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Influx of recent thymic emigrants into autoimmune thyroid disease glands in humans

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Summary

Autoimmune thyroid diseases (AITD) are considered as prototypic organ-specific autoimmune diseases, yet their underlying aetiology remains poorly understood. Among the various pathophysiological mechanisms considered, a failure of central tolerance has received little attention. Here we present evidence in favour of dysregulated thymic function playing a role in AITD. Flow-cytometric analyses conducted in peripheral blood lymphocytes from 58 AITD patients and 48 age- and-sex-matched controls showed that AITD patients have significantly higher blood levels of $CD4^+CD45RA^+$, $CD4^+CD31^+$ and CD4/CD8 double-positive T lymphocytes, all markers of recent thymic emigrants (RTE). In addition, the α-signal joint T cell receptor excision circles (TRECs) content (a molecular marker of RTEs) was higher in the group of AITD patients older than 35 years than in age-matched controls. This was independent from peripheral T cell expansion as assessed by relative telomere length. Comparisons of TREC levels in peripheral blood lymphocytes and intrathyroidal lymphocytes in paired samples showed higher levels within the thyroid during the initial 30 months of the disease, indicating an influx of RTE into the thyroid during the initial stages of AITD. Additionally, a lack of correlation between TREC levels and forkhead box P3 expression suggests that the intrathyroidal RTE are not natural regulatory T cells. These results uncover a hitherto unknown correlation between altered thymic T cell export, the composition of intrathyroidal T cells and autoimmune pathology.

Keywords: autoimmunity, human, T cells, thymus, tolerance

Introduction

Autoimmune disorders include a broad spectrum of clinical entities whose pathogenesis is mediated by an immune response directed at self-antigens. A variety of mechanisms, most of them invoking the failure of peripheral tolerance, have been proposed to explain the loss of tolerance to self-antigens. In contrast, failure of central T cell tolerance has, so far, received less attention. Among autoimmune disorders, organ-specific autoimmune diseases constitute a subgroup in which the autoimmune response is focused upon a particular tissue or cell type [1]. Hashimoto thyroiditis (HT) was the first organ-specific autoimmune disorder to be recognized as such [2]. Goitrous HT, together with atrophic autoimmune thyroiditis and Graves' disease, constitute one of the best-characterized groups of organ-specific autoimmune diseases, designated in general as autoimmune thyroid diseases (AITD) [3,4]. This group also includes other clinically less well-defined entities such as postpartum thyroiditis [5], isolated Graves' ophtalmopathy, thyroid-associated pretibial myxoedema [6] and focal lymphocytic thyroiditis [7].

Large linkage studies in families indicate a polygenic inheritance with no single locus playing a dominant role in AITD [8–10]. Recent genetic modelling based upon twin studies led to the conclusion that as much as the 79% of the predisposition to Graves' disease is attributable to genetic factors [10]. However, it has been difficult to quantify precisely the contribution of each gene or locus to the pathogenesis [11]. On one hand, genes in the human leucocyte antigen (HLA) class II region and the CTLA4, PTPN22 and CD40 genes may influence the balance between tolerance and autoimmunity through their influence on the regulation of the immune response. None of these genes, however, is specific to AITD, as they are also associated with other autoimmune diseases. On the other hand, polymorphisms in the regulatory regions of target autoantigens can affect disease susceptibility in a disease-specific manner (reviewed in [9]). This has been demonstrated most convincingly for

alleles of the insulin gene, which predispose to type 1 diabetes [12–15]), and more recently of the acetylcholine receptor gene, that predispose to myasthenia gravis [16]. The alleles that increase risk to disease reduce the level of thymic insulin and acetylcholine expression respectively, and presumably in this way lower the threshold for triggering an autoimmune response [16]. This is in accordance with the hypothesis of promiscuous gene expression in the thymus [17] and the threshold model of central tolerance [18], which postulates that small reductions in the level of expression of self-antigens in the thymus are sufficient to affect the efficacy of thymic deletion of autoreactive T cells. These reductions would tip the balance towards the development of autoimmune disease in a permissive genetic background [18].

Similarly, polymorphisms in thyroid autoantigen genes, i.e. thyroid hormone stimulating receptor (TSH-R), thyroglobulin and possibly thyroid peroxidase (TPO), could predispose to AITD. An elevated export of T cells specific for thyroid self-antigens from the thymus would lower the threshold for triggering of an autoimmune response to thyroid antigens. Concomitant factors, contributed possibly by the environment, may precipitate the disease. Iodine overload, interferon-α treatment, bone marrow transplantation, monoclonal antibody (mAb) Campath therapy and stress have, in fact, been shown to trigger AITD onset (reviewed in [19]).

In the above scenario it would be predicted that before and during the initial stages of AITD there would be an efflux of autoreactive T cells from the thymus to thyroid regional lymph nodes and/or the thyroid gland itself.

In this study we report that AITD patients, when compared with healthy controls, display elevated levels of recent thymic emigrants (RTE) in their peripheral circulation and more importantly among T cells infiltrating the thyroid, findings compatible with a continuous excessive release of self-reactive T cells by the adult thymus.

Material and methods

Patients

Thyroid patients attended the Endocrinology Clinic at the Hospital Universitari Germans Trias i Pujol and Hospital Universitari Vall d'Hebró, both affiliated to the Faculty of Medicine of the Universitat Autònoma de Barcelona. All patients in this study conformed to the usual clinical and laboratory criteria for the diagnosis of Hashimoto or Graves' disease. T4, free T4, T3 and TSH and thyroid antibody measurements supported the diagnosis. In some cases thyroid ultrasound and/or scintiscans were available. TPO and thyroglobulin autoantibodies were measured by means of an enzyme-linked immunosorbent assay (ELISA) test using a commercial kit (Orgentec, Mainz, Germany), normal range: 0–50 IU/ml and 0–100 IU/ml respectively. TSH-R autoantibodies were measured by means of a competitive ELISA test (DRG, Marburg, Germany; normal values <1 IU/l). Patients with Graves' disease were treated with carbimazole and propanolol and most had a normal thyroid function at the time of operation. The clinical and antibody data of the patients are given in Table 1. Patients who underwent surgical removal of the gland did not constitute a distinct clinical subgroup, as the surgical indication depended on a miscellaneous of reason: a large goitre, intolerance to methimazole and lack of compliance, among others.

Patients were selected by the criteria of definite diagnosis, availability of clinical and laboratory data and of the corresponding tissue and blood samples, and of having given their informed consent to participate in the study. As there were not samples available to carry all the studies in each of the patients, subgroups of patients were selected for each study. Table 2 shows the different patient subgroups. Patients of similar age and female/male ratio to ensure comparability of the results composed each subgroup. The number of patients is given in the legends. The institutional ethical boards of the two hospitals approved the project. Forty-eight blood samples from age- and sex-matched healthy donors were used as controls (Table 1).

Tissue and cell preparations

Thyroid tissue samples ($n = 41$) from patients were obtained from the histopathology department immediately after surgery. Blood samples were taken shortly before or at the operating theatre immediately prior to surgery. Thyroid glands were processed as described [20] and intrathyroidal lymphocytes (ITLs) were obtained by Ficoll density gradient separation of the total digest. Cells recovered from the interphase were washed and viability was > 90%, as assessed by acridine orange/ethidium bromide staining, in all the preparations used in this study. Cases and cells used in the different part of the study are given in Table 2.

Flow cytometry and immunofluorescence staining of tissue sections

Cell populations were studied by four-colour flow cytometry (FACSCanto, FACSDiva software; Becton Dickinson, San Jose, CA, USA) using fluorochrome-labelled mAbs to CD3, CD4, CD8, CD19, CD31, CD45RA and CD45RO (Pharmingen, San Diego, CA, USA).

In the analysis for RTE phenotypes, duplicate samples were always stained in parallel using CD4-fluorescein isothiocyanate (FITC), CD8-phycoerythrin (PE), CD45RA-Cy5.5 and CD31-allophycocyanin (APC) or CD8-FITC, CD4-PE, CD45RA-Cy5.5 and CD31-APC. This protocol aimed to ensure the consistency of the results by using always the FITC as labelling reagent for the main gating reagent (see Fig. 1).

Table 1. Main descriptive values for the study group.

Code	Disease	Gender	Age (years)	Evolution (months)	Tg antibodies (mU/ml)	TPO antibodies (mU/ml)	TSHR antibodies (mU/l)
AITD1	GD	F	19	36	302·2	938·6	29·1
AITD2	GD	M	24	84	201·0	379·0	2·8
AITD3	HT	F	24	46	–	–	n.d.
AITD4	HT	F	28	9	584·0	766·0	0·9
AITD5	GD	F	26	36	50·0	387·1	48·7
AITD6	GD	M	29	24	41·0	7000·0	0·9
AITD7	GD	F	39	36	90·3	101·0	47·5
AITD8	GD	F	30	12	137·0	65·2	31·6
AITD9	GD	F	26	24	239·0	124·3	8·8
AITD10	HT	F	33	n.d.	41·0	n.d.	33·0
AITD11	HT	F	45	36	41·0	85·1	0·9
AITD12	GD	F	40	n.d.	113·0	16·2	8·5
AITD13	GD	F	44	48	37·2	39·4	20·6
AITD15	GD	M	64	18	101·0	80·0	> 500·0
AITD16	GD	F	21	24	238·0	400·0	4·53
AITD17	GD	F	n.d.	46	n.d.	n.d.	n.d.
AITD18	GD	F	31	10	47·9	11·9	1·47
AITD19	GD	F	25	25	20·0	10·0	15·9
AITD20	GD	F	32	40	179·0	945·0	23·3
AITD21	GD	F	35	n.d.	41·0	25·0	75·0
AITD22	HT	F	67	48	50·0	387·1	–
AITD23	GD	F	51	60	35·6	256·5	n.d.
AITD24	GD	F	45	168	–	582·0	19·0
AITD25	GD	F	31	n.d.	82·5	47·4	18·7
AITD26	GD	F	23	32	212·0	1000·0	2·9
AITD27	GD	F	25	24	> 3000·0	> 1000·0	0·3
AITD28	GD	F	22	6	52·7	702·0	18·9
AITD29	GD	F	50	10	41·0	43·5	38·0
AITD30	HT	F	19	7	533·3	81·0	–
AITD31	GD	F	18	62	1965·0	1249·0	3·9
AITD32	GD	F	22	36	134·0	883·0	2·24
AITD33	GD	F	23	35	82·0	582·0	48·8
AITD34	GD	F	30	13	105·0	408·0	1·5
AITD35	GD	F	30	72	41·0	25·0	20·0
AITD36	GD	F	34	19	70·0	53·0	15·5
AITD37	HT	M	35	22	2564·4	1456·4	n.d.
AITD38	GD	F	37	18	175·0	532·0	96·0
AITD39	GD	F	37	n.d.	28·3	36·0	8·4
AITD40	HT	F	39	11	11·8	509·8	n.d.
AITD41	HT	F	39	122	137·0	110·8	–
AITD42	HT	F	39	5	167·0	297·0	n.d.
AITD43	GD	F	42	96	244·8	265·4	0·9
AITD44	HT	F	51	12	38·0	141·7	n.d.
AITD45	GD	F	55	97	79·1	111·0	17·2
AITD46	GD	F	57	n.d.	56·1	11·8	0·5
AITD47	GD	F	67	n.d.	> 3000·0	> 1000·0	15·1
AITD48	GD	F	14	2	20·5	13·9	36·0
AITD49	GD	F	21	60	598·0	1155·0	67·0
AITD51	GD	M	42	n.d.	1265·0	1001·0	85·9
AITD52	HT	F	21	8	774·0	105 000·0	0·9
AITD53	GD	F	37	n.d.	20·2	28·0	4·0
AITD54	GD	F	35	2	19·0	68·5	18·1
AITD55	GD	F	36	n.d.	26·7	40·1	n.d.
AITD56	GD	F	37	n.d.	32·3	31·2	n.d.
AITD57	GD	F	60	n.d.	20·2	33·2	15·5
AITD58	GD	F	74	33	41·0	494·9	32·3
AITD59	GD	F	32	n.d.	2·4	3·6	4·1
AITD60	GD	F	67	n.d.	398·8	566·7	71·3
AITD patients	12/46 (HT/GD)	53/5 (F/M)	35·7 (14–67)				
Controls		42/6 (F/M)	35·5 (19–70)				

AITD, autoimmune thyroid disease; GD, Graves–Basedow disease; HT, Hashimoto thyroiditis disease; n.d., not determined; Tg, thyroglobulin; TPO, thyroid peroxidase; TSHR, thyroid hormone stimulating receptor.

Table 2. Sample use distribution on the different experimental protocols. Consistency of age ratios.

	sjTRECs			CD45RA			CD31			CD4CD8	RTL		
CODE	PBLs	ITLs	PBL/ITLs paired	PBLs	ITLs	PBL/ITLs paired	PBLs	ITLs	PBL/ITLs paired	PBLs	PBLs	ITLs	PBL/ITLs paired
AITD61	+												
AITD48		+											
AITD16	+	+	+										
AITD28	+	+	+										
AITD32	+	+	+										
AITD33	+												
AITD2	+	+	+										
AITD4	+	+	+										
AITD34	+												
AITD8	+	+	+										
AITD25	+	+	+										
AITD18	+	+	+										
AITD10	+	+	+										
AITD21	+	+	+										
AITD37	+												
AITD39	+	+	+										
AITD40	+												
AITD41	+												
AITD7	+	+	+										
AITD42	+												
AITD12	+	+	+										
AITD43	+												
AITD24	+	+	+										
AITD60	+												
AITD44	+												
AITD45	+	+	+										
AITD46	+	+	+										
AITD60	+												
AITD1	+	+	+	+	+	+	+	+	+	+			
AITD30	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. *Continued*

	sjTRECs			CD45RA			CD31			CD4CD8	RTL		
CODE	PBLs	ITLs	PBL/ITLs paired	PBLs	ITLs	PBL/ITLs paired	PBLs	ITLs	PBL/ITLs paired	PBLs	PBLs	ITLs	PBL/ITLs paired
AITD52				+	+	+	+	+	+	+			
AITD49		+											
AITD26	+	+	+	+	+	+	+	+	+	+	+	+	+
AITD19	+	+	+	+	+	+	+	+	+	+			
AITD27	+	+	+		+			+			+	+	+
AITD3	+	+	+		+			+			+	+	+
AITD9	+	+	+	+			+			+	+	+	+
AITD5	+	+	+	+			+			+	+	+	+
AITD6	+	+	+		+			+					
AITD35	+	+	+	+			+			+	+	+	+
AITD20	+	+	+	+	+	+	+	+	+	+	+		
AITD59		+		+	+	+	+	+	+	+			
AITD36	+	+	+	+			+			+	+	+	+
AITD54				+			+			+			
AITD55				+			+			+			
AITD38	+	+	+		+			+			+	+	+
AITD53				+	+	+	+	+	+	+			
AITD56				+			+			+			
AITD51		+											
AITD13	+	+	+	+	+	+	+	+	+	+	+	+	+
AITD11	+	+	+		+			+			+	+	+
AITD17	+	+	+	+			+			+	+	+	+
AITD29	+	+	+		+			+			+		
AITD23	+	+	+	+	+	+	+	+	+	+	+	+	+
AITD14	+	+	+		+			+			+	+	+
AITD57				+			+			+			
AITD15	+	+	+	+			+			+	+	+	+
AITD22	+	+	+	+	+	+	+	+	+	+	+	+	+
AITD58				+			+			+			
age	32·9	35·9	34·8	34·1	35·4	35·3	38·3	35·4	35·3	38·3	36·1	36·3	36·3
	18–67	18–67	18–67	19–67	19–67	19–67	19–67	19–67	19–67	19–67	22–57	22–57	22–57
	n = 48	*n* = 41	*n* = 37	*n* = 22	*n* = 18	*n* = 11	*n* = 22	*n* = 17	*n* = 11	*n* = 22	*n* = 18	*n* = 16	*n* = 16

AITD, autoimmune thyroid disease; ITL, intrathyroidal lymphocytes; PBL, peripheral blood lymphocyte; RTL, relative telomere length; sjTRECs, signal joint T cell receptor excision circles.

The double indirect immunofluorescence technique was used to stain 4 μm cryosections. Primary mAbs were revealed with isotype-specific conjugates (Southern Biotechnology, Birmingham, AL, USA) following standard protocols and analysed using the Openlab® software (Improvision Ltd, Coventry, UK). Controls included the use of normal mouse serum instead of the primary monoclonal and omitting the first layer in the staining protocol.

For telomere length measurement, intrathyroidal T and B cells were separated in a FACSVantage sorter (Becton Dickinson) generating fractions of purity always in the range of 94–99%. These preparations will be designated hereafter as ITLs. Peripheral blood lymphocytes (PBLs) were obtained by Ficoll density gradient and purified using the same method.

Reverse transcription and quantitative real-time polymerase chain reaction

RNA was extracted using a Maxwell®-16 total RNA purification kit (Promega Corporation, Madison, WI, USA) and 2 μg RNA were retrotranscribed with oligo-(dT15–18mer) (Pharmacia-Biotech) and SuperScript-II® (Pharmacia-Biotech, Uppsala, Sweden). Real-time polymerase chain reaction (PCR) reactions to quantify forkhead box P3 (FoxP3), CD3 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expressions were performed in triplicate in a 96-well plate (LightCycler®-480; Roche, Mannheim, Germany) using a *Taq*Man® Universal PCR Master Mix and the Assay-on-Demand (*Taq*Man MGB probe; Applied Biosystems, Foster City, CA, USA) protocol following recommended procedures. For relative quantification the expression for each gene was normalized to the housekeeping gene GAPDH, following the $2^{-\Delta CP}$ method and referred as arbitrary units.

Signal joint T cell receptor excision circles measurement

Alpha signal joint [signal joint T cell receptor excision circles (sjTRECs)] levels were measured in gDNA from ITLs and PBLs using a real-time PCR adaptation of the original technique [21]. The standard for sjTRECs measurement was generated by amplification of thymic cDNA using specific primers. The sjTREC amplimer was purified, cloned, quantified and serial dilutions from 10e7 to 1 copy were stored until used. Quantitative real-time PCR for sjTRECs measurement was performed as follows: 200–800 ng of gDNA were subjected to amplification using a LightCycler® (Roche). Primers and the *Taq*Man probe were: sense 5′-CACATCCCTTTCAACCATGCT-3′; anti-sense 5′-GCC AGCTGCAGGGTTTAGG-3′ and 5′-FAM-ACACCTCTG GTTTTTGTAAAGGTGCCCACT-TAMRA-3′ respectively. Samples were subjected to an initial cycle of 600 s at 95°C, followed by 55 cycles of 2 s at 95°C and 20 s at 66°C. Fluorescence was monitored in single acquisition mode. All measurements were performed in duplicate samples and all amplification experiments were repeated at least once. When the readings from these four reactions gave a variation above 10%, the experiment was repeated. The lower limit of sensitivity was set at three copies. The results are expressed as sjTRECs per 10e4 $CD3^+$ T cells. To calculate this value, the mean of four sjTRECs determinations was corrected by the mean of beta-globin content in the same sample following the manufacturer's instructions (Roche).

Telomere measurement

Telomere length was determined by *in situ* hybridization using the PNA Kit/FITC for flow cytometry (Dako, Glostrup, Denmark). Results are given as relative telomere length (RTL) and calculated according to the following formula:

$$\%\,\mathrm{LTR} = \frac{\begin{array}{c}\text{MFI PNA-FITC } CD3^+ \left(\text{or } CD19^+\right) - \\ \text{MFI control-FITC } CD3_+ \left(\text{or } CD19^+\right) \times 2 \times 100\end{array}}{\begin{array}{c}\text{MFI PNA-FITC 1301 cell line} - \\ \text{MFI control-FITC 1301 cell line}\end{array}}$$

Statistics

Parametric (Student's *t*-test) and non-parametric (Mann–Whitney) tests were applied to normal and non-normal distributed data respectively, using the SPSS® software package (Microsoft Corp., Seattle, USA). A level of significance of 5% was used in all the statistical evaluations. Correlations were tested by Pearson's simple regression tests. When multiple comparisons were made, the Bonferroni correction was applied.

Results

Phenotypic markers associated with RTE are elevated in T lymphocytes from AITD patients at the time of operation

Because naive cell numbers decline with age, we first compared the proportion of $CD3^+CD45RA^+$ in PBL from AITD patients *versus* controls as a whole and as a function of age. There is a trend to have higher levels of naive T cells in AITD patients compared with controls, that are significant for the $CD4^+$ cells when analysed separately from the $CD8^+$ cells (63·027 ± 18·80 in AITDs *versus* 51·465 ± 13·47 in controls; $P = 0{\cdot}027$, Student's *t*-test) (Fig. 1). This slower decline of the naive T cell population in AITD patients could be attributed either to a higher thymic output (although RTE account for only 2–10% of the T cell pool at the age range of the studied population [22] or to an expansion of naive T cells in these patients.

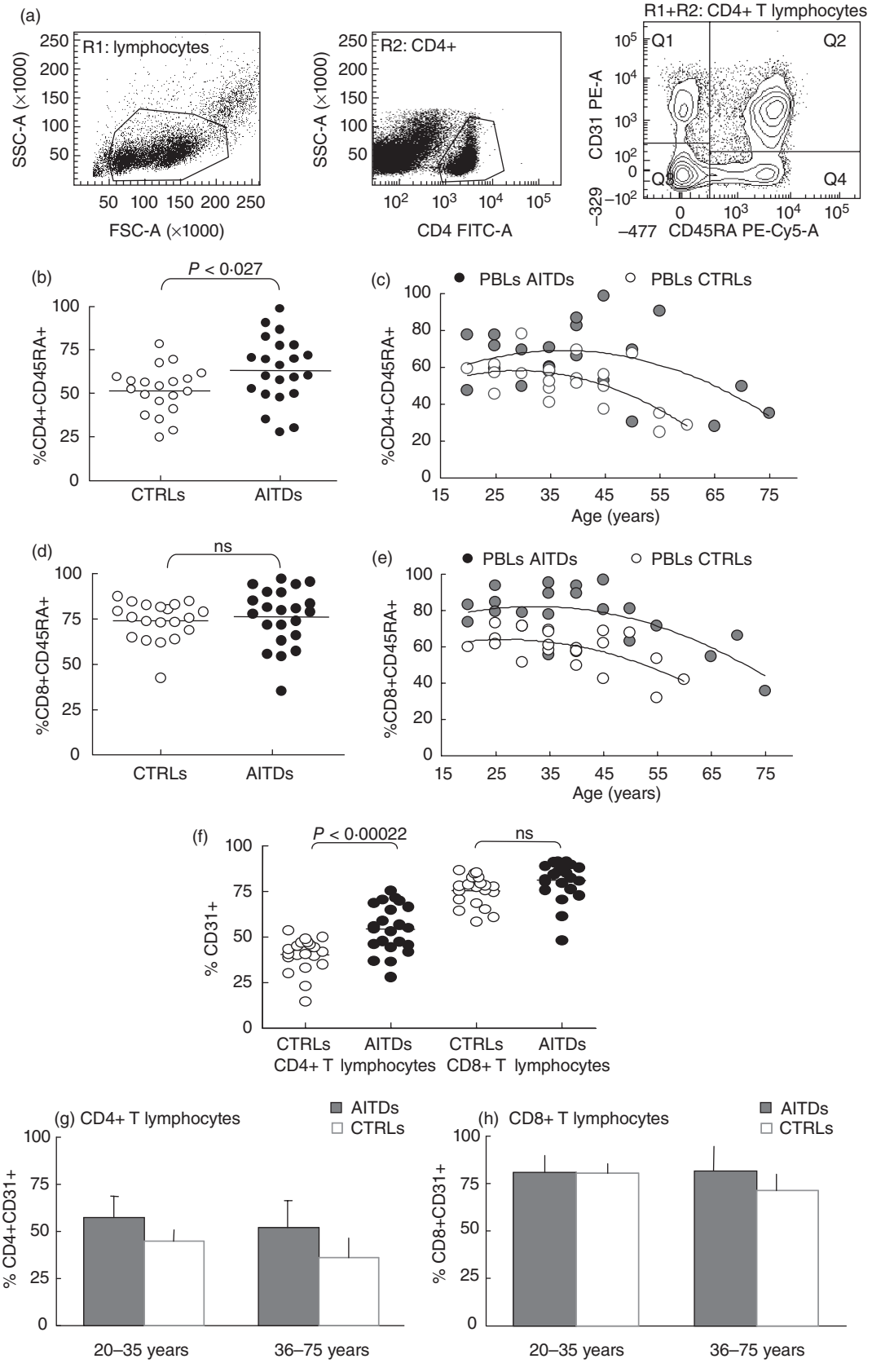
(a)
R1: lymphocytes
SSC-A (×1000)
FSC-A (×1000)
R2: CD4+
CD4 FITC-A
R1+R2: CD4+ T lymphocytes
Q1
Q2
Q3
Q4
CD31 PE-A
CD45RA PE-Cy5-A
(b)
P < 0·027
%CD4+CD45RA+
CTRLs
AITDs
(c)
PBLs AITDs
PBLs CTRLs
%CD4+CD45RA+
Age (years)
(d)
ns
%CD8+CD45RA+
CTRLs
AITDs
(e)
PBLs AITDs
PBLs CTRLs
%CD8+CD45RA+
Age (years)
(f)
P < 0·00022
ns
% CD31+
CTRLs CD4+ T lymphocytes
AITDs CD4+ T lymphocytes
CTRLs CD8+ T lymphocytes
AITDs CD8+ T lymphocytes
(g) CD4+ T lymphocytes
AITDs
CTRLs
% CD4+CD31+
20–35 years
36–75 years
(h) CD8+ T lymphocytes
AITDs
CTRLs
% CD8+CD31+
20–35 years
36–75 years

Fig. 1. Comparison of $CD3^+CD45RA^+$T lymphocytes in peripheral blood lymphocytes (PBLs) from AITD patients and control subjects in relation to age. (a) Flow cytometric analysis of the $CD4^+CD45RA^+$ and $CD8^+CD45RA^+$ subsets and the corresponding $CD31^+$ and $CD31^-$ subsets. The acquisition region (R1) was set up in the forward scatter (FSC) to side-scatter (SSC) plot excluding debris and dead cells (left panel); the $CD4^+$ or $CD8^+$ gate was set up in the CD4 (or CD8) FL1 to SSC plot (R2) (centre panel) and the events were analysed in the CD45RA (Cy5-A) to CD31 (PE-A) plot (right graph). CD31 staining clearly separated CD45 RA^+ cells into two subsets. The same protocol was applied to CD8 cells (not shown). (b) The proportion of the $CD4^+CD45RA^+$ cells among PBLs in the two groups is significantly different and higher in autoimmune thyroid disease (AITD) patients ($P < 0{\cdot}027$). (c) The percentage of $CD4^+CD45RA^+$ plotted in function of age showed a higher percentage in older AITD patients than in the controls of similar age. (d, e) In a similar analysis, the distribution of $CD8^+CD45RA^+$ cells was found not to be different in the control and AITD groups. (f) The percentage of $CD4^+CD45RA^+CD31^+$ cells is significantly higher in AITD patients than in controls, while it is not different for $CD8^+CD45RA^+CD31^+$. (g, h) Stratification by age shows a similar degree of differential distribution for $CD4^+CD45RA^+CD31+$ cells in both age groups but not for $CD8^+CD45RA^+CD31^+$ cells; AITD patients black dots ($n = 22$), controls empty dots ($n = 20$).

To investigate the frequency of RTE in AITD we measured CD31, CD4/CD8 double-positive cells and TRECs. CD31 has been advocated recently as a marker of RTE; not all $CD31^+$ cells are RTE, but almost of them are contained within this population [23,24]. AITD patients showed a significantly higher level of $CD31^+$ cells than controls [68·520 ± 11·06 ($n = 22$) *versus* 59·130 ± 8·72 ($n = 22$); $P = 0{\cdot}0036$]. This difference was due to the population of $CD4^+CD31^+$ cells, which was clearly more abundant in AITD (54·660 ± 13·04 *versus* 40·440 ± 9·40; $P = 0{\cdot}00022$) than in controls; for $CD8^+CD31^+$ cells this difference was in the limit of statistical significance (81·280 ± 10·59 *versus* 75·670 ± 8·21; $P = 0{\cdot}060$) (Fig. 1f). Comparing the groups after stratification by age, i.e. the 20–35-year and 36–75-year age-groups, $CD4^+CD31^+$ cells were found to be more abundant in both the two age groups (Fig. 1g), but for $CD8^+CD31^+$ cells in only the 36–75-year group (Fig. 1 h). Most interestingly, the proportion of $CD45RA^+CD31^+$ lymphocytes was consistently higher in peripheral blood than in ITL (see Fig. 2a and b). This applied to $CD4^+$ and $CD8^+$ cells ($n = 11$, $P = 0{\cdot}00017$ and $P = 0{\cdot}00007$ respectively). The analysis of sections stained for CD45RA and CD3 and CD45R0 and CD3 demonstrated the presence of a small but well-defined population of naive cells in the tissue (see Fig. 2c and d).

The minor subset of $CD3^+CD4^+CD8^+$ lymphocytes or CD4/CD8 double-positives, normally present in PBLs, is enriched in RTE [25]. Hence, this phenotype could serve as an additional surrogate marker for RTE. The comparison

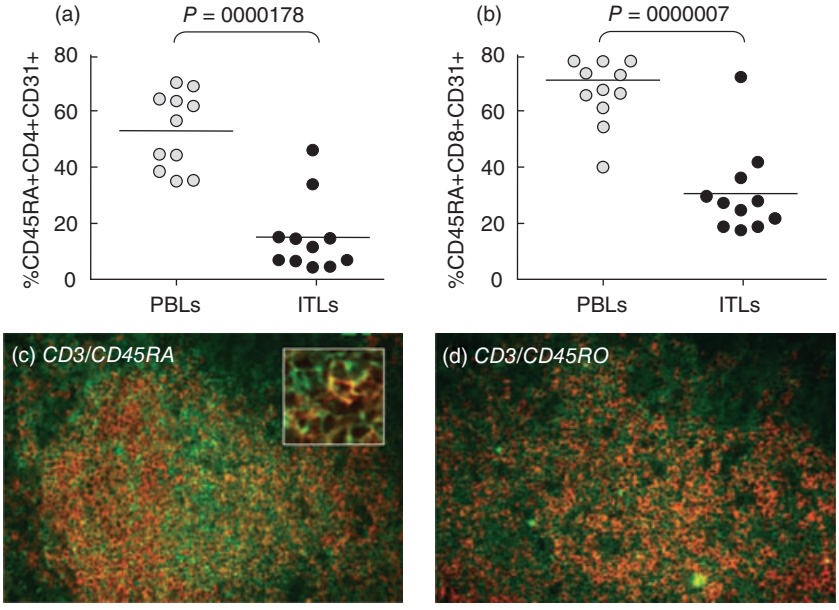

Fig. 2. Recent thymic emigrant lymphocytes (RTE) containing subset $CD45RA^+CD31^+$ in circulating peripheral blood cells (PBLs) and intrathyroidal lymphocytes (ITLs). (a) $CD45RA^+$ $CD4^+CD31^+$ cells in ITLs (black dots) and PBLs (grey dots) in autoimmune thyroid disease (AITD) patients. (b) $CD45RA^+CD8^+CD31^+$ cells in ITLs (black dots) and PBLs (grey dots) in AITD patients. Note that $CD31^+CD45RA^+$ are consistently higher in the PBLs than in ITLs between both $CD4^+$ and $CD8^+$ lymphocytes ($n = 11$). (c, d) Double immunofluorescence micrographs. Representative examples of Graves' disease thyroid cryostat sections stained for CD3 (green) and CD45RA (red) or CD45RO (red) at ×200. The inset shows a clear example of $CD3^+CD45RA^+$ cells adopting a yellow colour. In these areas of dense lymphocytic infiltration, there are patches in which naive ($CD3^+CD45RA^+$) T cells predominate clearly while in others (more often) memory cells ($CD3^+CD45RO^+$) predominate. This supports the view that there is an influx of naive T cells into the thyroid.

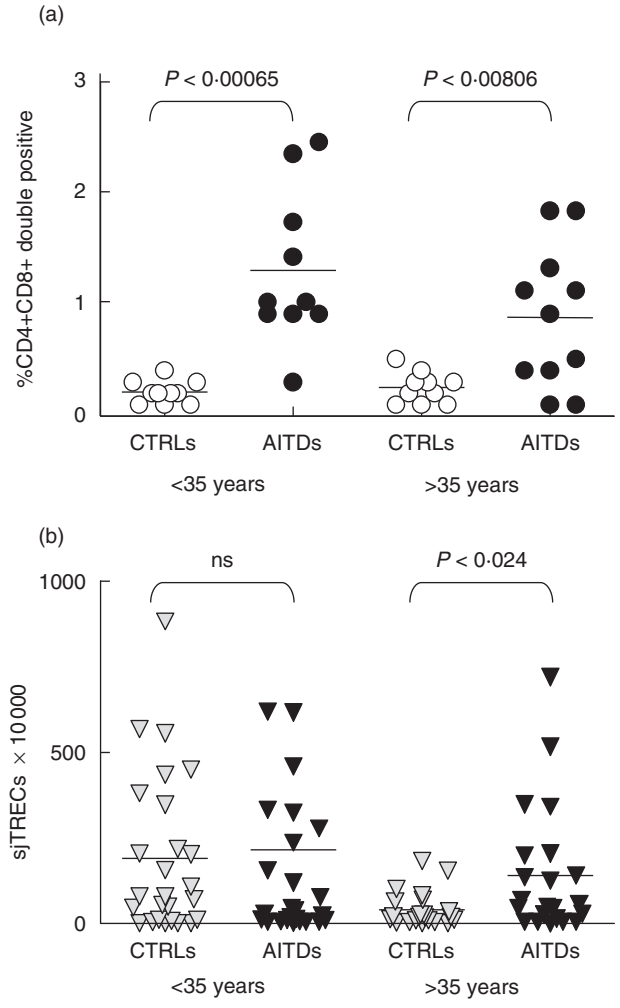

Fig. 3. Recent thymic emigrant (RTE) lymphocyte subset defined as CD4/CD8 double-positives and by T cell receptor excision circles (TRECs) in circulating lymphocytes (PBLs). (a) Levels of CD4/CD8 double-positive lymphocytes in PBLs from autoimmune thyroid disease (AITD) patients ($n = 21$, black dots) and control subjects ($n = 20$, empty dots) in the < 35 and > 35 years of age groups. Note a clearly different distribution, especially among younger patients. For details, see text. (b) Distribution of TREC levels in peripheral blood lymphocytes: in patients with AITD ($n = 48$) compared with control ($n = 48$) subjects. In patients with AITD older than 35 years of age, the levels of TRECs are significantly higher than in controls ($140{\cdot}17 \pm 187{\cdot}9$ *versus* $40{\cdot}45 \pm 50{\cdot}4$, $P = 0{\cdot}024$).

between AITD and controls (Fig. 3a) demonstrated a clear difference between both groups, which was maintained after age stratification (20–35 years, $1{\cdot}062 \pm 0{\cdot}66$ *versus* $0{\cdot}230 \pm 0{\cdot}12$, $P = 0{\cdot}00065$; 36–75 years, $1{\cdot}280 \pm 0{\cdot}67$ *versus* $0{\cdot}210 \pm 0{\cdot}10$, $P = 0{\cdot}00806$). The enumeration of the CD4/CD8 double-positive cells in ITL was attempted but found not feasible because of the low number of available ITLs and the high number of events that would have been necessary to acquire from each sample to reach reliable results.

The most widely used molecular marker for human RTE are TRECs [26–28]. TREC content in PBLs of AITD, compared with controls, showed a tendency to be higher in AITD patients but this difference was only significant in the > 35-year age group ($140{\cdot}17 \pm 187{\cdot}9$ *versus* $40{\cdot}45 \pm 50{\cdot}4$; $P = 0{\cdot}024$, see Fig. 3b). The TREC content in ITLs showed considerable interindividual variations among patients, as happens in PBLs, and they did not bear any correlation with gender, diagnosis, hormone or autoantibody status. ITL could not be obtained for comparison from normal thyroid glands as these are devoid of lymphocytes. Interestingly, when patients were classified according to the duration of clinical disease, a bimodal pattern was apparent. Patients who were operated within 30 months after the diagnosis, termed hereafter 'short course patients' (17 and 6–30; mean and range in months), had levels of intrathyroidal TRECs similar or even higher than those in their PBLs. On the other hand, most patients operated 30 months after diagnosis, hereafter termed 'long-course patients' (57 and 32–168), had lower levels of TRECs in the thyroid than in the blood. When these data were expressed as fold changes of ITL–TRECs to PBLs–TRECs this pattern became more clear, with positive or close to 1 values in most 'short-course patients' (fourfold over the average) and negative values for all but two 'long-course patients' (10-fold below the average) (Fig. 5). The comparison of short-course with long-course patients considering two categories of patients according to relative TREC distribution – (i) TRECs in ITLs > TRECs in PBLs and (ii) TRECs in ITLs < TRECs in PBLs – demonstrated a highly significantly difference in distribution (χ^2 test, $P = 5{\cdot}23 \times 10^5$). Thus the thyroid from short-course patients more often contained an excess of TRECs compared with PBLs than long-course patients. This result was not due to inclusion of cases with TRECs at the lower limit of detection, as can be seen in Fig. 4.

Analysis of telomeres and FoxP3 in PBLs and ITLs

In order to estimate the proliferative history of the peripheral T cell pool in patients and controls, we measured the RTL in PBLs from 18 AITD and 10 controls. These patients constitute a small but representative subgroup of our study population. In accordance with previous reports on physiological situations, telomeres were longer in B cells than T cells, reflecting the overall lower proliferative activity of B cells. The RTL was not different in patients from controls, either for circulating B or T lymphocytes (Fig. 5a and b). Interestingly, the comparison of telomere length between intrathyroidal T lymphocytes and the corresponding PBLs showed shorter telomeres in the former, indicating that intrathyroidal T lymphocytes had gone through more cycles of divisions than the circulating T cells or B lymphocytes. Overall, these findings do not support the possibility that the enrichment of T cells with a RTE phenotype in PBLs from AITD patients can be attributed to an enhanced expansion of T cells in the periphery.

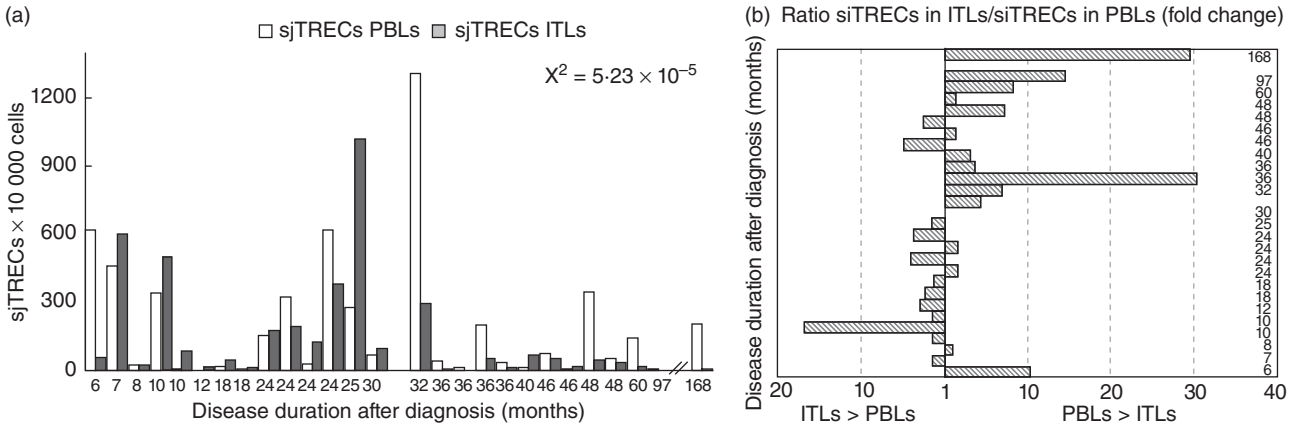

Fig. 4. Levels of signal joint T cell receptor excision circles (sjTRECs) in intrathyroidal (ITL) and circulating lymphocytes (PBLs) at the time of operation. (a) TRECS values in PBLs (grey bars) and ITLs (black bars) in relation to the duration of clinical symptoms prior to partial thyroidectomy. (b) To visualize the data more clearly in (a), values have been represented in the *x*-axis following opposite directions and expressed in fold differences: when TRECS in ITLs were > TRECS in PBLs, values are plotted as positive, while when TRECs in ITLs were < TRECs in PBLs, values are plotted as negative. In the *y*-axis, the duration of symptoms in months ($n = 37$). This representation helps to visualize how the patients operated with less than 30 months of symptoms have values of TRECs in ITLs often higher than in PBL, while in patients operated after more than 30 months of symptoms TRECs values in PBLs and ITLs are similar and decrease markedly for both with a tendency to zero values in the patients operated after 5 years of disease.

Because a selective enrichment of regulatory T cells (T_{reg}) has been described in thyroid autoimmune glands [29], we measured FoxP3 expression in relation to TREC levels in AITD glands in order to assess the contribution of this T cell subset to the TREC pool. While there was a clear correlation ($r = 0{\cdot}953744096$, $P = 0{\cdot}0003$) between the expression of CD3 and FoxP3, there was no correlation between the expression of FoxP3 and the number of TRECs (Fig. 5c and

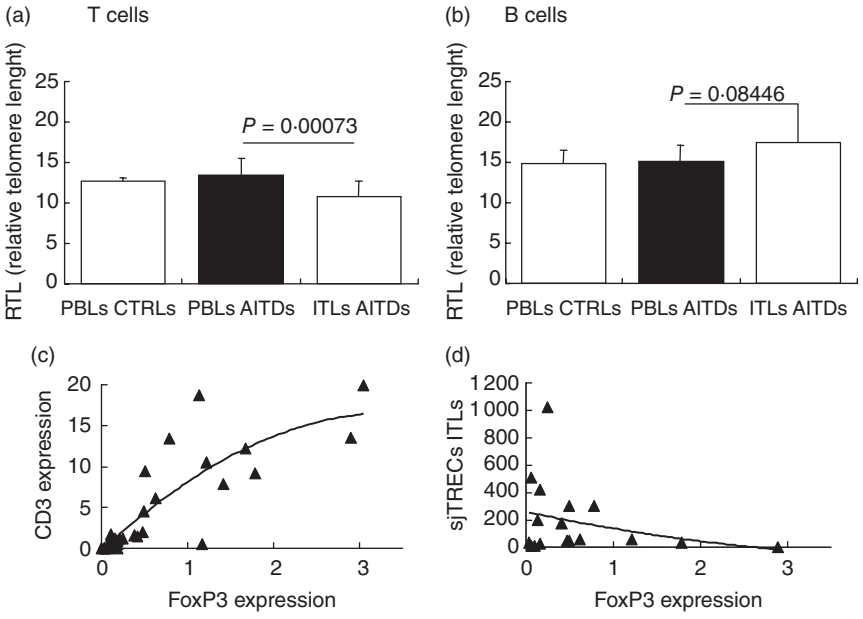

Fig. 5. Telomere length in T and B lymphocytes both circulating and intrathyroidal and forkhead box P3 (FoxP3) measurements mRNA in intrathyroidal lymphocytes. (a, b) Measurements of relative telomere length (RTL) in T and B lymphocytes from peripheral blood lymphocytes (PBLs) of autoimmune thyroid disease patients (AITD, $n = 18$) and controls ($n = 10$). In AITD patients RTL was also measured in intrathyroidal lymphocytes (ITLs, $n = 16$). Notice that in the circulating T cells from AITD patients there is no shortening of the telomeres, this arguing against their homeostatic expansion. (c, d) Correlation between FoxP3 expression with CD3 by quantitative real-time reverse transcription–polymerase chain reaction ($r = 0{\cdot}953744096$ $P = 0{\cdot}0003$) and with signal joint T cell receptor excision circles (sjTRECs) content (P not significant). Note that in spite of a good correlation of FoxP3 with CD3, there is no correlation of FoxP3 with sjTRECs, this arguing against regulatory T cells making up the population of recent thymic emigrants homing to the thyroid.

d). This result argues against the possibility that the RTE are comprised mainly of T_{regs}.

Discussion

In search of a phenotypic footprint of the immunological disorder(s) in AITD we analysed the peripheral and intrathyroidal T cell pool in AITD patients and healthy controls with respect to a set of phenotypic and functional parameters that reflect thymic T lymphocyte output. We found the following differences between the two groups: the peripheral pool of naive T cells and RTE was enlarged in AITD patients; this was not accompanied by evidence of an increase in homeostatic proliferation (i.e. telomere length); RTE, as assessed by a panel of phenotypic markers and TRECs, were enriched in the thyroid gland of patients during the initial phase of disease. Collectively, these data are compatible with a scenario characterized by an increased thymic output of T cells in adult AITD patients. These RTE enrich selectively in the thyroid gland, the underlying assumption being that at least part of them are reactive to thyroid-specific self-antigens, thus perpetuating the autoimmune pathology. This hypothesis would place the initial defect to the thymus, i.e. a failure of central tolerance. Given the obstacles to prove such a mechanism formally in humans, other interpretations of these data are not ruled out.

The higher level of naive $CD45RA^+CD3^+$ cells in AITD patients may be the result of (i) higher homeostatic expansion; (ii) a lower conversion rate into memory cells; (iii) a decreased death rate; (iv) a higher level of thymic export; and (v) a higher rate of conversion from the effector-memory cell compartment. As telomeres are not shorter in T cells from AITD patients that in the controls and TRECs are enriched rather than diluted, we consider the first two options unlikely. Similarly, a shift in the naive *versus* memory pool in favour of the former is not substantiated by the data, as the level of memory cells is not diminished. At present we cannot exclude a reduced death rate to account for the elevated levels of naive $CD45RA^+$ cells, but we consider higher thymic output the most probable explanation because of the data on $CD45RA^+CD31^+$ cells (see below).

The assessment of RTE in our study is based on three parameters, i.e. number of $CD45RA^+CD31^+$ and of CD4/CD8 double-positive lymphocytes and TRECs measurement. The concentration of TRECs in peripheral lymphocytes depends mainly on thymic output, the number of division that the cells have undergone after export from the thymus and their death rate. In steady state there is a good correlation between TRECs, thymic output and the concentration of RTE in peripheral blood, as demonstrated in animal models using *in vivo* cell-labelling techniques, thymectomy and congenic thymic grafting [28]. The progressive decline of TRECs with age in healthy subjects supports their reliability in humans. The surge in TRECs levels after successful bone marrow transplantation and anti-viral therapy in HIV^+ patients has demonstrated its value to monitor thymic function even in complex [28] situations [30–32]. After the age of 30 years there is wider interindividual variability in TREC levels, possibly also reflecting a higher variability in thymic function [33]. It is noteworthy that many AITD patients are in this age range.

T cell receptor excision circles concentrations have to be interpreted cautiously as a marker of thymic output in individuals suffering from a chronic immune or an autoimmune responses, because the rate of naive cell expansion and differentiation into memory cells, cell death and trapping into affected tissues are potentially confounding factors [28]. It is difficult to assess to what extent these caveats apply to AITD being a well-confined process, but the presumption is that it is not great.

Our TREC measurements are supported by the data on two additional, less commonly used RTE markers, CD31 and CD4/CD8 co-expression. CD31 is an adhesion molecule that T cells cease to express upon homeostatic activation via their TCR. Although CD31 is expressed by a large percentage of $CD45RA^+$ cells, the $CD31^+$ subset contains most RTE [23,24]. CD4/CD8 double-positive lymphocytes can be detected in the periphery at a very low level (< 2%), representing immature T cells that leaked out from the thymus prior to full maturation [25,34]. Interestingly, elevated levels of CD4/CD8 double-positive T cells have been described in myasthenia gravis, another organ-specific autoimmune disease, and their levels drop after thymectomy [25]. In our study concurrent higher levels of all three RTE markers – $CD45RA^+CD31^+$, CD4/CD8 double-positive lymphocytes and TRECs – argue for an increased output of T cells from the thymus of AITD patients. In at least another three autoimmune diseases, i.e. multiple sclerosis, rheumatoid arthritis and sytemic lupus erythematosus [35–37], the levels of TRECs have been investigated, but found reduced rather than elevated, thus suggesting that the mechanism proposed here does not apply to autoimmunity in general, but to a subset of diseases (see below).

Analysis of the ITLs is of particular interest, because there is evidence that the autoimmune response is driven, at least, in part, from the thyroid gland [20,38]. The lineages, state of activation, cytokine and chemokine production profiles of ITLs are therefore important parameters to understand AITD pathogenesis [20,39–41]. The finding that the levels of TRECs in ITLs tend to be higher in short-course patients than in long-course patients suggests that, during the early phase of disease, a proportion of the T cells infiltrating the glands are RTE. The enrichment of RTE within the thyroid gland is unexpected at first glance, as the site of inflammation is conducive to T cell proliferation, which should rather diminish TRECs levels. The dilution of TRECs as observed after 30 months of ongoing disease might indeed be a consequence of maintained local cell division. It remains crucial to demonstrate that the population of intrathyroidal RTE are specific to the thyroid self-antigens and that they drive the

autoimmune response actively. Even the thyroid-specific T cells that have been cloned from the thyroid glands [42,43] show poor proliferative capacity and it will be difficult to demonstrate that the small number of intrathyroidal RTE are autoreactive.

It has been reported that T_{regs} were increased in PBLs and, to a lesser extent, in ITLs of AITD patients [29]. The lack of correlation between TRECs levels and FoxP3 expression in ITLs argues against RTE belonging largely to the T_{reg} lineage. The phenotype of intrathyroidal T_{regs}, as reported by Marazuela *et al.* [29], suggested that they were of peripheral origin.

From the experiments presented here we propose that a failure of central tolerance resulting in an escape of T cells specific for thyroid autoantigens preconditions the development of AITDs, as it does in autoimmune polyendocrine syndrome (APS1) patients with autoimmune regulator gene mutations, but in a much less dramatic fashion. A similar scenario has already been advocated for two other organ-specific autoimmune diseases, namely type 1 diabetes mellitus and myasthenia gravis [11–16]. In both cases, allelic variants in the regulatory regions of target autoantigens affect the level of thymic antigen expression and thus, presumably, the threshold of central tolerance [18]. It remains to be shown whether such quantitative alterations, or rather qualitative changes of promiscuous expression of thyroid-restricted self-antigens, are associated with heightened susceptibility to AITDs (for review see [17]. In view of the re-appreciated role ascribed to central tolerance in preventing organ-specific autoimmunity, our hypothesis deserves further consideration and exploration.

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