

Hashimoto's thyroiditis is associated with peripheral lymphocyte activation in patients with systemic sclerosis

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Abstract

Objective

To investigate whether autoimmune thyroiditis [HT] (i.e., a TH1 disease) influences the pattern of peripheral lymphocyte activation in systemic sclerosis [SSc] (commonly regarded as a TH2 disease).

Methods

Twenty SSc patients, 6 with (SSc+HT+) and 14 without HT (SSc+HT-) and 20 controls were investigated for the intracellular content of IFN- γ and IL-4 in unstimulated and stimulated (25 ng/ml PMA and 1 μ g/ml ionomycin) CD4+ and CD8+ T lymphocytes.

Results

Under basal conditions the percentages of CD4+IFN- γ , CD4+IL-4+ and CD8+IFN- γ were significantly higher in the patients than the control subjects, no significant differences being detectable between the two patient subgroups. Upon PMA stimulation, the 20 SSc patients showed a higher percentage of CD4+IFN- γ + and CD8+IFN- γ + than the control subjects. In particular, the 14 SSc+HT- patients showed a higher number of CD4+IFN- γ + and CD4+IL-4+ cells, while the SSc+HT+ patients showed higher percentage of CD8+IFN- γ + cells. The latter patients showed a reduced percentage of CD4+IL-4+ cells and an increased percentage of CD8+IFN- γ + in comparison with the SSc+HT- patients.

Conclusion

Type-1 activation in the peripheral blood of SSc patients has been already pointed out by other authors and ourselves. This study shows that such activation mainly affects SSc patients with coexistent HT.

Key words

Hashimoto's thyroiditis, systemic sclerosis, Th1/Th2 cytokines, Tlymphocytes.

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Introduction

Differentiated human T lymphocytes can be subdivided into functionally distinct subsets based on their cytokine secretion profiles (1). The T-helper 1 (Th1) subset produces mainly IFN- γ which plays a critical role in the regulation of the cellular-mediated immune response, whereas the Th2 cells secrete IL-4, IL-5 and IL-10 which are involved in the modulation of antibody production and antigen-specific immunosuppression (2-4). Although the Th1/Th2 dichotomy is not as pronounced in humans as in animals, an imbalance between the two immune responses has been considered as an important pathogenetic feature of autoimmune and allergic diseases (5). Type-1 immune responses predominate in organ-specific autoimmune disorders such as encephalomyelitis, type-1 diabetes and Hashimoto's thyroiditis (HT), whereas the type-2 immune response characterises "systemic" autoimmune diseases (5). Systemic sclerosis (SSc) is a multisystem disease characterised by widespread vascular and microvascular damage and fibrosis of skin and internal organs (6). Although the pathogenesis of SSc is unclear, T lymphocytes are considered to play an important role (7, 8). SSc has been considered to be a Th2-mediated autoimmune disease, because of high levels of serum interleukin-4 (IL-4) and soluble CD30 and the pattern of cytokine production by T cell clones from involved skin (9,10). In the last few years, some authors (11-13) including ourselves (14, 15) have demonstrated Th1 along with Th2 activation in the peripheral blood of SSc patients. Since thyroid autoimmune disease is not an uncommon condition in SSc, serum positivity for thyroid autoantibodies having been reported in 20-40% of SSc patients (16, 17), we investigated whether the pattern of peripheral lymphocyte activation in SSc patients was influenced by the occurrence of thyroid autoimmunity.

Materials and methods

Subjects

Twenty patients consecutively admitted to the Rheumatology Unit of the Second University of Naples for SSc

between January and June 2001 were invited to take part in the study. All of them fulfilled the ACR criteria for classification of the disease (18). All of them underwent a pre-planned workup devoted to assess the extent of skin and internal organ involvement (Table I), as already reported (14). Out of the 20 SSc patients, 9 had diffuse cutaneous SSc (dSSc) and 11 limited cutaneous SSc (lSSc), according to the criteria of LeRoy *et al.* (19). All patients were screened for thyroid function and autoimmunity according to the standard clinical criteria (20). HT was diagnosed in patients with positivity for thyroperoxidase antibodies (TPOAb) and ultrasound features of thyroid immune involvement (20, 21). The inclusion criteria for the study were: 1) no steroids or other drugs that might influence the immune system in the last 12 months prior to enrolment; 2) no treatment with drugs known to induce thyroid autoimmunity and/or dysfunction (cytokines, lithium, amiodarone); 3) no pregnancy in the last 12 months prior to enrolment; 4) no smoking during the month prior to enrolment. Twenty healthy subjects, comparable in age (49.0 yr, range: 18-63) and sex ratio (18F, 2M), were recruited from the university staff as the control group. They were negative for thyroid autoantibodies and did not show clinical and biochemical findings suggestive of chronic disease. Informed consent was obtained from all subjects enrolled.

Methods

All samples were processed immediately after collection, and the biochemical assays were performed in batches on frozen aliquots. Serum free-thyroxine (FT4) and free-triiodothyronine (FT3) were tested by double antibody RIA (Technogenetics, Milan, Italy), while serum thyrotropin (TSH) was assayed by an immunoradiometric method (DIA-Sorin, Italy). Samples were assayed in duplicate for each hormone. The detection limit of the assays in SI and the intra-assay variation expressed as a coefficient of variation were: 1.2 pmol/l and 2.9% for FT3; 1.3 pmol/l, and 3.0% for FT4; 0.05 mU/l and 3.1% for TSH, respectively. In our laborato-

Table I. Demographical and clinical features of 20 patients affected by systemic sclerosis with (Gr. SSc+HT+) or without (Gr. SSc+HT-) Hashimoto's thyroiditis (HT). The data are expressed as frequencies or median and range.

Findings	SSc+HT- patients	SSc+HT+ patients	p-values
Cases	14	6	
Sex (F/M)	13/1	6/0	1
Age (years)	54 (16 – 60)	44 (41 – 55)	0.3
Disease duration*	8.8 ± 7.7	20.7 ± 6.8	0.004
Subsets (ISSc/dSSc)	5/9	6/0	0.01
Organ involvement			
Skin	14/14	6/6	1
Peripheral vascular system	14/14	6/6	1
Gut	9/14	4/6	1
Lung	14/14	5/6	0.30
Heart	5/14	4/6	0.34
Kidney	0/14	0/6	1

* From onset of the first symptom related to SSc (usually Raynaud's phenomenon).

ry, normal values were 3.8-7.7 pmol/l for FT3, 9.0-23.1 pmol/l for FT4 and 0.3-3.5 mU/l for TSH. Thyroglobulin antibodies [TgAb (negative < 100 U/ml)] were measured using the immunoradiometric assay (BioChem Immuno-System, Bologna, Italy) with intra-assay and limit of 3.9% and 5.0 U/ml, respectively. TPOAb (negative < 10 U/ml) were tested by an RIA kit (DIA-Sorin, Saluggia, Italy) with intra-assay variation and detection limit of 2.5% and 0.7 U/ml, respectively.

Peripheral blood mononuclear cells (PBMC) were obtained from whole blood by centrifugation over Ficoll (Amersham; Pharmacia Biotech). The membrane antigen expression was investigated using a panel of monoclonal antibodies (mAbs) directly coupled to fluorescein isothiocyanate (FITC), allophycocyanin (APC) or peridin chlorophyll protein (PerCP), including anti-CD3, -CD4, -CD8, -CD19, -CD16, -CD56 (Becton Dickinson, San Jose, CA). Stained cells were analysed using a FACSScan flow cytometer (Becton Dickinson, San Jose, CA). A minimum of 10,000 events was acquired and all analyses were carried out in duplicate. Data were processed using CellQuest software (Becton Dickinson, San Jose, CA).

Aliquots of PBMC in RPMI medium (500 µl for each well: 1x 10⁶ cells) were incubated for 4 hr at 37°C in a humidified atmosphere containing 5% CO₂, in the presence of activation reagent

[25 ng/ml PMAplus 1 µg/ml ionomycin (PMA+I: Sigma; St. Louis, MO)] and 10 µg/ml brefeldin-A (Sigma; St. Louis, MO) which inhibited cytokine secretion leading to their intracellular accumulation (22). Subsequently, the cells were incubated with PercP-labeled anti-CD3, FITC-labeled anti-CD4 or anti-CD8 mAbs for 20 min at 25°C. For the intracellular cytokine staining the cells were subsequently incubated for 30 min in the dark with phycoerythrin (PE) anti-IFN- and anti-IL-4 antibodies following permeabilisation with Lysing Solution (Becton Dickinson, San Jose, CA). The cells were washed once more before re-suspension in 2% paraformaldehyde prior to analysis on a FACS scan flow cytometer. The CD4+ and CD8+ cells were gated from CD3+ cells, and the intracellular cytokine expression was evaluated in each population separately, as already described (23). Intracellular expression of IL-4 and IFN- was analysed in resting and PMA-activated cells (Fig. 1).

Data were expressed as the median and ranges. Paired and un-paired data were compared using Wilcoxon's and Mann Whitney's tests, respectively. Comparisons over the three groups (control, patients with SSc+HT and patients with SSc alone) were performed using the Kruskal Wallis' test followed by a *post-hoc* Mann Whitney's test and Bonferroni's correction for multiple comparisons. Frequencies were com-

pared using the chi-square test, with Fisher's correction when appropriate. $P < 0.05$ was considered as significant.

Results

Out of the 20 SSc patients, 6 were found to have HT (Gr. SSc+HT+), whereas the remaining 14 were without thyroid autoimmunity (Gr. SSc+HT-) (Table I). In all 6 SSc+HT+ patients, thyroid autoimmune disease was diagnosed after SSc. The median duration of HT in these patients was 1.5 years (range: 0.5 – 5.0 yr). Four out of 6 SSc+HT+ patients had a clinical history of hypothyroidism [serum TSH above the normal range with thyroid hormones in or below the normal ranges] and were in treatment with l-thyroxine at enrolment. At this time serum TSH, FT4 and FT3 levels were in the normal ranges.

Table I shows the main epidemiological and clinical aspects of the 20 SSc patients divided into two groups according to the presence/absence of thyroid autoimmunity. The SSc+HT+ patients had ISSc more frequently than the patients with SSc alone (Table I).

SSc+HT- and SSc+HT+ patients showed no significant differences in CD3+, CD3+/DR+, CD4+, CD8+ CD19+ (B cells) cells and CD3-/56+/16+ cells (NK cells) (Fig. 2). In both patient groups, CD3-DR+ lymphocytes were more frequent than in the control subjects (Fig. 2). SSc+HT- patients but not SSc+HT+ patients were found to have fewer CD3-/56+/16+ cells than the control subjects (Fig. 2).

The frequencies of cytokine-producing CD4+ and CD8+ T cells were assessed before and after stimulation with PMA and ionomycin at the single cell level. Under basal conditions the frequencies of CD4+IFN- +, CD4+IL-4+ and CD8+IFN- cells were significantly higher in the patients than in the control subjects, without significant differences between the two patient subgroups (SSc+HT- and SSc+HT+) (Table II). As expected, PMA-stimulation induced a significant increase in cytokine expression in each lymphocyte subpopulation. Upon PMA activation, the whole SSc group showed higher number of IFN- producing

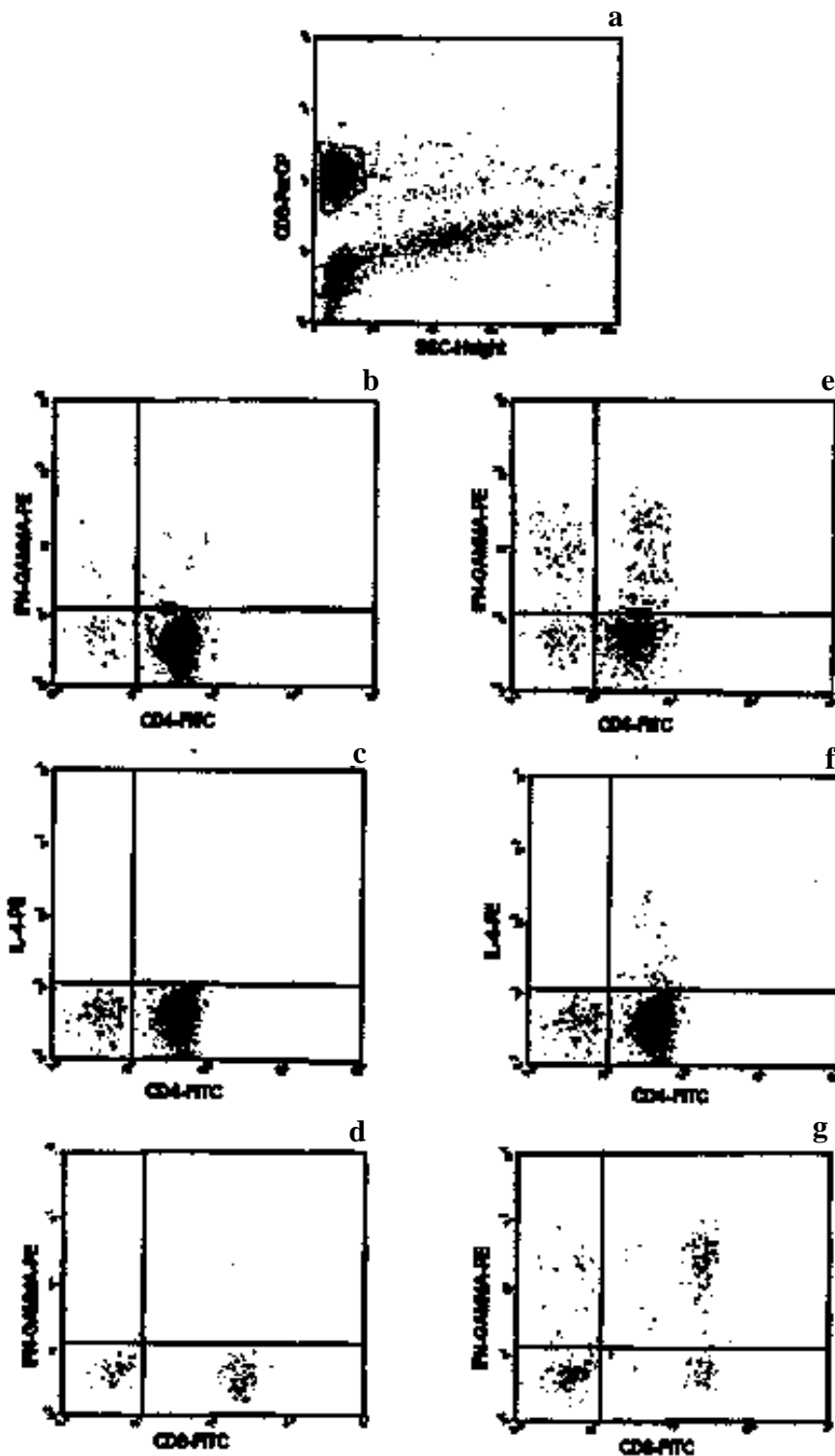


Fig. 1. Flow cytometry profile. The CD4+ and CD8+ cells were gated from CD3+ lymphocytes (a), and the intracellular cytokine expression (IFN- γ and IL-4) was analysed before (b-d) and after (e-g) stimulation with PMA(25 ng/ml) plus ionomycin (1 μ g/ml) in presence of 10 μ g/ml of brefeldin-A.

CD4+ (12.6%, range: 4.8-25.0 vs. 9.7%, range: 7.2-15.9; $p = 0.009$) and CD8+ T (16.7%, range: 8.1-30.2 vs. 11.9%, range: 4.9-17.6; $p = 0.003$) cells than the control subjects, without a sig-

nificant difference in CD4+IL-4+ cells (3.5%, range: 1.2-6.8 vs. 2.7%, range: 1.2-6.4; $p = 0.13$). Subdividing the SSc patients in relation to the presence or absence of HT, we

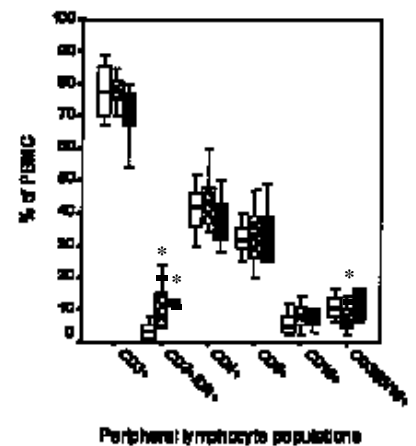


Fig. 2. Lymphocyte subpopulations in patients with systemic sclerosis (SSc) subdivided based on the presence (solid grey bars) or absence (shaded bars) of Hashimoto's thyroiditis in comparison to the control subjects (open bars). The data are expressed as median, the 25th and 75th percentiles as boxes, and the 3rd and 97th percentiles as whiskers. * $p < 0.05$ vs. control subjects.

found that the HT+ patients showed more IFN- γ producing CD8+ ($p = 0.007$) and less IL-4-producing CD4+ cells ($p = 0.004$) than the HT- patients, without a significant difference in CD4+IFN- γ + ($p = 0.40$) (Table II). With respect to the healthy subjects, the patients with SSc alone showed a higher number of CD4+IFN- γ + ($p = 0.01$) and of CD4+IL-4+ cells ($p = 0.03$), while those with SSc plus HT showed a higher percentage of CD8+IFN- γ + cells ($p < 0.001$) (Table II). No significant correlation was found between the duration of HT and the percentages of CD4+IFN- γ +, CD8+IFN- γ + and CD4+IL-4+ in SSc+HT+ patients. In the whole SSc population no significant differences in any cytokine-producing cells were demonstrated between lSSc and dSSc (Table III).

Discussion

In this study we provide evidence that SSC patients with HT present Th1 polarisation in the activation of both peripheral CD4+ and CD8+ T lymphocytes.

SSc has long been considered to be a Th2 disease (9, 10). Nevertheless, recent studies have shown both Th1 and Th2 immune activation in the peripheral blood of SSc patients (13-15). In our previous paper on this topic, we failed

Table II. Intracellular cytokine expression in CD4+ and CD8+ T cells analysed before and after PMA-stimulation in 20 patients with systemic sclerosis with [Gr. SSc+HT+ (6 cases)] or without [Gr. SSc+HT- (14 cases)] Hashimoto's thyroiditis (HT) in comparison to 20 healthy subjects. All the percentages calculated with respect to peripheral blood mononuclear cells (PBMC).

Cytokine-producing PBMC	Unstimulated PBMC			PMA-stimulated PBMC		
	SSc+HT-	SSc+HT+	Controls	SSc+HT-	SSc+HT+	Controls
CD4+IFN- +	1.5% (0.4 – 3.5)*	2.0% (1.1 – 3.2)*	0.8% (0.1 – 1.3)	13.0% (8.1 – 25.0)	12.6% (4.8 – 16.9)	9.7% (7.2 – 15.9)
CD4+IL-4+	0.9% (0.3 – 3.2) [§]	1.5% (1.2 – 3.6)*	0.5% (0.1 – 1.0)	4.1% (1.7 – 6.8)*	1.6% (1.2 – 3.8) [†]	2.7% (1.2 – 6.4)
CD8+ IFN- +	1.0% (0.3 – 2.2)*	1.2% (1.1 – 2.2)*	0.4% (0.1 – 0.9)	14.9% (8.1 – 27.3)*	24.9% (15.9 – 30.2)*	11.9% (4.9 – 17.6)

*p < 0.05 vs. control subjects; †p < 0.05 vs. SSc+HT- patients; §p = 0.05 vs. control subjects.

Table III. Intracellular cytokine patterns in the peripheral blood mononuclear cells (PBMC) from patients affected by systemic sclerosis (SSc) subdivided by disease extent [diffuse (dSSc) and localised (lSSc)] based on LeRoy *et al.* (ref. 19).

Cytokine-producing PBMC	lSSc patients	dSSc	p-values
Cases	11	9	
CD4+IFN- +	12.6% (4.8 – 25.0)	12.8% (8.6 – 24.0)	0.50
CD4+IL-4+	3.2% (1.2 – 4.4)	4.1% (1.7 – 6.8)	0.11
CD8+ IFN- +	24.9% (8.1 – 30.2)	14.4% (9.5 – 25.4)	0.07

to find any association between Th1 activation and any clinical features (15). Here, we demonstrate that Th1 activation is associated with HT, and SSc+HT+ patients more frequently have localized disease in which vascular damage is more evident. One could argue that different cytokine patterns could influence the clinical picture of SSc, Th2 activation being preferentially associated with fibroblast activation and Th1 cytokines with endothelial damage (13, 24). However, the fact that the cytokine pattern was correlated with HT but not with the disease extent would suggest that the type-1 polarization was mainly determined by thyroid disease rather than influenced by the clinical expression of SSc. Actually the small size of our study groups did not allow us to analyze the correlation between the heterogeneous clinical picture in SSc and the cytokine pattern of peripheral lymphocytes.

As in other models of T cell-mediated autoimmune diseases, cytokines play a pivotal role in the pathogenesis of HT, although with different effects in relation to the phase of disease (25). Various methods are currently employed to detect cytokine production and secretion in humans (23, 26, 27). Most of them, such as the ELISA systems, are highly specific but do not allow one to

measure cytokine production at the single cell level without the separation of cells *in vitro* (26, 27). In the present study, we used multi-parameter flow cytometry analysis to investigate the immune response separately for CD4+ and CD8+ lymphocytes among the total PBMC. Cytokine expression was measured before and after lymphocyte stimulation. Because unstimulated T-cells produce low amount of cytokines, *in vitro* stimulation is required (28). Moreover, the stimulation of lymphocytes could allow one to reproduce *in vitro* the *in vivo* activation of these cells by specific and non-specific stimuli. There are various means of "artificial" stimulation, such as polyclonal activation by lectin and lipopolysaccharide, monoclonal stimulation by specific antibodies (anti-TCR receptor, anti-CD3, anti-CD28), and pharmacological activation (29). Advantages and disadvantages have been pointed out for each of these (30, 31). Indeed, the use of stimulating monoclonal antibodies has proved invaluable for studying the TCR-mediated lymphocyte activation *in vitro*. However, they may not mimic the physiologic ligand-binding event with respect to epitope specificity, avidity, or valency (29). Furthermore, their functional effects could be dependent on the experimental conditions

(31). Pharmacological stimulus, as used in the present study, is not physiological but permits a rapid and reproducible stimulation of T cells by the activation of the transduction mechanisms used by the T-cell receptor (23, 29). Furthermore, the pharmacological stimulus permits us to detect even the low level expression of cytokine, as IL-4 (30).

In HT, the type-1 cytokines seem to play a predominant role in driving the immune response toward the cell-mediated damage of the thyroid gland (25, 32, 33), whereas type-2 cytokines would exert controlling inhibitory effects (25, 32, 33). We already demonstrated that type-1 activation is associated with the occurrence of Hashimoto's hypothyroidism, whereas the type-2 immune response occurs predominantly in euthyroid HTpatients (34). In agreement with this observation, in the present study our SSc-HT+ patients were hypothyroid with predominant type-1 activation, without any significant increase in the type-2 immune response. Cellular immune responses have been studied in the circulating and infiltrating lymphocytes of patients with thyroid autoimmune disease (32, 34). Since in organ-specific diseases the autoimmune state is reliably reflected in the target organ, analysis of the intra-thyroid lymphocytes is probably the best way to investigate the immunological status in patients with thyroid autoimmunity (32). At present, since thyroid fine-needle aspiration is not frequently performed in HT patients, the data available is primarily based on tissue obtained surgically, which probably does not reflect the situation *in vivo* (35). Therefore, the analysis of peripheral lymphocytes could

be the easiest manner to study the immunological status of the patients with thyroid autoimmunity. Indeed, most studies have found an activated phenotype in peripheral blood from HT patients, suggesting a generalised immune dysregulation in this disease (35-38). The occurrence of peripheral clinical manifestations in patients with thyroid autoimmunity has been reported, even when thyroid function is normal (39,40). In the present study we provide further evidence for the generalised activation of the immune system in this disease, because the peripheral cytokine patterns of SSc patients were markedly modulated by coexistent HT. However, we investigated just some of the aspects of the type-1 and type-2 immune responses. In particular, we did not investigate the effects of HT on the expression of chemokines, which play a critical role in the traffic and homing of lymphocytes in organ-specific autoimmune disease (35). Further studies will allow us to clarify whether HT could modulate the expression of homing molecules in SSc (13).

In our present study, HT was found to be confined to SSc patients with limited cutaneous disease. This is not always the case, even in our experience (41, 42). Nevertheless, our results should be considered to reflect the influence of HT on immune activation in lcSSc.

In conclusion, this study shows that the pattern of lymphocyte activation in patients with SSc is influenced by coexistent HT, and provides evidence that the latter may be a systemic disease sustained by a generalised activation of the immune system regardless of the antigen-specific immune response. Further studies will be needed to clarify whether a "systemic" approach to HT, a so-called "organ-specific" autoimmune disease, could guarantee the better management of affected patients, through the recognition of peripheral features of autoimmunity which could be independent of the functional impairment of the target organ.

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