# Increased Circulating Pro-Inflammatory Cytokines and Th17 Lymphocytes in Hashimoto's Thyroiditis

Nicté Figueroa-Vega, Manuel Alfonso-Pérez, Ignacio Benedicto, Francisco Sánchez-Madrid, Roberto González-Amaro, and Mónica Marazuela

Department of Immunology (N.F.-V., R.G.-A.), School of Medicine, Universidad Autónoma de San Luis Potosí, 78230 San Luis Potosí, S.L.P. México; Services of Endocrinology (N.F.-V., M.M.), Immunology (M.A.-P., F.S.-M.), and Molecular Biology Unit (I.B.), Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, 28006 Madrid, Spain; and CIBEREHD, Instituto de Salud Carlos III, Madrid, Spain

**Context:** Th17 lymphocytes play an important role in different chronic inflammatory and autoimmune conditions.

**Aim:** The aim of the study was to explore the status of Th17 cells in patients with autoimmune thyroid diseases (AITD).

**Design:** We assessed the serum levels and *in vitro* synthesis of IL-17 and IL-22 and of different cytokines (IL-6, IL-15, and IL-23) involved in the differentiation of Th17 cells in the peripheral blood and thyroid glands of 26 patients with AITD, eight with Graves' disease, and 18 with Hashimoto's thyroiditis (HT) as well as 10 healthy controls.

**Results:** We found enhanced levels of T cells synthesizing IL-17 and IL-22 in the peripheral blood from AITD patients, mainly in those with HT. In addition, a stronger expression of IL-17 and IL-22 and an enhanced number of IL-23R$^+$ cells was detected in thyroid glands from HT patients compared with Graves' disease or controls. Furthermore, increased concentrations of IL-6 and IL-15 were detected in sera from HT patients, whereas serum levels of IL-23 tended to be higher in these patients. Finally, an enhanced *in vitro* differentiation of T lymphocytes into Th17 cells induced by IL-23/IL-6 was observed in AITD patients. Accordingly, a strong induction of RORC2 gene was detected in lymphocytes from HT patients when stimulated with IL-23.

**Conclusion:** Our results indicate that there is an increased differentiation of Th17 lymphocytes and an enhanced synthesis of Th17 cytokines in AITD, mainly in HT. These phenomena may have an important role in the pathogenesis of thyroid autoimmunity. **(*J Clin Endocrinol Metab* 95: 953–962, 2010)**

CD4$^+$ T helper lymphocytes play a key role in the pathogenesis of inflammatory and autoimmune diseases via the production of distinctive sets of cytokines (1). On the basis of their pattern of cytokine synthesis, CD4$^+$ T helper cells were originally classified into Th1 and Th2 lymphocytes, which are involved in the cellular and humoral immune responses, respectively (2). A third subset of CD4$^+$ cells (Th3 lymphocytes) mainly synthesize TGF-$\beta$ and are considered as regulatory cells (3). Additional lymphocyte subsets have been identified, including Th9 (4) and Th17 cells (5–7). The latter lymphocytes are characterized by the synthesis of IL-17A, IL-17F, IL-21, and IL-22 (8) and express the transcription factors: retinoic acid receptor-related orphan receptor C2 (RORC2) and signal transducers and activators of transcription-3 (Stat-3), which have a critical role in their development (9, 10). Th17 cells are involved in the pathogenesis of chronic inflammatory conditions (11), including asthma (12), rheumatoid arthritis (13), psoriasis (14), and systemic lupus erythematosus (15).

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2009-1719 Received August 20, 2009. Accepted November 13, 2009.
First Published Online December 16, 2009

Abbreviations: Ab, Antibody; AITD, autoimmune thyroid diseases; $FT_4$, free $T_4$; GD, Graves' disease; HT, Hashimoto's thyroiditis; IFN, interferon; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; TFC, thyroidal follicular cell; Tg, thyroglobulin; TMC, thyroid mononuclear cell; TPO, antithyroperoxidase; Treg, T regulatory; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor.

Several cytokines are involved in the differentiation of human Th17 cells, mainly IL-21 and IL-23 as well as several proinflammatory cytokines, including IL-1$\beta$ and TNF-$\alpha$ (16–19). On the contrary, other cytokines exert a negative regulatory role on Th17 cell development, including interferon (IFN)-$\gamma$, and IL-27 (20). On the other hand, TGF-$\beta$ seems to have a dual role, inhibiting the generation of Th17 cells in the absence of other cytokines and promoting its differentiation in combination with IL-23, IL-6 and, likely, IL-1$\beta$ and TNF-$\alpha$ (21, 22). The proinflammatory cytokine IL-15 is also able to induce the synthesis of IL-17 by Th17 cells (23). Th17 cells synthesize IL-17A (also called IL-17) that exhibits a strong proinflammatory activity, inducing the synthesis of chemokines and proinflammatory cytokines (8, 11, 24, 25). Accordingly, the synthesis of IL-17 is increased in different inflammatory autoimmune conditions such as collagen-induced arthritis (26), rheumatoid arthritis (27), systemic sclerosis (28), psoriasis (29), and systemic lupus erythematosus (30). On the other hand, IL-22, mainly synthesized by Th7 cells, also has an important role in the pathogenesis of different inflammatory conditions (31).

Human autoimmune thyroid diseases (AITD), mainly including Hashimoto's thyroiditis (HT) and Graves' disease (GD), are characterized by reactivity to self-thyroid antigens (32). It has been proposed that the interaction of thyroidal follicular cells (TFCs), antigen-presenting cells, and autoreactive T cells results in an autoimmune response against thyroid antigens, which can be mediated by Th1 or Th2 lymphocytes (33). In the case of a Th1 response, a heavy/strong inflammatory infiltrate with destruction of thyroid gland occurs (HT), whereas in the case of a Th2 response, stimulatory anti-TSH receptor antibodies, an enhanced function of the gland and a variable degree of inflammation are observed (GD) (33–35). Autoreactive T lymphocytes and TFCs are able to synthesize different chemokines and cytokines, which favor the expansion of the intrathyroidal lymphocyte pool, exacerbating the autoimmune process (36–38).

We previously described that patients with AITD show an altered proportion and a defective function of T regulatory (Treg) cells in both the peripheral blood and thyroid gland, (39). Because there is a reciprocal negative regulation between Treg and Th17 lymphocytes, we hypothesized that patients with AITD show an expansion of Th17 cells and that these cells are involved in the pathogenesis of this condition. In this work, we found enhanced levels of Th17 cells and Th17 cytokines in patients with AITD, mainly those with HT. In addition, an increased capability of peripheral blood lymphocytes to differentiate *in vitro* into Th17 cells, with a strong expression of the RORC2 gene, was observed in these patients. These data suggest that Th17 cells may be involved in the pathogenesis of AITD.

**TABLE 1.** Clinical features of AITD patients included in the study (PBMC assays)

| | HT | GD | Range |
|---|---|---|---|
| n | 13 | 5 | |
| Gender (M/F) | 2/11 | 2/3 | |
| Age (yr) | 45.36 ± 13.20 | 45.80 ± 16.63 | |
| TSH (aeU/ml) | 9.75 ± 5.49 | 0.33 ± 0.63 | 0.3–4.94 |
| Free $T_4$ (ng/dl) | 0.83 ± 0.13 | 1.52 ± 1.30 | 0.7–1.48 |
| TPO-Ab (U/ml) | 584.09 ± 498.00 | 543.0 ± 514.64 | <344 |
| Tg-Ab (U/ml) | 1056.3 ± 2543.3 | <0.02 | <100 |
| TSHR-Ab (U/liter) | 0.054 ± 0.052 | 13.71 ± 16.6 | <2 |

Data correspond to the arithmetic mean ± SD. M, Male; F, female.

## Patients and Methods

### Individuals and samples

Eighteen AITD patients (32) (13 with HT and five with GD) were included in the study. Three of the five patients with GD had ophthalmopathy. Peripheral blood samples were obtained from all patients, and complete thyroid work-up was performed in them. Main clinical data of these patients are shown in Table 1. Serum free $T_4$ ($FT_4$), TSH levels, and titers of anti-TSH receptor (TSHR), antithyroglobulin (Tg) and antithyroperoxidase (TPO) antibodies (Abs) were measured as previously described (40). Ten age- and sex-matched healthy subjects were included as controls.

Thyroid glands were obtained from five patients with HT and three with GD who had relapsed after antithyroid drug treatment and were euthyroid under carbimazole therapy at the time of surgery; two of these patients had opthalmopathy. In addition, normal thyroid tissue was obtained from patients undergoing parathyroidectomy. These patients did not have evidence of thyroid or autoimmune disease (Table 2). Thyroid specimens were frozen, embedded in OCT medium (Ames, Miles Laboratories, Elkhart, IN) and stored at −80 C. All samples were taken in accordance with the regulations and approval of the Institutional Review Board of Hospital Universitario de la Princesa. In all cases, a written informed consent was obtained, and the local hospital bioethical committee approved this study.

### Immunohistochemistry

Thyroid tissue sections (5.0 $\mu$m thick) were mounted on glass slides (Dako Cytomation, Copenhagen, Denmark) and stained by an immunoperoxidase method as previously described (41). Briefly, endogenous peroxidase was inactivated, and Fc receptors blocked with diluted human AB serum. Then sections were incubated with anti-IL-17 (R&D Systems, Wiesbaden-Nordenstadt, Germany), anti-IL-22 (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-IL-18R (R&D Systems), goat anti-human polyclonal Ab, or an anti-IL-23R monoclonal antibody (mAb; R&D Systems). Next, sections were incubated with the proper horseradish peroxidase-conjugated secondary antibody (Envision; Dako Cytomation), washed, and developed with 3,3′-diaminobenzidine. Finally, sections were counterstained with Carazzi's hematoxylin and evaluated by at least two independent investigators.

**TABLE 2.** Clinical features of AITD patients included in the study (thyroid gland assays)

| | Normal | HT | GD | Range |
|---|---|---|---|---|
| n | 3 | 5 | 3 | |
| Gender (M/F) | 0/3 | 0/5 | 0/3 | |
| Age (yr) | 53.0 ± 10.8 | 44.0 ± 9.82 | 40.0 ± 15.7 | |
| TSH (aeU/ml) | 0.9 ± 1.1 | 4.5 ± 4.1 | 0.3 ± 0.7 | 0.3–4.94 |
| Free $T_4$ (ng/dl) | 0.9 ± 0.1 | 0.9 ± 0.11 | 1.3 ± 0.3 | 0.7–1.48 |
| TPO-Ab (U/ml) | Negative | 296.50 ± 336.76 | 215.78 ± 296.45 | <344 |
| Tg-Ab (U/ml) | Negative | 2168.8 ± 2821.8 | <0.02 | <100 |
| TSHR-Ab (U/liter) | Negative | Negative | 15.3 ± 17.2 | <2 |

Data correspond to the arithmetic mean ± SD. M, Male; F, female.

## Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation over Ficoll-Hypaque cushions (Biochrom AG, Berlin, Germany). Cell viability (trypan blue dye exclusion) was always greater than 95%.

To isolate thyroid mononuclear cells (TMCs), thyroid specimens were minced and then digested with collagenase (1.0 mg/ml; Roche Molecular Biochemicals, Roche, Mannheim, Germany) in Hanks' balanced-salt solution (BioWhittaker, Lonza, Verviers, Belgium) for 1 h at 37 C and passed through a steel mesh. Then cells were isolated by density-gradient centrifugation over Ficoll-Hypaque cushions, washed twice with PBS, and resuspended in complete RPMI 1640 culture medium (Life Technologies, Inc., Gaithersburg, MD). Cell viability was always higher than 95%.

## *In vitro* stimulation of T cells

For the detection of Th17 lymphocytes, PBMCs or TMCs were incubated in complete RPMI 1640 culture medium in the presence of 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich St. Louis, MO) and 1.0 μg/ml ionomycin (Sigma-Aldrich) or 100 ng/ml recombinant human IL-15 (PeproTech EC Ltd., London, UK) for 5 and 6 h, respectively, and with the addition of 5.0 μg/ml of Brefeldin A (Sigma-Aldrich). Then $IL\text{-}17^+$ cells were detected by flow cytometry, as described below.

PBMCs and TMCs were also cultured for 5 d with plate-bound anti-CD3 (T3b; 1.0 μg/ml) and anti-CD28 (10 μg/ml; Immunotech, Marseille, France) mAbs in the presence of anti-IFN-γ (5.0 μg/ml) and anti-IL-4 (5.0 μg/ml) blocking mAbs (R&D Systems), and different combinations of recombinant human IL-23 (20 ng/ml; R&D Systems), TGF-β (10 ng/ml; Peprotech), or IL-6 (20 ng/ml; Peprotech). Then cells were incubated with PMA and ionomycin, for 4 h in the presence of 5.0 μg/ml Brefeldin A (Sigma-Aldrich).

## Flow cytometry analysis

Cells were stimulated with PMA/ionomycin, washed, and immunostained with a CD3-Peridinin Chlorophyll Protein Complex (PerCP) and a CD8-fluorescein isothiocyanate mAb (BD PharMingen, San Diego, CA), fixed, and permeabilized and then incubated with a Phycoerythrin conjugated anti-IL-22 (R&D Systems) and an Alexa fluor 647-conjugated anti-IL-17 mAb (eBioscience, San Diego, CA). Isotype-matched Ab controls (BD PharMingen) were used in all procedures. Cells ($1–2 \times 10^5$) were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA), and results were expressed as the percent of $CD3^+CD8^-$ cells expressing IL-17 or IL-22.

## RNA isolation and real-time RT-PCR

Total RNA was isolated with the Ultraspec kit of Bioctecx Laboratories (Houston, TX). Reverse transcription was performed with random hexamers and the Moloney murine leukemia virus reverse transcriptase (Promega Biotech Ibérica, S.L., Madrid, Spain) in a GeneAmp PCR system 9700 thermal cycler (Perkin Elmer, Waltham, MA). Real-time RT-PCR was performed by duplicate using the StepOnePlus real-time PCR system using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and using the following primers: IL-17, sense, 5′-CCA CCT CAC CTT GGA AT-3′; antisense, 5′-GGA CCA GGA TCT CTT GCT-3′, IL-22, sense, 5′-TGA TAA CAA CAC AGA CGT TCG-3′, antisense, 5′-CTG CAT ATA AGG CTG GAA CC-3′. As internal control, we used Histone H3, with the following primers: sense, 5′-AAA GCCGCT CGC AAG AGT GCG-3′; antisense, 5′-ACT TGC CTC CTG CAA AGC AC-3′. Gene-specific amplification was confirmed by a single peak in melting curve analysis. Data were analyzed with the StepOne software version 2.0 (Applied Biosystems). In the case of RORC2, cDNA products were amplified by PCR with the following primers: sense, 5′-GCT AGG TGC AGA GCT TCA GG-3′, antisense, 5′-GGT GAT AAC CCC GTA GTG GA-3′. Amplification cycles (35) were as follows: 30 sec at 95 C, 30 sec at 57 C, and 45 sec at 72 C. Next, samples were incubated at 72 C for 7 min and cooled to 4 C. Finally, amplicons were electrophoresed on 2% agarose gel containing ethidium bromide (Bio-Rad) and visualized with an UV transilluminator.

## Cytokine quantification

Levels of IL-6, IL-15, IL-17, IL-22, and IL-23 were determined by ELISA (ImmunoTools GmbH, Friesoythe, Germany; and R&D Systems) using a Tecan Sunrise ELISA reader (Tecan, Spain). All determinations were performed by duplicate and the lower detection limits for IL-6, IL-15, IL-17, IL-22, and IL-23 were 0.70, 3.9, 15.0, 2.7, and 6.8 pg/ml, respectively.

To quantify the *in vitro* synthesis of cytokines (IL-17 and IL-22), PBMCs or TMCs were stimulated with anti-CD3/CD28 mAbs and/or PMA/ionomycin or with 100 ng/ml of recombinant human IL-15 in the presence or absence of blocking anti-IFN-γ/-IL-4 mAbs and recombinant cytokines (IL-23, TGF-β, IL-6), as described above. At the end of cell culture, cytokine concentration in cell-free supernatants was determined by ELISA.

## Statistical analysis

Data are showed as the arithmetic mean ± SD or mean ± SEM. For continuous variables, normality was assessed by the Kolmogorov-Smirnov test. Data were analyzed using both parametric and non-

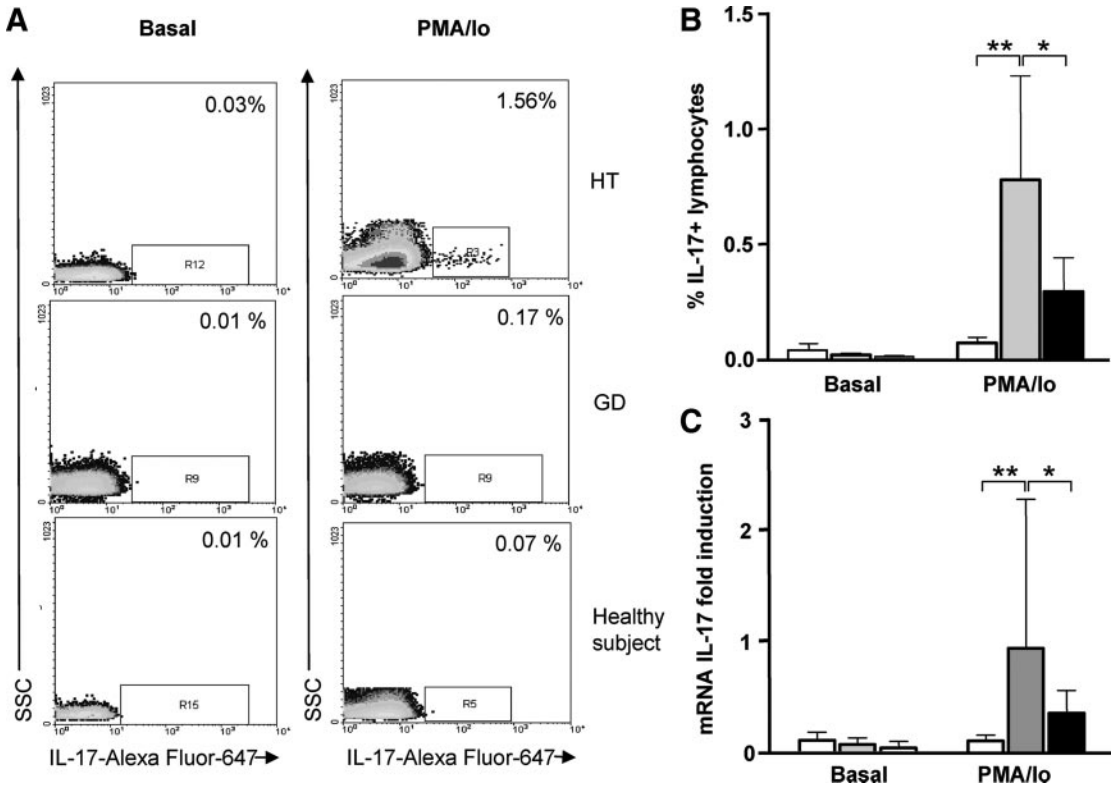


**FIG. 1.** IL-17 expressing cells in the peripheral blood from AITD patients. PBMCs from HT, GD, and healthy controls were incubated with PMA/ionomycin, stained for intracellular IL-17 and analyzed by flow cytometry. A, Representative dot plots from HT and GD patients and a control subject are shown. Numbers correspond to the percentage of $CD3^+CD8^-IL\text{-}17^+$ cells. B, Percentages of $IL\text{-}17^+$ cells in the peripheral blood of HT and GD patients, and healthy controls. Data correspond to the arithmetic mean ± SEM. *White bars* correspond to healthy controls, *gray bars* to HT, and *black bars* to GD. C, Levels of IL-17 mRNA in PBMCs from AITD patients and controls pretreated or not with PMA/ionomycin. IL-17 mRNA was detected by real-time PCR analysis. Data correspond to the arithmetic mean ± SEM of mRNA fold induction. *White bars* correspond to healthy controls, *gray bars* to HT, and *black bars* to GD. *, $P < 0.05$; **, $P < 0.01$. No significant differences were detected between GD patients and controls.

parametric tests. Mean group values were compared by one-way ANOVA and nonparametric ANOVA (Kruskal-Wallis test), and *post hoc* comparisons were carried out using the Bonferroni's and Dunn's test, respectively. Paired *t* test and Wilcoxon sum rank test were used to analyze two related samples. Analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL) and GraphPad Prism software version 5.0 (San Diego, CA).

## Results

### Th17 cells in the PBMCs from AITD patients

We first analyzed by flow cytometry the proportion of $CD3^+CD8^-IL\text{-}17^+$ lymphocytes in PBMCs from AITD patients (Fig. 1A). An increased percentage of Th17 cells was observed in HT patients compared with GD patients or healthy subjects (Fig. 1B). In addition, the percentage of $IL\text{-}17^+$ cells tended to be higher in GD patients compared with healthy subjects (Fig. 1B). However, no apparent differences were found between GD patients with and without ophthalmopathy (data not shown).

Real-time RT-PCR analysis showed enhanced levels of IL-17 mRNA in the PBMCs from HT patients, with lower expression in GD patients and healthy subjects (Fig. 1C and data not shown). However, IL-17 was not detected by ELISA in sera from any individual included in the study (data not shown). In addition, a very low number of $IL\text{-}17^+$ cells was detected in nonstimulated freshly isolated PBMCs from the three groups studied, with no significant differences among them (Fig. 1, A and B). Similar results were observed when the IL-17 mRNA was detected by real-time PCR (Fig. 1C).

In agreement with the above data, we found a modest but significant increased percentage of $CD3^+CD8^-IL\text{-}22^+$ T cells in the PBMCs from HT patients compared with GD or control individuals ($P < 0.05$ in both cases, Fig. 2, A and B). Accordingly, serum levels of IL-22 were significantly higher in HT patients compared with both GD patients and healthy volunteers ($P < 0.05$ in both cases, Fig. 2, A and C). However, additional flow cytometry analysis revealed that in most individuals only a small percentage of $IL\text{-}17^+$ cells also synthesized IL-22 and vice versa (data not shown).

### Th17 lymphocytes in thyroid glands from AITD patients

Immunohistochemical analysis of thyroid sections revealed a variable staining for IL-17 and IL-22, with a

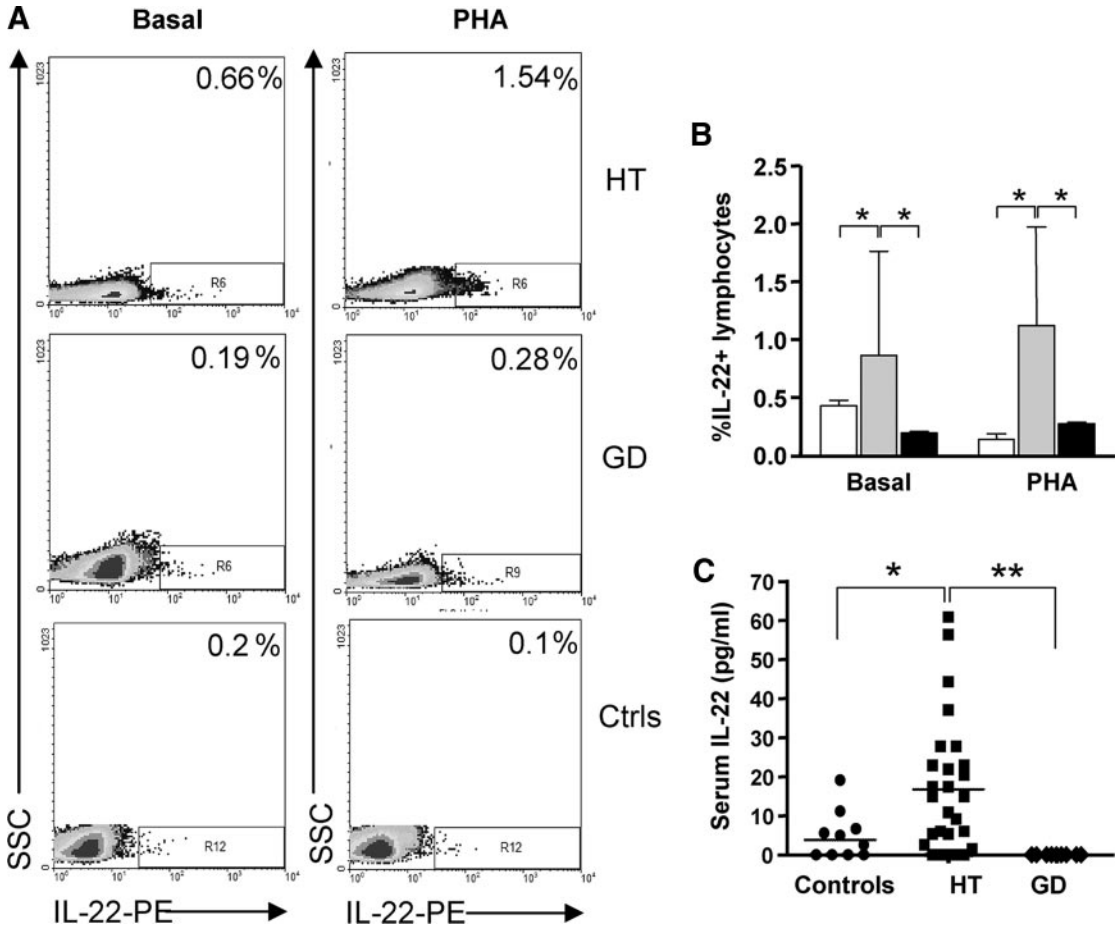


**FIG. 2.** IL-22 expressing cells in the peripheral blood from AITD patients. PBMCs from HT, GD, and healthy controls were incubated with PMA/ionomycin, stained for intracellular IL-22 and analyzed by flow cytometry. A, Representative dot plots from HT and GD patients and a control subject. Numbers correspond to the percentage of $CD3^+CD8^-IL\text{-}22^+$ cells. B, Data of the percent of $IL\text{-}22^+$ cells in the peripheral blood of HT and GD patients, and healthy controls are represented as the arithmetic mean ± SEM. *White bars* correspond to healthy controls, *gray bars* to HT, and *black bars* to GD. C, Serum levels of IL-22 were determined by ELISA in samples from AITD patients and healthy controls. *, $P < 0.05$; **, $P < 0.01$. No significant differences were detected between GD patients and controls.

stronger expression of these cytokines in patients with HT with active lesions, compared with GD and healthy controls (Fig. 3A). As expected, in HT glands $IL\text{-}17^+$ and $IL\text{-}22^+$ cells were mainly detected into the inflammatory cell infiltrate. IL-17 and IL-22 were almost undetectable in thyroid glands from GD patients and controls (Fig. 3A and data not shown). Accordingly, IL-17 and IL-22 mRNAs were detected in TMCs from HT but not GD patients or healthy controls (Fig. 3, B and C, and data not shown). In this regard, IL-15 induced a modest but significant differentiation of Th17 cells in the PBMCs from HT patients, whereas a similar effect was exerted by IL-23, alone or in combination with TGF-$\beta$ (Fig. 3B). In addition, IL-6 alone showed a greater effect on IL-17 cell differentiation compared with IL-15 or IL-23, and this effect was not augmented by the addition of TGF-$\beta$ (Fig. 3B). On the other hand, IL-15 did not induce the synthesis of IL-22 on TMCs from HT patients, whereas IL-23 or IL-23/TGF-$\beta$ exerted this effect (Fig. 3C). Finally, IL-6 and TGF-$\beta$ exerted a similar effect on the induction of $IL\text{-}22^+$ cells than IL-23/TGF-$\beta$ (Fig. 3C).

Additional immunohistochemical analysis showed a prominent expression of both IL-18 receptor and IL-23 receptor, which have been reported to be expressed by Th17 cells in the inflammatory cell infiltrates from HT thyroid specimens (Fig. 3D). In contrast, these cytokine receptors were not detected in GD or normal glands (data not shown).

## Serum levels of proinflammatory cytokines are increased in HT patients and are able to induce Th17 differentiation

Then we analyzed the levels of cytokines involved in Th17 cell differentiation, and we found that HT patients had significant increased serum concentrations of IL-6 compared with healthy subjects ($P < 0.05$, Fig. 4A). In addition, serum levels of IL-15 were also significantly augmented in HT patients compared with GD patients and controls ($P < 0.01$ in both cases, Fig. 4B). Finally, al-

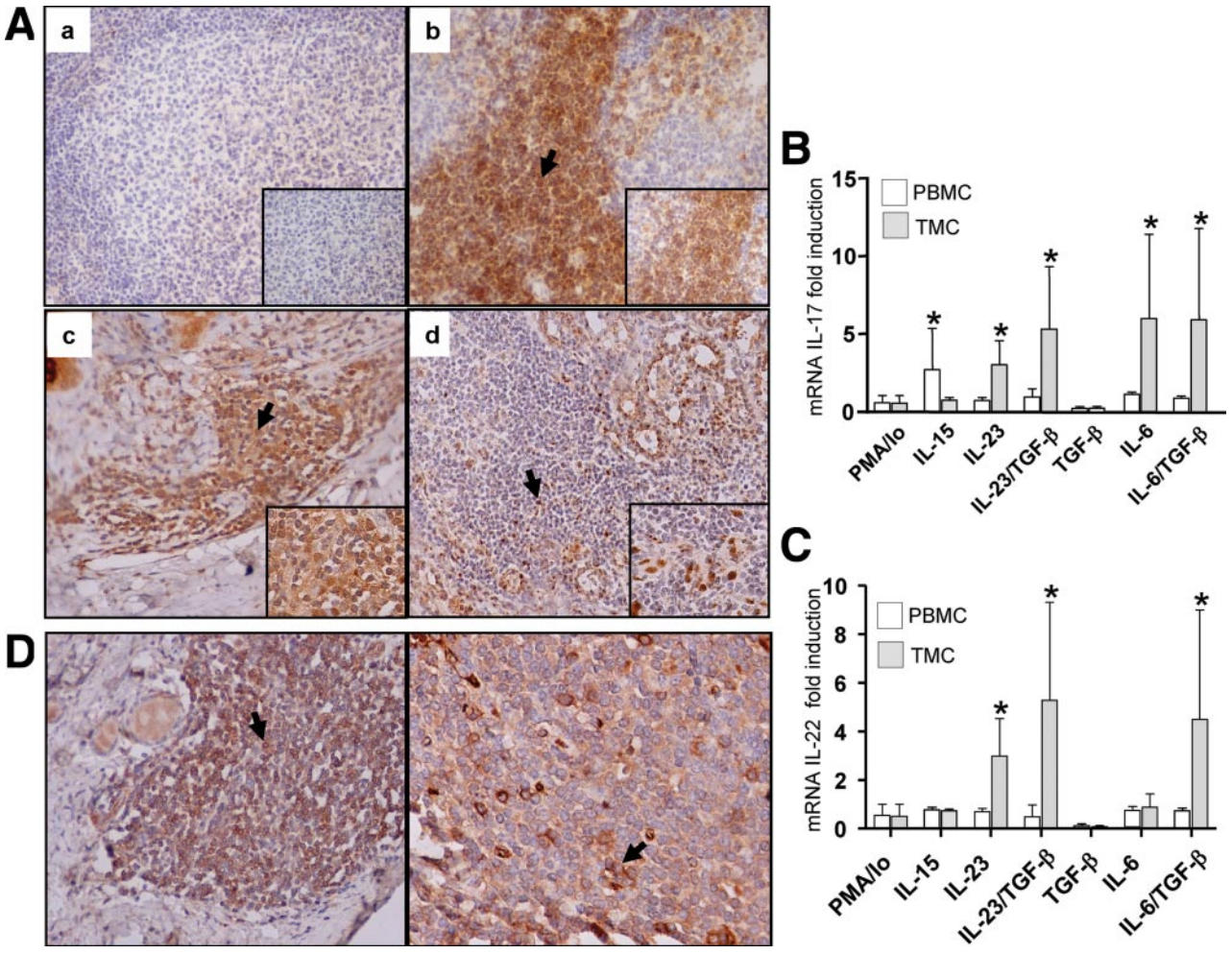


**FIG. 3.** IL-17 and IL-22 in thyroid tissue from AITD patients. A, Thyroid gland sections from an HT patient were stained for CD4, IL-17, or IL-22 by an immunohistochemical technique. a, Negative X63 control; b, CD4; c, IL-17; d, IL-22. Original magnification, ×250 and ×500 (*insets*). *Arrows* point to the inflammatory cell infiltrate. B and C, Detection of IL-17 and IL-22 mRNA in PBMCs and TMCs from HT patients after cell culture with the indicated stimuli. IL-17 and IL-22 mRNA was estimated by real-time RT-PCR. Data correspond to the arithmetic mean ± SEM of five patients with HT and three with GD. D, Immunohistochemical detection of IL-18R (*left panel*) and IL-23R (*right panel*) in thyroid sections from a patient with HT. Original magnification, ×250. *, $P < 0.05$ compared with PMA/ionomycin (Io).

though serum concentrations of IL-23 tended to be higher in HT patients in comparison with both GD patients and healthy subjects, this difference did not reach statistical significance (data not shown).

We also analyzed the *in vitro* differentiation of PBMCs and TMCs into IL-17- and IL-22-producing $CD4^+$ T cells (Fig. 4C). When cells were incubated with IL-15 alone, a modest differentiation effect was observed in cells from AITD patients (Fig. 4D). In contrast, IL-23 exerted a greater Th17 differentiation effect, which was higher in HT and GD patients compared with healthy controls (Fig. 4D). Interestingly, TGF-$\beta$ partially inhibited the inducing effect of IL-23, whereas IL-6, alone or in combination with TGF-$\beta$, enhanced the development of Th17 cells in HT and GD patients, and, to a lesser degree, healthy controls (Fig. 4D). These results were confirmed by RT-PCR analysis of the levels of IL-17 and IL-22 mRNA in the indicated cell cultures (Fig. 4E). In addition, different stimuli (mainly IL-23 and IL-6) were able to induce the release of IL-17 and IL-22 by cultured cells from AITD patients (data not shown). In contrast, all these stimuli were unable to induce the secretion of IFN-$\gamma$ (data not shown).

## Expression of RORC2 gene in cells from AITD patients

Finally, we assessed the expression, at mRNA level, of the RORC2 transcription factor, which plays a critical role in the differentiation of Th17 lymphocytes. As shown in Fig. 5, a strong induction of RORC2 mRNA was observed in cells from HT patients when they were stimulated with IL-23. In contrast, when cells were stimulated with TGF-$\beta$ alone, no induction of RORC2 was observed, but a prominently expression of Foxp3 was detected (Fig. 5, and data not shown). Furthermore, the addition of TGF-$\beta$ to IL-23 did not enhance the effect of the latter cytokine on RORC2 induction. On the other hand, IL-15

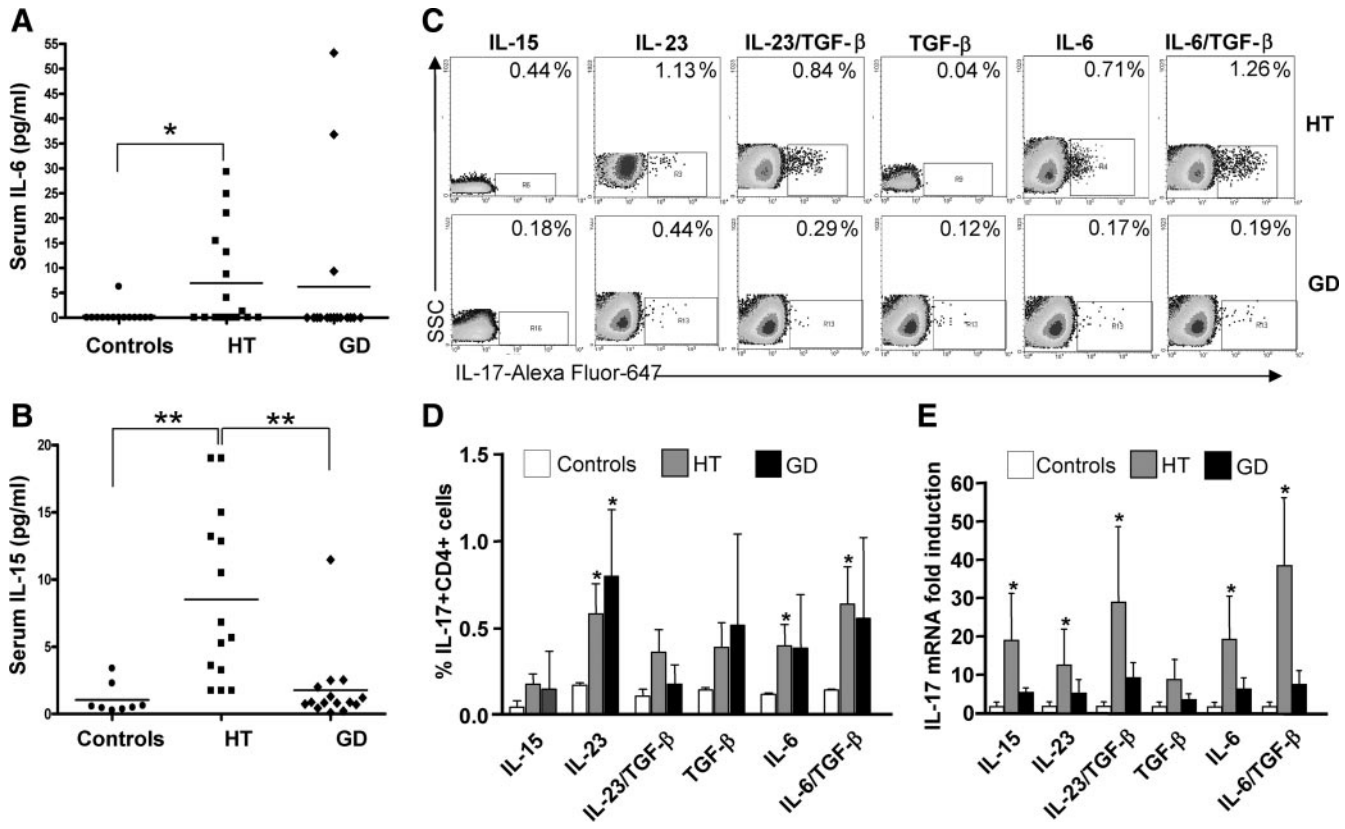


**FIG. 4.** Serum levels of proinflammatory cytokines are increased in HT and are able to induce the differentiation of Th17 cells *in vitro*. A and B, Serum levels of IL-6 and IL-15 were determined by ELISA in serum samples from AITD patients and controls. Mean group values were compared by one-way ANOVA and nonparametric ANOVA (Kruskal-Wallis test) and *post hoc* comparisons were carried out using the Bonferroni's and Dunn's test as appropriate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. C, *In vitro* differentiation of PBMCs into Th17 cells in patients with AITD (n = 13 with HT, n = 5 with GD). PBMCs were cultured under the indicated stimuli, and then IL-17$^+$ cells were analyzed by flow cytometry. Numbers correspond to the percent of IL-17$^+$ cells. D, *Bars* represent arithmetic mean ± SEM of the percent of IL-17$^+$ cells in AITD patients and controls. E, IL-17 mRNA levels in the cell cultures indicated in C. IL-17 mRNA was determined by real-time RT-PCR. *, $P < 0.05$ compared with controls.

as well as IL-6 was also able to induce RORC2 gene expression in cells from HT patients. However, the addition of TGF-$\beta$ to IL-6 did not enhance the effect of this cytokine on RORC2 expression. Finally, in PBMCs from GD patients, only IL-15 induced the expression of RORC2, whereas the other cytokines tested did not show any significant effect (Fig. 5). As expected, the different stimuli used did not induce the expression of the Th1- and Th2-specific transcription factors: Th1-specific T box transcription factor (Tbet), and GATA binding protein 3, respectively (data not shown).

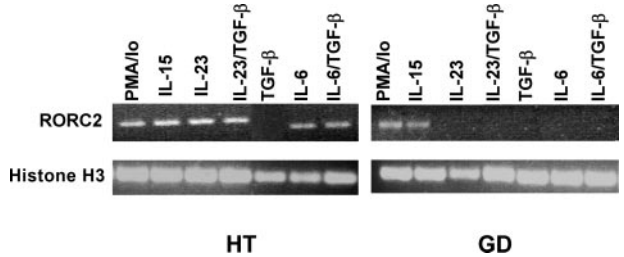


**FIG. 5.** Increased induction of RORC2 gene in PBMCs from AITD patients. PBMCs from patients with HT and GD were cultured under the indicated stimuli, and then the levels of RORC2 mRNA were determined by RT-PCR. Data from two representative patients, one with HT and one with GD, are shown.

## Discussion

In this work, we explored the possible involvement of Th17 cells in AITD. Th17 cells participate in the resistance against different extracellular bacteria and fungi and act as a link between the innate and adaptive immune response (42). In addition, Th17 lymphocytes are involved in the pathogenesis of different inflammatory and autoimmune conditions, including rheumatoid arthritis, psoriasis, and asthma (12–14). In these conditions, Th17 cells mainly exert their pathogenic effect through the release of IL-17, IL-17F, and IL-22, which in turn induce the synthesis of chemokines and proinflammatory cytokines by resident cells (11, 43).

We herein detected a higher proportion of Th17 cells and raised levels of Th17 cytokines in the peripheral blood and thyroid gland from patients with AITD, mainly those with HT. In addition, the peripheral blood lymphocytes from these patients showed an increased induction of RORC2 gene and an enhanced capability to differentiate *in vitro* into Th17 cells. Finally, high levels of IL-6 and IL-15 were detected in the sera from patients with HT.

Because it has been described that different cell subsets are able to synthesize IL-17 (44–47), we performed a flow cytometry analysis to detect $CD3^+CD8^-IL\text{-}17^+IL\text{-}22^+$ cells, which very likely correspond to classical Th17 lymphocytes. Our data indicate that patients with AITD, mainly those with HT, show enhanced levels of Th17 cells in their peripheral blood as well as the thyroid gland. We think that this is an interesting finding because it has been largely considered that HT is a Th1-mediated condition. Thus, our data suggest that, as in the case of rheumatoid arthritis or multiple sclerosis, Th17 may have a relevant role in the inflammatory phenomenon and tissue destruction seen in HT. This possibility adds an additional element to the complex pathogenesis of AITD, mainly because it has been proposed that there is a negative cross-regulation between Th1 cells and Th17 lymphocytes (48) and that these two cell subsets are under the control of Treg cells (49). In this regard, it has been recently reported that at least a subset of human $Foxp3^+$ Treg lymphocytes is able to synthesize IL-17 as well as express RORC2 (50). Because we previously described that there is an increased proportion of $Foxp3^+$ Treg cells in the peripheral blood and thyroid infiltrate of AITD patients (39), it is very likely that these cells correspond to the aforementioned Th17/Treg lymphocytes. However, our data on the defective function of Treg cells in AITD (39) suggest that in this condition, Th17/Treg cells mainly exert a proinflammatory effect, with a weak regulatory function.

The enhanced capability of freshly isolated PBMCs from HT patients to differentiate into Th17 cells is of interest. We think that this phenomenon could be related to the enhanced serum levels of IL-6 and IL-15 detected in these patients. In this regard, the important role of IL-6 in the induction of Th17 cell differentiation has been widely described (19). Likewise, the key effect of IL-15 on the development of Th17 lymphocytes (in combination with IL-6) has been also recently described (51). Although the cause of the enhanced levels of IL-6 and IL-15 in AITD remains to be determined, it is feasible that it could be related to genetic factors, mainly gene polymorphisms, which are associated with an enhanced transcription/translation rate. In this regard, different associations between single nucleotide polymorphisms of proinflammatory cytokine genes (*e.g.* TNF-$\alpha$) have been described in patients with AITD (52).

Although to the best of our knowledge this is the first report on the detection of Th17 cells and cytokines in thyroid glands from AITD patients, there is a recent article on the levels of these cells in the peripheral blood (53). In that work, Th17 lymphocyte levels were higher in AITD patients compared with healthy controls, and those patients with intractable GD showed a higher proportion of Th17 cells than patients with GD in remission. Even though we do not have a commendable explanation for our partially contradictory results, it is feasible that the apparent discrepancies between this study and our work could be due, at least in part, to the different genetic background of the individuals studied, the presence or absence of opthalmopathy, and the stage of the disease of the patients. Furthermore, it is worth mentioning that we have studied only a small number of thyroid glands because of the limited availability of these specimens. In addition, it is feasible that the resident lymphocyte populations of the thyroid glands studied could be affected by the long treatment history of GD patients. In any case, we consider performing a longitudinal study of Th17 cells in patients with AITD of interest, mainly because it has been suggested that it could occur a Th1-Th2 shift during the evolution of GD (54). Thus, whether there are variations in the commitment or differentiation of Th17 cells during the evolution of HT or GD remains an interesting question.

In summary, our data suggest that there is an enhanced differentiation of Th17 lymphocytes in AITD patients, mainly in those with HT, and that these cells might participate in the pathogenesis of the inflammatory phenomenon and tissue damage observed in this condition.

## Acknowledgments

We extend special thanks to our colleagues, Dr. E. Larrañaga and J. M. Bravo Lifante, for their kind provision of thyroid specimens. We also thank to Dr. Manuel Luque, Lía Nattero, and Alfonso Arranz for helping in recruitment of patients. We gratefully acknowledge the help of Dr. Abelardo Aguilera with statistical analysis. We also thank Dr. Luiz Stark Aroeira for critical review of the manuscript.

Address all correspondence and requests for reprints to: Mónica Marazuela, M.D., Ph.D., Service of Endocrinology, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, C/Diego de León 62, 28006 Madrid, Spain. E-mail: mmarazuela.hlpr@salud.madrid.org; or Roberto González-Amaro, M.D., Ph.D., Department of Immunology, School of Medicine, Universidad Autónoma de San Luis Potosí, Avenue V. Carranza 2405, C.P. 78230 San Luis Potosí, S.L.P., México. E-mail: rgonzale@uaslp.mx.

This work was supported by grants from Fondo para la Investigación Sanitaria 07/1119 and Fundación Mutua Madrileña (to M.M.); Programa de Biomedicina 2008-02635 from Ministerio de Ciencia e Innovación; Redes de Señalización Celular en Enfermedades de Etiología Inflamatoria from Comunidad Autónoma de Madrid; Fondo de Cooperación Internacional de Ciencia y Tecnología 95395 and Red Temática de Investigación Cooperativa en Enfermedades Cardiovasculares RD06/0014-0030 from Instituto de Salud Carlos III (to F.S.-M.). N.F.-V. was recipient of a scholarship from Consejo Nacional de Ciencia y Tecnología, México.

Disclosure Summary: The authors have no financial conflicts of interest.

## References


1. **Druet P, Sheela R, Pelletier L** 1996 Th1 and Th2 cells in autoimmunity. Chem Immunol 63:138–170
2. **Tato CM, Laurence A, O'Shea JJ** 2006 Helper T cell differentiation enters a new era: le roi est mort; vive le roi! J Exp Med 203:809–812
3. **Shevach EM** 2000 Suppressor T cells: rebirth, function and homeostasis. Curr Biol 10:R572–R5
4. **Soroosh P, Doherty TA** 2009 Th9 and allergic disease. Immunology 127:450–458
5. **Harrington LE, Mangan PR, Weaver CT** 2006 Expanding the effector CD4 T-cell repertoire: the Th17 lineage. Curr Opin Immunol 18:349–356
6. **Dong C** 2006 Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. Nat Rev Immunol 6:329–333
7. **Bettelli E, Korn T, Kuchroo VK** 2007 Th17: the third member of the effector T cell trilogy. Curr Opin Immunol 19:652–657
8. **Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, Basham B, Smith K, Chen T, Morel F, Lecron JC, Kastelein RA, Cua DJ, McClanahan TK, Bowman EP, de Waal Malefyt R** 2007 Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat Immunol 8:903–905
9. **Unutmaz D** 2009 RORC2: the master of human Th17 cell programming. Eur J Immunol 39:1452–1455
10. **Crome SQ, Wang AY, Kang CY, Levings MK** 2009 The role of retinoic acid-related orphan receptor variant 2 and IL-17 in the development and function of human CD4+ T cells. Eur J Immunol 39:1480–1493
11. **Ouyang W, Kolls JK, Zheng Y** 2008 The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28:454–467
12. **Traves SL, Donnelly LE** 2008 Th17 cells in airway diseases. Curr Mol Med 8:416–426
13. **Nistala K, Moncrieffe H, Newton KR, Varsani H, Hunter P, Wedderburn LR** 2008 Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. Arthritis Rheum 58:875–887
14. **Kryczek I, Bruce AT, Gudjonsson JE, Johnston A, Aphale A, Vatan L, Szeliga W, Wang Y, Liu Y, Welling TH, Elder JT, Zou W** 2008 Induction of IL-17+ T cell trafficking and development by IFN-gamma: mechanism and pathological relevance in psoriasis. J Immunol 181:4733–4741
15. **Garrett-Sinha LA, John S, Gaffen SL** 2008 IL-17 and the Th17 lineage in systemic lupus erythematosus. Curr Opin Rheumatol 20: 519–525
16. **Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ** 2007 Distinct regulation of interleukin-17 in human T helper lymphocytes. Arthritis Rheum 56:2936–2946
17. **Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F** 2007 Interleukins 1$\beta$ and 6 but not transforming growth factor-$\beta$ are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 8:942–949
18. **Liu Z, Yang L, Cui Y, Wang X, Guo C, Huang Z** 2009 IL-21 and TGF-$\beta$ are required for differentiation of human T(H)17 cells. Inflamm Bowel Dis 15:1133–1144
19. **Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR** 2007 IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 8:967–974
20. **Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, Villarino AV, Huang Q, Yoshimura A, Sehy D, Saris CJ, O'Shea JJ, Hennighausen L, Ernst M, Hunter CA** 2006 Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. Nat Immunol 7:937–945
21. **Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT** 2006 Transforming growth factor-$\beta$ induces development of the T(H)17 lineage. Nature 441:231–234
22. **Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B** 2006 TGF$\beta$ in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. Immunity 24:179–189
23. **Hwang SY, Kim HY** 2005 Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. Mol Cells 19:180–184
24. **Weaver CT, Hatton RD, Mangan PR, Harrington LE** 2007 IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25:821–852
25. **Korn T, Oukka M, Kuchroo V, Bettelli E** 2007 Th17 cells: effector T cells with inflammatory properties. Semin Immunol 19:362–371
26. **Lubberts E, Joosten LA, van de Loo FA, Schwarzenberger P, Kolls J, van den Berg WB** 2002 Overexpression of IL-17 in the knee joint of collagen type II immunized mice promotes collagen arthritis and aggravates joint destruction. Inflamm Res 51:102–104
27. **Paradowska A, Maśliński W, Grzybowska-Kowalczyk A, Łacki J** 2007 The function of interleukin 17 in the pathogenesis of rheumatoid arthritis. Arch Immunol Ther Exp (Warsz) 55:329–334
28. **Kurasawa K, Hirose K, Sano H, Endo H, Shinkai H, Nawata Y, Takabayashi K, Iwamoto I** 2000 Increased interleukin-17 production in patients with systemic sclerosis. Arthritis Rheum 43:2455–2463
29. **Arican O, Aral M, Sasmaz S, Ciragil P** 2005 Serum levels of TNF-$\alpha$, IFN-$\gamma$, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. Mediators Inflamm 2005:273–279
30. **Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW** 2008 Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. Clin Immunol 127:385–393
31. **Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, Nojima Y** 2005 Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. Arthritis Rheum 52:1037–1046
32. **Weetman AP** 2004 Autoimmune thyroid disease. Autoimmunity 3:337–340
33. **Weetman AP** 2004 Cellular immune responses in autoimmune thyroid disease. Clin Endocrinol 61:405–413
34. **Marazuela M** 1999 Lymphocyte traffic and homing in autoimmune thyroid disorders. Eur J Endocrinol 140:287–290
35. **Liblau RS, Singer SM, McDevitt HO** 1995 Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 16:34–38
36. **Paschke R, Schuppert F, Taton M, Velu T** 1994 Intrathyroidal cytokine gene expression profiles in autoimmune thyroiditis. J Endocrinol 141:309–315
37. **García-López MA, Sancho D, Sánchez-Madrid F, Marazuela M** 2001 Thyrocytes from autoimmune thyroid disorders produce the chemokines IP-10 and Mig and attract CXCR3+ lymphocytes. J Clin Endocrinol Metab 86:5008–5016
38. **Ajjan RA, Weetman AP** 2003 Cytokines in thyroid autoimmunity. Autoimmunity 36:351–359
39. **Marazuela M, García-López MA, Figueroa-Vega N, de la Fuente H, Alvarado-Sánchez B, Monsiváis-Urenda A, Sánchez-Madrid F, González-Amaro R** 2006 Regulatory T cells in human autoimmune thyroid disease. J Clin Endocrinol Metab 91:3639–3646
40. **Figueroa-Vega N, Sanz-Cameno P, Moreno-Otero R, Sánchez-Madrid F, González-Amaro R, Marazuela M** 2009 Serum levels of angiogenic molecules in autoimmune thyroid diseases and their cor-

relation with laboratory and clinical features. J Clin Endocrinol Metab 94:1145–1153

41. **Acevedo A, del Pozo MA, Arroyo AG, Sánchez-Mateos P, González-Amaro R, Sánchez-Madrid F** 1993 Distribution of ICAM-3-bearing cells in normal human tissues. Expression of a novel counter-receptor for LFA-1 in epidermal Langerhans cells. Am J Pathol 143:774–783
42. **Stockinger B, Veldhoen M, Martin B** 2007 Th17 T cells: linking innate and adaptive immunity. Semin Immunol 19:353–361
43. **Pène J, Chevalier S, Preisser L, Vénéreau E, Guilleux MH, Ghannam S, Molès JP, Danger Y, Ravon E, Lesaux S, Yssel H, Gascan H** 2008 Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. J Immunol 180:7423–7430
44. **Gu Y, Yang J, Ouyang X, Liu W, Li H, Yang J, Bromberg J, Chen SH, Mayer L, Unkeless JC, Xiong H** 2008 Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. Eur J Immunol 38:1807–1813
45. **Rachitskaya AV, Hansen AM, Horai R, Li Z, Villasmil R, Luger D, Nussenblatt RB, Caspi RR** 2008 Cutting edge: NKT cells constitutively express IL-23 receptor and RORgammat and rapidly produce IL-17 upon receptor ligation in an IL-6-independent fashion. J Immunol 180:5167–5171
46. **O'Brien RL, Roark CL, Born WK** 2009 IL-17-producing γΔ T cells. Eur J Immunol 39:662–666
47. **Huber M, Heink S, Grothe H, Guralnik A, Reinhard K, Elflein K, Hünig T, Mittrücker HW, Brüstle A, Kamradt T, Lohoff M** 2009 A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. Eur J Immunol 39:1716–1725
48. **Nakae S, Iwakura Y, Suto H, Galli SJ** 2007 Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. J Leukoc Biol 81:1258–1268
49. **Li MO, Wan YY, Flavell RA** 2007 T cell-produced transforming growth factor-β1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity 26:579–591
50. **Voo KS, Wang YH, Santori FR, Boggiano C, Wang YH, Arima K, Bover L, Hanabuchi S, Khalili J, Marinova E, Zheng B, Littman DR, Liu YJ** 2009 Identification of IL-17-producing FOXP3+ regulatory T cells in humans. Proc Natl Acad Sci USA 106:4793–4798
51. **Mathers AR, Janelsins BM, Rubin JP, Tkacheva OA, Shufesky WJ, Watkins SC, Morelli AE, Larregina AT** 2009 Differential capability of human cutaneous dendritic cell subsets to initiate Th17 responses. J Immunol 182:921–933
52. **Inoue N, Watanabe M, Nanba T, Wada M, Akamizu T, Iwatani Y** 2009 Involvement of functional polymorphisms in the TNFA gene in the pathogenesis of autoimmune thyroid diseases and production of anti-thyrotropin receptor antibody. Clin Exp Immunol 156:199–204
53. **Nanba T, Watanabe M, Inoue N, Iwatani Y** 2009 Increases of the Th1/Th2 cell ratio in severe Hashimoto's disease and in the proportion of Th17 cells in intractable Graves' disease. Thyroid 19:495–501
54. **Kocjan T, Wraber B, Repnik U, Hojker S** 2000 Changes in Th1/Th2 cytokine balance in Graves' disease. Pflugers Arch 440(Suppl 5): R94–R95