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ORIGINAL ARTICLE

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High-scale expansion of melanoma-reactive TIL by a polyclonal stimulus: predictability and relation with disease advancement

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Abstract The rationale of treating melanoma patients by infusion with tumor-infiltrating leukocytes (TIL) is to perform an adoptive therapy through injection of tumor-specific T cells. Nonetheless, methods currently used for ex vivo TIL expansion have not been evaluated for their efficacy to expand TAA-specific T cells. We have addressed this question here, using a culture method in which high TIL growth was induced by a polyclonal T cell stimulus. Intracellular cytokine assays were performed to measure the proportion of T cells responding to autologous tumor cells among the lymphocytes from lymph node biopsies (TIL) of 26 patients with stage III melanoma. The data show that TIL from 18 of these patients contained detectable amounts of tumor-specific T cells before expansion. Although they decreased somewhat in percent abundance during expansion, they were still present afterwards, ranging from 0.3 to 13.8%. Since a median number of 1.7×10^{10} TIL was obtained from these patients (starting from 3.6×10^6 TIL), a total amount of tumor-reactive cytokine-secreting TIL of between 2.8×10^6 and 1.12×10^9 was obtained in each case from 18 patients. The TIL populations from 8 patients did not contain tumor-reactive T cells: neither before expansion, nor after expansion. Lack of tumor-reactive TIL only occurs for patients bearing several tumor-invaded lymph

nodes (40%), but not for those having a single invaded lymph node. Therefore, high numbers of tumor-reactive T cells can be produced, through a polyclonal TIL stimulation, from most early stage III melanoma patients but from only about half of the patients with a more disseminated disease. For this last group, the possibility of getting tumor-reactive TIL can be predicted by checking the presence of these cells before expansion.

Key words Immunotherapy · Lymphocytes · Cytokines

Introduction

The adoptive transfer of TAA-specific CD8⁺ T cells was shown to induce tumor rejection in different animal tumor models [14, 15]. Although the mechanisms of this effect are far from being elucidated, data indicate that both lysis and cytokine secretion functions are critical for the anti-tumor effect of adoptively transferred T cells [1, 16]. Because human melanoma tumors frequently contain cytotoxic and cytokine-producing TAA-specific CD8⁺ T cells [8, 13, 17, 25], an approach based on high-scale expansion and infusion of tumor-infiltrating lymphocytes (TIL) has been proposed to treat melanoma patients [20, 25]. Interestingly, it was retrospectively shown that objective tumor regressions observed were frequently associated with TAA-specific or TAA-epitope-specific responses of the infused TIL [11, 21, 22].

Several culture methods have been designed to grow large numbers of human TIL for use in immunotherapy [2, 9, 24]. However, although the presence of tumor-reactive T cells among expanded TIL was reported in some cases, the efficacy of these methods to grow TAA-specific T cells or tumor-reactive T cells was not analyzed systematically, essentially because appropriate methods to measure these cells were not available. Such methods have now been developed. The most recent consists of using soluble HLA-peptide tetramers to label T cells specifically for a given epitope [18, 19]. However,

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this method is neither suitable to quantify T cells specific for unknown tumor epitopes, nor to screen polyclonal populations of highly diverse specificity. In contrast, both are possible through the detection of cytokine responses at the single cell level, using either the ELISPOT assay [7] or the intracellular cytokine labeling technique [10]. Here we used the latter technique to make a relative quantification of T cells reactive to autologous melanoma cells among melanoma-invaded lymph node lymphocytes (TIL) of stage III melanoma patients. This quantification was done among shortly-cultured TIL and among the same TIL after high-scale expansions for therapy, which were undertaken according to a culture method that we have described previously [9]. This method allows high, rapid and reproducible expansion of TIL populations, and most of these populations still exhibit specific lysis of autologous tumor cells after a 3-week expansion [9, 23]. The present study shows that tumor-reactive, cytokine-producing T cells can also be efficiently expanded by the same culture method.

Material and methods

Cell lines

Autologous melanoma cell lines were obtained by culturing small fragments of tumor-invaded lymph node biopsies, as described [5]. This was successful for 26 out of 44 patients. The LAZ 388 cell line, an Epstein Barr virus-transformed B-cell line, was a gift from Thierry Hercend. All cell lines were cultured in RPMI 1640 (Life Technologies, Cergy-Pontoise, France) containing 10% FCS (Eurobio, Les Ulis, France), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and 1 nM glutamine (Life Technologies).

TIL culture

TIL lines were produced in 'Good Manufacturing Practice' conditions in the Unit of Cellular and Genetic Therapy (CHRU, Nantes, France) according to a procedure described previously [9, 23]. Briefly, TIL were isolated by culturing cryopreserved fragments of stage III tumor-invaded LN in two 12-well tissue culture plates with X-Vivo 15 serum-free medium (Bio*Whittaker, Walkersville, Md., USA) containing 150 U/ml rIL2 (Eurocetus, Rueil-Malmaison, France) and 1nM glutamine (Bio*Whittaker) for 10–14 days. To perform high-fold expansion, 1.8×10^6 of these short-term-cultured TIL were plated at 300 viable lymphocytes/well with irradiated feeder cells into U-bottom microplates in 200 µl of rIL-2 medium. PHA-P (Difco, Detroit, Mi., USA) was added on day 0 (15 µg/ml). After 48 h, most PHA was removed by replacing the culture medium. Ten days later, lymphocytes were recovered from the culture plates, adjusted to 1×10^6 cells/ml in r-IL2 medium and transferred into culture trays for an additional 10 days. The final TIL harvest was performed by centrifuging, washing and suspending the TIL in 4% human serum albumin (LFB, Les Ulis, France). A second TIL expansion was performed within 1 month of the first one, starting from 1.8×10^6 cryopreserved short-term-cultured TIL. Aliquots of TIL suspensions injected to the patients were cryopreserved for the present study, which could be done retrospectively once the autologous tumor cell line had been established in culture.

Cytokine production

Samples of 1×10^5 TIL were stimulated by 3×10^5 stimulator cells (melanoma cells) in 200 µl of RPMI 1640 containing 10% FCS

and 10 µg/ml brefeldin A (Sigma, St. Louis, Mo., USA) in round-bottom 96-well plates. The cultures were incubated for 6 h at 37 °C in a 5% CO₂ humidified atmosphere. For intracytoplasmic cytokine staining, cells were then fixed 10 min at room temperature in a solution of PBS containing 4% paraformaldehyde (Sigma), washed and stored at 4 °C until labeling.

T-cell responses were considered significant when the mean fluorescence labeling of TIL stimulated by the autologous tumor cell line exceeded, by at least half a log, the mean fluorescence of the background responses of non-stimulated TIL and/or of TIL stimulated by an HLA-mismatched melanoma line. The value of 0.3% was considered as the significant threshold.

Flow cytometry analysis of intracellular cytokines

Stimulated TIL were fixed and stained for cytokines using the method described by Jung et al. [10]. Briefly, fixed cells were stained for 30 min at room temperature with the different mAbs at the concentration of 5 µg/ml, which was shown to give optimal staining. Anti-human cytokine mAbs (IFN-γ, TNF-α, IL-2 and GM-CSF) were purchased from Pharmingen. After two washes, cells were incubated with Fab'2 fragments of goat anti-mouse IgG (Bio-Atlantic, Nantes, France). Reagent dilutions and washes were performed with PBS containing 0.1% BSA and 0.1% saponin (Sigma). After staining, cells were resuspended in PBS and 5×10^3 events were analyzed with a FACScan flow cytometer, using Cell Quest software (Becton Dickinson, Grenoble, France).

Results

Quantification of autologous tumor-reactive T cells among short-term-cultured TIL

To evaluate the efficacy of a TIL expansion method to also expand TAA-specific T cells, we started measuring the fraction of TAA-specific TIL before expansion. Twenty-four populations of short-term-cultured TIL could be analyzed and as shown in Table 1 and Fig. 1A, 16 of these were found to contain a significant proportion of T cells secreting IFN-γ in response to autologous tumor cells. This fraction ranged from 0.3 to 17.9% (mean = 6%). Table 1 also shows that most IFN-γ-responding TIL populations also contained a similar percentage of TNF-α-responding tumor-specific T cells, ranging from 1.1 to 15.8%. GM-CSF-responding T cells were also present among the same TIL populations, although in lower proportions than IFN-γ-, and in most cases also than TNF-α-responding T cells. Sixteen TIL populations were analyzed for their capacity to secrete IL-2 in response to autologous tumor cells. Six of these populations contained detectable fractions of IL-2-secreting TIL, ranging from 0.3% to 4.8%.

Quantification of autologous tumor-reactive T cells among highly expanded TIL

For each patient, two independent high-fold expansions of short-term-cultured TIL (R1 and R2) were performed at a 1-month interval, using PHA and feeder cell stimulation [9]. As shown in Table 2 and Fig. 1B, C, 18 TIL populations, among the 26 analyzed, con-

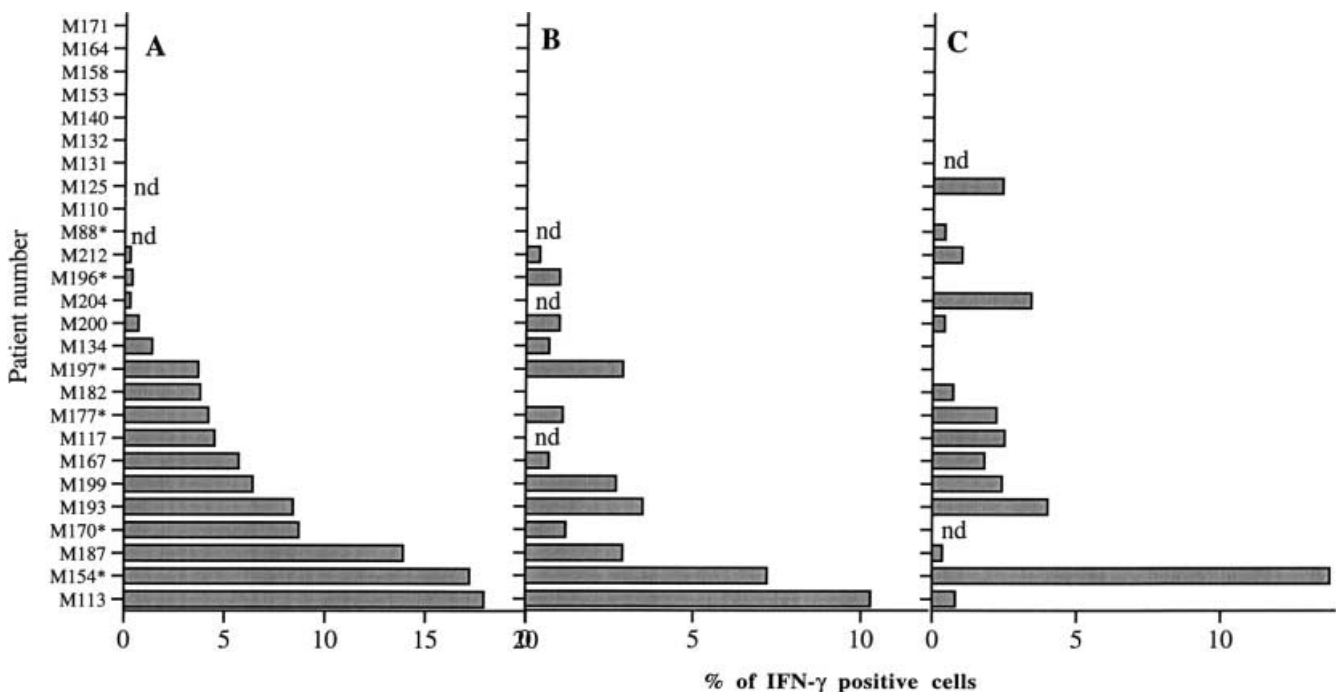
Table 1 Cytokine responses of short-term-cultured TIL to autologous melanoma cells. TIL were stimulated for 6 h by autologous melanoma cells in the presence of brefeldin A, then fixed, permeabilized, stained for cytokine production and analyzed by FACScan. (ND, not done)

Patient	% IFN- γ	% TNF- α	% IL-2	% GM-CSF
M88 ^a	ND	ND	ND	ND
M154 ^a	17.2	15.8	2.9	7.7
M170 ^a	8.7	2.5	ND	4.7
M177 ^a	4.2	1.6	0.5	2
M196 ^a	0.4	ND	2.6	ND
M197 ^a	3.7	ND	ND	ND
M110 ^b	—	—	ND	—
M113 ^b	17.9	8.1	ND	14.4
M117 ^b	4.5	—	ND	—
M125 ^b	ND	ND	ND	ND
M131 ^b	—	—	ND	ND
M132 ^b	—	—	—	—
M134 ^b	1.4	1.1	0.3	—
M140 ^b	—	—	ND	—
M153 ^b	—	—	—	—
M158 ^b	—	—	—	—
M164 ^b	—	—	ND	—
M167 ^b	5.7	3.5	—	1.4
M171 ^b	—	—	—	—
M182 ^b	3.8	1.8	—	2.4
M187 ^b	13.9	6.7	4.8	7
M193 ^b	8.4	5.5	—	1.4
M199 ^b	6.4	5.2	2.1	4.8
M200 ^b	0.7	2.8	—	2.3
M204 ^b	0.5	—	—	—
M212 ^b	0.3	—	—	—

^a Melanoma patients bearing only one invaded lymph node

^b Melanoma patients bearing more than one invaded lymph nodes

Fig. 1A–C Percentage of IFN- γ -positive cells detected in TIL populations by intracellular labeling in response to autologous melanoma cells. **A** Before expansion; **B** after the first expansion; **C** after the second expansion. (*Patients bearing only one invaded lymph node)



tained a detectable fraction of T cells, secreting IFN- γ and TNF- α in response to autologous tumor cells, after the first (R1), second (R2), or both ex-vivo expansions. Figure 2 shows some examples of IFN- γ intracellular labeling. Proportions of IFN- γ -secreting T cells ranged from 0.35 to 13.8%. The median level of these TAA-specific T cells among positive TIL was 2.7% ($n = 26$ for R1 + R2). Respectively, 12 out of 24 and 7 out of 19 expanded TIL populations contained T cells capable of secreting GM-CSF and IL-2, in response to autologous tumor cells.

Estimation of the amount of tumor-specific TIL obtained from each patient

The amounts of TIL obtained from each patient by the first (R1) and second (R2) TIL expansions are shown in Table 3. The average combined number of TIL obtained from each of these two expansion cultures was 1.7×10^{10} . Table 3 also shows the amounts of R1 and R2 TIL that exhibited a specific IFN- γ response to tumor cells. From these results we were able to calculate the total amount of tumor-reactive TIL obtained per patient. As shown in Table 3, high-fold expansions of TIL from eight patients failed to induce any detectable amount of tumor-reactive T cells. However, the data in Table 1 show that TIL from these patients did not contain detectable fractions of tumor-reactive TIL before expansion. For the other 18 patients, variable fractions of tumor-reactive T cells had been detected among TIL before expansion (see Table 1) and the two high-fold expansion cultures, R1 and R2, yielded a total amount of tumor-reactive TIL ranging from 2.8×10^6 to 1.12×10^9 .

Table 2 Percentages of highly expanded TIL reactive against autologous melanoma cells. TIL were stimulated for 6 h by autologous melanoma cells in the presence of brefeldin A, then fixed, permeabilized, stained for cytokine production and analyzed by FACScan. (ND, not done)

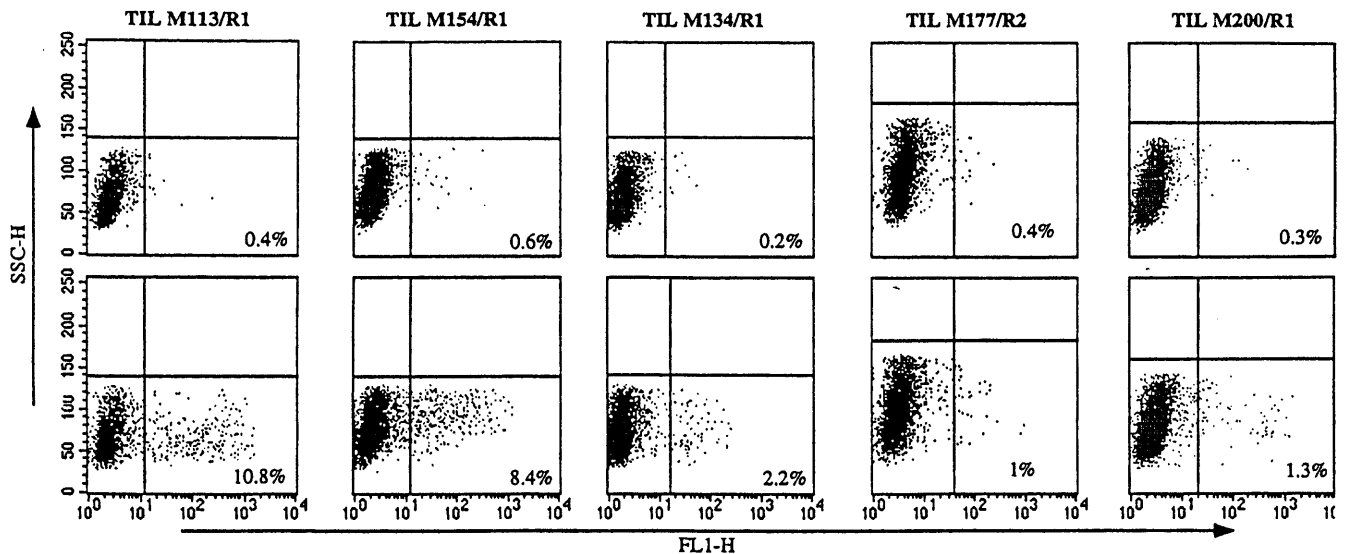
Patient	TIL R1 ^c				TIL R2 ^c			
	IFN- γ	TNF- α	IL-2	GM-CSF	IFN- γ	TNF- α	IL-2	GM-CSF
M88 ^a	ND	ND	ND	ND	0.4	0.7	ND	–
M154 ^a	7.2	7.1	2	2.9	13.8	ND	1.9	ND
M170 ^a	1.2	ND	ND	0.5	ND	ND	ND	ND
M177 ^a	1.1	ND	ND	ND	2.2	3.2	0.5	1.6
M196 ^a	1	3.1	0.8	ND	–	0.5	–	ND
M197 ^a	2.9	3.3	–	–	–	ND	ND	ND
M110 ^b	–	–	ND	–	–	–	ND	–
M113 ^b	10.3	5.8	1.8	4.4	0.8	ND	–	ND
M117 ^b	ND	ND	ND	ND	2.5	1.1	–	–
M125 ^b	–	–	–	–	2.4	2.4	–	1.6
M131 ^b	–	–	ND	–	ND	ND	ND	ND
M132 ^b	–	ND	ND	ND	–	ND	ND	ND
M134 ^b	0.7	2.3	–	0.4	–	1.7	–	0.7
M140 ^b	–	–	ND	–	–	ND	ND	ND
M153 ^b	–	–	–	–	–	–	–	–
M158 ^b	–	0.6	–	–	–	0.3	–	–
M164 ^b	–	–	ND	–	–	–	ND	–
M167 ^b	0.7	1	–	0.5	1.8	1.4	–	–
M171 ^b	–	–	–	–	–	–	–	–
M182 ^b	–	–	–	–	0.7	1.3	–	0.7
M187 ^b	2.9	8.7	0.3	–	0.35	1.1	–	ND
M193 ^b	3.5	0.8	–	ND	4	2.2	–	0.6
M199 ^b	2.7	0.6	0.8	0.9	2.4	1.6	–	1.3
M200 ^b	1	1.4	0.8	1.5	0.4	0.9	–	0.7
M204 ^b	–	–	–	–	3.4	1.8	–	1.6
M212 ^b	0.4	0.4	–	–	1	–	–	–

^a Melanoma patients bearing only one invaded lymph node

^b Melanoma patients bearing more than one invaded lymph node

^c R1 and R2 are the TIL populations obtained from the first and the second high- scale expansion, respectively

Fig. 2 Labeling of IFN- γ -secreting T cells among polyclonal TIL in response to autologous tumor cells. TIL were stimulated for 6 h by autologous tumor cells, at a ratio of 1: 3 respectively (*lower panel*), or by HLA-mismatched allogeneic tumor cells as a negative control (*upper panel*), in the presence of brefeldin A (10 μ g/ml). After fixation, TIL were stained with anti-IFN- γ mAb (5 μ g/ml) in the presence of saponin and analyzed by FACScan (5 $\times 10^5$ events)



Melanoma-specific responses are mainly elicited by CD8⁺ lymphocytes

We performed double staining of CD8 and CD4 IFN- γ on seven TIL populations in response to class II-positive autologous melanoma cell lines. As shown in Table 4, CD8-specific responses were found in higher

Table 3 Amounts of TIL and tumor-reactive TIL obtained through high-scale expansions

Patient	Total TIL ($\times 10^{-9}$)		Tumor-reactive TIL ($\times 10^{-6}$)		Total
	R1	R2	R1	R2	
M113 ^b	10	11.2	1030	89.6	1120
M154 ^a	5.5	3	396	414	810
M117 ^b	1.4	32	ND	800	800 ^c
M193 ^b	12	5.2	420	208	628
M204 ^b	9.5	16.5	0	561	561
M197 ^a	14	1	406	0	406
M167 ^b	10.9	14.2	76.4	255.6	332
M125 ^b	12	13.2	0	317	317
M187 ^b	8	10	232	35	267
M199 ^b	5	3.3	135	79	214
M177 ^a	2.5	6.4	27.5	140.5	168
M200 ^b	12.3	5.8	123	23	146
M212 ^b	7.8	11	31	110	141
M170 ^a	9	0.5	108	ND	108 ^c
M134 ^b	13.5	9	94	0	94
M196 ^a	8.6	14	86	0	86
M182 ^b	0.6	3.1	0	22	22
M88 ^a	6	0.7	ND	3	3 ^c
M110 ^b	1.1	1	0	0	0
M131 ^b	8.5	7	0	0	0
M132 ^b	8	12.2	0	0	0
M140 ^b	2.7	1.1	0	0	0
M153 ^b	7.4	9.3	0	0	0
M158 ^b	8	10.7	0	0	0
M164 ^b	10	17	0	0	0
M171 ^b	10	12	0	0	0

^a Melanoma patients bearing only one invaded lymph node^b Melanoma patients bearing more than one invaded lymph nodes^c Amount of specific TIL infused through only one TIL injection**Table 4** The proportions of CD4- and CD8-specific lymphocytes in TIL populations in response to autologous melanoma cell lines expressing HLA class II antigen. Shortly-cultured TIL were stimulated for 6 h by autologous melanoma cells in presence of brefeldin A, after which the cells were labeled with CD4-PE or CD8-PE antibodies and then fixed, permeabilized and stained for cytokine production

Patient	Proportion of CD4-specific T cells among tumor-specific lymphocytes	Proportion of CD8-specific T cells among tumor-specific lymphocytes
M125	4% (R2)	96% (R2)
M167	28% (R1)	72% (R1)
M170	0% (R1)	100% (R1)
M187	18% (R1)	82% (R1)
	16% (R2)	84% (R2)
M193	0% (R1)	100% (R1)
M197	0% (R1)	100% (R1)
	0% (R2)	100% (R2)
M204	40% (R2)	60% (R2)

proportions than CD4 responses for each tested population.

Discussion

We have undertaken, for the first time, a systematic quantification among melanoma patients of tumor-

reactive T cells that can be expanded for cellular therapy. This was possible retrospectively for 26 TIL-treated patients, after the autologous melanoma cell line had been derived from these patients. We used intracellular cytokine assays to measure TIL fractions that specifically responded to the autologous melanoma cells.

Eighteen of the TIL populations analyzed contained tumor-reactive T cells in proportions ranging from 0.3 to 17.9% before expansion. We showed that using a polyclonal TIL expansion method, based on the use of PHA and feeder cell stimulation, a fraction of these tumor-reactive T cells was systematically expanded. For the TIL populations that contained over 4% tumor-reactive T cells before expansion, increases in the numbers of these cells were observed in the two high-scale expansions performed. However, for some of the TIL populations, in which the fraction of tumor-reactive T cells was below 4% before expansion, a detectable growth of these cells was successful in only one out of the two TIL cultures. During expansion the initial fraction of tumor-reactive TIL usually decreased, but the final fraction was correlated with the fraction present before expansion. Therefore, checking TIL specificity before high-scale expansion allowed prediction of whether the recovery of tumor-reactive T cells would be feasible for an immunotherapy.

In the present study, the TIL of 8 out of 26 stage III melanoma patients apparently lacked fractions of tumor-reactive T cells detectable by the cytokine assays used. These populations derived from patients bearing more than one invaded lymph node. This suggests that either the presence or the reactivity of TAA-specific T cells inside melanoma-invaded lymph nodes could be influenced by the advancement of the disease. Indeed, we could obtain melanoma-specific TIL from the six patients bearing only one invaded lymph node and from only 12 out of 20 patients of the other group. However, this difference is not yet statistically significant due to the low representation by patients of the first group. If it were confirmed on a larger group of patients, this observation would directly support the existence of T cell inactivation mechanisms inside tumor-invaded lymph nodes.

Another new result from the present study was to allow a first estimation of the total amount of tumor-reactive T cells that can be obtained to treat melanoma patients via a given TIL expansion method. TIL production was performed here twice, at a 1-month interval, by two independent TIL cultures. Each expansion culture was initiated from 1.8×10^6 TIL, either fresh or thawed, which is a minimal TIL amount that can be easily obtained from melanoma biopsies. The median number of TIL obtained after two 3-week periods of culture was 1.7×10^{10} . The median amplification induced was therefore an approximately 5×10^3 -fold increase. The total amounts of tumor-reactive T cells obtained from these cultures were calculated from the percent of IFN- γ -responding T cells. These were quite variable, ranging from 2.8×10^6 to 1.12×10^9 . This is

probably a minimal estimation, however, since intracellular cytokine labeling fails to detect low-avidity T cells, and even some fractions of high-avidity T cells that are transiently refractory to cytokine production in vitro [3, 8].

As previously shown with melanoma-specific CTL clones, much lower fractions of TIL responded to autologous tumor cells by GM-CSF and IL-2 secretion than by IFN- γ and TNF- α secretion. This results in part from different antigen thresholds being necessary for the secretion by T cells of these cytokines [3, 4, 12]. These data therefore suggest that an important proportion of melanoma invaded lymph node lymphocytes specific for autologous tumor cells are of relatively low avidity for tumor cells. Avidity is considered to be an important parameter for the efficacy of T cell-dependent responses. Nonetheless, the potential role of the different cytokines secreted by CTL in the success of anti-tumor responses remains unclear.

The contribution of TAA-specific CTL in limiting tumor development has been established in animal models, in great part through adoptive transfers of specific T-cell populations [1, 14, 15, 16]. Since high numbers of TAA-specific T cells of defined specificity [6, 26] and high numbers of tumor-reactive TIL (the present results) can now be readily produced from melanoma patients, it will be feasible to ascertain their potential role in driving an efficient response against tumors by developing randomized adoptive transfer treatments.

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