

INNATE AND ACQUIRED IMMUNE SYSTEM IN PATIENTS DEVELOPING INTERFERON-ALFA-RELATED AUTOIMMUNE THYROIDITIS: A PROSPECTIVE STUDY.

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ABSTRACT

Objective. In this prospective study, we investigated whether the development of interferon-alfa (IFN- α)-related autoimmune thyroiditis (IFN-AT) was correlated with the sequential changes of cytokine pattern induced by the IFN- α in the peripheral lymphocytes.

Patients and methods. We enrolled 18 HCV+ patients who developed IFN-AT, of whom 8 with euthyroidism [IFN-AT(Eu)] and 10 patients with thyroid dysfunction [IFN-AT(Dy)]. Twenty HCV+ patients without IFN-AT acted as control group (Co-HCV+). Intracellular expression of IFN- γ and IL-4 was evaluated by multicolour flow-cytometry analysis in peripheral lymphocytes *in vitro* stimulated by PMA (25 ng/ml) and ionomycin (1 μ g/ml), in presence of monensin (5 μ M).

Results. At the appearance of thyroid disease, both IFN-AT(Eu) and IFN-AT(Dy) patients showed a significant increase of IFN- γ expression in CD3+CD56+ and CD3-CD56+ cells but not in CD4+ and CD8+ cells. At this time-point, IFN-AT(Eu) but not IFN-AT(Dy) patients showed also an increase of IL-4 expression in CD3+CD56+ cells and CD4+ cells. Six months later, IFN-AT(Eu) patients maintained high expression of IL-4 in CD4+ and CD3+CD56+ cells, without any further increase in IFN- γ expression. By contrast, IFN-AT(Dy) patients showed an increase of IFN- γ expression in CD4+ and CD8+ cells, with a concomitant decrease of IL-4 expression in CD4+ cells.

Conclusions. Type-2 immune response is activated early and specifically in patients with IFN-AT who remain euthyroid throughout the follow-up. In patients developing thyroid dysfunction, by contrast, is predominant the type-1 immune response that seems to occur earlier in innate than in acquired immune system.

INTRODUCTION

Thyroid disorders are a frequent side-effect of interferon-alfa (IFN- α) therapy [reviewed in Refs 1 and 2]. Positive thyroid antibodies with normal thyroid function tests is the commonest finding in patients treated with IFN- α whereas thyroid dysfunction is usually described in no more than 15% of all treated patients (3). Thyroid dysfunction may present as destructive thyrotoxicosis, Graves' thyrotoxicosis, and hypothyroidism. Indeed, destructive thyrotoxicosis and hypothyroidism are more frequent than Graves' hyperthyroidism, since only 20-25% of all patients with IFN-related thyrotoxicosis is due to Graves' disease induced by circulating thyroid receptor antibodies (TRAb) (3, 4). Destructive thyrotoxicosis and hypothyroidism have been considered as different expressions of the same disease at different time of development (5). It has been pointed out that IFN-related thyroid autoimmune disease (IFN-AT) may reproduce in its short-time and long-term-outcome the natural history of Hashimoto's thyroiditis (HT) (6). Indeed, IFN-AT has been proposed as a model for thyroid autoimmunity (7), although the immunological aspects of this relationship remain to be clarified.

So far, almost all studies have focused on the clinical aspects of IFN-AT, by the evaluation of thyroid autoantibody patterns and clinical pictures of thyroid dysfunction (3). However, there are limited data about the cellular immunological events accompanying the IFN-AT development. The main effect of IFN- α on the immune system is the enhancement of cell-cytotoxicity, very likely sustained by suppression of T helper 2 (Th2) and an increase in Th1 immune response (8-13). Although these effects seem to be transient (14), it has been suggested that the generalized Th1 activation induced by IFN- α may be important for the occurrence of thyroid autoimmunity (15). However, IFN- α was also shown to induce the production of type-2 cytokines (16, 17). In thyroid autoimmunity both Th1 and Th2 responses are found, although

with different reciprocal intensity in relationship with the clinical expression of the disease process (18-20). The type-1 response, in particular, seems to be dominant in hypothyroid patients with HT whereas type-2 immune response has been found in patients with thyroid autoimmunity and normal thyroid function (21). These differences were demonstrated at thyroid levels and in the peripheral lymphocytes (20, 21).

In this study we analysed the dynamics of peripheral lymphocyte responses during IFN- α plus ribavirin treatment in relation to the development of autoimmune thyroiditis. The evaluation of sequential modifications of Th1 and Th2 immune response in patients developing IFN-AT would provide information about the evolution of cellular immune response in the early phase of development of thyroid autoimmune process.

Subjects

The study group included 18 patients with HCV-related chronic hepatitis (10 females, 8 males, median age: 42 years, range: 21-56) who developed thyroid autoimmune disease during IFN- α plus ribavirin treatment. All of them were without thyroid disorders before the antiviral treatment. Twenty HCV+ patients who did not develop any thyroid disorder during IFN- α treatment were enrolled as control group (Co-HCV+ group). The Co-HCV+ subjects had comparable sex (13 females, 7 males) and age (44.5 years, range: 20-65) with the patients developing IFN-AT. The patients and the control subjects were retrospectively selected from a population of 90 patients affected by HCV-related chronic hepatitis who performed a full course of treatment with pegylated-IFN-alfa (1.5 μ g/kg/weekly subcutaneously) and ribavirin (1.0-1.2 g/day orally) in the period between 2002 and 2004. These patients were enrolled taking into account the following exclusion criteria: 1) thyroid autoimmunity and/or dysfunction before starting IFN- α treatment;

2) pregnancy in the latter 12 months prior the enrolment; 3) duration of IFN- α treatment shorter than 12 months. At the study entry, the both IFN-AT patients and Co-HCV+ subjects were asked for the presence of thyroid autoimmunity in their relatives. Thyroid function [serum thyrotropin (TSH), free-thyroxine (FT4) and free-triiodothyronine (FT3)] and autoimmunity [serum thyroglobulin (TgAb), thyroperoxidase (TPOAb) and TSH receptor antibodies (TRAb)] were evaluated at baseline and every 4 to 6 weeks for the first 6 months of treatment, and then every 12 to 15 weeks until the end of the treatment. The patients developing thyroid autoimmunity (23 cases) were selected on the basis of appearance of thyroid autoantibodies with or without thyroid dysfunction [thyrotoxicosis alone (suppressed serum TSH values, normal or high serum FT3 and FT4 values, a low radioiodine uptake and negative TRAb), hypothyroidism alone (high serum TSH values, low or normal serum FT4 and FT3 values), thyrotoxicosis followed by hypothyroidism, hyperthyroidism (suppressed serum TSH values, normal or high serum FT3 and FT4 values, a high radioiodine uptake and positive TRAb)]. For the purpose of this study, the patients with hyperthyroidism (2 cases) were excluded from the analysis. Moreover, 3 patients with IFN-AT were excluded because they stopped early the IFN- α treatment. Therefore, 18 patients IFN-AT were investigated in the present study.

In IFN-AT patients the immunological evaluations were performed on frozen samples drawn before starting antiviral treatment (T0), at the time of appearance of thyroid disease (T1) and six months later (T2). In Co-HCV+ patients the immunological evaluations were performed before commencing IFN treatment (T0), and at 3-5 (T1) and 9-11 month (T2) of the treatment.

Informed consent was obtained in each IFN-AT and Co-HCV+ patient and the study was approved by local Ethical Committee.

Methods

Serum FT4 and FT3 concentrations were measured by double antibody RIA (Technogenetics, Milan, Italy); serum TSH was assayed by an immunoradiometric method (DIA-Sorin, Italy). Samples were assayed in duplicate for each hormone. Normal ranges are as follows: TSH, 0.3-3.5 μ U/ml (SI: 0.3-3.5 mU/l), FT4, 7.0-17.9 pg/ml (S.I: 9.0-23.1 pmol/l), FT3, 2.5- 5.0 pg/ml (SI: 3.8-7.7 pmol/l). The sensitivity and the intra-assay coefficient of variation for TSH measurements were 0.05 μ U/ml (SI: 0.05 mU/l) and 3.1%, respectively.

TgAb (negative <100 U/ml) were measured using the immunoradiometric assay (BioChem ImmunoSystem, Bologna, Italy) with intra-assay and limit of 3.9% and 5.0 U/ml, respectively. TPOAb (negative <10 U/ml) were tested by RIA set (DIA-Sorin, Saluggia, Italy) with intra-assay and detection limit of 2.5% and 0.7 U/ml, respectively. Serum TRAb were measured with the DYNOfest TRAK human (BRAHMS AG, Berlin, Germany). This radioreceptor assay uses hTSHR on coated tubes, human antibodies for standard material, and expresses the results in international units (IU) based on a WHO standard (22). For TRAb values between 0.5 and 5.0 IU/l, intra-assay and inter-assay coefficients of variation were 9.6% and 13.0%, respectively. TRAb positivity was defined for values above 1.5 UI/l.

The immunological analyses were performed according to the procedure already described (21). Peripheral blood monuclear cells (PBMC) were obtained from peripheral blood collected in EDTA (15 ml) by density gradient centrifugation over Ficoll Histopaque-1077. The PBMC were re-suspended in aliquots of 2×10^6 cells in 1 ml of freezing mixture (50% fetal calf serum, 40% RPMI 1640, 10% dimethyl sulphoxide) and frozen at a rate of 1 °C/minute until the temperature reached -85 °C when they were transferred for long-term storage to liquid nitrogen.

The membrane antigen expression was investigated using a panel of monoclonal antibodies (mAbs) directly coupled to allophycocyanin (APC) or peridin chlorophyll protein

(PerCP), including anti-CD3, -CD4, -CD8 and -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). IgG1 isotype controls (Becton Dickinson, San Jose, CA) were utilised to establish background fluorescence.

The functional analysis consisted of evaluation of cytokine production from the above cell populations before and after pharmacological stimulation. Aliquots of PBMC in RPMI medium (500 μ l for each well: 1×10^6 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 PMA plus 1 μ g/ml ionomycin (PMA+I: Sigma; St. Louis, MO)] and in the presence of 5 μ M of monensin (Sigma; St. Louis, MO) which inhibited cytokine secretion leading to their intracellular accumulation (23). After 4 hours incubation, cell viability were assessed by Trypan-Blue exclusion. Subsequently, the cells were incubated with APC-labeled anti-CD4 or anti-CD8 mAb for 20 min at 25° C. After washing [phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% NaN₃] the PBMC cells were incubated [30 mins, room in the dark] with FACS permeabilising solution (Becton Dickinson, San Jose, CA) and conjugated anti-cytokine monoclonal antibodies [isothiocyanate (FITC) anti-IFN- γ and phycoerythrin (PE)- anti-IL-4 antibodies] (24). IFN- γ was investigated as marker of type-1 immune response, whereas IL-4 was detected as markers of type-2 response. CD8⁺ and CD3-CD56⁺ cells did not show detectable IL-4 even after stimulation, therefore this cytokine was studied only in CD4⁺ and CD3⁺CD56⁺. The cells were washed once more before re-suspension in 1% 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. CD56+ cells were subdivided in two supopulations (CD3+ and CD3-).

Data were presented as mean±SEM. Paired and un-paired data were compared using Wilcoxon's and Mann-Whitney's tests, respectively. Multiple comparisons were made by Friedman's and Kruskal Wallis' tests, with *post hoc* Bonferroni's correction. Frequencies were compared using Chi-square's test, with Fisher's correction, when appropriate. Statistical significance was assumed when the probability (p) was less than or equal to 0.05.

Results

In all IFN-AT patients thyroid disease appeared during the first 6 months of IFN- α treatment. In 10 of them, thyroid autoimmunity was accompanied by different degree of thyroid dysfunction [IFN-AT(Dy)] (Tab. 1), whereas 8 patients remained biochemically and clinically euthyroid [IFN-AT(Eu)] throughout the treatment, even if serum TSH concentrations decreased slightly in some of them (Tab. 2).

At the study entry, 2 Co-HCV+ subjects (10.0%) and 7 IFN-AT patients (38.9%) were aware that their relatives had autoimmune thyroid disease (Chi-square: 4.37; p=0.06). Before starting IFN- α treatment, patients developing IFN-AT with or without thyroid dysfunction showed no significant differences in cytokine pattern with respect to Co-HCV+ subjects who did not develop any thyroid abnormalities during IFN- α treatment (Fig. 1a, b).

IFN- α treatment induced a significant increase in IFN- γ + and IL-4 expression in all of the lymphocyte populations investigated, at higher extent in IFN-AT than in Co-HCV+ patients. IFN-AT(Dy) (10 cases) and IFN-AT(Eu) (8 cases) patients showed different sequential modifications of cytokine expression in the peripheral lymphocytes during the development of

thyroid disease. IFN-AT(Eu) patients showed a significant increase of IFN- γ in CD8⁺ (Fig. 2b), CD3⁺CD56⁺ (Fig. 2c) and CD3⁻CD56⁺ (Fig. 2d) cells, but not in CD4⁺ cells (Fig. 2a). However, the patients with IFN-AT(Dy) showed a significant increase of IFN- γ ⁺ expression in all of lymphocyte populations (Fig. 2a-d). In CD3⁺CD56⁺ and CD3⁻CD56⁺ cells we found no significant difference in IFN- γ ⁺ expression between IFN-AT(Dy) and IFN-AT(Eu) patients. By contrast, IFN-AT(Dy) patients showed higher IFN- γ ⁺ expression in CD4⁺ and CD8⁺ cells than IFN-AT(Eu) patients. In CD3⁺CD56⁺ (Fig. 2c) and CD3⁻CD56⁺ (Fig. 2d) cells the increase of IFN- γ expression was earlier than that found in CD4⁺ cells in which the IFN- γ expression increased during the 6 months after the diagnosis of IFN-AT (Fig. 2a). In CD8⁺ cells, IFN- γ expression increased progressively throughout the period of the study (Fig. 2b).

At diagnosis of thyroid disease (T1), both IFN-AT(Eu) and IFN-AT(Dy) patients showed higher expression of IFN- γ ⁺ in CD3⁺CD56⁺ (Fig. 2c) and CD3⁻CD56⁺ (Fig. 2d) cells as compared with the Co-HCV⁺ subjects, whereas the IFN- γ ⁺ expression in CD4⁺ (Fig. 2a) and CD8⁺ (Fig. 2b) cells of both IFN-AT(Eu) and IFN-AT(Dy) remained comparable to the control subjects. At this time-point, IFN-AT(Eu) and IFN-AT(Dy) patients showed no significant differences in CD4⁺IFN- γ ⁺, CD8⁺IFN- γ ⁺, CD3⁺CD56⁺IFN- γ ⁺ and CD3⁻CD56⁺IFN- γ ⁺ cells (Fig. 2a-d).

Six months after the diagnosis of thyroid disease (T2), both IFN-AT(Eu) and IFN-AT(Dy) patients showed higher IFN- γ ⁺ expression than the Co-HCV⁺ subjects in CD8⁺ (Fig. 2b), CD3⁺CD56⁺ (Fig. 2c) and CD3⁻CD56⁺ (Fig. 2d) cells, whereas in CD4⁺ cells the IFN- γ ⁺ expression increased only in IFN-AT(Dy) but not in IFN-AT(Eu) patients (Fig. 2a). At this time-point, IFN-AT(Dy) showed higher numbers of CD4⁺IFN- γ ⁺ (Fig. 2a) and CD8⁺IFN- γ ⁺ (Fig. 2b)

cells as compared with IFN-AT(Eu), without any significant difference in CD3+CD56+IFN- γ + (Fig. 2c) and CD3-CD56+IFN- γ + (Fig. 2d) cells.

The IFN-AT(Eu) patients showed a significant increase of IL-4 expression in both CD4+ (Fig. 3a) and CD3+CD56+ (Fig. 3b) cells at diagnosis of IFN-related thyroid disease. At this time-point, the patients with thyroid dysfunction had lower expression of IL-4 in the both cell populations as compared with the euthyroid patients (Fig. 3a, b). This difference was still present 6 months after the diagnosis of thyroid disease, especially in CD4+ cells (Fig. 3a).

The Th1/Th2 ratio in CD4+ and CD3+CD56+ cells increased significantly during IFN- α treatment in IFN-AT(Dy) but not in IFN-AT(Eu) patients (Fig. 4a, b). In IFN-AT(Eu) patients the Th1/Th2 ratio remained significantly lower than the control HCV+ patients who did not develop any thyroid disease during IFN- α treatment (Fig. 4a, b).

Discussion

This prospective study shows that the sequential modifications of type-1 and type-2 immune responses induced by IFN- α treatment in peripheral lymphocyte of HCV+ patients are in relation to the development of thyroid autoimmune disease in its different clinical expressions.

Following the original description of IFN-related hypothyroidism (25), several studies have demonstrated that thyroid disease is a frequent side effect of IFN- α treatment (1-3). In the previous studies the immunological evaluations of IFN-AT were limited to the analysis of serum thyroid autoantibody patterns (2). To our knowledge this is the first study analysing the lymphocyte immune response in patients with IFN-AT.

Various methods are currently employed to detect cytokine production and secretion in humans (24, 26, 27). In the present study, we used the multi-parameter flow cytometry analysis

to investigate the immune response separately for CD4+ and CD8+ lymphocytes among the total PBMC. Because, unstimulated T-cells produce low amount of cytokines, *in vitro* stimulation is required (28). Moreover, the stimulation of lymphocytes could allow to reproduce *in vitro* the *in vivo* activation of these cells by specific and unspecific stimuli. There are various means of “artificial” stimulation, and advantages and disadvantages have been described for each of them (29, 30). 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 T-cell receptor (24). Furthermore, the pharmacological stimulus permits to evidence the expression of low degree expressed cytokine, as IL-4 (29). Finally, the pharmacological stimulus gave us the opportunity to evaluate the immune response in NK cells which are not provided of antigen-specific receptors.

Cellular immune responses have been studied in circulating (21, 31-33) and infiltrating lymphocytes (18-20, 34-36) 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 manner to investigate the immunological status in the patients with thyroid autoimmunity (37). However, a prospective study as the ours would force to perform repeated cytological evaluations that would have been not justifiable and not easily feasible. Therefore, the analysis of peripheral lymphocytes result to be the easiest manner to study the immunological status of the patients with thyroid autoimmunity. Indeed, previous studies found an activated phenotype in peripheral blood from HT patients (21, 31, 32, 38), suggesting a generalised immune dysregulation in such disease (39).

According to the Th1/Th2 paradigm, the clinical expressions of autoimmune thyroid disease are sustained by different polarization of immune response (19, 20). Here, we studied the patients with IFN-related autoimmune thyroiditis which resembles Hashimoto’s thyroiditis (6, 7).

Our study confirms previous observations that both Th1 and Th2 immune responses are activated in autoimmune thyroiditis, the former being more evident in presence of thyroid dysfunction and the latter more pronounced in euthyroidism (21). The dynamic model of IFN-related thyroid autoimmune disease allowed us to observe that Th2 activation in euthyroid patients was an early event during the development of the disease. Throughout the 6 months following the IFN-AT diagnosis, the patients with euthyroidism maintained high Th2 activation, whereas the patients with thyroid dysfunction showed a decrease in Th2 immune response with a concomitant increase in Th1 immune response. A similar outcome was already found in women developing post-partum thyroiditis with thyroid dysfunction in whom the physiological Th2/Th1 shift occurred early during the post-partum period (32). Future prospective studies will clarify whether the different time-dependent activation of Th1 and Th2 immune responses occurs also in IFN-related hyperthyroidism, as already suggested in patients with Graves' disease (40).

Innate and acquired immune system have synergic effects in the control of immunological tolerance in adult subjects (41). It is well known that innate cells [Natural killer (NK) and NKT cells] provide an early defence, serving to prevent replication and dissemination of infection before efficient adaptive responses are activated. However, new insights into innate immunity propose major roles for innate responses in driving, shaping and even regulating adaptive immune responses (42). Recent studies using animal models suggest that the innate response is instrumental in determining whether or not an autoimmune reaction will occur (43, 44). A type-1 bias in NK cells was demonstrated in experimental models of autoimmune diabetes and encephalomyelitis (44-46). Here, we found a type-1 and type-2 activation of peripheral NK and NKT CD56⁺ cells. The type-1 activation of innate immune system occurred at the same extent in patients with euthyroidism and in those with thyroid dysfunction, whereas the type-2 activation occurred specifically in patients who remained euthyroid for the whole period of follow-up as

that observed in CD4+ cells. It is intriguing that the type-1 activation in CD56+ cells was earlier than that observed in CD4+ cells, according to the experimental evidence of an early involvement of innate immune system in the development of autoimmunity (43). One could argue that the type-1 polarization in NK and NKT cells is an important factor for the development of thyroid autoimmunity but it not capable alone to drive the damage process of thyroid gland. By contrast, the type-1 activation of CD4+ and CD8+ T cells may be critical for inducing the destruction of thyroid parenchyma, as previously suggested in other experiences (47-49).

According to this model, innate and acquired immune system may play different roles in the thyroid autoimmune process. Future studies will be needed to demonstrate whether the specific modulation of IFN- γ (Th1) and IL-4 (Th2) polarization of NK, NKT, CD4+ and CD8+ cells may modify the evolution of autoimmune process in patients with thyroid autoimmunity.

Legend to tables and figures:

Table 1: Thyroid function [serum thyrotropin (TSH: $\mu\text{U/ml}=\text{mU/l}$) and free-thyroxine (FT4: $\text{pg/ml}=\text{pmol}/1.287$)] and autoimmunity [thyroglobulin (TgAb) and thyroperoxidase (TPOAb) antibodies] in 10 patients [4 males (M) and 6 females (F)] who developed IFN-related autoimmune thyroiditis (IFN-AT) with thyroid dysfunction. All of these patients were positive for thyroid autoantibodies. Three patients developed desctructive thyrotoxicosis (DT) alone, 2 patients hypothyroidism (Hypo) alone and 5 patients developed DT followed by Hypo. In this table we report the biochemical data (serum TSH and FT4 values) at the appearance of thyroid disease (T1) and 6 months later (T2).

Table 2: Thyroid function [serum thyrotropin (TSH: $\mu\text{U/ml}=\text{mU/l}$) and free-thyroxine (FT4: $\text{pg/ml}=\text{pmol}/1.287$)] and autoimmunity [thyroglobulin (TgAb) and thyroperoxidase (TPOAb) antibodies] in 8 patients [4 males (M) and 4 females (F)] who developed IFN-related autoimmune thyroiditis (IFN-AT) without thyroid dysfunction. All of these patients were positive for thyroid autoantibodies and they maintained serum TSH and free-thyroxine FT4 in the normal ranges throughout the follow-up. In this table we report the biochemical data (serum TSH and FT4 values) at the appearance of thyroid disease (T1) and 6 months later (T2).

Fig. 1: Intracellular IFN-gamma expression (1a) in peripheral CD4+, CD8+, CD3+CD56+ and CD3-CD56+ lymphocytes and IL-4 expression (1b) in CD4+ and CD3+CD56+ cells in IFN-related autoimmune thyroiditis (IFN-AT) analysed separately for patients with euthyroidism [IFN-AT(Eu): 8 cases] and for those with thyroid dysfunction [IFN-AT(Dy): 10 cases] in comparison with the control HCV+ subjects (Co-HCV+: 20 cases).

We report the data at the start of IFN-alfa treatment. The data are expressed as mean±SEM.

Fig. 2: Outcome of intracellular IFN-gamma expression in peripheral CD4+ (2a), CD8+ (2b), CD3+CD56+ (2c) and CD3-CD56+ (2d) lymphocytes in IFN-related autoimmune thyroiditis (IFN-AT) analysed separately for patients with euthyroidism [IFN-AT(Eu): 8 cases] and for those with thyroid dysfunction [IFN-AT(Dy): 10 cases] in comparison with the control HCV+ subjects (Co-HCV+: 20 cases) during IFN-alfa treatment. We report the data at start of IFN-alfa treatment (T0), at the appearance of thyroid disease (T1) and 6 months later (T2). In Co-HCV+ subjects, T1 and T2 correspond to 3-5 months and 9-11 months of treatment, respectively. The data are expressed as mean±SEM. ^a, p<0.05 vs T0; ^b, p<0.05 vs. T0 and T1; ^c, p<0.05 vs. Co-HCV+; ^d, p<0.05 vs. Co-HCV+ and IFN-AT(Eu). Multiple comparisons were made by Friedman's (for repeated measures) and Kruskal Wallis' (for un-paired data) tests, with *post hoc* Bonferroni's correction.

Fig. 3: Outcome of intracellular IL-4 expression in peripheral CD4+ (3a), and CD3+CD56+ (3b) lymphocytes in IFN-related autoimmune thyroiditis (IFN-AT) analysed separately for patients with euthyroidism [IFN-AT(Eu): 8 cases] and for those with thyroid dysfunction [IFN-AT(Dy): 10 cases] in comparison with the control HCV+ subjects (Co-HCV+: 20 cases) during IFN-alfa treatment. We report the data at start of IFN-alfa treatment (T0), at the appearance of thyroid disease (T1) and 6 months later (T2). In Co-HCV+ subjects, T1 and T2 correspond to 3-5 months and 9-11 months of treatment. The data are expressed as mean±SEM. ^a, p<0.05 vs T0; ^b, p<0.05 vs. T0 and T1; ^c, p<0.05 vs. Co-HCV+; ^d, p<0.05 vs. Co-HCV+ and IFN-AT(Eu); ^e, p<0.05 vs. Co-HCV+ and IFN-AT(Dy). Multiple

comparisons were made by Friedman's (for repeated measures) and Kruskal Wallis' (for un-paired data) tests, with *post hoc* Bonferroni's correction.

Fig. 4: Outcome of Th1/Th2 ratio in peripheral CD4+ (4a), and CD3+CD56+ (4b) lymphocytes in IFN-related autoimmune thyroiditis (IFN-AT) analysed separately for patients with euthyroidism [IFN-AT(Eu): 8 cases] and for those with thyroid dysfunction [IFN-AT(Dy): 10 cases] in comparison with the control HCV+ subjects (Co-HCV+: 20 cases) during IFN-alfa treatment. We report the data at start of IFN-alfa treatment (T0), at the appearance of thyroid disease (T1) and 6 months later (T2). In Co-HCV+ subjects, T1 and T2 correspond to 3-5 months and 9-11 months of treatment. The data are expressed as mean±SEM. ^a, p<0.05 vs T0; ^b, p<0.05 vs. T0 and T1; ^c, p<0.05 vs. Co-HCV+; ^d, p<0.05 vs. Co-HCV+ and IFN-AT(Eu); ^e, p<0.05 vs. Co-HCV+ and IFN-AT(Dy). Multiple comparisons were made by Friedman's (for repeated measures) and Kruskal Wallis' (for un-paired data) tests, with *post hoc* Bonferroni's correction.

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Table 1

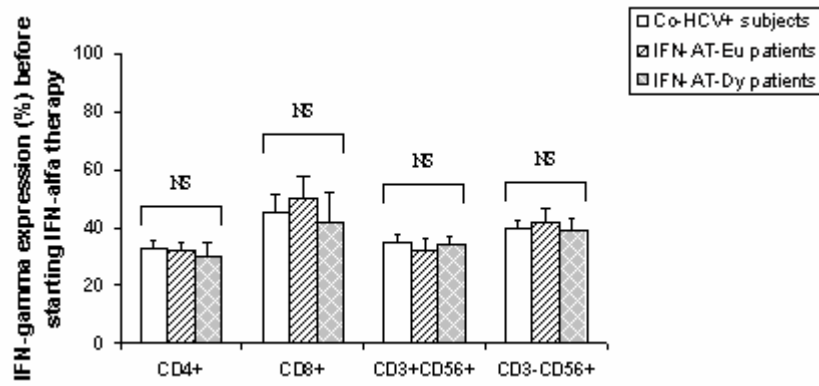
Pts	Sex	Pattern Abs	IFN-AT at the appearance (T1)		IFN-AT 6 months after diagnosis (T2)		Description of IFN-AT
			TSH (μU/ml)	FT4 (pg/ml)	TSH (μU/ml)	FT4 (pg/ml)	
1	M	TgAb alone	0.1	18.9	3.2	7.8	Overt DT
2	M	TgAb alone	<0.05	23.3	22.4	5.7	Overt DT → overt Hypo
3	M	TgAb+TPOAb	1.5	12.3	7.5	7.7	Mild Hypo
4	M	TgAb alone	0.2	15.9	15.3	5.0	Mild DT → overt Hypo
5	F	TgAb alone	0.1	18.3	2.2	11.3	Overt DT
6	F	TgAb+TPOAb	<0.05	25.2	19.6	4.3	DT → overt Hypo
7	F	TgAb+TPOAb	0.3	15.9	25.5	3.5	Mild DT → overt Hypo
8	F	TgAb+TPOAb	2.4	12.1	28.7	4.7	Overt Hypo
9	F	TgAb+TPOAb	<0.05	20.9	12.8	6.1	Overt DT → overt Hypo
10	F	TgAb+TPOAb	1.2	12.8	8.9	8.3	Mild Hypo

Table 2

Pts	Sex	Pattern Abs	IFN-AT at the appearance (T1)		IFN-AT 6 months after diagnosis (T2)	
			TSH (μ U/ml)	FT4 (pg/ml)	TSH (μ U/ml)	FT4 (pg/ml)
1	M	TgAb alone	0.9	14.4	2.3	9.5
2	M	TgAb alone	0.5	14.8	2.8	9.7
3	M	TgAb alone	1.0	13.0	1.3	11.3
4	M	TgAb alone	0.8	13.6	1.9	10.5
5	F	TgAb alone	0.6	15.9	3.0	8.8
6	F	TgAb+TPOAb	1.5	12.1	1.8	10.2
7	F	TgAb+TPOAb	1.8	13.5	2.5	12.3
8	F	TgAb+TPOAb	1.1	13.9	1.6	12.7

Fig. 1

(a)



(b)

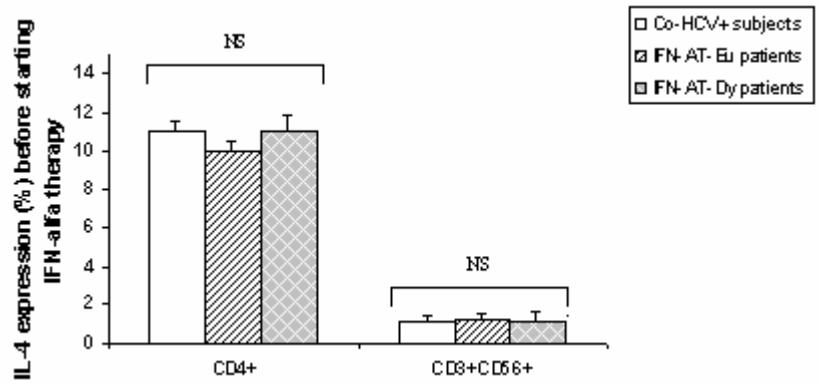
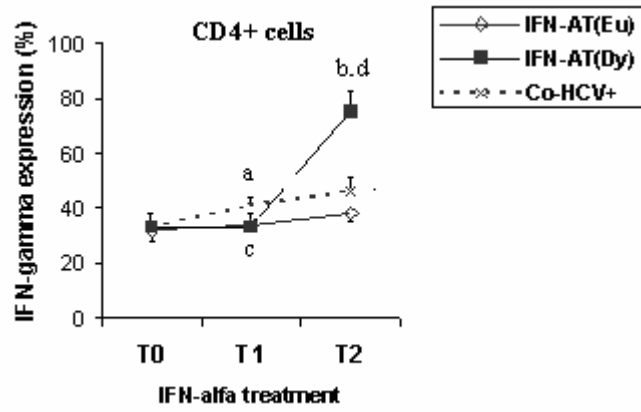
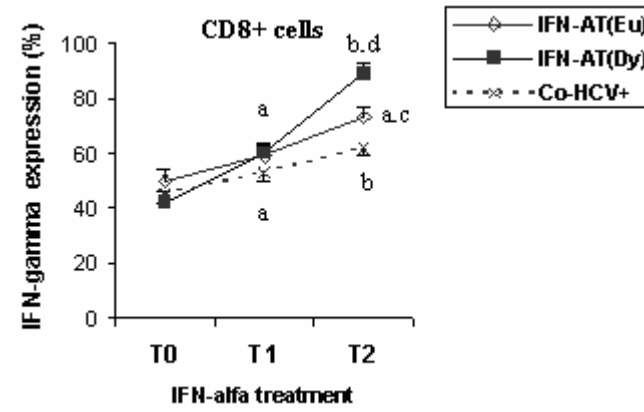


Fig. 2

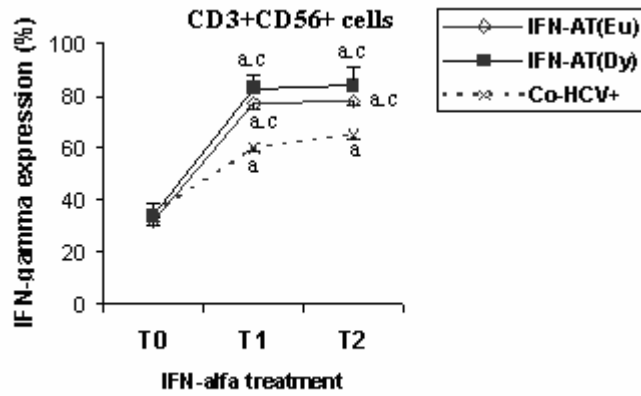
(a)



(b)



(c)



(d)

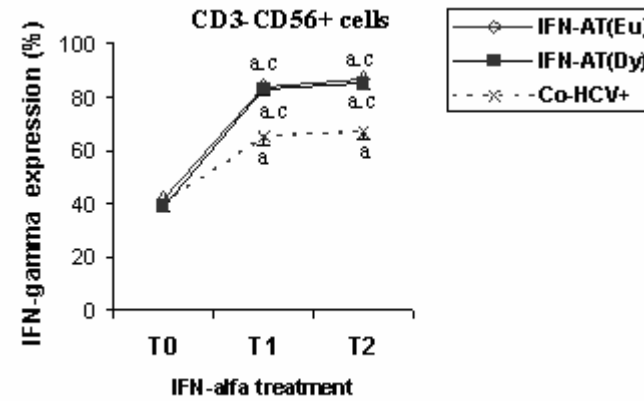
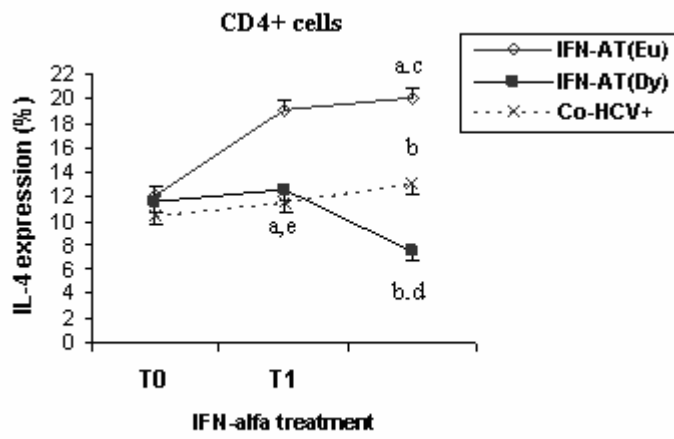


Fig. 3

(a)



(b)

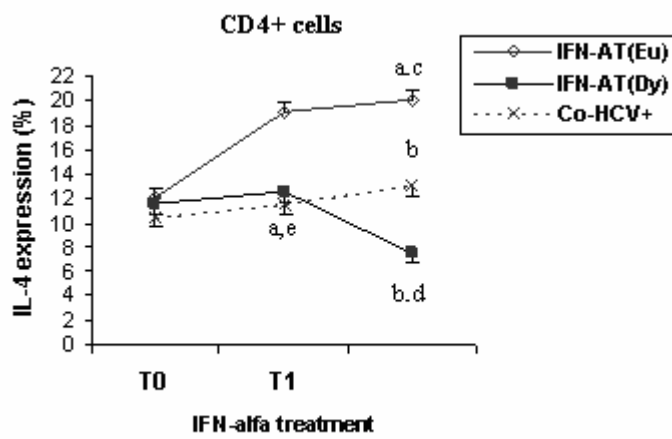
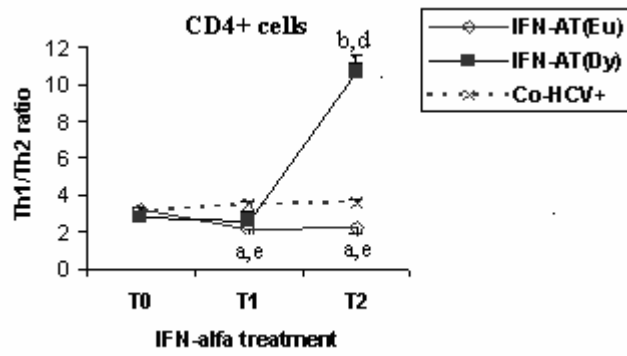


Fig. 4

(a)



(b)

