able to kill through the Fas pathway, as well as the perforin cytolytic way (16). The latter finding confirms the interpretation of our data. The phenotypic asset of peripheral lymphocytes is used as a diagnostic factor because it parallels disease in many solid tumors. In our study, the prevalence of a different lymphocytic subset in autoimmune thyroid disease and papillary carcinoma with abundant lymphocytic infiltrate has proved diagnostic reliability. We think that because of their non–invasiveness and relative simplicity, peripheral lymphocytic studies constitute a useful tool in the decision making for thyroid surgeons when facing the diagnostic problem of nodules arising in patients with chronic thyroiditis with a suspicion of differentiated cancer and could be used in the follow-up of patients with thyroiditis.

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