

Analysis of orbital T cells in thyroid-associated ophthalmopathy

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SUMMARY

Thyroid-associated ophthalmopathy (TAO) has a major effect on the two compartments of the retro-orbital (RO) space, leading to enlargement of the extraocular muscles and other RO tissues. T lymphocyte infiltration of RO tissue is a characteristic feature of TAO and there is current interest in whether these T cells are specifically and selectively reactive to RO tissue itself. We recently established 18 T cell lines (TCL) from RO adipose/connective tissue of six patients with severe TAO by using IL-2, anti-CD3 antibodies and irradiated autologous peripheral blood mononuclear cells (PBMC) to maintain the growth of T cells reactive to autologous RO tissue protein fractions. Here we report on the phenotype characteristics and cytokine gene expression profiles of these orbital TCL and on their immunoreactivity to the organ-specific thyroid antigens thyrotropin receptor (TSH-R), thyroidal peroxidase (TPO) and thyroglobulin (TG). Flow cytometry revealed that 10 TCL were predominantly of CD4⁺ phenotype, three being mostly CD8⁺ and five neither CD4⁺ nor CD8⁺. Analysis with reverse transcriptase-polymerase chain reaction (RT-PCR) of cytokine gene expression revealed both Th1- and Th2-like products in all TCL: IL-2 product (in 17 TCL), interferon-gamma (IFN- γ) ($n = 10$), tumour necrosis factor-beta (TNF- β) ($n = 15$), IL-4 ($n = 12$), IL-5 ($n = 17$), IL-6 ($n = 13$), TNF- α ($n = 12$) and IL-10 ($n = 4$). Reactivity to thyroid antigens was observed only in two TCL, the other 16 being uniformly unreactive. Although 10 out of 18 RO tissue-reactive TCL were predominantly CD4⁺ there were no significant relationships between TCL phenotype, cytokine gene profile, magnitude of reactivity to RO tissue protein or the (rare) occurrence of thyroid reactivity. The findings of both Th1- and Th2-like cytokine gene expression in all RO tissue-reactive TCL support the concept that TAO is a tissue-specific autoimmune disease, distinct immunologically from the thyroid, and involving both T cell and B cell autoimmune mechanisms in disease pathogenesis.

Keywords thyroid-associated ophthalmopathy orbital T cell lines cytokine profiles thyroid antigens

INTRODUCTION

Enlargement of the two compartments of the retro-orbital (RO) space, namely the extraocular muscles and the RO adipose/connective tissue, leads to an increase of total orbital volume. Thus, proptosis and lid retraction are prominent features in patients with ophthalmopathy associated with autoimmune thyroid disease [1,2]. Histological examination of RO tissue samples demonstrates mononuclear cell infiltration comprising mainly activated T cells, with a few B cells, macrophages and mast cells in patients with thyroid-associated ophthalmopathy (TAO) [3]. Cytokines released by activated immunocompetent cells stimulate RO fibroblasts to proliferate and to secrete glycosaminoglycans, leading to oedema and finally fibrosis [4–7]. Expression of intercellular adhesion

molecule-1 (ICAM-1), HLA-DR and heat shock proteins (hsp) by orbital fibroblasts may also be induced by T cell-derived cytokines [8,9]. The phenotypes of T cells in the RO tissue of patients with TAO has been studied using mainly markers for the CD4⁺ and CD8⁺ antigens, with inconsistent results [3,10,11]. Both cell-mediated and humoral mechanisms have been postulated to be involved in the immunopathogenesis of TAO [8,12]. The few studies concerning cytokine profiles of T lymphocytes from RO tissue of patients with TAO produced various findings [11,13–15].

Factors that initiate T cell activation and an orbital immune reaction are unknown. Many putative orbital antigens have been already described and may be an immunological target in TAO [16–18]. On the other hand, known thyroidal antigens may be an antigenic link between TAO and the associated thyroidal disorder [19]. Analysis of circulating populations of peripheral blood mononuclear cells (PBMC) poorly reflect the principal pathogenic

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events within the orbit. Therefore, we established orbital adipose connective tissue-derived T cell lines (TCL) from patients with TAO which react to autologous RO tissue protein fractions, shown in previous investigations [16,17]. In the present study, we were interested in the phenotype characteristics and cytokine gene expression profiles of these orbital TCL and in their immunoreactivity to organ-specific thyroid antigens.

PATIENTS AND METHODS

Subjects

Pieces of orbital adipose/connective tissue were obtained under sterile conditions from six euthyroid patients with severe TAO undergoing orbital decompression surgery (Table 1). All patients received steroids, and three were additionally irradiated. Immunosuppressive therapy was terminated at least 2 months before surgery. Written consent was obtained from all patients and the study was approved by the local Ethics Committee.

Antigen preparation and protein electroelution

Preparation of RO protein fractions was performed as previously described [16,17]. RO adipose/connective tissues were snap-frozen immediately in liquid nitrogen. Tissue was thawed in 10 vol of lysis buffer containing a mixture of protease inhibitors (aprotinin, phenylmethylsulfonylfluoride, and ϵ -aminocaproic acid) and non-ionic detergent (2% Nonidet P-40; INC Pharmaceuticals, Costa Mesa, CA), homogenized in a Polytron mechanical blender (Brinkmann Instruments, Westbury, NY), and incubated on ice for 30 min. Homogenates were centrifuged at 15 000 g for 15 min to remove cell debris. The supernatant from this step was retained for use in SDS-PAGE, which was performed using a linear 5–20% polyacrylamide gradient separating gel (11 × 11 × 0.15 cm) and a 4% stacking gel in a vertical gel apparatus [20]. SDS-treated protein samples were added at a concentration of 300 μ g protein in 50- μ l aliquots/lane. The gel was run at 35 mA for 3 h under reducing conditions with mercaptoethanol.

To recover the separated proteins in soluble form, a Blotolutor (Biometra, Göttingen, Germany) was used [21]. They were electroeluted for 1 h under constant current (0.8 mA/cm²) to a masterplate containing 22 × 24 wells filled with 5 mmol/l Tris buffer pH 10.4. Each row of the 22 different molecular mass protein fractions was pooled, lyophilized, resuspended in 200 μ l PBS, and sterilized with ultraviolet light (312 nm) for 5 min.

Table 1. Clinical data of six patients with thyroid-associated ophthalmopathy (TAO)

Patient	Age (years)	Sex	Duration of TAO (months)	Therapy of TAO before decompression surgery*	Thyroid therapy
1	35	F	7	S (5)	Methimazole
2	46	F	15	S (4), T (12)	None
3	50	F	10	S (3), R (6)	Methimazole
4	56	F	6	S (3), R (4)	Methimazole
5	59	F	9	S (2)	Methimazole
6	48	M	12	S (5), R (6)	None

F, Female; M, male; S, steroids; R, radiotherapy; T, thyroidectomy.

* Interval between end of treatment and surgery in months.

Preparation of T cell lines

PBMC were separated from heparinized venous blood by Ficoll gradient centrifugation. As previously described [17], *in vivo* activated, orbital tissue-reactive T lymphocytes were selected by cultivating small pieces of RO adipose/connective tissue in medium (RPMI 1640; 10% AB⁺, Rh⁺ human serum) containing IL-2 (20 ng/ml) for 10 days, followed by growth stimulation with anti-CD3 antibodies (30 ng/ml; CD3-UCHT1-IOT-3b; Dianova, Hamburg, Germany), thereby retaining antigen specificity of the T cells [22]. Every 10 days anti-CD3 antibodies and irradiated autologous PBMC (5 × 10⁴; 40 Gy) were added to the cell lines as feeders. Every 3 days IL-2 medium was changed. In order to generate sufficient numbers of T cells for various investigations, six to eight culture passages were needed.

Phenotype analysis

Phenotypes of the TCL were determined by immunofluorescence staining with specific MoAbs. After transfer of the lymphocytes into round-bottomed tubes (10⁵ cells/tube) and washing with PBS containing 0.1% bovine serum albumin (BSA), the cells were incubated with FITC- and PE-conjugated MoAb against CD3, CD4 and CD8 (each 2.5 μ g/ml; Immunotech, Marseille, France) for 30 min at 4°C in the dark. Lymphocytes were washed twice with PBS containing 0.1% BSA and 0.1% NaN₃, diluted in fixation buffer containing 0.5% formaldehyde, and analysed on a fluorescence-activated cell sorter (FACS analysis; Becton Dickinson Immunocytometry Systems, Mountain View, CA). Ten-thousand scatter-gated cells were analysed in each sample. The frequency and fluorescence profile of the cells were determined with logarithmic signal amplifiers.

mRNA preparation and cDNA synthesis

After restimulation of orbital T cells (10⁵) with anti-CD3 MoAb, IL-2 medium and feeders for 5 days to induce cytokine expression, total mRNA was prepared from cultivated TCL using a modified guanidinium thiocyanate/phenol/chloroform extraction method (RotiQuick kit; Roth, Germany) according to the manufacturer's instructions. In order to analyse IL-2 expression, TCL were stimulated for 6 h with anti-CD3 MoAb without IL-2 or feeders, in microtitre plates coated with anti-CD28 MoAb (1 μ g/well, overnight, 4°C; Pharmingen, Germany) and washed twice in PBS before isolation of total mRNA. Samples were used immediately to synthesize cDNA or stored at -70°C. One microgram of total RNA obtained from each TCL was used to generate cDNA by reverse transcription (RT) with oligo-desoxythymidine primer (cDNA synthesis kit; Boehringer, Mannheim, Germany).

Polymerase chain reaction amplification of cytokine cDNA

PCR was performed in 50 μ l amplification reaction containing 5 μ l of 10 × buffer with 20 mM Tris-HCl pH 8.55, 16 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 2.0 U of *Taq* DNA polymerase, 0.2 mM each dNTP (all from AGS, Heidelberg, Germany), 25 pmol of the corresponding primer pairs, 1 μ l of cDNA and autoclaved deionized water. Primers were chosen according to references [23–27] (Table 2). Amplifications were carried out using cycles of 94°C for 30 s, primer corresponding annealing temperature for 30 s, and 72°C for 1 min. Thirty-five cycles were performed to amplify T cell-specific CD3- δ and eight different cytokine products. As positive control, PBMC from a normal donor were cultivated in RPMI 1640, 10% human AB⁺ serum, and stimulated with 2 μ l/ml phytohaemagglutinin (PHA; Biochrom, Germany) for 72 h. Cells were separated

Table 2. Oligonucleotide sense (S) and antisense (AS) primers used for polymerase chain reaction (PCR) amplification of CD3- δ and cytokines cDNA

Cytokine	Oligonucleotide primers	Annealing temperature	Ref.
CD3- δ	S 5'-ctggacctgggaaaacgcac	58°C	[23]
	AS 5'-gtactgagcatcatctcgatc		
IL-2	S 5'-aactcctgtcttgattgca	54°C	[24]
	AS 5'-gtgttgagatgatgctttgac		
IFN- γ	S 5'-atgaaatatacaagttatatac	46°C	[25]
	AS 5'-ttactgggatcctcttcgacctgaaacagcat		
TNF- α	S 5'-atgagcactgaaagcatgatc	56°C	[25]
	AS 5'-tcacagggaatgatcccaagtagacctgccc		
TNF- β	S 5'-aaacctgctcaccctcatt	56°C	[26]
	AS 5'-tggatcacaccattcttctggg		
IL-4	S 5'-caactttgtccacggacac	54°C	[24]
	AS 5'-tccaactgactctggttg		
IL-5	S 5'-aggatgctctgcatcttgag	54°C	[24]
	AS 5'-ctattatccactcgggttc		
IL-6	S 5'-aactccttcccacaagcg	54°C	[25]
	AS 5'-tggactcgaggaactcctt		
IL-10	S 5'-atgccccaagctgagaaccaagaccagac	60°C	[27]
	AS 5'-tctcaagggtgctggctcagctatccca		

by centrifugation and used to prepare total RNA and cDNA, as described above. Control reaction without cDNA and with total RNA, in which RT was omitted in order to exclude a possible amplification of contaminating genomic DNA, were carried out in parallel, and were consistently negative (data not shown). Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis, visualized by staining with ethidium bromide, and their size was verified by comparison with a 100-bp ladder. Gels were permanently recorded using an automatic video system (Eagle Eye; Stratagene, La Jolla, CA).

T lymphocyte proliferation assay

TCL or PBMC were cultivated at a cell density of 1×10^4 or 1.5×10^5 cells per well, respectively, in 96-well round-bottomed plates containing 200 μ l culture medium (RPMI 1640; 10% AB⁺, Rh⁺ human serum and antibiotics) at 37°C in a humid atmosphere comprising 95% air and 5% CO₂, together with irradiated autologous feeders (4×10^4 ; 40 Gy) and autologous protein fractions of

different molecular masses obtained from SDS-PAGE or thyroidal proteins in different concentrations as antigen [16,17]. The following thyroidal antigens were used: recombinant human thyrotropin receptor (TSH-R), recombinant human thyroidal peroxidase (TPO) in soluble form [28], and native human thyroglobulin (TG; Elias, Freiburg, Germany). Triplicate cultures were set up, and incubation was for 5 days. Eighteen to 24 h before termination, ³H-thymidine (1 μ Ci, 925 GBq/mmol; Amersham Buchler, Braunschweig, Germany) was added to each well, cultures were harvested and thymidine incorporation was measured in a beta counter. Control cultures without orbital or thyroidal antigens, with tuberculin (Tb; 66 U/well; Behring, Marburg, Germany) as negative control, and with PHA (0.3 μ g/well) as positive control, were included in all assays. Results were expressed as stimulation index (SI) calculated as (ct/min (lymphocytes + antigens)/ct/min (controls)) and Δ ct/min (ct/min (lymphocytes + antigens) - ct/min (controls)). An SI > 3.0 was taken as positive response. Proliferative responses were compared using Student's *t*-test.

RESULTS

Phenotype analysis of orbital T cell lines

In all 18 orbital TCL derived from six patients with TAO, the T cell marker CD3⁺ could be detected in almost 100% of cases. TCL from all patients except patient 2 consisted predominantly of CD4⁺ cells (Table 3). Ten out of 18 TCL were mostly CD4⁺. The percentage of these CD4⁺ helper/inducer cells of each total TCL population ranged between 46% and 92% with a mean of 78.5%. Three TCL, nos 1 (patient 1), 8 (patient 2), and 15 (patient 4), were predominantly CD8⁺. Low numbers of CD4⁺CD8⁺ cells were detected in all samples. Three TCL, derived from patient 2, and two TCL (patient 3) were mainly CD4⁻CD8⁻. All remaining TCL contained a low proportion of CD4⁻CD8⁻ cells.

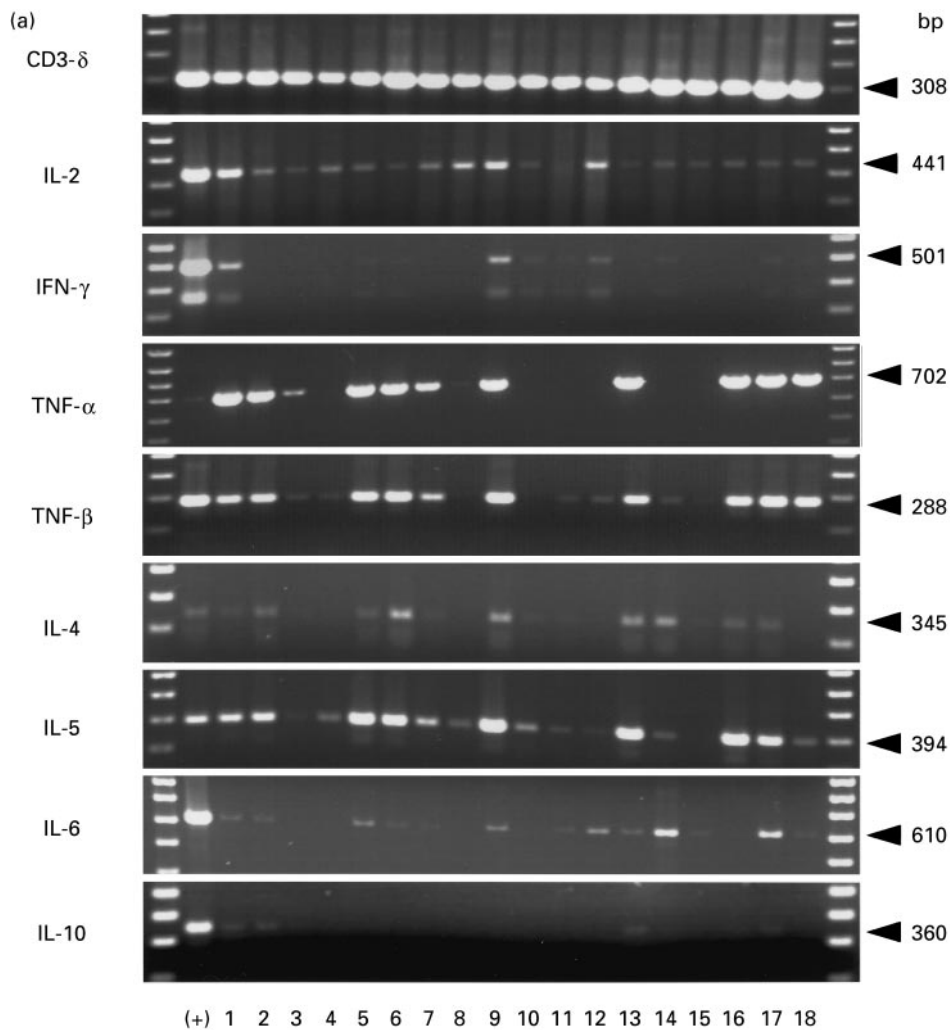
Cytokine profile of orbital T cell lines

We analysed the cytokine gene expression using RT-PCR. Each cDNA transcript of the predicted size could be detected in the positive control (X), prepared from stimulated PBMC from a normal subject (Fig. 1a,b). Consistent amplification of CD3- δ cDNA, which was used as an internal control for studies on T cell transcripts, could also be detected in all TCL samples from six patients with TAO, demonstrating an intact mRNA preparation and the integrity of T lymphocytes as a specific T cell marker. The

Table 3. Phenotype determination by FACS analysis of 18 orbital tissue-derived T cell lines (TCL) from six patients with thyroid-associated ophthalmopathy (TAO)

Patient	1				2				3				4		5		6	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CD3 ⁺	100	100	100	100	100	100	99	100	99	100	100	100	100	99	100	100	100	100
CD3 ⁺ CD4 ⁺	34	88	66	72	5	0.3	0.6	2.2	72	8	92	16	91	91	12	46	82	85
CD3 ⁺ CD8 ⁺	58	0.5	27	18	23	0.1	15	97	4	32	6	15	5	5	71	42	15	11
CD3 ⁺ CD4 ⁺ CD8 ⁺	2.6	11	2.3	1.7	0.3	0	0.2	0.2	2.3	0.9	1.2	8.2	1.3	2.1	4.4	7.6	1.8	2.4
CD3 ⁺ CD4 ⁻ CD8 ⁻	4.5	0.2	4	6.8	71	99.7	84	0.3	21	58	0.5	60	2	1.3	12	4.3	1	1.3

Cells were stained with FITC-/PE-conjugated MoAbs against CD3, CD4, and CD8. Results are expressed as percentage (%) of positive cells in the different TCL.



(b)

Patient		1				2				3				4			5		6
T cell line	(X)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CD3- δ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IL-2	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
IFN- γ	+	+	-	-	-	+	+	-	-	+	+	+	+	-	+	-	-	+	+
TNF- α	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	-	+	+	+
TNF- β	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	+
IL-4	+	+	+	-	-	+	+	+	-	+	+	+	-	+	+	-	+	+	-
IL-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
IL-6	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+	+	-	+	+
IL-10	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-

Fig. 1. Cytokine gene expression of 18 T cell lines (TCL) derived from orbital tissue of six patients with thyroid-associated ophthalmopathy (TAO) using reverse transcription-polymerase chain reaction (RT-PCR). Following cDNA synthesis, all TCL (lanes 1–18) and as positive control cDNA prepared from stimulated peripheral blood mononuclear cells (PBMC) of a normal donor (X), were amplified by PCR simultaneously for each cytokine-specific oligonucleotide primer pair. Orbital TCL were generated from six patients with TAO (patient 1, lanes 1–4; patient 2, lanes 5–8; patient 3, lanes 9–12; patient 4, lanes 13–15; patient 5, lanes 16 and 17; patient 6, lane 18). Lanes right and left sideways represent the DNA molecular weight markers. Only the expected range of base pairs (bp) size with visualized transcripts after electrophoretic separation are shown (a). Additionally, positive signals are demonstrated by + and negative by – (b).

generation of cytokine DNA products was variable, although the RT-PCR technique provides qualitative information only. Transcripts of IL-2, interferon-gamma (IFN- γ) and tumour necrosis factor-beta (TNF- β) typically produced by Th1 lymphocytes were amplified in all 18 TCL. IL-2 cDNA could be detected in all samples except TCL no. 11, whereas IFN- γ was detectable in 10 TCL. All six patients were positive for IFN- γ cDNA transcript products. TNF- β was found in all TCL except nos 8, 10 and 15. Amplification of TNF- α , produced by Th1 but also by Th2 lymphocytes, was generated from cDNA of 12 TCL. All analysed TCL were also positive for at least one of the Th2 cytokines. cDNA transcripts for IL-4, IL-5 and IL-6 were each demonstrated in 10 TCL. IL-4 was detectable in 12 TCL, IL-5 in all TCL except no. 15, and IL-6 in 13 TCL. IL-10 cDNA could be amplified in four samples. In TCL nos 8 and 15 with high predominance of CD8⁺ cells (97% and 71%, respectively; Table 3) cDNA transcripts for IFN- γ , TNF- β , IL-4 and IL-10 could not be amplified, and cDNA for IL-5 (TCL no. 8, patient 2) and IL-6 (TCL no. 15, patient 4) gave only very weak signals. No cytokine transcripts were amplified from orbital TCL mRNA without performing cDNA synthesis (data not shown), thereby excluding the presence of contaminating genomic DNA as a source of template.

Immunoreactivity to thyroidal antigens

Immunological responses of 18 orbital TCL to known thyroidal antigens are summarized in Table 4. In no case was specific proliferation demonstrated. None of the TCL showed significant proliferation when incubated with TG (SI < 3.0 in all cases). TPO stimulated two TCL from two different patients, with SI values of 5.4 (TCL no. 8, patient 2) and 5.1 (TCL no. 15, patient 4), respectively. The same two TCL responded to TSH-R (SI 8.0 and 4.3, respectively). Reaction of TCL was not dependent on the concentrations of the antigens used. None of the other orbital TCL was stimulated by the thyroidal antigens used (SI < 3.0). Orbital TCL did not proliferate when incubated with Tb, but showed an increased proliferation with PHA as positive control.

Correlations between various characteristics of orbital T cell lines
Previous investigations showed that incubation of orbital-derived TCL from six TAO patients with autologous protein fractions obtained from RO adipose/connective tissue resulted in markedly elevated T cell proliferation with proteins of 6–10 and 19–26 kD. The results are shown in Table 5 expressed in SI and Δ ct/min. Incubation of the two RO adipose/connective protein fractions with autologous PBMC from the investigated TAO patients induced a significant proliferation of lymphocytes from patient 6. PBMC from patients 2 and 4 responded to the protein fraction 6–10 kD and from patient 5 to the protein fraction 19–26 kD. Comparison between the magnitude of reactivity to RO tissue proteins, TCL phenotype, cytokine gene profile or the rare occurrence of thyroid reactivity showed no significant relationships. Furthermore, no correlation of the TCL characteristics was found with previous treatments of the TAO patients, e.g. orbital irradiation or steroid therapy.

DISCUSSION

In previous investigations, we were able to establish orbital adipose/connective tissue-derived TCL from six patients with severe TAO that react to autologous protein fractions of RO tissue, as well as of cultured autologous orbital fibroblasts [16,17]. In the present study, we analysed these 18 orbital TCL in order to study their phenotype, functional and immunological properties. Since the majority of T cells infiltrating RO space are presumed to be activated [3], T lymphocytes were selected from surgical material and cultivated in IL-2-conditioned medium. With this procedure, *in vivo* activated T cells that already express IL-2 receptor were stimulated. After cell amplification with anti-CD3 antibodies, TCL showed specific responses to orbital antigens [16,17], thus maintaining their antigen specificity.

Immunohistological analysis in TAO patients showed a predominance of CD4⁺ phenotype T cells in extraocular muscle biopsies [3] and RO adipose/connective tissue [10]. In comparison,

Table 4. Reactivity of orbital T cell lines (TCL) from six patients with thyroid-associated ophthalmopathy (TAO) to known thyroidal antigens

Patient	1			2			3			4			5			6			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
hTSH-R	1*	1.1	0.9	1.0	0.8	1.0	0.8	0.4	3.3	1.2	1.2	0.6	0.9	1.0	0.4	1.6	1.0	2.1	0.6
	0.1*	0.9	0.5	1.2	1.1	0.7	0.9	0.6	3.9	0.8	1.1	0.8	1.3	0.5	0.7	4.3	0.5	0.6	0.4
	0.01*	1.1	0.9	1.5	0.8	1.2	0.9	0.6	8.0	0.5	0.9	0.9	0.6	0.6	0.9	1.3	1.7	0.7	0.5
TPO	10*	0.8	1.0	1.0	1.1	0.9	0.7	0.5	2.6	1.5	1.1	0.9	0.8	1.0	1.1	5.1	1.5	0.7	0.5
	1*	0.4	0.5	1.0	0.9	1.0	1.0	0.7	2.0	0.6	1.0	1.0	1.1	0.8	0.7	2.2	0.8	0.4	0.5
	0.1*	0.4	0.6	1.0	0.7	1.2	0.7	0.8	5.4	0.9	0.9	0.8	0.3	1.3	0.7	0.9	2.8	0.4	0.5
TG	100*	1.0	1.7	0.9	1.1	1.7	0.9	1.2	2.9	1.6	1.0	0.9	0.6	0.9	0.8	1.7	1.4	0.4	0.8
	10*	1.0	0.5	0.9	1.3	1.3	0.7	0.5	1.0	2.1	0.9	0.8	0.9	0.6	1.2	1.3	1.0	1.5	0.4
	1*	0.5	0.7	1.2	0.9	1.5	1.0	1.0	1.9	1.6	1.2	0.7	1.3	0.8	0.7	2.1	0.7	0.3	0.5
Tb	66†	0.9	2.1	0.8	0.2	1.9	1.1	0.1	0.4	0.8	0.9	0.6	0.8	0.2	0.5	2.2	0.4	0.6	0.6
PHA	300*	39.8	172.2	16.9	22.2	502.5	11.5	208.8	139.9	19.0	72.5	78.3	94.2	200.2	84.4	76.3	56.4	176.7	15.0

Results are expressed as mean stimulation index (SI) for triplicate cultures in the lymphocyte transformation assay (³H-thymidine incorporation). Variations among triplicate cultures were <15%. SI of more than 3.0 was taken as significant. hTSH-R, Recombinant human TSH receptor; TPO, recombinant human thyroidal peroxidase; TG, native human thyroglobulin; Tb, tuberculin; PHA, phytohaemagglutinin.

* Concentration of stimulating agents (ng/well).

† Concentration of stimulating antigens (U/well).

Table 5. Response of orbital T cell lines (TCL) and peripheral blood mononuclear cells (PBMC) from six patients with thyroid-associated ophthalmopathy (TAO) to autologous orbital protein fractions 6–10 and 19–26 kD, respectively

Patient	1				2				3				4			5		6	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
T cell line																			
6–10 kD	SI	65	44	171	87	11	14	16	21	30	54	33	67	11	6	4	12	5	53
	Δ ct/min	7232	4171	20570	8944	2090	3029	3150	4420	3496	12764	2144	7298	962	1063	1191	1970	1013	7917
19–26 kD	SI	85	30	21	43	4	3	3	7	17	41	5	54	4	4	8	3	8	33
	Δ ct/min	9492	2813	2420	4368	627	466	420	1326	1933	9851	285	5861	277	558	2464	384	1884	4884
PHA	SI	1	1	7	29	7	4	20	18	92	21	408	89	167	56	28	5	1	83
	Δ ct/min	63	48	785	2912	1182	772	5259	3757	10989	4940	27263	9731	16123	10750	9713	638	88	12449
Tb	SI	1	1	2	1	1	1	1	1	3	2	1	1	1	2	1	1	1	2
	Δ ct/min	<0	<0	121	<0	24	56	<0	15	354	329	<0	31	<0	110	46	49	<0	234
PBMC																			
6–10 kD	SI		2				34			3				7		5		32	
	Δ ct/min		53				5059			54				198		165		2484	
19–26 kD	SI		1				3			1				3		24		12	
	Δ ct/min		<0				307			16				73		893		879	
PHA	SI		22				19			126				226		63		66	
	Δ ct/min		1118				2812			3916				6876		2473		5456	
Tb	SI		1				1			19				19		20		2	
	Δ ct/min		<0				<0			563				498		765		65	

Results are expressed as mean stimulation index (SI) and Δ ct/min for triplicate cultures in the lymphocyte transformation assay (3 H-thymidine incorporation). Variations among triplicate cultures were <15%. Tb, Tuberculin; PHA, phytohaemagglutinin.

mainly CD8⁺ phenotype profiles were reported on RO adipose/connective tissue-derived TCL [11], and RO T cell clones displayed almost equal proportions of CD4⁺ and CD8⁺ cells [13]. Recently, TCL derived from extraocular muscles were demonstrated to be predominantly CD4⁺ [15]. In our laboratory, previous phenotype screening of 62 orbital T cell clones revealed a CD4/CD8 ratio of 8:2, compared with a normal ratio of 2:1 in PBMC [29]. In the present study, phenotype analysis of the 18 RO adipose/connective tissue-derived TCL showed that 10 of the TCL were predominantly CD4⁺ lymphocytes, while only three were mainly CD8⁺. Five TCL were CD3⁺CD4⁺CD8⁻. The phenotype of RO T cells might depend more on the stage of disease than on the origin of the biopsy material like extraocular muscles or adipose/connective tissue. Although our TCL prepared from the same individual had different phenotypes, indicating that both CD4⁺ and CD8⁺ lymphocytes were present in the RO space at the same time, the predominance of CD4⁺ cells in the investigated TCL favours a predominantly T helper cell-mediated immune process. Since the number of TCL studied was low, it is not possible to provide definitive information about the nature of the tissue infiltrates.

T helper cells are characterized by their profile of cytokine production. In patients with TAO, but not in controls, the presence of IL-1, TNF- α and IFN- γ has been observed in orbital tissue [30]. Furthermore, in investigation of orbital fat/connective tissue by RT-PCR the presence of IL-2, IL-4 and IL-10 but not of IFN- γ in two out of five patients with TAO was reported, supporting a humoral autoimmunity [14]. Recently, analysis of extraocular muscle biopsies showed a predominance of IL-4 gene expression, and cultured TCL derived from the same tissue released a mixture of IL-2, IL-4, IFN- γ , IL-10 and TNF- β [15]. In another study, orbital fat/connective tissue-derived T cell clones of four TAO

patients were established, and secretion of cytokines was measured *in vitro* [13]. In contrast, only a small proportion of clones secreted IL-4 and IL-5, whereas the majority secreted IL-2 and IFN- γ , suggesting a predominance of Th2-mediated cytolytic T cells in the orbit [13]. Investigated by RT-PCR, our TCL showed cytokine gene expression of Th1 cells, IL-2, IFN- γ and TNF- β . In each of the 18 TCL at least one of these cytokines were demonstrable, and most were positive for two or all three cytokines. Strong evidence for a Th2 response was also found in our patients: Th2 cytokines were detectable in all 18 orbital TCL, and most TCL expressed IL-4, IL-5 and IL-6 simultaneously. IL-6, positive in 13 TCL, is a known B and T cell activator and its over-expression has been associated with the production of antibodies [31]. IL-10, a potent immunosuppressant of macrophage function and cell-mediated immunity, with a wide array of immunostimulatory actions on B cells and autoantibody production [32], does not discriminate between a Th1 or Th2 profile. In humans, this cytokine is produced by both Th1 and Th2 cells as well as by B cells and stimulated macrophages [33]. Gene expression of TNF- α , that is predominantly of macrophage origin under *in vivo* circumstances, and is produced by Th1 and Th2 cells as well [23], was found in 12 out of 18 TCL. As recently shown for thyroid-derived lymphocytes [34], orbital T cells from patients with TAO are also producers of TNF- α .

In this study, gene expression of cytokines in 18 orbital TCL derived from adipose/connective tissue of TAO patients has only been demonstrated qualitatively. Although a low contamination of PBMC in analysis of RO tissue cannot be excluded and the two major types of T cell subsets are negatively interactive [35], we found gene expression of both Th1- and Th2-mediated cell activity without a preference for one or the other autoimmune process. As recently shown for the neonatal immune system [36–38], T helper

cell reaction in an autoimmune disorder changes during the course of the disease and is never purely antibody- or T cell-driven. The cytokine profile and the type of autoimmune activity in the RO space may depend on the activity of TAO and may change during the course of the eye disease, thus explaining the apparent differences in the investigations. In addition, TCL cytokine gene expression could depend on the origin of the lymphocytes, either from the extraocular muscles or the orbital adipose/connective tissue.

The relationship between TAO and thyroid autoimmunity is unclear, and thyroidal antigens may also play a role in T cell immunity of the RO space [39]. Known thyroidal antigens such as TSH-R, TG and TPO, all well studied as immunological targets in Graves' disease and Hashimoto's thyroiditis, did not lead to disease-specific proliferation of our 18 TCL. Only two TCL, that consisted predominantly of CD8⁺ cells with a Th1 cytokine profile, were stimulated by TSH-R and TPO. In comparison with non-specific stimulation with PHA, responses were very low and not antigen dose-dependent, suggesting that only small proportions of the cell lines were responsive. It seems that T lymphocytes directed against these antigens are not predominant in the RO space. Although our T lymphocytes showed no guiding reaction pattern against known thyroidal antigens, cross reactivity with a thyroid antigen cannot be excluded.

Comparisons of the response to RO protein fraction, phenotype or cytokine profile of our investigated TCL were without significance. Previous treatment, e.g. orbital irradiation or steroid therapy of the TAO patients, from whom the TCL were derived, showed no significant influence on the characteristics of these TCL. Investigation of organ-specific T cells from an early stage of disease without previous immunosuppressive treatment would be useful to show the initial situation in TAO.

In conclusion, while results derived from the study of 18 TCL from six patients with TAO may not reflect the usual inflammatory reaction in the RO space, they suggest that infiltrating immunological cells in TAO are T lymphocytes, comprising mainly cells of the helper phenotype. Both immunological immune mechanisms, involving antibodies and cytolytic T lymphocytes, respectively, were demonstrated by cytokine gene expression of orbital derived TCL. These findings of RO tissue-reactive TCL support the concept that TAO is a tissue-specific autoimmune disease, immunologically distinct from the thyroid.

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