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ORIGINAL ARTICLE

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Randomized trial of adoptive transfer of melanoma tumor-infiltrating lymphocytes as adjuvant therapy for stage III melanoma

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Abstract The aim of this study was to demonstrate the interest of using tumor-infiltrating lymphocytes (TIL) as adjuvant therapy for stage III (regional lymph nodes) melanoma. After lymph node excision, patients without any detectable metastases were randomly assigned to receive either TIL plus interleukin-2 (IL-2) for 2 months, or IL-2 only. The primary endpoint was determination of the duration of the relapse-free interval. Eighty-eight patients determined as eligible for treatment were enrolled in the study. After a median follow-up of 46.9 months, for the study population the analysis did not show a significant extension of the relapse-free interval or overall survival. However, a significant interaction ($P < 0.001$) was found between the treatment and the number of invaded lymph nodes. In the group with only one invaded lymph node, the estimated relapse rate was significantly lower ($P_{adjusted} = 0.0285$) and the overall survival was increased ($P_{adjusted} = 0.039$) in the TIL + IL-2 arm compared with the IL-2 only arm. No differences between the two arms, either as regards the duration of disease-free survival or overall survival, were noted in the group with more than one invaded lymph node whatever the number of invaded lymph nodes. Treatment was compatible with normal daily activity. This study demonstrates for the first time that the efficiency of TIL in stage III melanoma (AJCC) is directly related to the number of invaded lymph nodes, indicating that tumor burden might be a crucial factor in the efficacy and/or in vitro expansion of T cells specific for autologous tumor antigen, a finding which could be of value in future vaccine development for the treatment of melanoma.

Keywords Adjuvant therapy · Immunotherapy · Melanoma

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Introduction

The incidence of malignant melanoma has been increasing during recent years [19]. The efficiency of chemotherapy remains limited, with a mean response rate of 15% and a short duration of response (mean of 6 months) [2]. The immunological characteristics of melanoma have been extensively studied for several years, and are now being utilized to develop approaches to immunotherapy. Thus adoptive immunotherapy for melanoma is a therapeutic approach in which effector cells such as tumor-infiltrating lymphocytes (TIL) with anti-tumor reactivity can be transferred to a patient to mediate the regression of existing tumors. The rationale for this approach is that melanomas are frequently infiltrated by cytotoxic and cytokine-producing $CD8^+$ T cells specific for autologous tumor-associated antigens (TAA) [3], and that the number of such T cells is probably a critical factor in determining their capacity to eradicate melanoma cells. In some animal models, the adoptive transfer of high numbers of TIL can indeed mediate tumor rejection [6, 7, 8, 12]. Furthermore, in advanced-stage (stage IV AJCC) melanoma patients, clinical trials using TIL combined with interleukin-2 (IL-2) have shown

a response rate of 35% [4, 5]. However, independent studies have concluded that the response rates of TIL + IL-2-treated melanoma patients were not different from those of subjects treated with IL-2 only [10, 11]. Interestingly, it was retrospectively shown that objective tumor regression was more frequently associated with TAA-specific responses of the injected TIL [10].

Recently, we have demonstrated the feasibility and tolerance of home-based adoptive immunotherapy in 6 patients with stage IV melanoma using TIL plus low doses of subcutaneous (s.c.) IL-2. The TIL used in this study were produced within a period of 6 weeks [20]. Tolerance was excellent, and positive responses were obtained in 4 out of 6 patients. However, the responses were of short duration, which is one major problem of immunotherapeutic trials on cancer at the metastatic stage. In this regard, one critical point regarding the clinical efficiency of TIL might be the extent of tumor burden. The therapeutic approaches involving stimulation of the immunological system are indeed expected to be more efficient in the context of limited tumor burden. Regarding the latter, the use of adoptive TIL transfer could be more relevant in an adjuvant situation.

Our study was based on the hypothesis that adjuvant treatment with TIL and s.c. IL-2 could be effective in AJCC stage III (palpable regional lymph nodes) melanoma patients who have not yet shown clinical evidence of metastases. We carried out a randomized open trial to assess treatment with TIL + IL-2 in patients with regional melanoma lymph node metastases, but without any detectable visceral metastases. In the present work, the primary aim was to check the effect of TIL + IL-2 treatment on relapse-free survival in comparison to IL-2-treated patients.

Materials and methods

Trial design

This prospective randomized trial was a home-based study. Patients aged between 18 and 75 years had to meet the following criteria for inclusion: histologically proven primary cutaneous melanoma without any prior systemic adjuvant therapy; clinically apparent N1 regional lymph node recurrence occurring at any interval aftersurgery for primary melanoma of any depth (T1–4N recurrent M0); no sentinel node dissection previously carried out; absence of visceral metastases verified by physical examination, chest radiography, liver echography and brain-chest-liver computed-tomography (CT) scan; no history of other types of cancer except basal cell carcinoma; white blood cell count over 4×10^9/l, hemoglobin levels over 11 g/dl; creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase concentrations less than 1.5 times those of the upper limit of normal; written patient consent. Contraception was required in women of childbearing age. Randomization was carried out as soon as the histology was confirmed.

After the histology had been confirmed, patients were randomly assigned to receive either two injections of TIL (the first about 6 and the second about 10 weeks post-surgery, according to the duration of the expansion) combined with IL-2 (Proleukin; Chiron) or IL-2 only. IL-2 treatment began 6 weeks after lymph node resection in the control arm at the same doses. TIL were injected on the same day as that on which IL-2 treatment was started in the combined arm. IL-2 consisted of an s.c. injection of 6×10^6 IU/m^2 per day, 5 days a week for 2 weeks. The same dose and duration of IL-2 treatment was used for the second injection of TIL performed 1 month later. After 2 months adjuvant therapy, patients received no other treatment. Only a regular follow-up was performed.

The trial was performed at only one center to ensure the reproducibility of lymph node excision. This study was approved by the ethical committee of Nantes (Pays de La Loire). Moreover, TIL were obtained from a specific genetic and cellular therapy unit (UTCG; CHRU, Nantes, France) under good manufacturing practice conditions.

Our TIL culture method has been previously reported [9]. Briefly, TIL were isolated by culturing cryopreserved fragments of stage III melanoma-invaded lymph nodes in two 12-well tissue culture plates with X-vivo 15 medium (Biowhittaker, Walkersville, Md) containing 150 IU/ml rIL-2 (Chiron, France) and 1 nM glutamine (Biowhittaker) for 10 to 14 days. To perform high-fold expansion, 1.8×10^6 of short-term culture TIL were plated at 300 viable lymphocytes/well with irradiated feeder cells into U-bottomed microplates in 200 µl rIL-2 medium. Phytohemagglutinin (PHA; Difco, Detroit, Mich.) was added on day 0 (15 µg/ml). After 48 h, most of the PHA was removed by replacing the culture medium. Ten days later, lymphocytes were removed from the culture plates, adjusted to 1×10^6 cells/ml in rIL-2 medium and transferred into 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 1 month of the first from frozen short-term culture TIL. Thus for each patient TIL production was carried out twice, at a 1-month interval, with two independent TIL cultures. Each culture expansion was initiated from 1.8×10^6 TIL, a minimal amount that can be easily obtained from any melanoma lymph node biopsy. The median number of TIL obtained after two 3-week periods of culture was 1.7×10^{10}. The total amount of TIL obtained from the first (R1) and second (R2) TIL expansion for each patient varied between 0.22 and 3.34×10^{10} cells.

Flow cytometry analysis confirmed that only T lymphocytes were present in all cases (100% CD3-positive cells).

The 88 TIL expansions (two expansions for each of the 44 patients) were all performed successfully, and then injected into the patients. No technical problems were noted during these expansions and all the bacteriological controls were negative.

Follow-up

In both treatment groups, clinical examination, full blood counts and biochemical analyses were repeated every 15 days for the first 2 months, then every 2 months for 18 months, and finally every 3 months. Liver echography and brain-chest-liver CT scan were performed every 6 months.

The date and site of first recurrence as well as the date and cause of death were recorded. Adverse events were noted and the WHO toxicity scale was used to grade their severity.

Statistical analysis

The primary endpoint was the duration of the disease-free interval. The secondary endpoint was overall survival. To keep to a minimum the number of patients required, and to ensure earlier termination of the study, it was carried out as a sequential trial using the triangular test [22]. An improvement, i.e. 70% disease-free interval, was expected for the experimental group, against 50% disease-free interval for the control group at 2.5 years. Inspections were carried out every 8 events. A working significance level of 0.05 against the one-sided alternative was required, with a power of 0.90.

The Kaplan-Meier estimates and log rank tests were used for the main efficacy analysis. The log-likelihood ratio test was used to assess different factors. The Cox model was used to adjust treatment comparison on baseline characteristics known to have a prognostic significance, namely: Breslow thickness (< 1.5 mm; > 1.5 mm), capsular breaking, number of detectable regional nodes (1, > 1), sex, age (< 50 yr, 50 yr).

Assumption of proportional hazards was checked for all factors. All interactions were tested. Because of a high interaction between treatment and the number of invaded lymph nodes, an additional analysis was performed. Determination of the alpha level was based on the Bonferroni adjustment. A P value of 0.0345 or lower was considered to indicate statistical significance in the final analysis [17]. Analyses were performed using BDMP7.0, Splus2000 and PEST 3.

Results

Main efficacy analysis

The trial started on December 1, 1994. The seventh sequential analysis was performed on May 11, 1999, by which time there had been 28 relapses in the TIL group, and a similar number of relapses in the IL-2 group (Fig. 1). As shown in Fig. 2, there was no significant difference between the 2 groups ($P = 0.608$), and patient recruitment was terminated.

Long-term analysis

The following analysis was based on data collected in June 2000, when all patients had completed 18 months or more of follow-up.

Study population

Eighty-eight patients were enrolled in the study, with 44 subjects in each group; all patients received one of the two treatments and were evaluated. The median follow-up was 46.9 months (range: 18.2–76.5 months) for the control group and 45.1 (range: 18.6–75.7 months) for the TIL group. The two groups were well balanced for major prognostic factors of melanoma (Table 1).

Disease-free survival

Twenty-nine recurrences were recorded in the TIL + IL-2 group and 31 in the control group. The median for relapse-free interval was 9.9, $CI_{95\%}$ (3.93–23.05) months for the TIL group and 9.21,

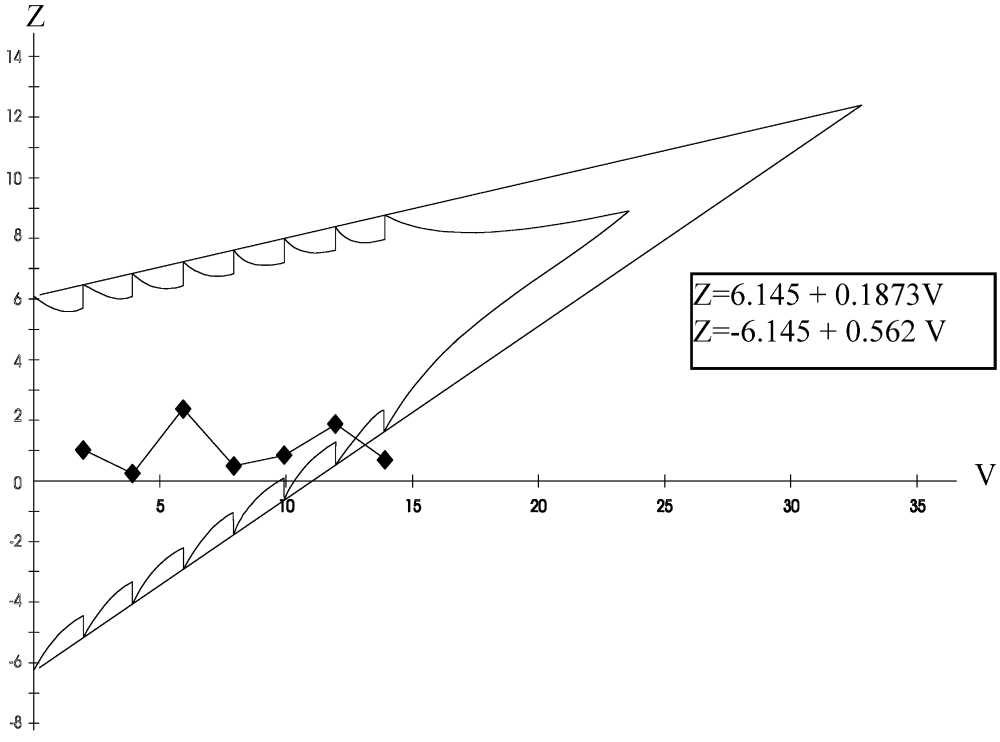

Fig. 1. Design of triangular test and results of seventh sequential analysis. At seventh analysis, the lower triangular boundary was crossed, showing no significant difference in relapse between the two groups ($P = 0.608$)

DATE	Recurrences	TIL	IL2
June 13,1995	8	3/10	5/11
November 16,1995	16	8/14	8/15
June 17,1996	24	10/19	14/21
Mars 23,1997	32	16/24	16/28
November 20,1997	40	19/32	21/33
August 7,1998	48	23/40	25/39
May 11,1999	56	28/44	28/44

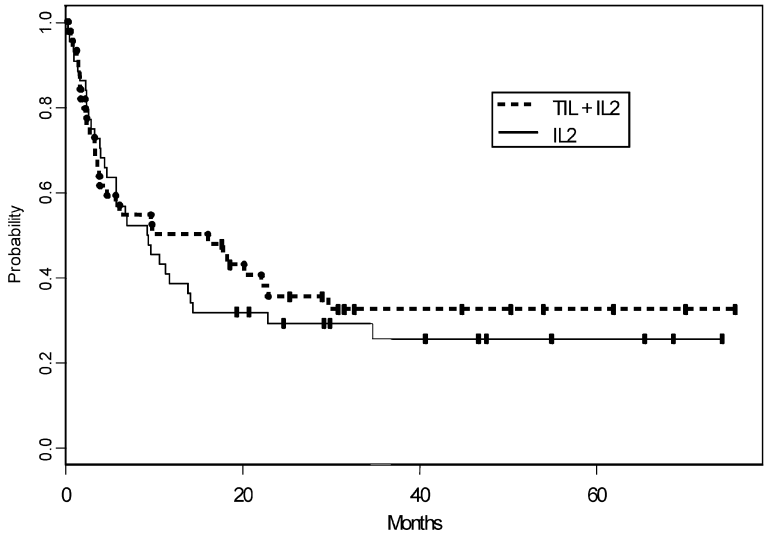

Fig. 2. Relapse-free survival in the TIL + IL-2 group: this difference was not significant ($P = 0.63$; log-rank test)

$CI_{95\%}$ (4.69–14.13) months for the control group. This difference was not significant ($P = 0.55$, log rank test). None of the baseline characteristics adjusted (Breslow thickness, capsular breaking, sex, age, Clark stage) significantly increased or decreased the hazard ratio of relapse in the overall group except for the number of detectable tumor-invaded regional nodes (log-likelihood ratio test < 0.001; Table 2). The analysis of all interactions showed a significant relation between treatment and the number of nodes ($P < 0.01$; Table 3). This means that the effect of the treatment differed according to the number of invaded lymph nodes.

In the group with only one invaded node ($n = 34$), the estimated relapse rate was lower for the patients treated with TIL + IL-2 (5/15) than for the control group (13/19) ($P = 0.019$) (Fig. 3). The log-likelihood of a Cox's model, including all baseline characteristics (sex, Breslow thickness, Clark stage) and treatment was 44.64. In the other group, the log likelihood of the same model but without treatment was 48.25. This difference means that there was a significant effect of treatment in the group with one tumor-invaded node ($\chi_2 = 7.23$; $P = 0.007$).

Table 1. Patient characteristics according to treatment group

		TIL + IL-2 ($n = 44$)	IL-2 ($n = 44$)
Primary tumor site	Head	7	0
	Trunk	20	22
	Limbs	16	20
	Extremities	1	1
Breslow	< 1.5	9 (0.8 ± 0.1)	9 (1.2 ± 0.1)
	> 1.5	31 (4.3 ± 0.5)	33 (3.6 ± 0.3)
Invaded nodes	= 1	15	19
	> 1	29	25
Capsular breaking	Yes	23	22
	No	20	21

In the group with more than one tumor-invaded node ($n = 54$), the estimated hazard function for relapse showed that there were 24 relapses among the 29 patients treated by TIL + IL-2, and 18 among the 25 patients in the IL-2 control group. The difference was not significant (log rank test; $P = 0.36$; Fig. 4). The Cox's model adjusted on the others factors did not show an effect of treatment in the group with more than one invaded node ($\chi_2 = 0.25$; $P = 0.62$). There was no difference between two, three or more invaded lymph nodes. With more than one invaded lymph node, no interaction between TIL and relapse-free survival was noted.

Overall survival

Twenty-four patients died out of the 44 in the TIL + IL-2 group, and 26 out of the 44 in the control group. The median survival time was 29.53 ± 9.61 months for the TIL + IL-2 group and 19.96 ± 4.04 months for the control group. This difference was not significant ($P = 0.63$, log rank test; Fig. 5).

None of the adjusted baseline criteria significantly increased or decreased the hazard ratio of relapse in the overall group or in each group except for the number of detectable regional nodes (log-likelihood ratio test for the overall group: 0.001) (Table 3).

There was a significant interaction between treatment and the number of tumor-invaded nodes ($P < 0.01$). Treatment was then assessed in each group of patients by the log-rank test.

In the group with only one tumor-invaded node ($n = 34$), the estimated survival rate was higher for the patients treated with TIL + IL-2 (11/15) than for the control group (7/19) (log rank test; $P = 0.026$; Fig. 6). The same result was found with a Cox's model adjusted for all factors. The comparison of log likelihood between the model with and without treatment showed a significant effect of treatment in the group with one invaded node ($\chi_2 = 6.68$; $P = 0.009$).

In the group with more than one tumor-invaded node ($n = 54$), there was no difference in survival between the TIL + IL-2 group

Table 2. *P* values of different factors according to the judgement criteria

Covariate	Disease-free interval Hazard ratio ($CI_{95\%}$) *P*	Overall survival Hazard ratio ($CI_{95\%}$) *P*
Age	0.98 (0.96; 1.00) $P = 0.10$	0.99 (0.97; 1.02) $P = 0.63$
Sex	0.97 (0.58–1.61) $P = 0.91$	0.921 (0.52; 1.61) $P = 0.77$
Breslow thickness (< 1.5 mm; = 1.5 mm)	1.3 (0.672; 2.61) $P = 0.41$	1.08 (0.53; 2.23) $P = 0.83$
Clark stage (< 4; = 4) 6 missing data items	1.26 (0.74; 2.14) $P = 0.39$	1.12 (0.63; 1.98) $P = 0.69$
Capsular breaking	1.41 (0.84; 2.34) $P = 0.18$	1.55 (0.87; 2.74) $P = 0.13$
Node ulceration	1.19 (0.70–2.02) $P = 0.51$	1.29 (0.72; 2.3) $P = 0.38$
No. of detectable regional nodes	1.10 (1.04; 1.16) $P < 0.0001$	1.11 (1.05; 1.17) $P = 0.0001$

and the IL-2 control group (log-rank test; $P = 0.87$; Fig. 7). The Cox's model adjusted on the other factors did not show an effect of the treatment (χ_2 test, $P = 0.28$). There was no difference between two, three or more tumor-invaded lymph nodes. With more than one invaded lymph node, no interaction between TIL and overall disease-free survival was noted.

Table 3. Relapse percentage according to treatment and number of invaded nodes (1 or >1). Data obtained in June 2000

	TIL + IL-2	IL-2	All
1 invaded node	5/15 (33.3%)	13/19 (68.42%)	18/34 (52.94%)
>1 invaded node	24/29 (82.75%)	18/25 (72%)	42/54 (77.77%)
All	29/44 (65.90%)	31/44 (70.45%)	60/88 (68.18%)

Safety

Among the 44 patients who received IL-2 + TIL, all subjects experienced at least one adverse effect, and in all cases except one, this was related to IL-2. In only one patient, it was related to TIL with a fever that became apparent about 1 h after TIL injection, disappearing spontaneously after 2 h. All the 44 patients in the IL-2 arm experienced at least one adverse effect. These adverse effects were IL-2-related, and were always grade 1 or 2. No patient presented grade 3 or 4 toxicity. There was no drug-related mortality. No patient withdrew from the trial as a result of one or several adverse events. The most frequently occurring grade 1 or 2 adverse events were those usually observed with low doses of IL-2: asthenia (100%), influenza-like symptoms (100%), headache ($n = 78$; 88%), nausea and vomiting ($n = 38$; 43%), dizziness ($n = 21$; 23%), depression ($n = 12$; 14%). No cardiovascular symptoms, hematological toxicity grade 3 or 4, or increase in liver enzyme levels grade 3 or 4 were noted. However, in all patients either erythema or inflam-

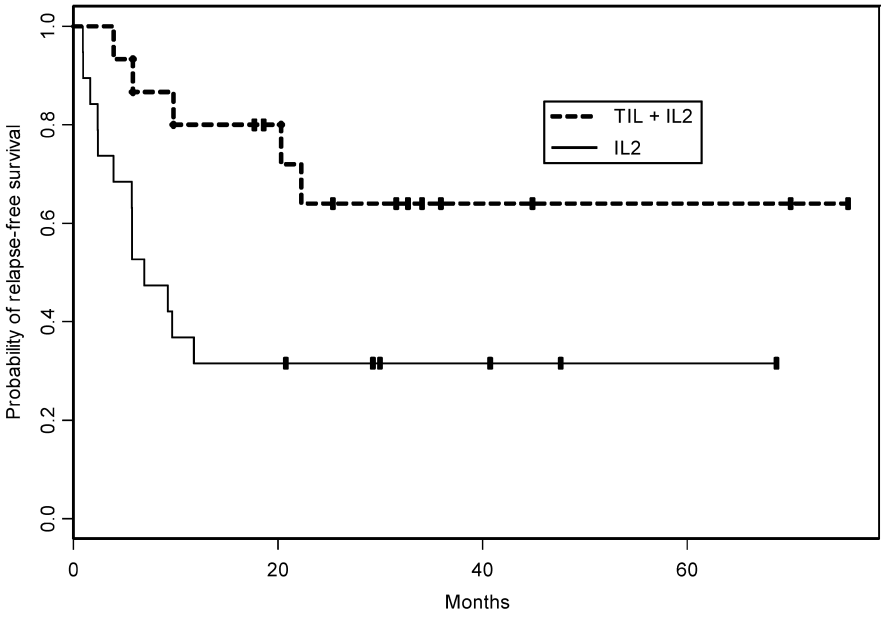

Fig. 3. In the group with only one invaded lymph node, the estimated relapse rate was lower for the patients treated with TIL + IL-2 than for the IL-2 control group ($P_{adjusted} = 0.0285$; power = 50.4%)

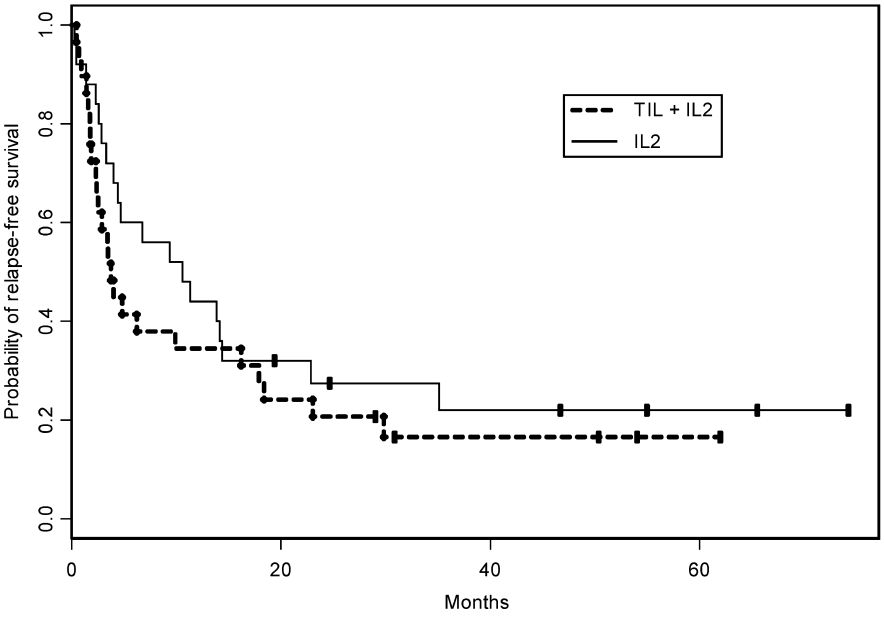

Fig. 4. In the group with more than one invaded lymph node, the estimated relapse rate between the TIL + IL-2 group and IL-2 group was not significant ($P = 0.36$)

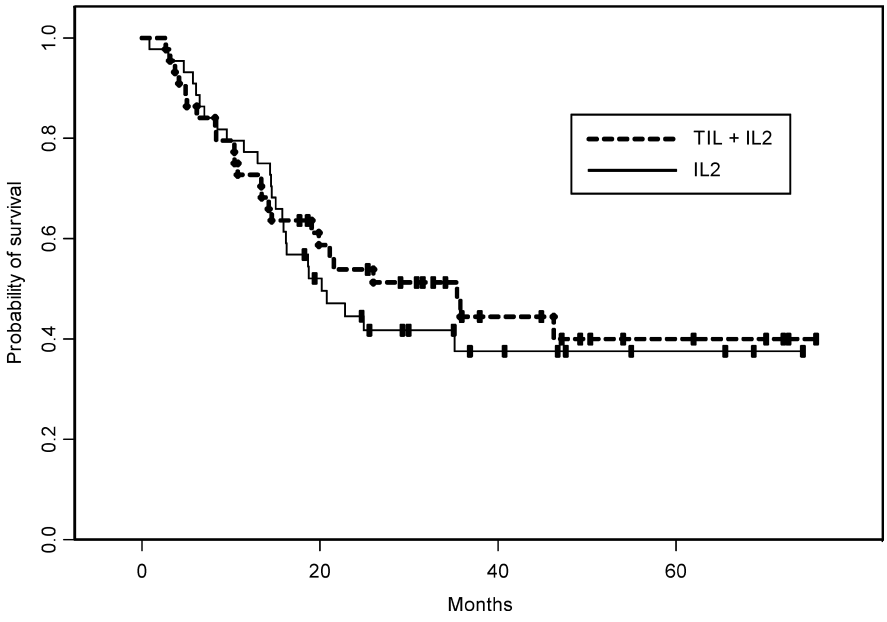

Fig. 5. The overall survival rate between the TIL + IL-2 group and IL-2 group was not significantly different ($P = 0.367$; log-rank test)

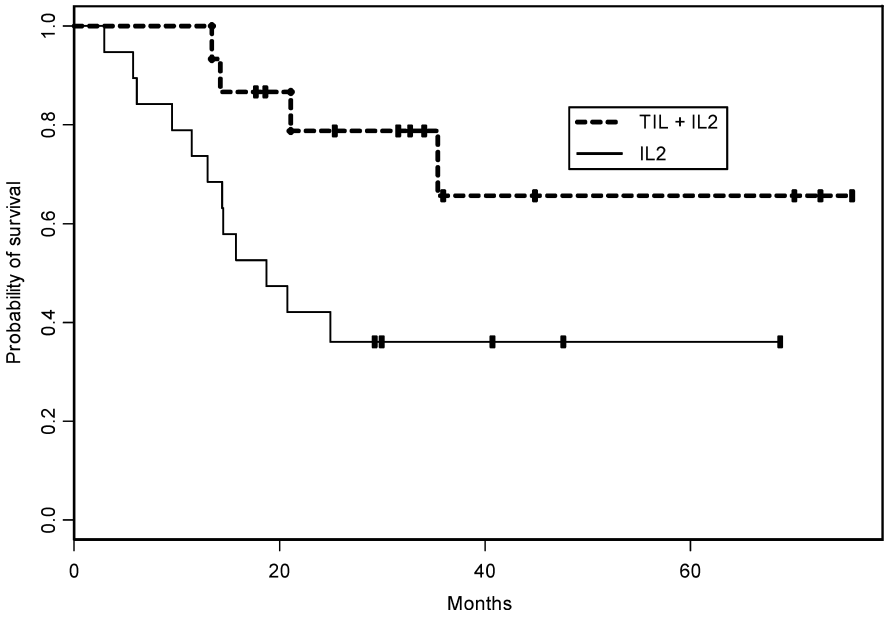

Fig. 6. In the group with only one invaded lymph node, the overall survival rate was greater for the patients treated with TIL + IL-2 than for the IL-2 control group ($P_{adjusted} = 0.039$; power = 54%)

matory nodules could be observed at the site of the IL-2 injection, which regressed within about 3 weeks.

Results and discussion

In the present work, we analyzed the effect on relapse-free survival and overall survival in stage III melanoma of TIL + IL-2 used as adjuvant treatment before any cutaneous or visceral metastases became apparent. Patients treated with TIL + IL-2 and controls treated with IL-2 only were well balanced for major prognostic factors of melanoma (Table 1): age, sex, Breslow thickness, Clark level, number of invaded lymph nodes, and capsular infraction. Another important point concerning the methodology was that the lymph node excisions were always performed by the same surgical team, thus avoiding an external influence in the frequency of relapses between the two arms related to the technique of lymph node excision.

Thus, considering the overall population with stage III melanoma and lymph node involvement, we have shown that adjuvant administration of TIL plus s.c. low doses IL-2 before cancer progression to the metastatic stage does not significantly improve the duration of relapse-free interval or overall survival. After a mean follow-up of 46.9 months, the median survival time was increased in the TIL + IL-2 group, with 29.53 ± 9.61 months compared to 19.96 ± 4.04 months in the IL-2 group, but without statistically significant value.

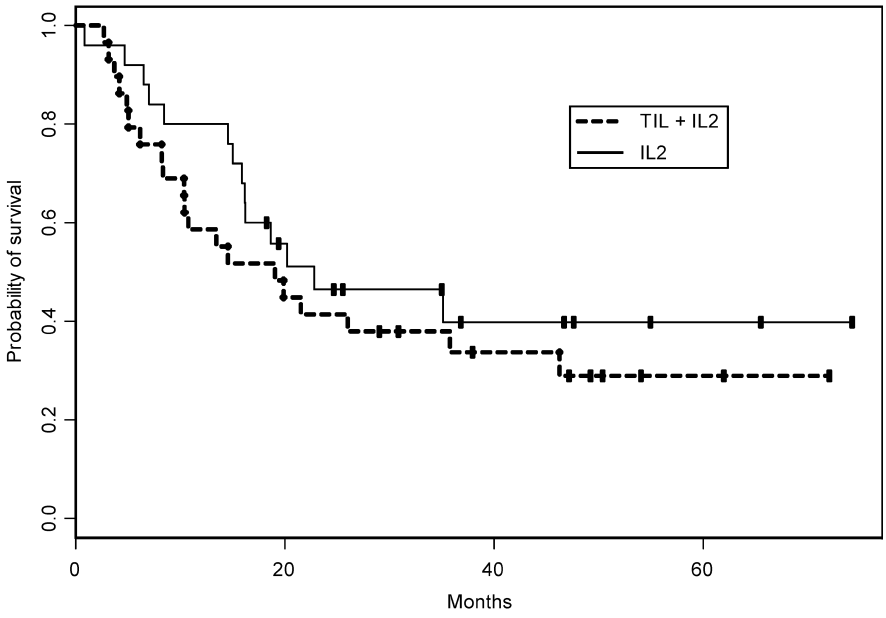

Fig. 7. In the group with more than one invaded lymph node, there was no difference in overall survival between the TIL + IL-2 and the IL-2 control group ($P = 0.87$)

Nevertheless, when we investigated the interactions between the different prognostic factors for melanoma, a significant interaction between the efficiency of TIL transfer and the number of invaded lymph nodes was shown both for relapse-free survival and overall survival. In patients with only one tumor-invaded lymph node, a significant increase in relapse-free survival ($P = 0.019$) and overall survival ($P = 0.026$) was indeed obtained in the TIL + IL-2 arm compared to the IL-2 arm. These results are independent of the usual major prognostic factors for melanoma, i.e. Breslow, Clark, sex, age and capsular infraction. The total R1 and R2 amounts of TIL injected in each patient varied between 0.22 and 3.34 10^{10} cells, with a median of 1.7 10^{10} cells. We also found a lack of relation between the number of TIL administered to each patient and the duration of relapse-free survival or overall survival. In order to validate our statistical analysis, we compared our conclusions and those obtained by the approach proposed by Matthews and Altman [11]. The individualization between one or more than one invaded lymph node is based on the AJCC classification, bearing in mind that the number of invaded lymph nodes is considered as a prognostic factor. Our study clearly demonstrates that the interaction between clinical results and TIL was only present for the one lymph node category.

Furthermore, this adjuvant treatment with TIL plus s.c. low doses of IL-2 over a 2-month period was very well tolerated, with only grade 1 or 2 toxicity. The main adverse effects were the inflammatory nodes at the site of injection and asthenia that always remained mild. No patient terminated treatment due to an adverse event. Except for the TIL injection, which necessitated one day's hospitalization, the treatment was administered at home with the help of a nurse and was compatible with normal daily activity of short duration.

To our knowledge, the long-term effect of an adoptive transfer of TIL melanoma used as an adjuvant regimen for the treatment of stage III melanoma has never been discussed previously. Our results indicate that the extent of the disease, which in our study was represented by the number of tumor-invaded lymph nodes could be a crucial factor in determining the efficiency of TIL treatment.

Three main hypotheses could explain the difference in the efficiency of TIL treatment according to the number of invaded lymph nodes. Firstly, a low tumor burden could be essential for the efficiency of TIL activity against melanoma cells. Secondly, the TAA-specific T cells could be more frequent at the early stage of lymph node invasion and thus the expansion taking place at this stage of the disease would be more efficient in providing high amounts of tumor-specific lymphocytes. Interestingly, according to this hypothesis, we have previously reported that a lack of tumor-reactive TIL production by expansion mainly occurs in patients with several tumor-invaded lymph nodes, but rarely in subjects with a single invaded node [13]. The results obtained from in vitro experiments are comparable to our present clinical data, and suggest that the tumor-reactive TIL mainly present in melanoma lesions during the early stages, disappear with the progression of the disease. Such a decrease (or disappearance) of specific tumor-reactive TIL with the progression of the disease might be induced by chronic stimulation of the reactive T cells by tumor-derived antigenic peptides, leading to antigen-mediated cell death or anergy of specific reactive T cells [1]. A third hypothesis to explain the difference in the efficiency of the TIL according to the number of invaded lymph nodes could be related to the development of immunological escape mechanisms by the melanoma cells with the progression of the disease, such as the loss of expression of molecules implicated in the activation of

specific TIL. These molecules could be HLA class I antigens, tumor antigens [16], or some integrins whose expression has been noted to decrease with the progression of the disease [21]. Another mechanism of immunological escape could be the production by melanoma tumor cells of cytokines inhibiting the activation of reactive T cells, such as IL-10 or alpha-MSH with the spread of this disease [18].

In conclusion, our results strongly suggest an interaction between TIL as adjuvant treatment in stage III melanoma patients and the number of invaded lymph nodes. This means that the effect of the treatment differed according the number of invaded lymph nodes. This strongly suggests that T cells specific for autologous TAA are present at an early stage in the development of this disease, and indicates the possible interest of obtaining melanoma-reactive TIL from early-stage lesions as nodes in transit and even as sentinel lymph nodes. This information could be crucial for the future development of vaccination treatment in melanoma patients.

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