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ORIGINAL ARTICLE

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Therapeutic efficacy of melanoma-reactive TIL injected in stage III melanoma patients

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Abstract Adoptive therapy for cancer using tumor-infiltrating lymphocytes (TIL) has mainly been investigated in cancer patients with advanced stage disease. The limited clinical success has not been encouraging, although this might be explained by poor TIL specificity and/or high tumor burden. To re-evaluate the effectiveness of adoptive therapy, we analyzed the capacity of tumor-reactive TIL injection in preventing the further development of disease in stage III melanoma patients after complete tumor resection. A phase II/III randomized trial was performed on 88 melanoma patients, who received autologous TIL plus interleukin-2 (IL-2) or IL-2 only. The duration of relapse-free survival was analyzed, taking into account the immunological specificity of injected TIL and the number of metastatic lymph nodes removed before treatment. Kaplan-Meyer analysis revealed that the injection of tumor-reactive TIL was statistically correlated with prolonged relapse-free survival in patients with only one metastatic lymph node. Therefore, improved clinical outcome could be obtained after adoptive therapy by selecting appropriate groups of patients and monitoring the specificity of the injected TIL populations.

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Introduction

The efficacy of adoptive T-cell therapy for tumors was first shown in transplanted animal tumor models, using tumor-infiltrating lymphocytes (TIL), or specifically sensitized T lymphocytes [9, 18, 22] and has recently been confirmed in animal tumor systems that better model human malignancies [8, 10]. In humans fairly good evidence for the efficacy of T cell adoptive therapy has been obtained for hematopoietic cancers through the targeting of viral antigens [11, 24]. Nonetheless, a formal demonstration of the effectiveness of this approach for solid tumors has still to be made. Adoptive therapy of melanoma has involved the transfer of TIL, or recently of melanosomal protein-specific T cell clones, and interleukin-2 (IL-2). Significant tumor regression rate and vitiligo induction have been reported by Rosenberg's group following extensive clinical trials, suggesting a certain efficacy of these treatments [21, 23]. Nonetheless, independent clinical studies, also using TIL and IL-2, have yielded very limited if any clinical results [5, 7]. These mixed clinical results might reflect the heterogeneity of the injected TIL populations, e. g. in terms of activation status and/or frequency of tumor-specific T cells obtained by different culture methods and/or from different tumor samples. In support of this, when tumor antigen specificity of the injected TIL was analyzed, some correlation between objective regression and the presence of melanosomal protein-specific T cells among TIL was reported [16]. To re-address the effectiveness of adoptive T cell therapy in melanoma patients, we first designed a culture protocol allowing efficient expansion of tumor-specific TIL derived from small tumor or metastatic lymph node samples [14]. We recently demonstrated that tumor-reactive TIL, when present in metastatic lymph nodes, were systematically maintained

after ex-vivo expansion using this strategy [20]. Nonetheless, we also showed in the same study that about one-third of the metastatic lymph nodes from stage III melanoma patients did not contain detectable amounts of tumor-reactive T cells, so that expanded TIL were devoid of such T cells [20].

A clinical trial using TIL obtained by this culture method was previously performed on 6 patients and led to tumor regression in 4 cases [27]. Therefore, a randomized phase II/III protocol was designed to directly address the therapeutic efficacy of such TIL, in an adjuvant setting following tumor resection. Eighty-eight stage III (according to the criteria of the American Joint Committee on Cancer, AJCC) melanoma patients were randomly included in this trial. Half of the study group, i.e. 44 patients, received two injections of autologous expanded TIL together with injections of low-dose IL-2. The other 44 subjects received IL-2 only. We retrospectively analyzed the presence of melanoma-reactive T cells among the TIL injected in 27 patients, from whom an autologous melanoma cell line was available [20], and carried out Kaplan-Meyer analysis to investigate whether the injection of tumor-reactive TIL had any influence on relapse-free survival in these patients.

Materials and methods

Melanoma patients

The patients under study had been included in an immunotherapy protocol performed between 1997 and 1999. This protocol was a randomized study designed to compare the efficacy of TIL + IL-2 versus IL-2 injections in patients suffering from stage III malignant melanoma (i.e. lymph node metastases without distant metastases, as described by the AJCC). Forty-four patients were randomly included in the group "IL-2 only" and 44 in the group "TIL + IL-2". For all the patients, the treatment was performed within 6–10 weeks of metastatic lymph node resection as follows: one daily subcutaneous (s.c.) injection of IL-2 (6 million IU; Chiron), from days 1 to 5 and from days 8 to 12 each month for 2 months. Patients included in the group "TIL + IL-2" also received two intravenous injections of autologous ex-vivo expanded TIL on day 1 of treatment, and one month later. Clinical and paraclinical examinations were recorded at each follow-up (every 2 months until month 18, every 3 months until month 36, and then every 4 months until month 60). Evaluation of the therapeutic benefit of tumor-reactive TIL injection was based on the duration of relapse-free survival, established at a time ranging from 2 to 5 years following treatment.

TIL and melanoma cell lines

TIL lines were produced in accordance with good manufacturing practice conditions at the Unit of Cellular and Genetic Therapy (CHRU, Nantes, France) according to a previously described procedure [14, 20]. Briefly, short-term cultured TIL were isolated by culturing cryopreserved fragments of stage III metastatic lymph nodes in two 12-well tissue culture plates with X-vivo 15 serum-free medium (Biowhittaker, Walkersville, Md.) containing 150 IU/ml rIL2 (Eurocetus, Rueuil-Malmaison, France) and glutamine (1 nM; Biowhittaker) for 10–14 days. Therapeutic ex-vivo expanded TIL were derived as follows: 1.8×10^6 short-term cultured TIL were plated at 300 viable lymphocytes/well with irradiated feeder cells (allogeneic PBL and B-EBV cells) into U-bottomed microplates in 200 µl of rIL-2 medium. PHA-P ((15 µg/ml; Difco, Detroit, Mich.) was added on day 0. Ten days later, lymphocytes were recovered from the culture plates, adjusted to 1×10^6 cells/ml in r-IL2 medium and transferred to culture trays for an additional 10 days. The final TIL harvest was obtained by centrifuging, washing and suspending the TIL in 4% human serum albumin (LFB, Les Ulis, France). A second TIL expansion was performed within one month of the first, starting from 1.8×10^6 cryopreserved short-term cultured TIL. Aliquots of TIL suspensions injected in the patients were cryopreserved to study their tumor specificity, which was carried out later once the autologous tumor cell line had been established in culture. The autologous melanoma cell lines were successfully established from 40/88 tumor samples (27 from TIL + IL-2-treated patients [20], and 13 from IL-2 only patients). They were established within 6 to 12 weeks from the same tumor fragments used to derive the TIL.

Evaluation of the tumor-reactive TIL fraction

The fraction of tumor-reactive TIL was determined from the measurement of the fraction of interferon-gamma (IFN-γ)-secreting T cells among TIL stimulated by the autologous melanoma cell line, as described previously [20]. Briefly, samples of 1×10^5 TIL were stimulated for 6 h by 3×10^5 stimulator cells (melanoma cells) in 200 µl of RPMI 1640 containing 10% fetal calf serum (FCS) and 10 µg/ml brefeldin A (Sigma, St Louis, Mo.). For intracytoplasmic cytokine staining, cells were fixed for 10 min, then washed and stored at 4°C until staining. Negative controls were performed with non-stimulated TIL and TIL stimulated with an allogeneic HLA-mismatched melanoma line. Fixed TIL were stained for IFN-γ production using the method described by Jung et al. [15]. Briefly, TIL were stained for 30 min at room temperature with anti-human IFN-γ monoclonal antibody (mAb) (Pharmingen, San Diego, Calif.), at a concentration of 5 µg/ml in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1% saponin (Sigma). After 2 washes, cells were incubated with Fab' 2 fragments of goat anti-mouse IgG (Bio-Atlantic, Nantes, France). After staining, cells were resuspended in PBS and 5×10^3 events were analyzed by FACScan flow cytometer using CellQuest software (Beckton Dickinson, Grenoble, France). 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. A value of 0.3% was considered as the significance threshold.

Statistical analysis of therapeutic efficacy of melanoma-specific TIL

A total of 40 patients from whom the autologous melanoma cell line was available were included in the statistical analysis. Twenty-seven of these patients had received TIL + IL-2 and 13 patients had received IL-2 only. Variables used for the analysis were the duration of relapse-free survival (interval from day 1 of treatment to relapse diagnosis), the presence or absence of tumor-reactive T cells among highly expanded injected TIL or, for IL-2-only treated patients, the presence or absence of tumor-reactive T cells among short-term cultured TIL. The correlation between the duration of relapse-free survival and injection of melanoma-reactive TIL was estimated using the Kaplan-Meyer method in the 27 patients who received TIL + IL-2. We also compared the duration of relapse-free survival in 13 patients treated by IL-2 only, according to the presence of melanoma-specific TIL in their tumors before treatment. Finally, we analyzed the relapse-free interval according to the injection of melanoma-reactive TIL and the number of metastatic lymph nodes. A P value of 0.05 was considered significant. All analyses were performed with the StatView 5.0.1 statistical package for MacOS (SAS Institute Inc., San Francisco, Calif.).

Results

Relapse-free survival of melanoma patients treated by TIL and IL-2 was correlated with the presence of tumor-reactive lymphocytes in injected TIL

Autologous melanoma lines were obtained from 27 patients treated by TIL and IL-2, and the corresponding ex-vivo expanded TIL were analyzed for their ability to secrete IFN-γ in response to autologous melanoma cells. As reported previously [20], and shown in Table 1, 19 out of 27 patients received after the first (E1) and/or the second (E2) TIL cell culture preparations containing melanoma-reactive T cells ranging from 0.4% to 13.8% (mean: 2.8 ± 3). The relapse status of the 27 patients analyzed is shown in Table 1. Ten out of 19 patients that had been injected with melanoma-reactive TIL did not relapse, while 7 out of 8 patients that had been injected with preparations without tumor-reactive cells relapsed. Fig. 1 illustrates the duration of relapse-free survival in the two groups of patients. Kaplan-Meyer statistical analysis showed that the duration of relapse-free survival was significantly longer for the group of patients that received tumor-reactive TIL ($P = 0.0375$).

Table 1. Fractions of tumor-reactive lymphocytes in large-scale expanded TIL from melanoma-invaded lymph nodes of 27 patients treated by TIL + IL-2

Patient	% IFN-γ-positive lymphocytes[b]		Relapse-free survival[c]
	E1[a]	E2	
M88*	ND	0.4	+
M113	10.3	0.8	–
M117	ND	2.5	+
M125	0	2.4	–
M134	0.7	0	+
M154*	7.2	13.8	+
M167	0.7	1.8	–
M170*	1.2	ND	+
M177*	1.1	2.2	+
M180*	5	4.5	+
M182	0	0.7	+
M187	2.9	0.35	+
M193	3.5	4	–
M196*	1	0	–
M197*	2.9	0	+
M199	2.7	2.4	–
M200	1	0.4	–
M204	0	3.4	–
M212	0.4	1	–
M110	0	0	–
M131	0	0	–
M132	0	0	–
M140	0	0	+
M153	0	0	–
M158	0	0	–
M164	0	0	–
M171	0	0	–

*Melanoma patients bearing only one metastatic lymph node
[a]E1 and E2 were **TIL** populations obtained and reinjected to the patient from respectively the first and the second ex-vivo expansions
[b] Percentages of IFN-γ secreting **TIL** were estimated by intracellular labeling. **TIL** were stimulated 6 h by autologous melanoma cells in presence of brefeldin A. Then, cells were fixed, permeabilized, stained for cytokine production and analyzed on a FACScan. Percentages are calculated after deduction of the background obtained on an allogeneic mismached melanoma line
[c]Relapse-free survival of patients 30 months after lymph node resection

The presence of tumor-reactive T cells in metastatic lymph node was not correlated with the duration of relapse-free survival in patients treated by IL-2 only

Since tumor-reactive T cells from metastatic lymph nodes could be systematically expanded by the culture method used, subjects who received tumor-reactive TIL were those patients whose metastatic lymph nodes contained tumor-reactive T cells. Therefore, the above analysis did not permit the determination of which of these parameters influenced the duration of relapse-free survival. This question was addressed indirectly by analyzing the relapse status of IL-2-treated patients according to the presence or absence of tumor-reactive T cells in their metastatic lymph nodes. We investigated the presence of tumor-specific T cells among TIL from 13 IL-2-treated patients from whom autologous melanoma cell lines were available. As shown in Table 2, melanoma-specific TIL fractions, ranging from 0.6% to 8.1% (mean: 3.1 ± 2.9), were detected among the TIL from 8 of these patients. Fig. 2 illustrates the duration of relapse-free survival for these patients, according to the presence or absence of tumor-reactive TIL in their resected tumor-invaded lymph nodes. Kaplan-Meyer statistical analysis indicated that the duration of relapse-free survival was not statistically different between these two groups of patients ($P = 0.55$). Thus, the duration of relapse-free survival of melanoma patients treated by IL-2 was not correlated with the presence of tumor-reactive T cells in the metastatic lymph nodes before treatment.

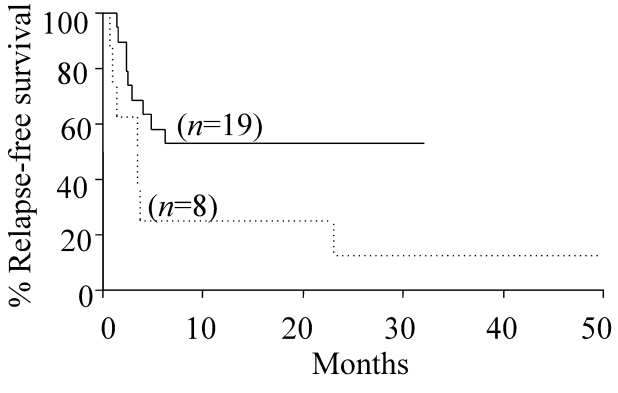

Fig. 1. Relapse-free interval in 27 melanoma patients treated with IL-2 and TIL containing (—) or not containing (·····) MAA-specific lymphocytes

Table 2. Fractions of tumor-reactive lymphocytes in TIL from melanoma-invaded lymph nodes of 13 patients treated by IL-2

Patient	% IFN-γ[a]	Relapse-free survival[b]
M101	7.9	–
M120	8.1	–
M128	0.8	–
M136*	0.6	–
M137*	1.9	–
M183*	0.9	+
M147	3.3	–
M203*	1.7	–
M119*	0	–
M122	0	–
M138*	0	–
M208*	0	+
M210	0	–

*Patients with only one metastatic lymph node
[a]Percentages of IFN-γ-secreting TIL were estimated by intracellular labeling. Short-term cultured TIL were stimulated for 6 h by autologous melanoma cells in the presence of brefeldin A. Then the cells were fixed, permeabilized, stained for cytokine production and analyzed by FACScan. Percentages were calculated after deduction of the background obtained on an allogeneic mismatched melanoma line
[b]Relapse-free survival of patients 30 months after lymph node resection

The therapeutic efficacy of melanoma-specific TIL was not correlated with the amount of tumor-specific lymphocytes injected

The amount of tumor-specific T cells injected in each melanoma patient from the first (E1) or the second (E2) culture was calculated. As shown in Fig. 3, the 19 patients injected with tumor-reactive T cells received between 2.8 million and 1.12 billion melanoma-reactive TIL (mean: 352 million). As shown in Fig. 3, there was no correlation between the duration of relapse-free interval and the number of tumor-specific TIL infused. Thus, the length of relapse-free survival of TIL + IL-2 treated-patients was correlated with the presence of tumor-reactive lymphocytes among the injected TIL, but the minimal number of cells required for such an effect could not be established.

The injection of melanoma-specific lymphocytes in an adjuvant setting increased the duration of relapse-free survival in patients with only one metastatic lymph node

Since a relatively high proportion of patients treated by tumor-reactive TIL relapsed (10/19), we tried to find another parameter that could affect relapse status. Tumor burden is usually considered critical for the success of immunotherapy. We therefore decided to perform an analysis of relapse-free survival taking into account both the disease extent, evaluated on the basis of tumor lymph node invasion (one or more than one lymph node), and the injection of tumor-reactive TIL. Such an analysis was not feasible for the 27 patients treated by TIL + IL-2, because all patients from this group bearing only one metastatic lymph node also received melanoma-reactive TIL (Table 1). We therefore compared the status of the 19 patients treated by tumor-reactive TIL + IL-2 to that of the 44 patients in the IL-2-treated group. Statistical analysis clearly showed that the duration of relapse-free survival was increased by the injection of melanoma-reactive TIL in patients with one metastatic lymph node ($P = 0.0306$; Fig. 4A), but not in patients with several tumor-invaded lymph nodes ($P = 0.6015$; Fig. 4B).

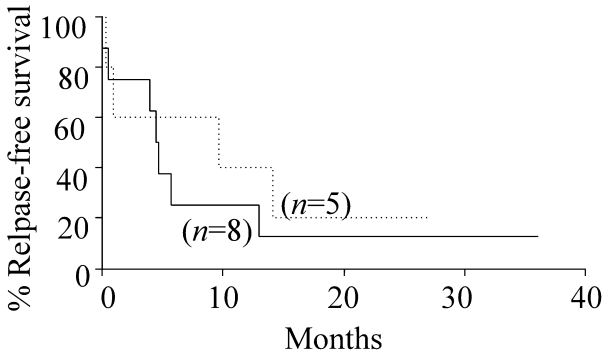

Fig. 2. Relapse-free interval in 13 melanoma patients treated with IL-2, with lymph nodes containing (—) or not containing (·····) MAA-specific lymphocytes

Discussion

We have shown here that the duration of relapse-free survival was longer for TIL-treated patients who received tumor-reactive TIL than for those patients receiving TIL with no detectable tumor reactivity. In order to clearly determine the therapeutic efficacy of the treatment with melanoma-reactive TIL, we investigated whether the presence of such lymphocytes before treatment had any influence on the duration of relapse-free survival. This hypothesis was ruled out by the fact that the presence of tumor-reactive T cells in the tumor-invaded lymph nodes of IL-2-treated patients did not affect the duration of relapse-free survival in these patients. Thus, relapse-free survival of TIL-treated patients was correlated with the infusion of ex-vivo expanded tumor-reactive T cells. To our knowledge, this is the first strong evidence of the efficacy of adoptive T cell therapy for a human tumor that does not express viral antigens.

IFN-γ secretion in response to autologous melanoma cells was used in this study to evaluate melanoma-specific TIL. IFN-γ is a cytokine that appears to contribute significantly to anti-tumor responses [1, 2, 17, 19, 25]. It is secreted by most $CD8^+$ and $CD4^+$ Th1 T cells above a minimal threshold of activation [6, 13]. Therefore, only tumor-specific T cells of minimal avidity were taken into account in the present study. It is possible that this mode of evaluation of TIL reactivity may be critical in showing a significant effect of tumor-specific T cell transfer. In support of this, a recent study has demonstrated IFN-γ^+ but not IFN-γ^- T cells from tumor-immunized mice

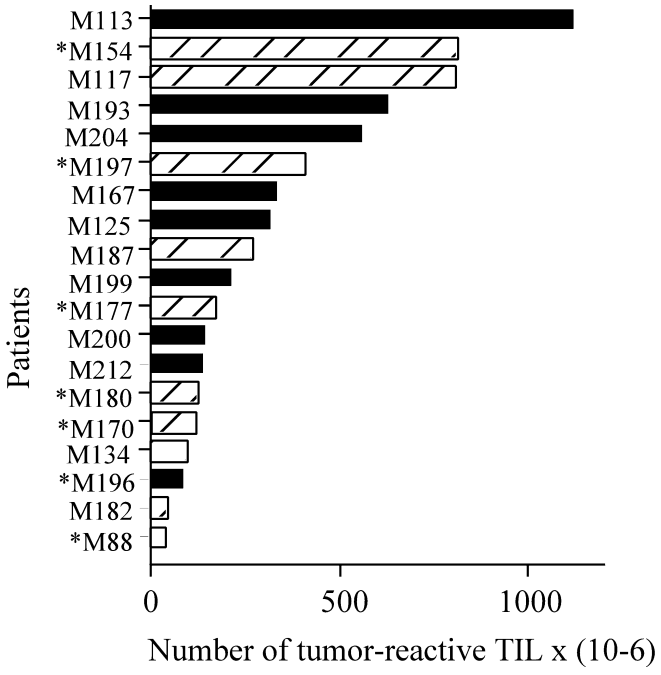

Fig. 3. Number of tumor-reactive TIL injected in 19 patients in the group TIL + IL-2. *Hatched bars* indicate relapse-free patients 30 months after treatment. *Indicates patients with only one melanoma-metastatic lymph node

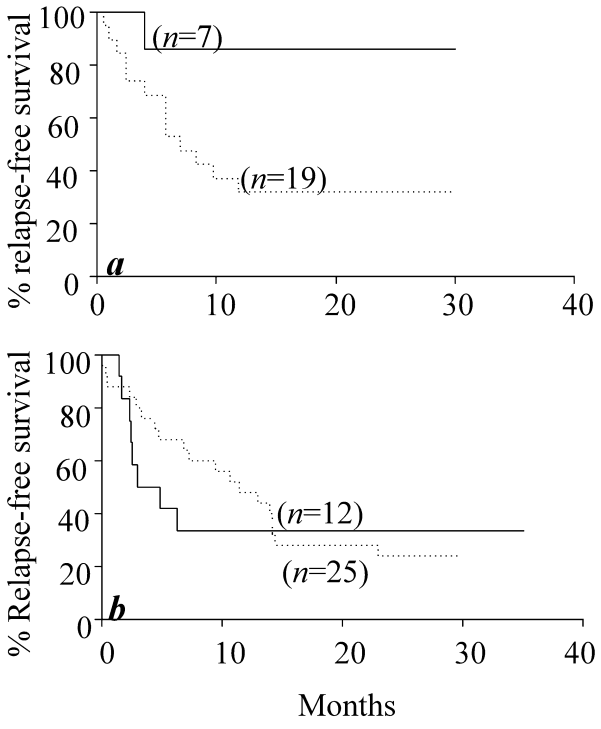

Fig. 4. **A** Relapse-free interval in melanoma patients with only one metastatic lymph node treated with tumor-reactive TIL + IL-2 (—) or with IL-2 (·····). **B** Relapse-free interval in melanoma patients with more than one metastatic lymph node treated with tumor-reactive TIL + IL-2 (—) or with IL-2 (·····)

are cytolytic and mediate tumor rejection upon adoptive transfer [3].

An important but unsolved question encountered by clinicians involved in adoptive therapy protocols is that regarding the definition of the optimal number of tumor-specific T cells to be injected in patients. In the present study, the duration of relapse-free survival of patients treated with tumor-reactive TIL was not correlated with the amount of injected tumor-reactive TIL. Three million tumor-reactive TIL, a number much lower than that currently expected to be effective, were injected in the present study in a relapse-free patient. This absence of correlation may be due to the functional diversity of TIL populations. In this regard, we have recently reported the widely diverse antigen and epitope specificity of melanoma TIL [4]. The number of tumor-reactive TIL required for a therapeutic benefit might therefore be different according to their specificity and avidity. So far it has thus not been possible to determine the minimal number of tumor-reactive T cells to be injected to obtain a therapeutic response as far as this strategy is concerned.

Another important issue regarding adoptive therapy is that of the respective roles of CD4 and CD8 tumor-reactive T cells in anti-tumor responses. Although melanoma-responding TIL are predominantly $CD8^+$ T cells, we have observed that when melanoma lines express HLA class II molecules, some of these tumors also contained tumor-reactive $CD4^+$ TIL [20]. We have previously reported that melanoma lines from 9 out of the 27 TIL-treated patients under study expressed HLA class II molecules and that 4 out of the 7 corresponding TIL contained a detectable fraction of tumor-reactive $CD4^+$ T cells, ranging from 0.1% to 2% [20]. Therefore, at least 4 patients in this study received $CD4^+$ tumor-reactive TIL. Such a small number of patients does not permit definitive conclusions to be drawn on the potential contribution of tumor-reactive $CD4^+$ T cells to the improvement of relapse-free survival. Although this question has been often addressed in many different animal tumor models, the importance of $CD4^+$ T cell contribution and its precise role in anti-tumor responses has not yet been fully elucidated [12, 26, 28].

Although we have provided statistical evidence for the therapeutic efficacy of tumor-reactive TIL injection, about 50% of the patients treated with such TIL relapsed, suggesting that another parameter was involved. In an attempt to establish whether disease extent might be this parameter, we tried to perform a multivariate analysis for the 27 TIL-treated patients, taking into account both the number of tumor-invaded lymph nodes (one or several) and the injection of tumor-reactive TIL. Unfortunately this was not possible, because all the patients with one metastatic lymph node hadreceived tumor-specific TIL. In contrast, tumor-reactive IFN-γ-producing TIL were not detected in 8 out of the 19 patients with several metastatic lymph nodes, thus suggesting that tumor-reactive TIL might disappear in the course of tumor progression [20]. Relapse-free survival of patients treated by tumor-specific TIL was therefore compared with that of patients treated by IL-2

according to the number of metastatic lymph nodes present. The results of this analysis showed that the injection of tumor-reactive TIL improved the duration of relapse-free survival exclusively for patients at the earliest stage of the disease (one tumor-invaded lymph node). A statistical analysis of relapse-free survival and overall survival of the entire patient cohort confirmed this result, i.e. the therapeutic benefit of TIL injection only for the patients with one metastatic lymph node (manuscript in preparation).

In conclusion, this study provides for the first time strong evidence that adoptive immunotherapy using tumor-reactive TIL and low doses of IL-2 is an effective treatment for stage III melanoma patients with a limited invasion of lymph nodes in an adjuvant setting.

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