Clinical & Experimental Immunology
The Journal of Translational Immunology

British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12557

Altered balance between self-reactive T helper (Th)17 cells and Th10 cells and between full-length forkhead box protein 3 (FoxP3) and FoxP3 splice variants in Hashimoto's thyroiditis

B. Kristensen,*† L. Hegedüs,† H. O. Madsen,‡ T. J. Smith§ and C. H. Nielsen*

**Institute for Inflammation Research, Department of Infectious Diseases and Rheumatology, ‡The Tissue Typing Laboratory, Department of Clinical Immunology, Copenhagen University Hospital Rigshospitalet, Copenhagen, †Department of Endocrinology and Metabolism, Odense University Hospital, Odense, Denmark, and §Departments of Ophthalmology and Visual Sciences, Kellogg Eye Center, and Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA*

Accepted for publication 18 November 2014
Correspondence: C. H. Nielsen, Institute for Inflammation Research, Department of Infectious Diseases and Rheumatology, Copenhagen University Hospital Rigshospitalet, Copenhagen, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark.
E-mail: claus.henrik.nielsen@regionh.dk.

Summary

T helper type 17 (Th17) cells play a pathogenic role in autoimmune disease, while interleukin (IL)-10-producing Th10 cells serve a protective role. The balance between the two subsets is regulated by the local cytokine milieu and by the relative expression of intact forkhead box protein 3 (FoxP3) compared to FoxP3Δ2, missing exon 2. Th17 and Th10 cell differentiation has usually been studied using polyclonal stimuli, and little is known about the ability of physiologically relevant self-antigens to induce Th17 or Th10 cell differentiation in autoimmune thyroid disease. We subjected mononuclear cells from healthy donors and patients with Hashimoto's thyroiditis (HT) or Graves' disease (GD) to polyclonal stimulation, or stimulation with human thyroglobulin (TG), human thyroid peroxidase (TPO), or *Escherichia coli* lipopolysaccharide (LPS). TPO and LPS induced increased differentiation of naive $CD4^+CD45RA^+CD45R0^-$ T cells from HT patients into Th17 cells. Th10 cell proportions were decreased in HT after polyclonal stimulation, but were comparable to those of healthy donors after antigen-specific stimulation. Taken together, our data show that an increased Th17 : Th10 ratio was found in HT patients after stimulation with thyroid-specific self-antigens. We also observed an elevated baseline production of IL-6 and transforming growth factor (TGF)-β1 and of mRNA encoding FoxP3Δ2 rather than intact FoxP3. This may contribute to the skewing towards Th17 cell responses in HT.

Keywords: Graves' disease, Hashimoto's thyroiditis, interleukins, regulatory T cells, Th17 cells

Introduction

Autoimmune thyroid disease (AITD) includes Hashimoto's thyroiditis (HT) and Graves' disease (GD), which are characterized by the breakdown of immune self-tolerance towards thyroid antigens followed by lymphocytic infiltration of the thyroid gland (reviewed in Weetman [1]). In HT, T cells cause the destruction of thyroid epithelial architecture leading to hypothyroidism. In contrast, GD is characterized by generation of autoantibodies against the thyroid stimulating hormone receptor (TSHR). These stimulate thyrocytes and promote thyroid hormone overproduction and result in hyperthyroidism [1]. Immune responses against two major thyroid self-antigens, thyroglobulin (TG) and thyroid peroxidase (TPO), are frequently associated with both HT and GD, as is evidenced by the high prevalence of anti-TG and anti-TPO antibodies [1].

$CD4^+$ T cells play essential roles in adaptive immune responses by virtue of their production of cytokines. Mosmann and colleagues demonstrated that $CD4^+$ T helper (Th) cells can be subdivided into Th1 and Th2 cells on the basis of distinct cytokine profiles [2–4]. Signature Th1 cytokines include interferon (IFN)-γ, while interleukin-4 (IL-4) production represents the Th2 prototype [2–4]. Substantial evidence links Th1 cells to the pathogenesis of AITD [5–9]. Relatively recent insights have revealed a third Th cell subset that generates IL-17 [10]. These, designated Th17 cells, play important roles in the clearance of extracellular pathogens [11]. Th17 cells have been associated with several autoimmune diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel disease,

 © 2014 British Society for Immunology,

neuromyelitis optica and multiple sclerosis [11–13]. Recent studies have demonstrated increased proportions of Th17 cells in peripheral blood from patients with HT or GD following polyclonal stimulation [13–16].

Regulatory T cells (T_{regs}) represent a fourth $CD4^+$ T cell subset that is critical in maintaining tolerance to self-antigens [17]. T_{regs} can be classified as either natural T_{regs} (nT_{regs}), which leave the thymus as mature cells [17,18], or as inducible T_{regs} (iT_{regs}), which are generated from naive $CD4^+$ T cells in the periphery after antigenic stimulation [18] (reviewed in [19]). nT_{regs} are identified as $CD4^+$ T cells expressing the transcription factor forkhead box protein 3 (FoxP3) and expressing high levels of the IL-2 receptor α-chain, CD25 [20–22]. iT_{regs} are distinguished by their production of IL-10 or transforming growth factor (TGF)-β [18,19]. However, iT_{regs} may also acquire expression of FoxP3/CD25 [23], while nT_{regs} may produce IL-10/TGF-β [24–26], making the two subsets difficult to distinguish. In a few studies, the term 'Th10' cells has been applied to IL-10-producing $CD4^+$ T cells, irrespective of their origins [27]. Grouping HT and GD together as AITD, Glick *et al.* found equal numbers of $CD25^{hi}$ $FoxP3^+$ T_{regs} circulating in individuals with AITD and healthy controls [28]. Pan *et al.* reported similar findings in GD [29]. Previously, we demonstrated that the thyroid self-antigens, TG and TPO, could induce IL-10 production in human mononuclear cells (MNCs) [30–32].

A high degree of 'plasticity' exists between Th17 cells and iT_{regs}. Low concentrations of TGF-β in combination with IL-6, IL-21 or IL-1β drive Th17 differentiation from naive Th cells (Th0 cells) [11,33–36]. In the absence of the aforementioned proinflammatory cytokines, TGF-β drives iT_{reg} differentiation [11,37]. Also, $CD25^{hi}FoxP3^+$ Th cells within the memory cell ($CD45R0^+$) compartment [38] can be converted into Th17 cells [39,40]. IL-23 may be important in maintaining Th17 cells once fully differentiated [41], as well as in acquisition of full Th17 pathology [42].

Studies on T_{regs} and Th17 have generally involved polyclonal stimulation, typically with phorbol 12-myristate 13-acetate (PMA)/ionomycin [13–16]. Th17-cell differentiation following more physiologically relevant stimulation, such as with self-antigens, has yet to be examined. One study used microbial pathogens as a stimulus and found, surprisingly, that *Candida albicans* and *Staphylococcus aureus* both prime Th17 cells with co-production of IFN-γ and IL-10, respectively, depending on whether IL-1β or IL-2 was present [43]. Little is known about the ability of self-antigens to induce IL-17 and IL-10 production by human Th cells. We have demonstrated previously that TG induces IL-10 production by $CD4^+$ T helper cells with a $CD45R0^+$ phenotype [31], and that other self-antigens, such as myelin basic protein, induce IL-17 production in cultured peripheral blood mononuclear cells (PBMCs) from healthy controls [44]. To our knowledge, no studies have addressed the polarization of human $CD4^+$ T cells into Th17 cells driven by thyroid self-antigens, or examined the balance between Th17 cells and Th10 cells in healthy individuals and those with AITD.

Here we report that TG and TPO can induce IL-17 and IL-10 in circulating $CD4^+$ T cells from patients with AITD and those from healthy donors. We also assessed the induction of IL-6-producing $CD4^+$ T cells. Finally, we determined whether the self-reactive Th17 and Th10 cells represent reactivated memory cells or differentiate *de novo* from the pool of circulating naive Th cells.

Materials and methods

Patients

The study included 10 patients with HT (defined as serum TSH above 10 IU/l, and serum TPO antibodies > 100 U/l and absence of TSHR antibodies) and 11 patients with GD (defined as a suppressed serum TSH with increased serum freeT4 (FT4) and freeT3 (FT3), elevated serum TSHR antibodies, diffuse uptake on thyroid scintigraphy and ultrasound demonstrating diffuse hypoechogenicity), attending the endocrinology out-patient clinic at Odense University Hospital between August 2012 and October 2013. All patients were diagnosed within 3 years of study participation, with the exception of one HT patient diagnosed in 2006. Clinical characteristics for the patients at the time of blood collection are shown in Table 1. Eight HT

Table 1. Patient characteristics.

	Graves' disease ($n = 11$)	Hashimoto's thyroiditis ($n = 10$)
Age (years)		
Median	45·0	45·5
Interquartile range	27–77	24–74
Gender (% females)	72·7	90·0
Thyroid hormone levels		
TSH (mIU/l)[†]	2·8 ± 6·4	4·7 ± 2·9**
T3 (nmol/l)[‡]	2·6 ± 1·1	1·7 ± 0·5*
T4 (nmol/l)[§]	117·9 ± 51·6	87·5 ± 26·2
T4 uptake (nmol/l)[¶]	1·0 ± 0·2	1·0 ± 0·2
FT3[††]	2·8 ± 1·5	1·6 ± 0·2**
FT4[††]	122·8 ± 53·4	85·0 ± 13·1
Autoantibodies		
Anti-TSHR (kIU/l)[‡‡]	7·1 ± 8·5	Negative
Anti-TPO (kIU/l)[§§]	327·9 ± 721·5	1379·7 ± 1092·5

All thyroid hormone and autoantibody levels are taken at the time of immunological studies. [†]Normal range: 0·3–4·0 mIU/l; [‡]normal range: 1·3–2·2 nmol/l; [§]normal range: 60–130 nmol/l; [¶]normal range: 0·6–1·2 nmol/l; [††]FT3 or FT4 were defined as T3 or T4 divided by T4 uptake; [‡‡]positive >1·0 kIU/l; [§§]normal range: 2·1–9·8 kIU/l. *$P < 0·05$; **$P < 0·01$ between Hashimoto's thyroiditis (HT) and Graves' disease (GD) patients. TSH = thyroid stimulating hormone; TSHR = thyroid stimulating hormone receptor; TPO = thyroid peroxidase.

patients were receiving levothyroxin [median = 75 μg/day, interquartile range (IQR) = 50–100 μg/day], while nine GD patients were receiving methimazole (median = 15 mg/day, IQR = 5–20 mg/day) at the time of blood collection. The duration of anti-thyroid treatment at the time of blood collection varied from 2 weeks to 8 years. Fifteen anonymous healthy donors with no history of autoimmune disease (11 females, four males, median age 46 years) attending the Blood Bank at Copenhagen University Hospital served as controls. The study was approved by the Ethical Committee from the Region of Southern Denmark (project no. 28699) and followed the guidelines outlined in the Declaration of Helsinki. Written informed consent was obtained from all included patients.

Biochemistry

Serum TSH was measured by solid-phase, two-site chemiluminescent immunometric assay on an Immulite 2000 equipment (Siemens, Erlangen, Germany). The limit of detection for TSH was 0·004 mIU/l. T4 and T3 were measured using time-resolved fluoroimmunoassays based on competitive binding to T3- or T4-specific antibodies, respectively, on AutoDELFIA equipment (Perkin Elmer/Wallac, Turku, Finland). The limit of detection was 0·3 mmol/l for T3 and 5·0 mmol/l for T4. FT3 and FT4 were defined as T3 or T4 divided by T4 uptake. Anti-TPO antibodies were measured by solid-phase time-resolved fluoroimmunometric assays (AutoDELFIA), and anti-TSHR antibodies were measured by DynOtest TRAK human radio receptor assay (Brahms Diagnostica, Berlin, Germany). The limits of detection for anti-TPO antibodies and anti-TSHR antibodies were <1·0 kIU/l and 0·3 U/l, respectively.

Reference ranges were 0·3–4·0 mIU/l for TSH, 1·3–2·2 nmol/l for T3, 60–130 nmol/l for T4, 0·6–1·2 nmol/l for T4 uptake, 2·1–9·8 kIU/l for anti-TPO antibodies. Samples were regarded as positive for anti-TSHR antibodies if above 1·0 kIU/l.

Cells and serum

Venous blood was collected in tubes with or without heparin (BD Vacutainer Systems, Plymouth, UK). For isolation of PBMCs, blood samples were centrifuged over LymphoPrep™ (Axis-Shield, Oslo, Norway) at 1200 ***g***, without brake, for 30 min. The cells were washed twice in phosphate-buffered saline (Gibco/Invitrogen Life Technologies, Carlsbad, CA, USA) by spinning at 400 ***g*** for 10 min. All subsequent centrifugations were carried out at 400 ***g***. Human serum isolated from healthy male donors of blood group AB (AB serum) was purchased from Lonza (Basel, Switzerland; cat. no. 14-490E) and used as the serum source in all experiments.

Antibodies

Cells were stained using a combination of the following antibodies: anti-CD4 peridinin chlorophyll (PerCP) (cat. no. 345–770), anti-CD45RA fluorescein isothiocyanate (FITC) (cat. no. 555-488), anti-CD45R0 allophycocyanin (APC) (cat. no. 559–865), anti-IL-6 phycoerythin (PE) (cat. no. 340–527), anti-IL-17A PE (cat. no. 560-436) or IL-10 PE (cat. no. 559-330). All antibodies were purchased from BD Biosciences (San Jose, CA, USA) and were used according to the manufacturer's instructions.

Antigens

Human TG (MW 660 kDa) purified from thyroid tissue was purchased from Aviva Systems Biology (San Diego, CA, USA; cat. no. OPSA10707) and was found to contain endotoxin using the Limulus amebocyte lysate assay (QCL-1000 chromogenic LAL; Lonza; cat. no. 50-647U). Endotoxin was removed using Triton X-114 as described previously [45]. Human reconstituted TPO (MW 18·6 kDa) was a generous gift from Marlena Godlewska (Medical Centre of Postgraduate Education, Warsaw, Poland). *Escherichia coli* lipopolysaccharide (LPS) O111:B4 strain (Sigma Aldrich, St Louis, MO, USA; cat. no. L2630) was used as a foreign control antigen.

PBMC cultures

PBMCs were inoculated onto flat-bottomed 96-well Nunc Microtitre Nunclon plates (Fisher Scientific, Loughborough, UK) at a density of 5×10^5 cells/well. These were stimulated with TG (30 μg/ml), TPO (30 μg/ml) or *E. coli* LPS (50 ng/ml) in RPMI-1640 medium containing L-glutamine (Gibco/Invitrogen Life Technologies), gentamicin (50 μg/ml; Lonza) and 30% (v/v) human AB serum (Lonza) to a final volume of 100 μl per well. One well per donor was stimulated with anti-CD3/anti-CD28 micro-Dynabeads® (Life Technologies). Unstimulated cells (no antigen) served as negative controls. Cultures were incubated in a humidified 5% CO_2 incubator at 37°C for 18 or 48 h.

Cytokine measurements

For IL-17A and IL-6, brefeldin A (cat. no. 420601; Biolegend, San Diego, CA, USA) was added (1 μl/well) to PBMCs after the initial 6 h of antigen stimulation. After a further 12 h incubation, cells were stained with anti-CD4 PerCP, anti-CD45RA FITC and anti-CD45R0 APC. For intracellular staining, cells were fixed and made permeable using CytoFix/CytoPerm (cat. no. 554–722; BD Biosciences, San Jose, CA, USA) and stained with anti-IL-17A PE or anti-IL-6 PE. For IL-10 staining, cultures were incubated for

 © 2014 British Society for Immunology,

48 h and brefeldin A was added during the final 6 h. Culture supernatants were collected after 18 h and assessed for levels of IL-1β, IL-6 and TGF-β1 by Luminex (Austin, TX, USA). Multiplex beads were supplied by Bio-Rad (Hercules, CA, USA).

Flow cytometry

After stimulation, cells were washed and resuspended in phosphate-buffered saline and stained. A FACS Canto (BD Biosciences) flow cytometer with argon laser (488 nm) and helium–neon laser (633 nm) excitation was used to acquire fluorescence, and the FACS Diva software (BD Biosciences) was used for analysis.

RNA purification and cDNA synthesis

Following stimulation with TG, TPO or *E. coli* LPS, mRNA was extracted, purified, and isolated using Qiashredder (cat. no. 79654; Qiagen, Hilden, Germany) and RNeasy Mini Kit (cat. no. 74104; Qiagen). cDNA was synthesized as described previously [46]. The reaction conditions were as follows: 42°C for 30 min, 99°C for 5 min and 4°C for 10 min [47].

Quantitiative reverse transcription–polymerase chain reaction (qRT–PCR)

qRT–PCR was used for quantification of mRNA encoding FoxP3 (referred to as total FoxP3, comprising all isoforms) or the FoxP3Δ2 isoform, specifically as described [46]. CD4 served as the house-keeping gene, as its expression is invariant with respect to stimulation [48]. The cycle threshold (Ct) values were averaged and normalized against the corresponding CD4 values (ΔCt).

Statistics

Comparisons between each patient group and healthy controls were conducted using the two-tailed Mann–Whitney *U*-test. Differences between HT and GD patients were considered subordinate. Correlation between cytokine production and thyroid hormones, TSH or TSHR-antibody levels was evaluated using Spearman's rank correlation coefficient. All analysis was carried out using GraphPad Prism version 6·00 (GraphPad Software, La Jolla, CA, USA). Raw *P*-values are presented in the figures, and Bonferroni-adjusted *P*-values (raw *P*-values multiplied by 2) are given in the text body. Adjusted *P*-values < 0·05 were considered significant.

Results

Induction of IL-17-, IL-10- and IL-6-producing Th cells by anti-CD3/anti-CD28 stimulation

PBMCs from 15 healthy controls, 11 patients with GD and 10 with HT were isolated and stimulated with anti-CD3/anti-CD28 micro-Dynabeads®. The frequencies of Th cells producing IL-17, IL-10 or IL-6 were examined by flow cytometry, following the gating strategy shown in Fig. 1a,b. The frequency of Th cells was similar in all three donor groups as determined by flow cytometry (data not shown).

The stimulation provoked differentiation of 5 per 10 000 Th cells in HT patients, 0·4 per 10 000 Th cells in GD patients and 10 per 10 000 Th cells into Th17 cells in cultures derived from healthy controls (Fig. 1c). Neither of the patient groups deviated significantly from the healthy controls. A positive correlation was observed in HT patients between circulating TSH levels and the proportion of committed Th17 cells, as determined by the proportion of Th17 cells induced by anti-CD3/anti-CD28 beads ($Rs = 0{\cdot}78$, $P = 0{\cdot}048$; data not shown).

A smaller proportion of Th10 cells was induced in cultures from HT patients (10 per 10 000 Th cells) than in cultures from healthy donors (44 per 10 000 Th cells, adjusted *P*-value = 0·028), as shown in Fig. 1d. The proportion in GD was intermediate (24 per 10 000 Th cells). No significant associations were observed between FT3 or FT4 and Th10 differentiation.

Polyclonal activation failed to yield IL-6 producing Th cells in any donor group (Fig. 1e).

Induction of Th17 cells by thyroid self-antigens and *E. coli* LPS

We next examined the induction of cytokine-producing Th cells by the thyroid self-antigens TG and TPO (Fig. 2). *E. coli* LPS, which is known to expand pre-existing Th17 cells, was used as a foreign control antigen [49]. To distinguish between naive and memory Th cells, PBMC cultures were co-stained with anti-CD45RA and anti-CD45R0 antibodies (Fig. 2a). Before cultivation, similar ratios of naive and memory Th cells were found in the three donor groups (GD: 33 *versus* 29%; HT: 29 *versus* 34%; and healthy controls: 33 *versus* 29%, respectively) (data not shown).

TG induced IL-17 production in 8·4 per 10 000 naive Th cells from HT patients, 4·8 per 10 000 naive Th cells from GD patients and 2·8 per 10 000 naive Th cells from healthy donors (Fig. 2b,c). The corresponding proportions in the memory cell compartment were 4·0 per 10 000 Th cells from HT patients, 2·1 per 10 000 Th cells from GD patients and 5·3 per 10 000 Th cells from healthy donors (Fig. 2d). These proportions did not differ significantly between the groups.

TPO induced IL-17 production in 2·8 per 10 000 naive Th cells from individuals with HT, but in no cells from GD patients or healthy donors (adjusted $P = 0{\cdot}016$ *versus* HT patients), as shown in Fig. 2c. In the memory cell compartment, TPO failed to induce IL-17 production in all three groups (Fig. 2d).

E. coli LPS stimulated IL-17 production in naive Th cells from HT (11·9 per 10 000 cells) but failed to do so in this

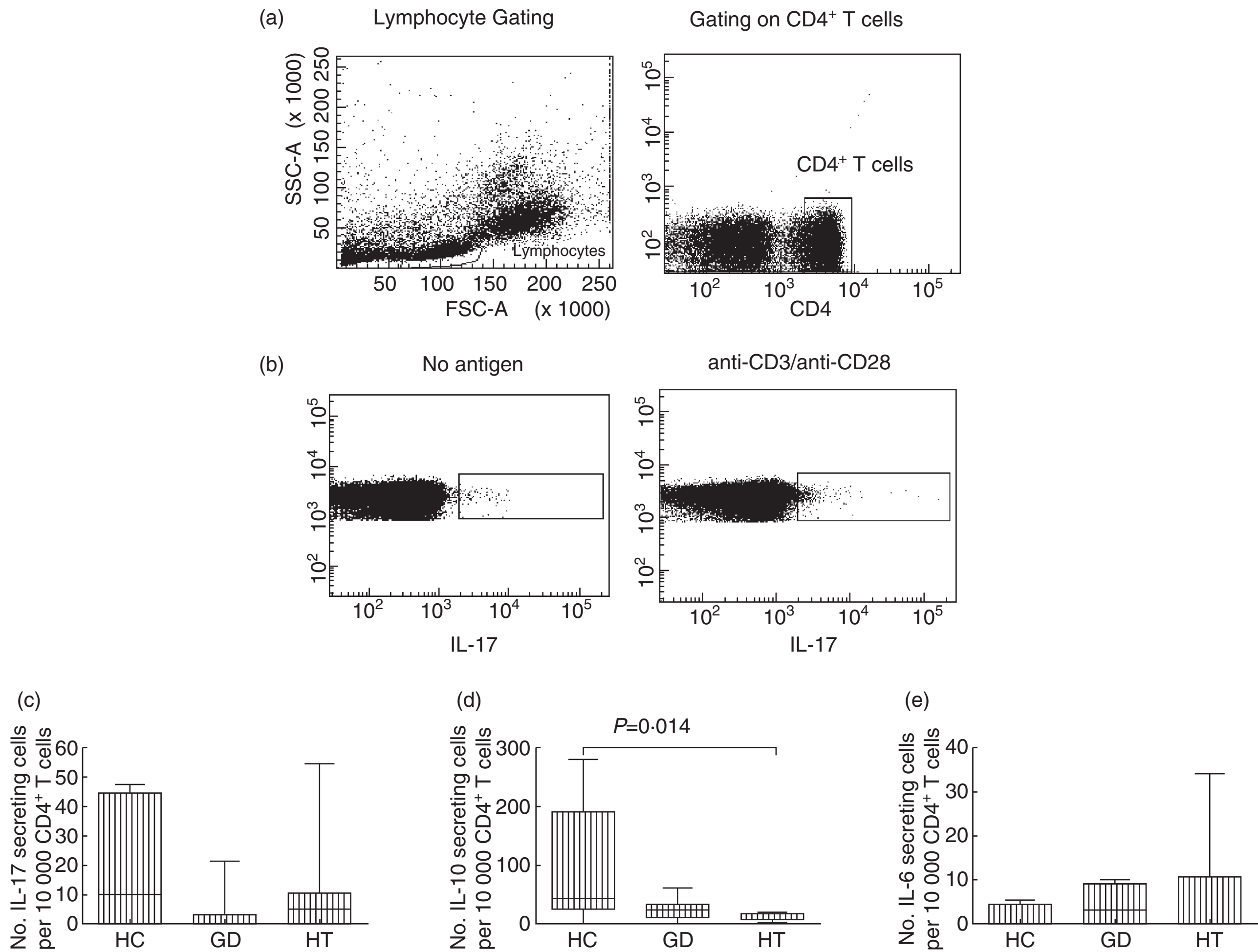

Fig. 1. Quantification of T helper (Th)17-, Th10- and interleukin (IL)-6-producing Th cells in subjects with autoimmune thyroid disease (AITD) and healthy controls. Peripheral blood mononuclear cell (PBMC) cultures were stimulated with anti-CD3/anti-CD28 micro-Dynabeads and stained for intracellular cytokine content. (a) Within a morphological lymphocyte gate (left panel) $CD4^+$ Th cells were gated (right panel). (b) Cytokine-producing cells were defined as positive when appearing within the gate shown. The left panel shows the background (unstimulated $CD4^+$ Th cells), while the right panel demonstrates $CD4^+$ Th cells stimulated with anti-CD3/anti-CD28. (c) The proportion of Th17 cells, (d) Th10 cells and (e) IL-6-producing Th cells are shown for eight healthy controls (HC), seven patients with Graves' disease (GD) and seven patients with Hashimoto's thyroiditis (HT). The respective proportions observed in the absence of a stimulating antigen have been subtracted. Box-plots indicate median, interquartile range (box) and range (whiskers). Raw *P*-values are shown (Mann–Whitney *U*-test); negative net values were included in calculations.

compartment from GD patients or healthy donors (adjusted $P = 0{\cdot}014$, HT *versus* healthy donors), as shown in Fig. 2c. The proportion of memory Th cells induced by *E. coli* LPS was greater in HT (19·2 per 10 000 cells) than in healthy controls (1·4 per 10 000 cells; adjusted $P = 0{\cdot}027$; Fig. 2d).

Induction of Th10 cells by thyroid self-antigens and *E. coli* LPS

To examine induction of Th10 cells, PBMCs were stimulated with TG, TPO or *E. coli* LPS for 48 h.

TG induced IL-10 production in 2·6 per 10 000 naive Th cells from HT patients, no naive Th cells from GD patients and 1·9 naive Th cells from healthy controls (Fig. 3a). Moreover, IL-10 production was induced by TG in 5·5 per 10 000 memory Th cells from HT patients, 1·2 per 10 000 memory Th cells from GD patients and 2·6 per 10 000 memory Th cells from healthy donors. None of these responses were significantly different in the three donor groups.

TPO and *E. coli* LPS induced Th10 cells uniformly in the three donor groups. The medians ranged from 0 to 3·5-induced Th10 cells per 10 000 Th cells in both the naive and memory cell compartments (Fig. 3a,b). Notably, a negative association was observed between circulating anti-TSHR antibody levels in GD patients and the proportion of TPO-induced Th10 cells in the naive Th compartment (Fig. 3c).

 © 2014 British Society for Immunology,

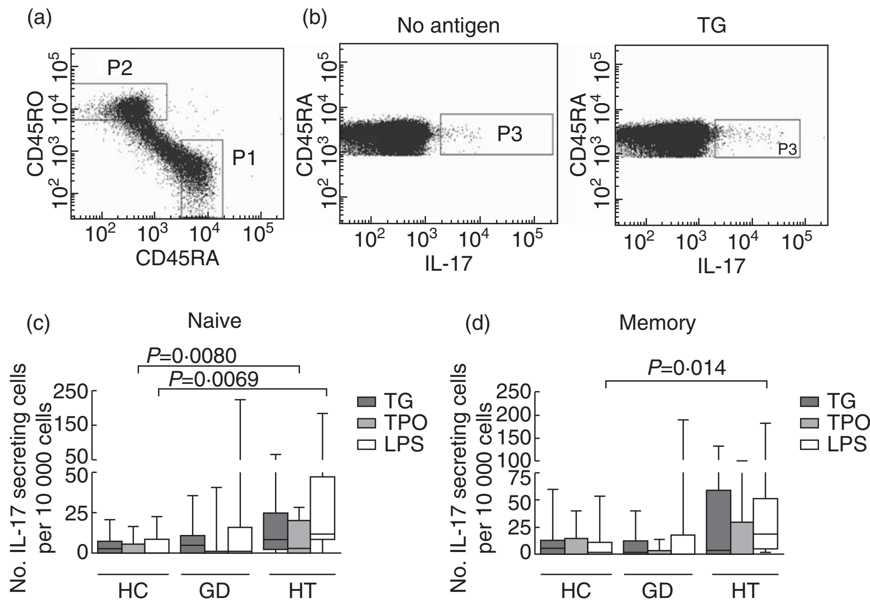

Fig. 2. T helper (Th)17 cell differentiation driven by thyroid self-antigens. Peripheral blood mononuclear cells (PBMCs) were incubated with thyroglobulin (TG), thyroid peroxidase (TPO) or *Escherichia coli* lipopolysaccharide (LPS) for 18 h, and $CD4^+$interleukin (IL)-17^+ Th cells were assessed by flow cytometry. (a) Naive and memory $CD4^+$ Th cells were identified as $CD45RA^+CD45R0^-$ events (P1) and $CD45RA^-CD45R0^+$ events (P2), respectively. (b) $Th17^+$ cells were identified as events within the P3 gate. Left panel shows naive unstimulated $CD4^+$ Th cells (no antigen); right panel shows naive cells stimulated with thyroglobulin (TG). The same gating strategy was applied to TPO- and LPS-stimulated cells. (c) Proportions of naive $Th17^+$ cells in PBMC cultures derived from healthy controls (HC, $n = 15$), donors with Graves' disease ($n = 11$) and donors with Hashimoto's thyroiditis ($n = 10$). (d) Corresponding proportions of memory $Th17^+$ cells are shown. Proportions observed in the absence of stimulating antigen have been subtracted. Box-plots indicate median, interquartile range (box) and range (whiskers). Raw *P*-values are shown (Mann–Whitney *U*-test); negative net values were included in calculations.

Relationship between antigen-induced Th17 and Th10 cells

Naive Th cells from HT donors differentiated preferentially into Th17 cells after stimulation with TG, TPO or *E. coli* LPS. The Th17 : Th10 ratio was 6·73 after stimulation with TG, 4·43 after stimulation with TPO and 3·73 after stimulation with *E. coli* (Fig. 4a). The corresponding ratios in donors with GD were 1·63, 1·54 and 0·26, respectively. By contrast, naive Th cells from healthy controls differentiated preferentially towards the Th10 phenotype rather than Th17. After stimulation with TG, the Th17 : Th10 ratio was

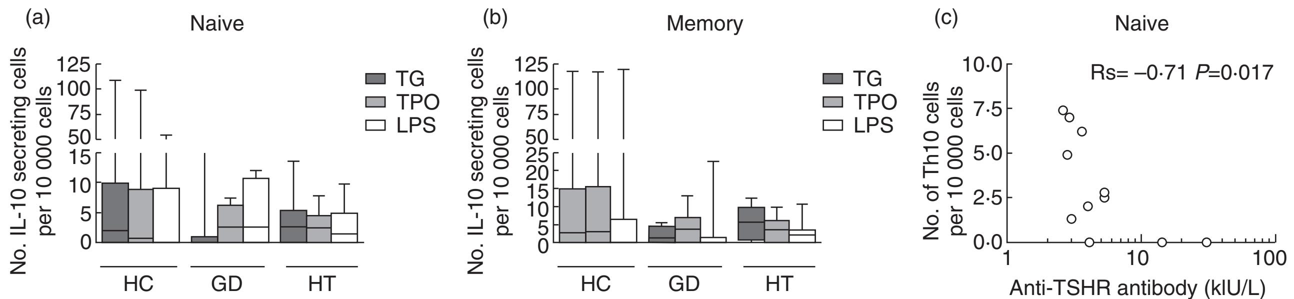

Fig. 3. T helper (Th)10 cell differentiation driven by thyroid self-antigens. Peripheral blood mononuclear cells (PBMCs) were incubated with thyroglobulin (TG), thyroid peroxidase (TPO) or *Escherichia coli* lipopolysaccharide for 48 h, and $CD4^+$ Th cells were stained intracellularly for IL-10 and detected by flow cytometry. (a) Proportions of naive $Th10^+$ cells are shown for healthy controls (HC, $n = 15$), patients with Graves' disease (GD, $n = 11$) and patients with Hashimoto's thyroiditis (HT, $n = 10$). (b) Corresponding proportions of memory $Th10^+$ cells are shown. Corresponding proportions observed in the absence of antigen have been subtracted. (c) The proportion of naive $Th10^+$ cells in GD patients ($n = 11$) after TPO stimulation is shown against circulating levels of anti- thyroid stimulating hormone receptor (TSHR) antibodies (kIU/l). Spearman's rank correlation coefficient (*Rs*) and level of significance are shown; negative net values were included in calculations. Box-plots indicate median, interquartile range (box) and range (whiskers).

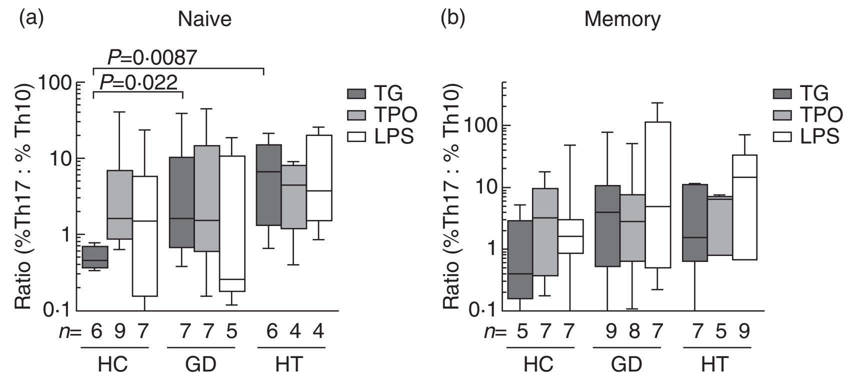

Fig. 4. Relationship between antigen-induced T helper (Th)17 cells and Th10 cells. Shown are ratios of antigen-induced Th17 cells (as shown in Fig. 2) divided by the corresponding proportions of Th10 cells (as shown in Fig 3). Ratios were calculated only in cases where both factors were >0. The number of observations (*n*) is shown below the *x*-axis. Box-plots indicate median, interquartile range (box) and range (whiskers). HC = healthy controls; GD = Graves' disease; HT = Hashimoto's thyroiditis. Raw *P*-values are shown (Mann–Whitney *U*-test).

0·46 (adjusted $P = 0{\cdot}017$ *versus* HT patients and $P = 0{\cdot}044$ *versus* GD patients). Positive Th17 : Th10 ratios were found in healthy donors after stimulation with TPO or *E. coli LPS* (1·64 and 1·50, respectively).

Similar tendencies were found in the memory Th cell population, although statistical significance was not reached (Fig. 4b).

Th17-related cytokines in culture supernatants

To examine whether the local cytokine milieu determined the propensity of naive Th cells to differentiate to Th17 cells (Figs 2c and 4), TGF-β1, IL-6 and IL-1β concentrations were assessed in conditioned medium from antigen-stimulated cell cultures.

The TGF-β1 levels were significantly higher in cultures from HT and GD patients than in those from healthy donors, regardless of antigenic stimulus (adjusted $P = 0{\cdot}0004$–$0{\cdot}04$; Fig. 5a). Stimulation with TG, TPO or *E. coli* LPS failed to enhance TGF-β1 levels in any group.

Although variable, baseline IL-6 production was higher in cultures from HT patients than those from healthy donors (adjusted $P = 0{\cdot}038$; Fig. 5b), while cultures from GD patients had baseline levels similar to those of healthy donors. Only in cultures from healthy donors did incubation with TG or TPO induce IL-6 above the basal level ($P = 0{\cdot}019$ and $P = 0{\cdot}0002$, respectively). *E. coli* LPS induced IL-6 in all donor groups above the basal levels ($P < 0{\cdot}0001$–$0{\cdot}0022$), with no differences between groups.

The baseline production of IL-1β did not differ between the donor groups (Fig. 5c). Only in the control group did TG and TPO induce IL-1β production ($P = 0{\cdot}032$ and $P = 0{\cdot}015$, respectively). TG-induced IL-1β in the GD group was lower than that in healthy donors (adjusted $P = 0{\cdot}0098$; Fig. 5c), while the TPO-induced IL-1β production tended to be lower among HT patients than among healthy donors ($P = 0{\cdot}060$). As anticipated, a dramatic increase in IL-1β production was observed following stimulation with *E. coli* LPS in all donor groups ($P < 0{\cdot}0001$–$0{\cdot}0022$), but neither of the patient groups differed from healthy donors in this respect (Fig. 5c).

Measurement of total FoxP3 and the splice variant FoxP3Δ2

The induction of total FoxP3 mRNA and mRNA encoding the splice variant FoxP3Δ2 was examined after stimulation with anti-CD3/anti-CD28 beads (Fig. 6a,b), as well as after antigen-specific stimulation (Fig. 6c,d). While the baseline expression of total FoxP3 mRNA was similar in the three donor groups (Fig. 6a,c), both patient groups displayed higher constitutive expression of FoxP3Δ2 than did healthy donors (adjusted $P = 0{\cdot}012$ in both cases; Fig. 6b,d).

While stimulation with TG or TPO had no significant effect on the total FoxP3 expression or on the expression of FoxP3Δ2 among cells from HT patients, both self-antigens significantly increased FoxP3Δ2 expression among GD patients ($P = 0{\cdot}029$ in both cases; Fig. 6c,d). Moreover, stimulation with TPO significantly increased the expression of total FoxP3 ($P = 0{\cdot}014$) as well as of FoxP3Δ2 ($P = 0{\cdot}011$) in healthy donors. Stimulation with *E. coli* LPS failed to increase the expression of total FoxP3 or FoxP3Δ2 in HT patients, but robustly increased the expression of both total FoxP3 and FoxP3Δ2 in cells from GD patients ($P = 0{\cdot}029$ in both cases) and healthy donors ($P = 0{\cdot}0003$ in both cases; Fig. 6c,d).

Regardless of whether stimulated or not, FoxP3Δ2 constituted approximately 30% of the total FoxP3 expressed in cells from healthy donors, compared to 59–67% for HT patients and 65–73% for GD patients ($P = 0{\cdot}008$ for all comparisons; Fig. 6e,f).

Discussion

Th17 cells are thought to play a pathogenic role in organ-specific autoimmune diseases [11]. In contrast, T_{regs}, including Th10 cells, exert immunoregulatory actions and in so doing protect against autoimmune disease manifestations [28,29]. The induction of Th17 and Th10 cells by disease-relevant self-antigens has not been characterized previously in AITD, nor has the balance between them. It is well known that Th0 cells can differentiate into Th17 cells or IL-10 producing iT_{regs} under the influence of TGF-β in

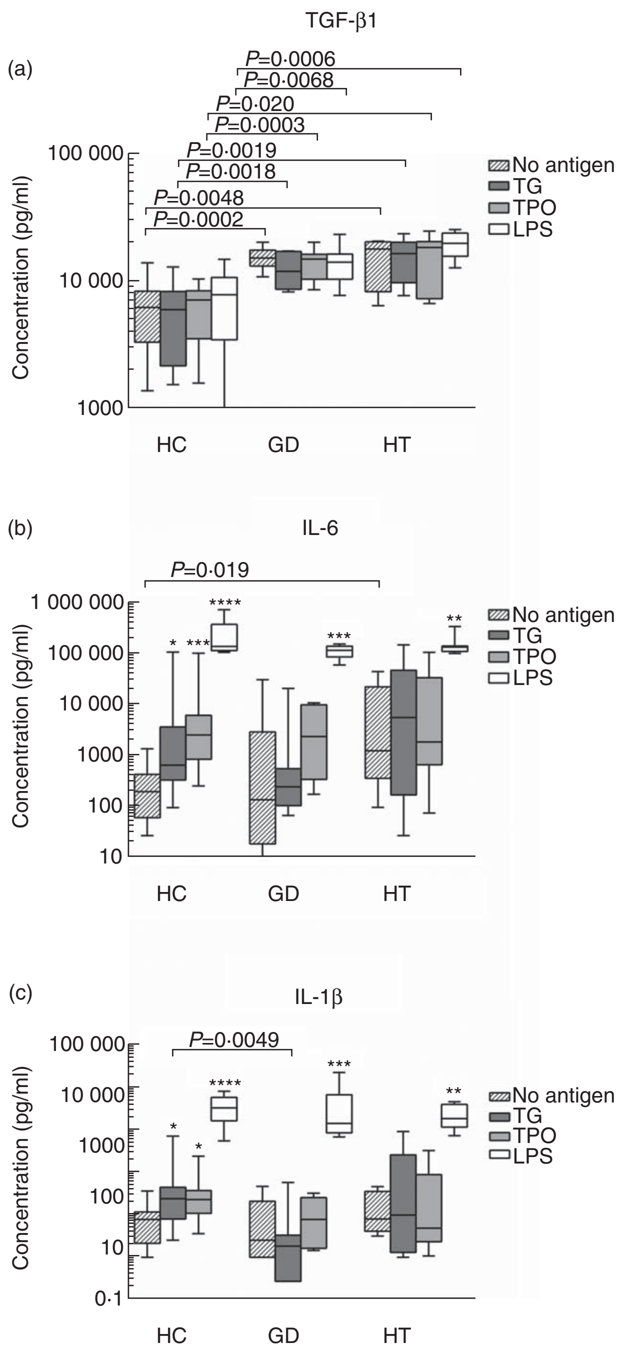

Fig. 5. Cytokine production induced by thyroid antigens. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with thyroglobulin (TG), thyroid peroxidase (TPO), *Escherichia coli* lipopolysaccharide or were unstimulated. Concentrations of (a) transforming growth factor (TGF)-β1, (b) interleukin (IL)-6, (c) IL-1β in culture supernatants derived from healthy controls (HC, $n = 11$), donors with Graves' disease (GD, $n = 8$) or donors with Hashimoto's thyroiditis (HT, $n = 6$) were measured using the Luminex platform. Box-plots indicate median, interquartile range (box) and range (whiskers). Brackets show differences between groups with the corresponding raw *P*-values (Mann–Whitney *U*-test). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ within groups.

concert with other cytokines [11,33–36]. Conversely, $FoxP3^{+}CD25^{hi}$ T_{regs} displaying $CD45RA^{-}CD45R0^{+}$ can also take on the Th17 phenotype [38]. We therefore investigated whether TG or TPO could drive Th17 and Th10 responses by naive and memory Th cells. It should be noted that the term Th10 cells, used in this report, may comprise iT_{regs}/Tr1 cells [18] as well as IL-10 producing nT_{regs} [17,23,26] and even a subset of Th17 cells [40] which may also produce IL-10. Our staining protocol did not allow distinction between these subsets. The possibility exists that a proportion of Th17/Th10 cells reported here produce both cytokines.

Studies of Th17 cells often employ phorbol myristate acetate (PMA)/ionomycin or anti-CD3/anti-CD28-coated beads to induce IL-17 production. Using this strategy, earlier studies have detected an increased abundance of circulating Th17 cells in individuals with HT or GD compared to healthy donors [13–16]. Our findings are congruent with these findings, but failed to reach statistical significance. However, within the HT group, we identified a positive correlation between TSH levels and anti-CD3/anti-CD28-induced Th17 cells. Importantly, fewer Th10 cells were induced by anti-CD3/anti-CD28 in HT-derived cell cultures than in those derived from healthy donors, suggesting that impairment of Th10 responses could also be associated with HT. Induction of Th17 cells by disease-relevant thyroid self-antigens has, to our knowledge, not been studied before.

A modest differentiation of naive Th cells into Th17 cells was observed in all three donor groups following stimulation with TG. In contrast, TPO induced Th17 cells only in cultures derived from HT patients, and only in the naive Th cell compartment. The increase of Th17 cells in cultures from HT patients was also observed in LPS-stimulated naive and memory Th cells. Thus, the increased propensity of Th cells from HT patients to differentiate into Th17 cells following stimulation is not restricted to activation provoked by self-antigens. It is noteworthy that LPS, acting as a Toll-like receptor-4 (TLR-4) ligand on antigen-presenting cells, has been reported to expand precommitted Th17 cells rather than causing Th17 differentiation [49].

Both TG and TPO induced equivalent numbers of Th10 cells in cultures from healthy donors and HT patients. Concordantly, a study by Glick *et al.*, who grouped HT and GD together, found equal numbers of $CD25^{hi}FoxP3^{+}$ T_{regs} among AITD patients and healthy donors [28]. Congruent findings were reported by Pan *et al.* when investigating subjects with GD [29]. Our subjects exhibited a skewed balance between Th17 and Th10 cells, rather than a reduction in antigen-specific Th10 cells.

The skewing of Th cells from HT patients towards differentiation into Th17 cells rather than Th10 cells may rely upon an increased propensity to produce Th17-promoting cytokines during antigen presentation, or upon intrinsic Th cell factors. Among the cytokines that have been implicated

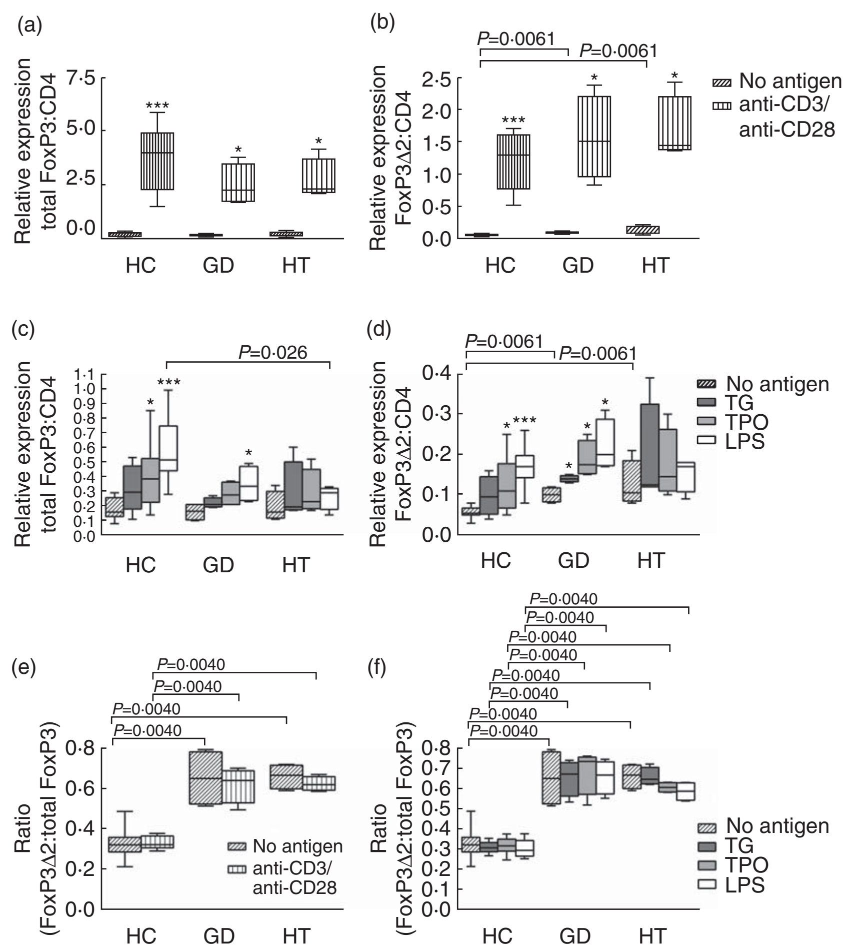

Fig. 6. Expression of total forkhead box protein 3 (FoxP3) and FoxP3Δ2 mRNA following antigenic and polyclonal stimulation. (a,b) Peripheral blood mononuclear cells (PBMCs) from healthy controls (HC; $n = 8$), patients with Graves' disease (GD, $n = 4$), and patients with Hashimoto's thyroiditis (HT, $n = 4$) were incubated for 18 h with anti-CD3/anti-CD28-coated beads. (c,d) Alternatively, thyroglobulin (TG), thyroid peroxidase (TPO), or *Escherichia coli* lipopolysaccharide (LPS) were used as stimuli. PBMCs receiving no antigenic stimulation (no antigen) served as the negative control. RNA was purified and quantitative reverse transcription–polymerase chain reaction (qRT–PCR) performed using CD4 as housekeeping gene. The expression of CD4 did not alter between the patients or healthy controls as determined by flow cytometry (data not shown). Expression of (a,c) total FoxP3 or (b,d) FoxP3Δ2, relative to the expression of CD4, was determined. Ratios of FoxP3Δ2 over total FoxP3 are shown after (e) anti-CD3/anti-CD28 stimulation or (f) antigenic stimulation. Box-plots indicate median, interquartile range (box) and range (whiskers). Brackets show differences between groups with the corresponding raw *P*-values (Mann–Whitney *U*-test). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ within groups.

in Th17 cell differentiation [11,33–36], we quantified TGF-β1, IL-6 and IL-1β in culture supernatants. Notably, higher concentrations of TGF-β1 were found in cultures derived from donors with HT than in those from healthy donors, irrespective of antigenic stimulus. Also, higher constitutive levels of IL-6 were found in conditioned medium from unstimulated HT-derived cultures than in those from healthy donors. TGF-β and IL-6 have been highlighted as the differentiation factors for Th17 cells [11,34,35] which may account, at least in part, for the skewing of HT-derived Th cells towards Th17-cell differentiation.

As an intrinsic determinant, we looked for abnormalities in the expression of FoxP3 and the splice variant FoxP3Δ2 in patient-derived cells. FoxP3Δ2 lacks exon 2, the encoded protein of which plays a critical role in maintenance of immune homeostasis. Mutations within this exon are associated with immune-mediated, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans [50,51]. It is not known whether full-length FoxP3 and FoxP3Δ2 are co-expressed or expressed in different cells. We found that, irrespective of whether or not PBMCs were stimulated with antigen, 59–67% and 65–73% of the FoxP3 transcripts expressed by cells from HT or GD patients, respectively, encoded the FoxP3Δ2 isoform, compared to approximately 30% in healthy individuals. The full-length FoxP3 is known to suppress the transcription factors retinoic acid-related orphan receptor (ROR)γt, RORα, nuclear factor of activated T cells (NFAT) and nuclear factor-kappa B (NF-κB) signalling, while FoxP3Δ2 is incapable of these trophic effects [47,52–55]. Because RORγt is the essential transcription factor for Th17 cells, and RORα is expressed highly in TGF-β/IL-6-induced Th17 cells, the relative over-expression of FoxP3Δ2 of AITD patients may compromise inhibition of Th17 differentiation.

Some of the patients had received anti-thyroid drugs prior to participation in this study. Anti-thyroid drugs may inhibit lymphocyte function [56,57], in part as a consequence of NF-κB inhibition [58]. It is also possible that the thyroid status of a patient affects the immune responses [59]. The low power of this study did not allow adjustment for relevant covariates including the effect of medication or thyroid hormones on the observed cytokine responses. Thus, an influence of these parameters cannot be excluded. Moreover, in view of the small sample sizes, the findings reported here should be interpreted cautiously and regarded as hypothesis-generating.

 © 2014 British Society for Immunology,

In conclusion, our findings demonstrate for the first time an increased frequency of thyroid antigen-specific Th17 cells in patients with HT in the naive Th cell compartment, while the abundance of Th10 cells appears to remain unaltered. We propose that the tendency of naive Th cells from HT patients and, to a lesser extent, GD patients to differentiate into Th17 cells could result from an increased expression of FoxP3Δ2 and diminished inhibition of RORγt. Furthermore, elevated constitutive TGF-β1 and IL-6 production in these patients could contribute to a skew towards the Th17 phenotype. Studies on larger cohorts of subjects will be necessary before these possibilities are reported conclusively.

Acknowledgements

B. K. was supported by the Novo Nordisk Foundation (94-101-12676) and Aase and Ejnar Danielsen Fond. L. H. was supported by an unrestricted grant from the Novo Nordisk Foundation. T. J. S. was supported by National Institutes of Health grants EY008976, EY011708 and DK063121 as well as by the Centre for Vision grant EY007003 from the National Eye Institute. T. J. S. was also supported by an unrestricted grant from Research to Prevent Blindness and the Bell Charitable Foundation. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Disclosure

The authors declare no financial or commercial conflicts of interest.

Author contributions

B. K. performed the experiments, C. H. N. and L. H. designed the study, C. H. N. and H. O. M. provided the experimental supplies and B. K., C. H. N., L. H. and T. J. S. wrote the paper.

References

1 Weetman AP. Determinants of autoimmune thyroid disease. Nat Immunol 2001; **2**:769–70.

2 Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986; **136**:2348–57.

3 Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; **7**:145–73.

4 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996; **17**:138–46.

5 Del Prete GF, Tiri A, Mariotti S, Pinchera A, Ricci M, Romagnani S. Enhanced production of gamma-interferon by thyroid-derived T cell clones from patients with Hashimoto's thyroiditis. Clin Exp Immunol 1987; **69**:323–31.

6 Guo J, Rapoport B, McLachlan SM. Balance of Th1/Th2 cytokines in thyroid autoantibody synthesis *in vitro*. Autoimmunity 1999; **30**:1–9.

7 Ajjan RA, Watson PF, McIntosh RS, Weetman AP. Intrathyroidal cytokine gene expression in Hashimoto's thyroiditis. Clin Exp Immunol 1996; **105**:523–8.

8 Nielsen CH, Moeller AC, Hegedus L, Bendtzen K, Leslie RGQ. Self-reactive CD4^{+} T cells and B cells in the blood in health and autoimmune disease: increased frequency of thyroglobulin-reactive cells in Graves' disease. J Clin Immunol 2006; **26**: 126–37.

9 Colin IM, Isaac J, Dupret IP, Ledant T, D'Hautcourt JL. Functional lymphocyte subset assessment of the Th1/Th2 profile in patients with autoimmune thyroiditis by flow cytometric analysis of peripheral lymphocytes. J Biol Regul Homeost Agents 2004; **18**:72–6.

10 Harrington LE, Hatton RD, Mangan PR *et al*. Interleukin-17 producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; **6**:1123–32.

11 Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. Immunol Rev 2008; **223**:87–113.

12 Kurts C. Th17 cells: a third subset of CD4+ T effector cells involved in organ-specific autoimmunity. Nephrol Dial Transplant 2008; **23**:816–9.

13 Shi Y, Wang H, Su Z *et al*. Differentiation imbalance of Th1/Th17 in peripheral blood mononuclear cells might contribute to pathogenesis of Hashimoto's thyroiditis. Scand J Immunol 2010; **72**:250–5.

14 Nanba T, Watanabe M, Inoue N, Iwatani Y. Increases of the Th1/Th2 cell ratio in severe Hashimoto's disease and in the proportion of Th17 cells in intractable Graves' disease. Thyroid 2009; **19**:495–501.

15 Figueroa-Vega N, Alfonso-Perez M, Benedicto I, Sanchez-Madrid F, Gonzalez-Amaro R, Marazuela M. Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis. J Clin Endocrinol Metab 2010; **95**:953–62.

16 Peng D, Xu B, Wang Y, Guo H, Jiang Y. A high frequency of circulating Th22 and Th17 cells in patients with new onset Graves' disease. PLOS ONE 2013; **8**:e68446.

17 Morris GP, Brown NK, Kong YM. Naturally-existing CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. J Autoimmun 2009; **33**: 68–76.

18 Marazuela M, Garcia-Lopez MA, Figueroa-Vega N *et al*. Regulatory T cells in human autoimmune thyroid disease. J Clin Endocrinol Metab 2006; **91**:3639–46.

19 Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. Immunol Rev 2006; **182**:68–79.

20 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; **299**:1057–61.

21 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4^{+}CD25^{+} regulatory T cells. Nat Immunol 2003; **4**:330–6.

22 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). Breakdown of a single mechanism of

self-tolerance causes various autoimmune diseases. J Immunol 1995; **155**:1151–64.

23 Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. $CD25^{-}$ T cells generate $CD25^{+}FOXP3^{+}$regulatory T cells by peripheral expansion. J Immunol 2004; **173**:7259–68.

24 Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. *Ex vivo* isolation and characterization of $CD4^{+}$ $CD25^{+}$ T cells with regulatory properties from human blood. J Exp Med 2001; **193**:1303–10.

25 Green EA, Gorelik L, McGregor M, Tran EH, Flavell RA. $CD4^{+}CD25^{+}$ T regulatory cells control anti-islet $CD8^{+}$ T cells through TGF-β–TGF-β receptor interactions in type 1 diabetes. Proc Natl Acad Sci USA 2003; **100**:10878–83.

26 Collison LW, Pillai MR, Chaturvedi V, Vignali DAA. Regulatory T cell suppression is potentiated by Target T cells in a cell contact, IL-35- and IL-10-dependent manner. J Immunol 2009; **182**:6121–8.

27 Sundstedt A, Hoiden I, Rosendahl A, Kalland T, van Rooijen N, Dohlsten M. Immunoregulatory role of IL-10 during superantigen-induced hyporesponsiveness *in vivo*. J Immunol 1997; **158**:180–6.

28 Glick AB, Wodzinski A, Pingfu F, Levine AD, Wald DN. Impairment of regulatory T-cell function in autoimmune thyroid disease. Thyroid 2013; **23**:871–8.

29 Pan D, Shin YH, Gopalakrishnan G, Hennessey J, De Groot LJ. Regulatory T cells in Graves' disease. Clin Endocrinol (Oxf) 2009; **71**:587–93.

30 Nielsen CH, Hegedus L, Rieneck K, Moeller AC, Leslie RGQ, Bendtzen K. Production of interleukin (IL)-5 and IL-10 accompanies T helper cell type 1 (Th1) cytokine responses to a major thyroid self-antigen, thyroglobulin, in health and autoimmune thyroid disease. Clin Exp Immunol 2007; **147**:287–95.

31 Nielsen CH, Galdiers MP, Hedegaard CJ, Leslie RGQ. The self-antigen, thyroglobulin, induces antigen-experienced $CD4^{+}$ T cells from healthy donors to proliferate and promote production of the regulatory cytokine, interleukin-10, by monocytes. Immunology 2009; **129**:291–9.

32 Langkjær A, Kristensen B, Hansen BF, Schultz H, Hegedus L, Nielsen CH. B-cell exposure to self-antigen induces IL-10 producing B cells as well as IL-6 and TNF-α producing B-cell subsets in healthy humans. Clin Immunol 2012; **145**:1–10.

33 Yang L, Anderson DE, Baecher-Allan C *et al*. IL-21 and TGF-beta are required for differentiation of human T(H)17 cell. Nature 2008; **454**:350–2.

34 Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 2007; **8**:942–9.

35 Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammaT. Nat Immunol 2008; **9**:641–9.

36 Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGF-beta in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells. Immunity 2006; **24**:179–89.

37 Bettelli E, Carrier Y, Gao W *et al*. Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells. Nature 2006; **441**:235–8.

38 Ayyoub M, Deknuydt F, Raimbaud I *et al*. Human memory FOXP3 Tregs secrete IL-17 *ex vivo* and constitutively express the Th17 lineage-specific transcription factor RORgammaT. Proc Natl Acad Sci USA 2009; **106**:8635–40.

39 Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human $CD25^{high}$ $FOXP3^{+}$ regulatory T cells differentiate into IL-17-producing cells. Blood 2008; **112**:2340–52.

40 Beriou G, Costantino CM, Ashley CW *et al*. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 2009; **113**:4240–9.

41 Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. J Autoimmun 2008; **31**:252–6.

42 McGreachy MJ, Bak-Jensen KS, Chen Y *et al*. TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. Nat Immunol 2007; **8**:1390–7.

43 Zielinski CE, Mele F, Aschenbrenner D *et al*. Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. Nature 2012; **484**:514–9.

44 Hedegaard CJ, Krakauer M, Bendtzen K, Lund H, Sellebjerg F, Nielsen CH. T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. Immunology 2008; **125**:161–9.

45 Lui S, Tobias R, McClure S, Styba G, Shi Q, Jackowski G. Removal of endotoxin from protein preparations. Clin Biochem 1997; **30**:455–63.

46 Ryder LR, Woetmann A, Madsen HO *et al*. Expression of full-length and splice forms of FOXP3 in rheumatoid arthritis. Scand J Rheumatol 2010; **39**:279–86.

47 Ryder LR, Bartels EM, Woetmann A *et al*. FOXP3 mRNA splice forms in synovial $CD4^{+}$ T cells in rheumatoid arthritis and psoriatic arthritis. APMIS 2011; **120**:387–96.

48 Mane VP, Heuer MA, Hillyer P, Navarro MB, Rabin RL. Systematic method for determining an ideal housekeeping gene for real-time PCR analysis. J Biomol Tech 2008; **19**:342–7.

49 McAleer JP, Liu B, Li Z *et al*. Potent intestinal Th17 priming through peripheral lipopolysaccharide-based immunization. J Leukoc Biol 2010; **88**:21–31.

50 Kobayashi I, Shiari R, Yamada M *et al*. Novel mutations of FOXP3 in two Japanese patients with immune dysregulation, polyendocrinopathy, enteropathy, X linked syndrome (IPEX). J Med Genet 2001; **38**:874–6.

51 Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. J Allergy Clin Immunol 2007; **120**:744–50.

52 Krejsgaard T, Gjerdrum LM, Ralfkiaer E *et al*. Malignant Tregs express low molecular splice forms of FOXP3 in Sezary syndrome. Leukemia 2008; **22**:2230–9.

53 Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF-κB to repress cytokine gene expression and effector functions of T helper cells. Proc Natl Acad Sci USA 2005; **102**:5138–43.

54 Du J, Huang C, Zhou B, Ziegler SF. Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. J Immunol 2008; **180**:4785–92.

55 Zhou L, Lopes JE, Chong MM *et al*. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature 2008; **453**:236–40.

56 McGregor MA, Petersen MM, McLachlan SM, Rooke P, Rees Smith B, Hall R. Carbimazole and the autoimmune response in Graves' disease. N Engl J Med 1980; **303**:302–7.

57 Weiss I, Davis TF. Inhibition of immunoglobulin-secreting cells by antithyroid cells. J Clin Endocrinol Metab 1981; **53**:1223–8.

58 Humar M, Dohrmann H, Stein P *et al.* Thionamides inhibit the transcription factor nuclear factor-κB by suppression of Rac1 and inhibitor of κB kinase α. J Pharmacol Exp Ther 2008; **324**:1037–44.

59 Volpe R. The immunomodulatory effects of anti-thyroid drugs are mediated via actions on thyroid cells, affecting thyrocyte–immunocyte signaling: a review. Curr Pharm Des 2001; **7**:451–60.