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Original Paper

Phenotypic and Functional Characterisation of Tumour-infiltrating Lymphocytes Derived from Thyroid Tumours

R.S. Lee,[1] M. Schlumberger,[2] B. Caillou,[3] F. Pages,[1] W.H. Fridman[1] and E. Tartour[1]

[1]Laboratoire d'Immunologie Clinique, Unité INSERM 255 Institut Curie, 26 Rue d'Ulm, 75231 Paris Cedex 5; and [2]Département de Médecine Nucléaire and [3]Département de Pathologie, Institut Gustave Roussy, 94805 Villejuif Cedex, France

The natural history of thyroid tumours and the hyper-reactivity of the immune system in patients with thyroid cancer suggest that immune surveillance may play a role in the control of this disease. A study was therefore undertaken to analyse the phenotypic and functional features of tumour-infiltrating lymphocytes (TILs) derived from thyroid tumours. In a series of experiments, it was found that, in contrast to TILs derived from patients with melanoma or renal cell carcinoma, thyroid TILs could be efficiently expanded *in vitro* only in the presence of allogeneic EBV transformed B (B. EBV) cells. Indeed, only one of the seven thyroid-derived TILs grew *in vitro* without feeder cells, whereas all 16 thyroid-derived TILs could be expanded in the presence of allogeneic B. EBV feeder cells. Phenotypic analysis of these TILs revealed a frequent *in vitro* expansion of an unusual T cell population that expressed both the CD4 and CD8 markers. Indeed, it was demonstrated that in five of 14 TILs in short-term culture (< day 23) and four of 11 TILs in long-term culture (> day 40), a lymphocyte population that coexpressed CD4 and CD8 antigen accounted for more than 15% of the total TIL population. This double-positive T cell population was not observed in TILs derived from melanoma or renal cell carcinoma. Thyroid-derived TILs also displayed an intense cytolytic activity against NK-sensitive tumour targets with 10 of 11 TILs exhibiting significant cytotoxicity towards the NK-sensitive K562 cell line. Six of 11 TILs were also cytotoxic towards autologous tumour, but when cold target inhibition with K562 was performed with three cultures, unlabelled K562 completely inhibited lysis of autologous tumour cells. A significant expansion of CD3+CD56+ T cells in the different TIL populations may explain this high level of NK-like cytotoxicity. In conclusion, TILs derived from thyroid tumours could be efficiently expanded *in vitro* under certain culture conditions. Different strategies must be explored to enhance their specific tumour autologous specificity, however, before they can be used in immunotherapy protocols.

Key words: thyroid tumour, tumour-infiltrating lymphocytes, immunophenotype
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INTRODUCTION

THYROID CANCER is recognised as a radiation-related malignant disease [1], and this was recently supported by evidence of an increased incidence of thyroid cancer among children from areas contaminated by the Chernobyl accident [2]. Genomic abnormalities, such as *RET* or *TRK* translocation and *RAS* point mutations, have also been implicated in thyroid carcinogenesis [3].

Correspondence to R.S. Lee.
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Good prognosis and the slow growth of this type of tumour, particularly papillary thyroid carcinoma, however, even in the presence of distant metastases, and the frequent finding of occult carcinoma at autopsy [4] suggest that immune surveillance may play a role in the natural history of this disease. Hyper-reactivity of the immune system in patients with thyroid cancer was demonstrated by a frequent autoimmune thyroid disease associated with papillary thyroid carcinoma [5–6]. Antithyroglobulin and antithyroid microsomal autoantibodies may also be found in the serum of patients with thyroid cancer [7]. This autoimmune process in the context of

thyroid carcinoma may reflect an active antitumour immune response, as tumour antigens recognised by T lymphocytes are often also expressed by normal cells [8]. In patients with melanoma, a local depigmentation resulting from antimelanocyte autoimmune reactions, vitiligo, has been associated with a clinical response to immunotherapy [9] and a favourable prognosis [10–11].

Follicular and anaplastic carcinomas of the thyroid can be aggressive with progressive and disseminated disease unresponsive to treatment with isotopes and/or hormones. Patients with these forms of the cancer may benefit from new therapeutic approaches, such as immunotherapy. The potential value of adoptive immunotherapy using tumour-infiltrating lymphocytes (TILs) for the treatment of patients with cancer has already been emphasised [12]. This study was undertaken to ascertain whether TILs, derived from thyroid tumour biopsies, can be established in long-term culture with interleukin (IL)2, and to characterise the phenotype and functional activity of these lymphocytes.

MATERIALS AND METHODS

Patients

Fresh thyroid tumour tissues were obtained from 16 patients, all of whom underwent surgery at the Institut Gustave Roussy, Villejuif. The ages of the patients ranged from 21 to 69 years. The histopathological data for each biopsy specimen are shown in Table 1.

Culture of TILs

Freshly excised tumour tissues were washed twice with RPMI-1640 (Bio-Whittaker, Walkerville, Maryland, U.S.A.), minced with scalpels into 3–5 mm pieces, and stirred into 40 ml of RPMI-1640 medium containing 3600 units of deoxyribonuclease, 50 mg of collagenase and 125 units of hyaluronidase (Sigma) for 1–2 h at 37°C as previously described [13]. The resulting cell suspension was filtered through a Nitex mesh, washed twice and suspended in X-VIVO-15 medium (Bio-Whittaker) with 20% of lymphokine-activated killer cell supernatant and human recombinant IL2 (200 units/ml). The cell suspension was cultured at 3–5 × 10^5 cells/ml in 75 cm^2 bottles (Becton Dickinson, France). In some experiments, irradiated allogeneic EBV-transformed B (B. EBV) cells (100 Gy, 5 × 10^5 cells/ml) were used as feeder cells.

Table 1. Patient characteristics

Patient	Sex	Age (years)	Diagnosis
1	F	43	Follicular adenoma
2	F	43	Follicular adenoma
3	F	57	Follicular adenoma
4	F	59	Oncocytic carcinoma
5	F	50	Follicular adenoma
6	F	69	Follicular adenoma
7	F	46	Follicular adenoma
8	F	47	Follicular adenoma
9	M	38	Papillary carcinoma
10	F	39	Papillary carcinoma
11	F	21	Follicular adenoma
12	F	68	Papillary carcinoma
13	M	48	Follicular adenoma
14	M	47	Adenoma with oncocytic cells
15	M	57	Follicular adenoma
16	F	27	Papillary carcinoma

Tumour cell lines

Thyroid primary tumour cells were cultured in the 'Alpha Modification of MEM' proline-free medium (Eurobio, Les Ulis, France) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The thyroid origin of neoplastic cultured cells was confirmed by immunohistochemical analysis with MAbs against thryoglobulin and cytokeratin (data not shown). The growth of the tumour cell lines was slow and long-term culture for more than 3 months could not be established.

Immunophenotyping

The TILs were labelled by direct immunofluorescence with MAbs directly linked to either fluorescein isothiocyanate or phycoerythrin. The following antibodies were used: anti-CD3, anti-CD4, anti-CD8 and anti-CD56; they were purchased from Becton Dickinson (Mountain View, California, U.S.A.). Non-specific irrelevant conjugated mouse antibodies were used as negative controls; 5 × 10^5 cells/tube were incubated with the antibody for 30 min at 4°C, then washed twice with phosphate-buffered saline. The analyses were performed on a FACSCAN 440 (Becton Dickinson).

Western blot analysis for CD3-ζ chain expression

T lymphocytes from day 0 preparations were enriched by a Ficoll-hypaque gradient followed by monocyte and NK cell depletion using microbeads conjugated with anti-CD14 and anti-CD56 MAb (IO bead Immunotech, Marseille France). These T-TIL preparations were then lysed by incubation in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptine) for 15 min at 4°C. The lysate was centrifuged at 12000 rpm for 20 min at 4°C, and the supernatant was then collected. A 2X SDS sample buffer was added to the supernatant which was then boiled for 5 min and electrophoresed or stored at −70°C until use. SDS-PAGE was performed at room temperature for 1 h at 200 V. The samples were applied to 15% polyacrylamide gels in a minislab gel system (Bio-Rad, Richmond, California, U.S.A.). Gels were washed in transfer buffer (0.192 M glycine, 25 mM Tris base pH 8.3, 20% ethanol) and transferred to a nitrocellulose membrane (BA85, 0.45 μm, Schleicher & Schüll, Darsel, Germany) using the transblot cell (Bio-Rad). Nitrocellulose was saturated for 1 h at 37°C in 20 mM Tris pH 7.4, 150 mM NaCl (Western Buffer, WB), 5% BSA and 0.1% Tween 20, and incubated for 18 h at 4°C with anti-TCR ζ antiserum (387; kindly provided by Dr I. Engel, NIH, Bethesda, Maryland, U.S.A.) at a 1/15000 dilution and anti-CD3-ε (A 452; Dako Co., Trappes, France) at a 1/3000 dilution. The membranes were washed with WB, 0.1% Tween 20, and incubated for 1 h with anti-rabbit immunoglobulin coupled with horseradish peroxidase (Amersham, Les Ulis France). After washing with WB and 0.1% Tween 20, the filters were incubated with the Western blotting reagent ECL (Amersham, Les Ulis, France) and chemoluminescence was detected by exposure of the filters to X-Omat AR films (Kodak). Autographic films were scanned using densitometry.

Cytotoxicity assays

Cultured tumour cell targets were labelled with 100 μCi of sodium chromate (159 mCi/mg; Compagnie Oris Industrie, Gif sur Yvette, France) for 1 h at 37°C. Cells were then washed twice in RPMI-1640, counted and aliquoted at 2.5×10^3 targets/well in 96-well U-bottom plates (Costar), into which the effector cells had been previously aliquoted at predetermined concentrations. Controls included labelled target cells incubated in culture medium alone (spontaneous release) and labelled target cells incubated with 1 N HCl (maximum release). Plates were incubated in 5% CO_2 in air at 37°C for 4 h, after which the microtitre plates were centrifuged for 10 min at 2500 rpm. One hundred microlitres of supernatant per well was then harvested and the radioactive contents measured in a gamma counter (LKB, Les Ullis, France). The percentage-specific lysis was determined according to the formula:

$$\frac{\text{experimental mean cpm} - \text{spontaneous release mean cpm}}{\text{maximal mean cpm} - \text{spontaneous release mean cpm}} \times 100.$$

In some experiments, to discriminate between a NK-like and tumour autologous specific cytotoxicity, a 40-fold excess of unlabelled K562 cells (NK-sensitive) were mixed with labelled target cells.

RESULTS

1. *Ability to grow TILs from thyroid tumour biopsy specimens*

In the first study, two culture conditions for the development of TILs were compared, differing only by the presence or absence of allogeneic B. EBV feeder cells. Only one of the seven TIL samples grew *in vitro* without feeder cells, whereas all TILs could be expanded in the presence of allogeneic B. EBV feeder cells (Table 2). These experiments were performed using 200 units/ml IL2, but even with higher doses of IL2 (1000 units/ml), these lymphocytes could not be cultured in the absence of feeder cells. The number of T cells present in tumour samples after enzymatic digestion ranged from 10 to 20%. Therefore, as controls, five tumour biopsy specimens were selected from patients with melanoma and six from those with renal cell carcinomas (RCC) with T cell infiltrates at the same order of magnitude described above for thyroid tumours. TILs grew *in vitro* in the absence of B. EBV allogeneic feeder cells in four of five (80%) and four of six (67%) biopsy samples from patients with melanoma and renal cell carcinoma, respectively. The three TILs that failed to grow in the absence of feeder cells were not cultured under other conditions.

Table 2. In vitro *culture of tumour-infiltrating lymphocytes (TILs) derived from thyroid tumour (Thyr), melanoma (Mel) and renal cell carcinoma (RCC)*

	Initial percentage of T lymphocytes in tumour*	Feeder cells† in culture of TILs		Days in culture after *in vitro* expansion
		Absent	Present	
Thyr1	20	–	+	42
Thyr2	19	–	+	>23
Thyr3	ND	–	+	67
Thyr4	10	–	+	63
Thyr8	11.5	–	+	42
Thyr9	15	–	+	>42
Thyr15	ND	+	+	49
Mel1	13	+	ND	38
Mel2	12	–	ND	–
Mel3	18	+	ND	58
Mel4	12	+	ND	>42
Mel5	14	+	ND	51
RCC1	11	–	ND	–
RCC2	10	+	ND	48
RCC3	16	+	ND	>27
RCC4	13	+	ND	64
RCC5	14	–	ND	–
RCC6	10	+	ND	56

TILs were cultured with human recombinant interleukin (IL)2, as described in Materials and Methods.
*Analysis of T cell infiltration in tumours was performed by cytofluorometric analysis using anti-CD3 (ϵ) MAb; † irradiated allogeneic EBV-transformed B cells were used as feeder cells; ND, not done.

2. *Phenotypic analysis of TILs*

(*a*) *Cell surface markers expressed on TILs after* in vitro *expansion.* The phenotypic profile of the thyroid-derived TILs after short-term (< day 23) (Figure 1a) and long-term (> day 40) culture (Figure 1b) were analysed. A mixed phenotype composed of both CD4+ and CD8+ T lymphocytes was observed in which the CD8+CD3+ population usually constituted the predominant population. In only one case (Thyr 5) was a selective expansion of CD3+CD4+ lymphocytes demonstrated (Figure 1a). During the course of culturing, an increase in CD8+ T cells was seen that was associated with a decrease in NK cells (CD56+CD3–) (Figure 1b). TILs derived from benign (adenoma) or malignant (carcinoma) thyroid tumours did not appear to give rise to different phenotypic profiles.

Surprisingly, surface antigen analysis revealed that a considerable fraction of TILs displayed an unusual surface phenotype. Indeed, short-term (11/14) and long-term cultures (7/11) both showed that the CD56+ T cell populations (CD3+CD56+) represented more than 15% of the total number of lymphocytes. In addition, the percentage of CD3+ cells appeared to outnumber those of CD4+ plus CD8+. In fact, we demonstrated that in five of 14 TILs in short-term culture and four of 11 TILs in long-term culture, a T lymphocyte population that coexpressed CD4 and CD8 antigen accounted for more than 15% of the total TIL population (Figure 1).

As controls, the CD3+CD56+ and CD4+CD8+CD3+ populations were shown to represent less than 6% of the total peripheral blood lymphocytes from five control patients and five patients with cancer (data not shown). Among TILs from five non-thyroid tumours (three melanomas and two RCC) grown in the absence of feeder cells, the CD3+CD56+ population was also shown to be expanded in two of five cases (Table 3). In contrast, an increase in the double positive (DP) population (CD3+CD4+CD8+) could not be demonstrated in any of the five TILs in the absence of feeder cells (Table 3).

Whether the presence of feeder cells during culture could explain this unusual phenotype was then examined. The phenotypic profile of each of the five TILs derived from non-thyroid tumours and cultured with or without feeder cells were compared. In four of five cases, an increase in CD56 expression and CD4-CD8 coexpression was observed when

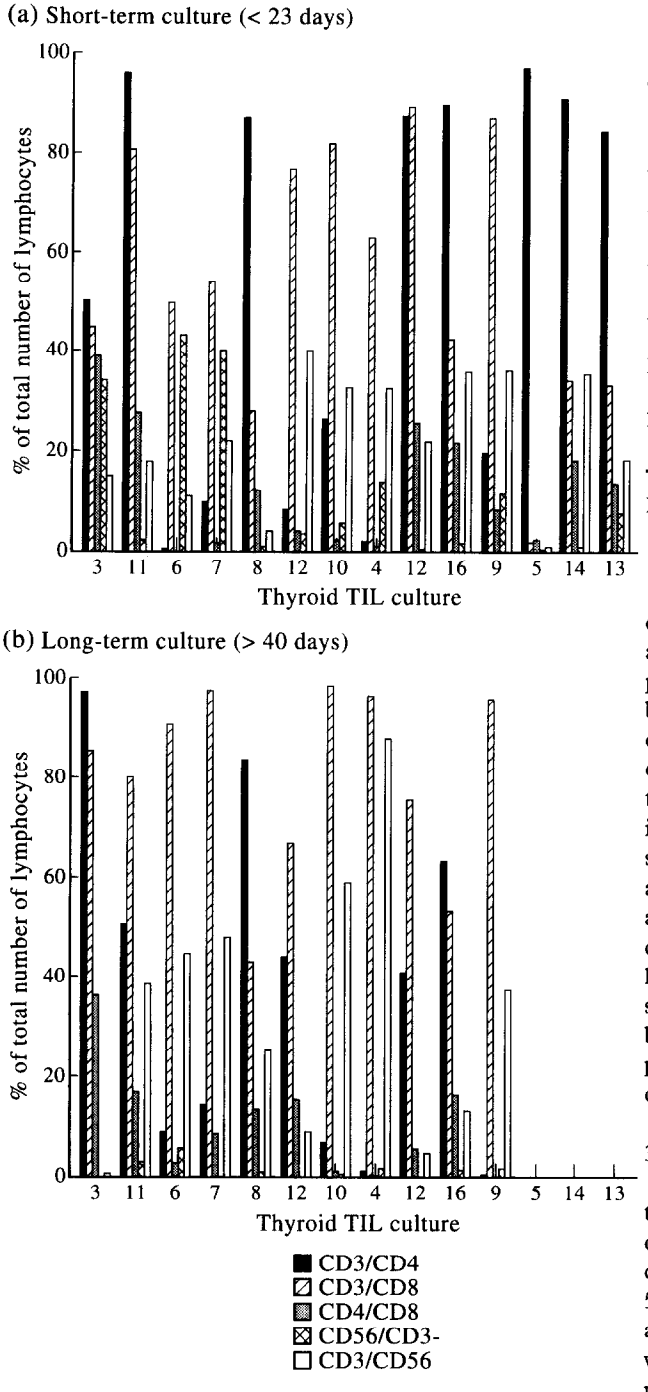

Figure 1. Phenotypic profile of tumour-infiltrating lymphocytes (TILs) derived from thyroid tumours. Surface phenotype of the thyroid-derived TILs after short-term (< 23 days) (a) and long-term (> 40 days) culture (b).

feeder cells were added to the culture. However, the DP population reached a percentage of more than 15% of the total T cell population in only one case (mel B).

(*b*) *Expression of the CD3-ζ chain by TILs prior to culture.* It has recently been reported that TILs from RCC and colorectal carcinoma show a marked decrease in CD3-ζ chain expression [14–16]. This defect was associated with an impaired cytotoxic function in these T cells. Western blot analysis of CD3-ζ was therefore performed in nine lysates of purified T-TILs

Table 3. Influence of feeder cells in culture on the phenotypic profile of tumour-infiltrating lymphocytes (TILs) derived from non-thyroid tumours

	Feeder cells during TIL culture	CD3+CD56+ T cells (%)	CD4+CD8+ T cells (%)
Mel A	–	3.2	11.2
	+	30.9	11.8
Mel B	–	2.6	2.8
	+	4.2	15.8
Mel C	–	21.6	0.4
	+	34.2	5.5
RCC A	–	31.5	1.7
	+	43.1	4.8
RCC B	–	7.1	6
	+	1.4	0.1

RCC, renal cell carcinoma; mel, melanoma.

derived from thyroid tumours. These included five follicular adenomas and four papillary carcinomas. Results were compared to a pool of T-PBL from healthy individuals. As it has been demonstrated that the expression of CD3-ε is relatively constant in the TIL population [14], the analysis of the expression of this chain was included as a control. In this way, the ζ/ε ratio enabled us to compare the level of ζ chain, irrespective of the number of T cells present in different samples. No significant variation in the ζ/ε ratio was observed, as it varied from 0.8 to 1.4 when an arbitrary value of 1 was assigned to normal T-PBL (Figure 2). Daudi lymphoma B cells were included as a negative control and the Jurkatt T cell line was included as a positive control (Figure 2 and data not shown). Before mixing the two antibodies in the same Western blot, it was verified that each antibody alone revealed only one protein of the expected size, i.e. 16 kD for ζ and 22 kD for ε on a reducing 15% gel (data not shown).

3. *Cytotoxicity of TILs grown in culture*

TILs grown as bulk culture and expanded with rIL2 were tested for cytotoxicity against various targets (K562, autologous and allogeneic tumour cells). Ten of 11 TILs exhibited cytotoxicity (> 20% of lysis at an effector/target ratio of 50/1) towards K562 (NK-sensitive), whereas eight of 11 lysed allogeneic tumour cells (Figure 3). All of these experiments were performed on short-term TIL cultures, but the same profile was demonstrated in long-term culture. As six of 11 TILs were also cytotoxic towards autologous tumour, cold target inhibition with K562 was performed in three cases (Thyr 9, 1, 4) where sufficient material was available. In all of the cases tested, unlabelled K562 completely inhibited lysis of autologous tumour cells (data not shown).

CD3+CD56+ T cells and CD56+CD3– NK cells have been reported to mediate non-MHC-restricted cytotoxicity. An attempt was therefore made to relate the TIL phenotypic profile with cytotoxic activity. Indeed, the only TIL (No. 8) in which the CD56+ T cell population and CD56+CD3– NK cells represented less than 15% of the total number of lymphocytes was the one that did not exhibit NK cytotoxicity (Figure 3). No correlation with specific or non-specific cytotoxicity could be demonstrated for the TILs with increased CD4+CD8+ T cells (data not shown).

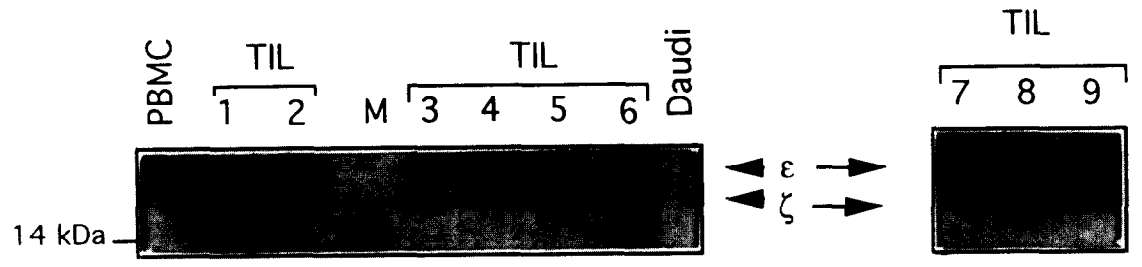

Figure 2. Western blot analysis of CD3-ζ chain expression in nine tumour-infiltrating lymphocytes (TILs) derived from thyroid tumours. The Daudi lymphoma B cell line and a pool of normal PBMC (peripheral blood mononuclear cells) were included as negative and positive controls, respectively. Note that TILs 1–9 do not correspond with cell cultures Thyr 1–9, but are representative blots from five follicular adenomas and four papillary carcinomas.

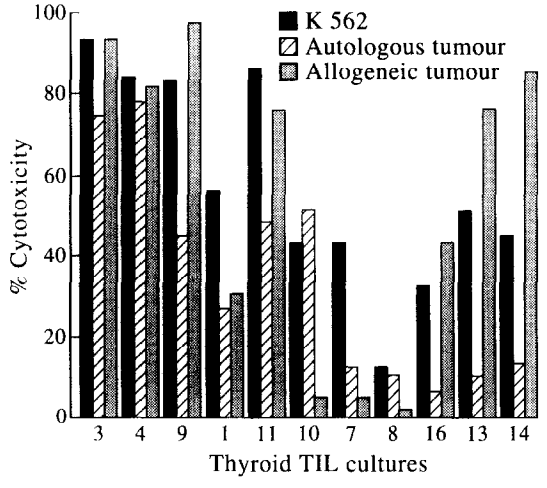

Figure 3. Cytotoxicity of tumour-infiltrating lymphocytes (TILs) derived from thyroid tumours. Cytotoxicity of effector cells was assayed in a 4 h ^{51}Cr release assay at an effector to target ratio of 50:1. These experiments were performed on short-term TIL cultures.

DISCUSSION

We have previously shown that TILs derived from melanoma and RCC could be developed in 70% of specimens [13, 17]. In this study using the same culture protocols, only one of seven (14%) thyroid-derived TILs was expanded *in vitro*. This low outgrowth yield was also observed in the development of TILs derived from breast cancers [17]. The number of lymphocytes infiltrating tumour cells could not account for the variability in TIL expansion between different tumours, as a high frequency of TIL expansion was seen with melanoma and RCC matched with thyroid tumours for the number of initial T cell infiltrates (Table 2). However, when B. EBV allogeneic feeder cells were added to the culture, 100% of TILs derived from thyroid tumours could be grown *in vitro* (Table 2). To our knowledge, this is the first characterisation of TILs derived from thyroid tumours, as Bagnasco and colleagues only studied T cell clones from papillary carcinomas [18].

In an extensive analysis of 142 TILs derived from various tumours, including breast cancer, but not thyroid tumour, Maleckar and colleagues found that TILs could be generated *in vitro* in more than 70% of cases when stimulated by autologous tumour antigen [19]. Unfortunately, the growth rate of the thyroid cell line was too slow to allow their use as feeder cells.

From these different observations, it seems that some tumours, such as thyroid or breast cancer, are dependent on feeder cell stimulation for their *in vitro* expansion. It may be that lymphocytes derived from these tumours are not primed *in vivo* and require a cosignal that is provided by feeder cells to proliferate. This was supported by phenotypic analysis of fresh T lymphocytes isolated from thyroid tumour infiltrates that did not express activation markers (CD25, class II antigen) of T cells [18]. Furthermore, an aberrant expression of class II antigen was demonstrated in more than 50% of thyroid tumours [20]. This may lead T cells to a state of anergy in the absence of a costimulatory signal delivered by the tumour cells [21]. Secretion of TGFβ1 by thyroid tumour cells may also be involved in a local immunosuppression at the tumour site [3]. Lastly, as it was recently reported that T cells from patients with colorectal and renal cancer often exhibited a defect in ζ-CD3 chain expression that may be associated with lymphocyte dysfunction [14–16], the expression of the ζ-CD3 chain was investigated in fresh lymphocytes derived from these thyroid tumours. We did not find a significant variation in ζ-CD3 chain level in T lymphocytes derived from thyroid tumours (Figure 2). This discrepancy may be related to the difference between the stage of tumour analysed, as we studied benign or primary malignant thyroid tumours, whereas only malignant tumours, both primary and metastatic, were tested by other groups.

TILs derived from thyroid tumours displayed an intense cytolytic activity against NK-sensitive tumour targets. It is noteworthy that this activity was observed in both short-term and long-term cultures (Figure 3 and data not shown) which differed from previous observations claiming that early activity against NK- and LAK-sensitive targets often disappeared in the later stages of culture [19, 22]. In papillary cancer tumour infiltrates, 95% of T cell clones also exhibited NK activity [18]. This high level of NK-like cytotoxicity may be explained by the use of B. EBV cells as feeder cells, which have been shown to elicit a strong NK-like cytotoxicity [23].

As some bulk TIL cultures exhibited lytic activity against both NK-like targets and autologous tumours, we tried to segregate antitumour CTL from NK-like effectors by cold target inhibition with K562. In all three experiments, lysis of autologous tumours by TILs was completely inhibited by an excess of unlabelled K562, which suggests that NK cell clones were mainly responsible for autologous cytotoxicity. The lack of antiautologous tumour CTL may be explained by the absence of autologous tumour cells used for stimulation. It seems that the frequency of T cell clones, derived from peripheral blood lymphocytes and able to lyse fresh thyroid tumour

cells, is very low [5]. We tried to relate this profile of cytotoxicity with the TIL phenotype, as expansion of CD56+ T cells in short-term and long-term TIL cultures was demonstrated (Figure 1). This expansion did not seem to be peculiar to thyroid tumours, as we found that 40% of TILs derived from melanoma also exhibited an increase in this population, and CD56+ T lymphocytes varied from 10 to 34% in TILs derived from cervical carcinoma [24]. This CD56+ T cell population may be involved in non-MHC-restricted cytotoxicity, as we found that the only TIL that did not exhibit NK activity was also the only one in which the CD56+ T cell population and CD56+CD3- NK cells represented less than 15% of the total number of lymphocytes (Figures 1 and 3). Among the 10 TILs that exerted NK activity, NK cells exceeded 10% of total cells in only two cases. Although we could not rule out that a small number of NK cells was sufficient to generate high level non-MHC-restricted cytotoxicity, it seems more likely that other T cell populations, such as the increased CD56+CD3+ T cells, were responsible for this type of cytotoxicity. This is in accordance with a previous report showing that expression of CD56 antigen on T lymphocytes is usually correlated with non-MHC-restricted cytotoxicity [25]. Similarly, Barnaba and colleagues reported that when CD56+ T cells were purified from the same CD4+ T cell clones that recognised hepatitis virus, the high-density CD56 clone had a stronger cytotoxic activity and produced higher levels of IFNγ than cells with low-density CD56 [26]. In contrast, Lanier and colleagues reported that CD56 antigen on cultured T cell lines was not directly correlated with MHC- or non-MHC-restricted cytotoxic function [27]. A direct role of CD56 antigen in cytotoxicity function could be excluded by experiments showing that anti-CD56 MAb did not inhibit cytotoxic activity of NK or CD56+CD3+ T cells [28].

Phenotypic analysis of our TIL population also revealed a frequent *in vitro* expansion of an unusual T cell population that expressed both the CD4 and CD8 markers. This population appeared early in the TIL culture but, in the absence of detailed phenotypic analysis of a fresh TIL population, it was not clear whether the development of these DP T cells (CD3+CD4+CD8+) had already occurred *in vivo* or whether they were selected by the culture conditions. It is noteworthy that an increase in these DP T cells was also observed among intrathyroid lymphocytes in patients with autoimmune thyroid disease [29]. The possible mechanisms underlying this phenomenon remain obscure. It has been shown that IL4 mediates CD8 induction in the human CD4+ T cell clone [30]. Therefore, high levels of IL4 secreted by thyroid TIL or produced by B. EBV feeder cells *in vitro* [31] could generate this rare population. This was reinforced by the experiments demonstrating an increase in these DP T cells when TILs derived from non-thyroid tumours were cultured in the presence of B. EBV allogeneic cells (Table 3). However, the rise of DP T cells was less marked than that observed for TILs derived from thyroid tumours. The function of these DP T cell clones has yet to be established. During T cell development in the thymus, DP T cells to predominantly seen as immature cortical thymocytes that give rise to mature CD4+CD8- or CD4-CD8+ single positive T cells [32]. Increased numbers of DP T cells have also been observed in the joint fluid of patients suffering from juvenile rheumatoid arthritis [33] and in peripheral blood lymphocytes of patients with myasthenia gravis [34]. In patients with leprosy, T cell clones often coexpress the CD4 and CD8 antigen and exert a suppressive activity on reactive helper T cells [35].

In conclusion, the present study demonstrates that TILs derived from thyroid tumours can only be efficiently expanded *in vitro* in the presence of feeder cells, such as allogeneic B. EBV cells, unlike TILs derived from other tumour models. The absence of specific tumour cytotoxicity associated with thyroid TILs does not argue in favour of their use in immunotherapy protocols, as the clinical response after TIL administration was correlated with the capacity of these TILs to lyse specifically autologous tumour targets *in vitro* [12]. However, one group recently reported the generation of memory T cells recognising peptides derived from mutated p21 *RAS* in a patient with follicular thyroid carcinoma [36]. To enhance tumour autologous specificity of TILs without the need of autologous thyroid tumour cell line expansion, direct *in vitro* immunisation of TILs by potential thyroid tumour antigen, such as mutated *RAS* peptide, may therefore represent a promising alternative strategy.

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