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CLINICAL STUDY

Type-1 response in peripheral $CD4^+$ and $CD8^+$ T cells from patients with Hashimoto's thyroiditis

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Abstract

Objective: In this study we performed single-cell analysis of the intracellular cytokine expression in peripheral $CD4^+$ and $CD8^+$ lymphocytes from patients with Hashimoto's thyroiditis (HT) to investigate the type-1 response separately for the two lymphocyte sub-populations.
Design and methods: Twenty-nine patients affected by HT and 20 healthy subjects, matched for sex and age, were enrolled. After the analysis of the lymphocyte sub-populations, the intracellular content of interferon-γ (IFN-γ) in phorbolmyristate acetate (PMA)-stimulated $CD4^+$ and $CD8^+$ lymphocytes was assayed. Moreover, the $CD4^+$ lymphocytes were also evaluated for the intracellular expression of IL-4.
Results: No significant differences in $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes were found between HT patients and control subjects. However, the HT patients showed higher numbers of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$ (*t*-test, $P \leq 0.001$) cells than the control subjects. Analysing the intracellular expression of IFN-γ and IL-4 in relation to thyroid function, we found that the euthyroid patients (*18 cases*) showed more expression of IL-4 in $CD4^+$ lymphocytes than the control subjects, without any significant modification of IFN-γ expression in $CD4^+$ and $CD8^+$ lymphocytes. However, the hypothyroid patients (*11 cases*) showed an increase of IFN-γ expression in both $CD4^+$ and $CD8^+$ lymphocytes with respect to the control subjects and the euthyroid patients. Moreover, the expression of IL-4 in $CD4^+$ cells from hypothyroid patients was significantly lower than that seen in the euthyroid cases and comparable to that found in the control subjects.
Conclusions: Our study has demonstrated that the peripheral $CD4^+$ and $CD8^+$ T lymphocytes from the HT patients show a type-1 activation strictly correlated to the occurrence of hypothyroidism. Further studies will be needed to clarify the exact role of peripheral lymphocytes in HT and whether they could provide a reliable marker of thyroid immune involvement.

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Introduction

Differentiated human T lymphocytes can be subdivided into functionally distinct subsets based on their cytokine secretion profiles (1–3). The T-helper 1 (Th1) subset produces mainly interferon-γ (IFN-γ), which has a critical role in the regulation of the cellular-mediated immune response; whereas the Th2 cells secrete interleukin-4 (IL-4), IL-5 and IL-10, which are involved in the modulation of antibody production and antigen-specific immunosuppression (1–3). In animal models of autoimmune diseases, Th1 cells mediate tissue damage and disease progression whilst Th2 cells are associated with disease remission and suppression of the immune response (4).

In thyroid autoimmunity both Th1 and Th2 responses are found (5–9), although with different reciprocal intensity in relationship with the clinical expression of the disease process (9). The type-1 response, in particular, seems to be dominant in Hashimoto's thyroiditis (HT), suggesting the physiopathological importance of a cytotoxic process in this disease (9–13). Although experimental and clinical evidence suggests that both the $CD4^+$ and $CD8^+$ T compartments play a critical role in the development of autoimmune thyroiditis (14–18), a functional analysis of cytokine expression has been performed separately for the two lymphocyte sub-populations in only a few cases (9, 10). The widespread use of flow cytometry has fundamentally affected the analysis of the structure and lineage of the cells that comprise the immune

system. In particular, intracellular cytokine staining has played an important role in defining functional subsets in mixed T-cell populations, allowing both the frequency of cells producing specific cytokines to be estimated and, to some extent, the levels of expression to be compared (19).

In this study we have performed single-cell analysis of the intracellular cytokine expression, and especially the type-1 response separately in the peripheral $CD4^+$ and $CD8^+$ lymphocytes from patients with HT.

Materials and methods

Subjects

This study was performed on 29 patients (20 females, 9 males; mean age: 39.0±3.7 years, range: 17–58) affected by HT who were admitted to our hospital between January and December 2001. The inclusion criteria were: (1) positivity for anti-thyroglobulin and/or anti-peroxidase antibodies (TgAb and TPOAb respectively) at high titres; (2) negativity for anti-TSH receptor antibodies (TRAb); (3) thyroid ultrasound imaging suggestive of a chronic thyroiditis (20); (4) no clinical history of hyperthyroidism; (5) no treatment with drugs known to induce thyroid autoimmunity and/or dysfunction (cytokines, lithium, amiodarone); (6) no pregnancy in the last 12 months prior to enrolment; and (7) non-smoker during the month prior to enrolment. The control group comprised 20 healthy subjects recruited from this establishment, matched for sex and age with the HT patients. At enrolment, acute and chronic systemic diseases, other than HT, were excluded both in the patients and the control subjects. Thyroid function (serum free thyroxine (FT_4), free tri-iodothyronine (FT_3) and thyrotrophin (TSH)) and autoimmunity (serum TgAb, TPOAb and TRAb) were assayed in all 49 subjects. We also evaluated the lymphocyte sub-populations and the intracellular content of IFN-γ and IL-4 of phorbolmyristate acetate (PMA)-stimulated peripheral T lymphocytes. The analysis of the intracellular cytokine expression was performed separately for $CD4^+$ (IFN-γ and IL-4) and $CD8^+$ (IFN-γ) lymphocytes. The biochemical and immunological analysis was performed on fresh aliquots of blood drawn after a 12 h fast.

Methods

All samples were processed immediately after collection. Serum FT_4 and FT_3 were tested by double antibody RIA (Technogenetics, Milan, Italy), while serum TSH was assayed by an immunoradiometric method (DIA-Sorin, Saluggia, Italy). Samples were assayed in duplicate for each hormone. The detection limit of the assays in SI units and the intra-assay variation expressed as coefficient of variation were: 1.2 pmol/l and 2.9% for FT_3; 1.3 pmol/l and 3.0% for FT_4; 0.05 mU/l and 3.1% for TSH respectively. In our laboratory, normal values were 3.8–7.7 pmol/l for FT_3, 9.0–23.1 pmol/l for FT_4 and 0.3–3.5 mU/l for TSH. TgAb (negative < 100 U/ml) were measured using the immunoradiometric assay (BioChem ImmunoSystem, Bologna, Italy) with an intra-assay coefficient of variance and detection limit of 3.9% and 5.0 U/ml respectively. TPOAb (negative < 10 U/ml) were tested by RIA set (DIA-Sorin) with an intra-assay and detection limit of 2.5% and 0.7 U/ml respectively. TRAb (negative < 10 U/ml) were tested by radioreceptor assay (Henning, Berlin, Germany).

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 coupled directly to peridin chlorophyll protein (PerCP) and fluorescein isothiocyanate (FITC), including anti-CD3, -CD4 and -CD8 (Becton Dickinson, San Jose, CA, USA). Stained cells were analysed using a FACSscan flow cytometer (Becton Dickinson). A minimum of 10 000 events were acquired and all analyses were carried out in duplicate. Data were processed using CellQuest software (Becton Dickinson). IgG1 isotype controls (Becton Dickinson) were utilised to establish background fluorescence.

Aliquots of PBMC in RPMI medium (500 μl for each well: 1×10^6 cells) were incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2, in the presence of activation reagent (25 ng/ml PMA plus 1 μg/ml ionomycin (PMA+I: Sigma, St Louis, MO, USA)) and in the presence of 10 μg/ml of brefeldin-A (Sigma), which inhibited cytokine secretion leading to their intracellular accumulation (21). Subsequently, the cells were incubated with FITC-labelled anti-CD4 or anti-CD8 monoclonal antibodies 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-γ and PE-anti-IL-4 antibodies following permeabilisation with Lysing Solution (Becton Dickinson). The cells were washed once more before re-suspension in 1% paraformaldehyde prior to analysis on a FACS scan flow cytometer. The T lymphocytes were gated on the basis of right-angle light scatter (SSC) and CD3 phenotype (Fig. 1). The gated data were plotted in graphs of cytokine vs CD4 and vs CD8 (Fig. 1). The quadrants were set according to the unstimulated cells (control). The cytokine-positive population was calculated from the quadrant analysis by subtracting the unstimulated cytokine-positive population from the stimulated one as an internal control.

Data are expressed as means±S.D. Unpaired samples were compared using the *t*-test. Multiple groups were compared using ANOVA with Bonferroni's *post hoc* tests. Percentages were compared using the χ^2 test, with Fisher's correction when appropriate. $P < 0.05$ was considered as significant.

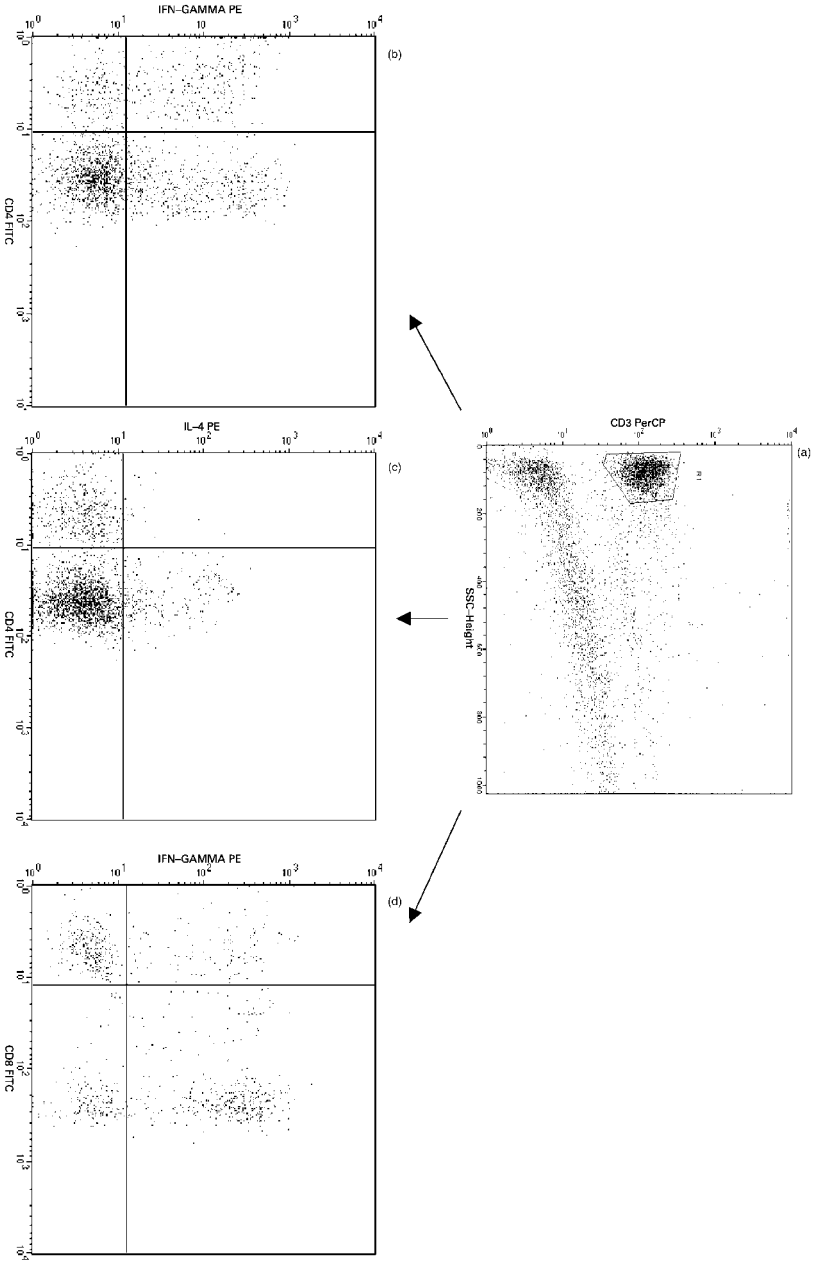

Figure 1 Flow cytometry profile. The T lymphocytes were gated on the basis of right-angle light scatter (SSC) and CD3 phenotype (a). The gated data were plotted as a cytokine vs CD4 (b and c) and vs CD8 (d).

Results

At enrolment, all 29 patients with HT were positive for TgAb and/or TPOAb, but all were negative for TRAb (Table 1). Eleven of the HT patients had a clinical history of hypothyroidism and they were treated with L-thyroxine at enrolment. The mean duration of this treatment was 3.0±1.0 years (range: 2–6). All 20 control subjects were euthyroid and negative for thyroid autoantibodies.

No significant difference in $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes was found between HT patients and control subjects (Table 2). Similarly, the CD4/CD8 ratio was not significantly different between the two groups (Table 2). However, the HT patients showed a higher number of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$ cells than the control subjects (Table 2). Analysing the intracellular expression of IFN-γ and IL-4 in relation to the thyroid function, we found that the euthyroid patients (*18 cases*) showed more expression of IL-4 in $CD4^+$ lymphocytes than the control subjects, without any significant modification of IFN-γ expression in $CD4^+$ or $CD8^+$ lymphocytes (Fig. 2). However, the hypothyroid patients (*11 cases*)

Table 1 Demographic and clinical data for the 29 patients affected by HT in comparison to the 20 control subjects at enrolment. The data are expressed as means±S.D.

Variables	Control group	Study group	*P* values
No. of cases	20	29	
Sex (F/M)	15/5	(20/9)	0.7
Age (years)	44.1±10.6	43.3±9.3	0.8
Serum TSH values (mU/l)	1.6±0.4	1.8±0.7	0.2
Serum FT_4 values (pmol/l)	16.4±2.2	15.2±3.2	0.1
Serum FT_3 values (pmol/l)	4.6±0.2	4.5±0.4	0.2
Serum TgAb levels (U/ml)	53±12	2.011±754	**<0.001**
Serum TPOAb levels (U/ml)	4.2±0.8	3.011±1.645	**<0.001**
Serum TRAb levels (U/l)	2.6±0.80	3.3±1.1	0.3

showed an increase of IFN-γ expression in both $CD4^+$ and $CD8^+$ lymphocytes with respect to the control subjects and the euthyroid patients (Fig. 2). Moreover, the expression of IL-4 in $CD4^+$ cells from hypothyroid patients was significantly lower than that seen in the euthyroid cases and comparable to that found in the control subjects (Fig. 2). The Th1/Th2 ratio in the $CD4^+$ cells was 3.1±1.1 in the euthyroid and 7.3±5.2 in the hypothyroid patients ($P = 0.002$).

Discussion

This study has demonstrated that patients with HT show a type-1 response that involves both the peripheral $CD4^+$ and $CD8^+$ lymphocytes. Moreover, we observed that the cytokine expression in these lymphocyte sub-populations was also different in relation to the occurrence of thyroid dysfunction.

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 (22, 23). Various methods are currently employed to detect cytokine production and secretion in humans (19, 24, 25). Most of them, such as the ELISA systems and the cytometric bead arrays, are highly specific but do not allow the measurement of cytokine production at the single-cell level unless separation of cells *in vitro* is performed (24, 25). In the present study, we used the multi-parameter flow cytometry analysis to investigate the immune response separately for the $CD4^+$ and $CD8^+$ lymphocytes among the total PBMC. As unstimulated T cells usually produce no measurable cytokines (26), *in vitro* stimulation is required. 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 (27). Advantages and disadvantages have been described for each of these methods (27, 28). 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 physiological ligand-binding event with respect to epitope specificity, avidity or valency (27). Furthermore, their functional effects could be dependent upon the various experimental conditions (29). The 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 (19, 27). Furthermore, the pharmacological stimulus allows the study of cytokines that are expressed at low levels, such as IL-4 (28).

Cellular immune responses have been studied in circulating (6, 12, 18) and infiltrating lymphocytes (6–10) of the patients with thyroid autoimmune disease. Since in the organ-specific diseases the reliable reflection of the autoimmune state lies in the target organ, the analysis of the intra-thyroid lymphocytes is probably the best way to investigate the immunological status in the patients with thyroid autoimmunity (30). Actually, as thyroid fine-needle aspiration is not frequently performed in HT patients, the data available

Table 2 Immunological data for the 29 patients affected by HT in comparison with those for 20 healthy subjects. All values are expressed as a percentage of the peripheral blood mononuclear cells (PBMC). The data are expressed as means±S.D.

Immunological parameters	Control subjects (20 cases)	Patients with HT (29 cases)	*P* values
$CD4^+$ PBMC	42.0±6.3	40.5±6.9	0.4
$CD8^+$ PBMC	31.4±4.3	33.1±9.1	0.5
$CD4^+/CD8^+$ PBMC	1.4±0.3	1.3±0.5	0.8
$CD4^+$IFN-γ^+ PBMC	11.8±2.4	17.3±5.3	**0.02**
$CD4^+$IL-4^+ PBMC	3.1±1.1	4.5±1.5	**0.009**
$CD8^+$IFN-γ^+ PBMC	12.3±3.4	20.3±7.8	**<0.001**

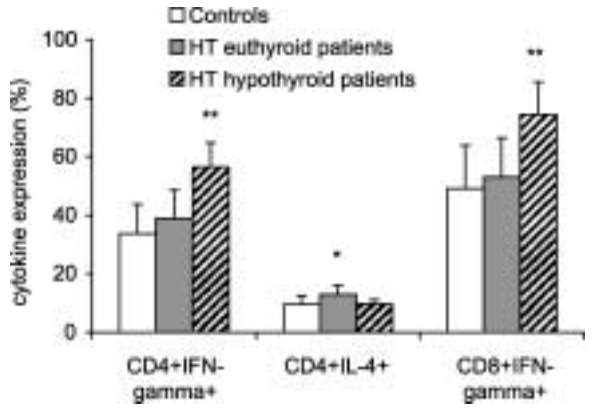

Figure 2 Intracellular cytokine expression in $CD4^+$ and $CD8^+$ T cells analysed separately for euthyroid (18 cases) and hypothyroid (11 cases) HT patients in comparison with the control subjects (20 cases). $^*P < 0.05$ vs control subjects; $^{**}P < 0.05$ vs control subjects and euthyroid HT patients. The data are expressed as means±S.D.

are mostly obtained from surgical tissue that probably does not reflect the situation present *in vivo* (30). Therefore, the analysis of peripheral lymphocytes could be the easiest way 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 disregulation in such diseases (30, 31). According to this finding, our patients showed a peripheral intra-lymphocyte cytokine pattern that was different from the healthy subjects, suggesting the existence in the peripheral compartment of markers of the autoimmune process. The fact that our patients were without coexistent acute or chronic diseases has enabled us to correlate the cytokine abnormalities to the manifestation of thyroid disease.

T lymphocytes are effectors of tissue damage in many autoimmune diseases and are also involved in the regulation of the autoimmune responses by the production of many cytokines. Although the coexistence of Th1- and Th2-type responses has been demonstrated in patients with HT (9), experimental (32) and clinical (5, 6, 12, 33) evidence suggests that the type-1 cytokines play a dominant role in pathogenesis of the disease process. In the present work, we have demonstrated that the type-1 lymphocyte activation can occur in peripheral lymphocytes, as well as in the thyroid gland (9). The single-cell analysis showed an involvement of the both $CD4^+$ and $CD8^+$ lymphocyte compartments in the type-1 activation. An interesting finding was that this activation occurred specifically in the patients with hypothyroidism. In those with normal thyroid function there was predominantly a hyper-expression of IL-4 in the $CD4^+$ lymphocytes. Such a result suggests a different immunological status for euthyroid and hypothyroid HT patients, and is in agreement with the experimental evidence demonstrating an important role of Th2 cytokines in the control of the Th1-mediated autoimmune process (1, 34–36). The fact that our hypothyroid patients were on long-term L-thyroxine treatment suggests that the immunological abnormalities described could not be caused by impairment of thyroid function, as has been suggested previously (12). In this view, the peripheral lymphocytes might express specific markers reflecting the degree of organ involvement in HT. The frequency of specific responding T cells for any given antigen is too low to be studied without expansion by specific stimulation. Therefore, the Th1 polarisation demonstrated in our hypothyroid patients could have reflected an involvement of non-specific T-cell compartments in the thyroid autoimmune disease. Although the few autoreactive cells have a critical role in driving the autoimmune process (37), there is experimental evidence to suggest that it is difficult to induce autoimmune disease without the contribution of the non-specific infiltrate (37–39).

In conclusion, our study has demonstrated that the peripheral $CD4^+$ and $CD8^+$ T lymphocytes from the HT patients show a type-1 activation strictly correlated to the occurrence of hypothyroidism. Further studies will be needed to clarify the exact role of peripheral lymphocytes in HT and whether they could provide a reliable marker of thyroid immune involvement.

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