Cancer Therapy: Clinical

Clinical Responses in a Phase II Study Using Adoptive Transfer of Short-term Cultured Tumor Infiltration Lymphocytes in Metastatic Melanoma Patients

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Abstract

Purpose: Adoptive cell therapy with autologous tumor-infiltrating lymphocytes (TIL) has shown promising results in metastatic melanoma patients. Although objective response rates of over 50% have been reported, disadvantages of this approach are the labor-intensive TIL production and a very high drop-out rate of enrolled patients, limiting its widespread applicability.

Previous studies showed a clear correlation between short TIL culture periods and clinical response. Therefore, we used a new TIL production technique using unselected, minimally cultured, bulk TIL (Young-TIL). The use of Young-TIL is not restricted to human leukocyte antigen (HLA)-A2 patients.

The purpose of this study is to explore the efficacy and toxicity of adoptively transferred Young-TIL following lympho-depleting chemotherapy in metastatic melanoma patients, refractory to interleukin-2 and chemotherapy.

Experimental Design: Young-TIL cultures for 90% of the patients were successfully generated, enabling the treatment of most enrolled patients. We report here the results of 20 evaluated patients.

Results: Fifty percent of the patients achieved an objective clinical response according to the Response Evaluation Criteria in Solid Tumors, including two ongoing complete remissions (20+, 4+ months) and eight partial responses (progression-free survival: 18+, 13+, 10+, 9, 6+, 4, 3+, and 3 months). All responders are currently alive. Four additional patients showed disease stabilization. Side effects were transient and manageable.

Conclusion: We showed that lympho-depleting chemotherapy followed by transfer of short-term cultured TIL can mediate tumor regression in 50% of metastatic melanoma with manageable toxicity. The convincing clinical results combined with the simplification of the process may thus have a major effect on cell therapy of cancer. *Clin Cancer Res; 16(9); 2646–55. ©2010 AACR.*

Cutaneous metastatic melanoma patients have a medium survival of merely 6 to 10 months (1). Interleukin (IL)-2 and dacarbazine, the only two Food and Drug Administration–approved drugs, are very limited in their effectiveness and mediate a clinical response in just 10%

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of the patients (2). Only seven percent of the IL-2–treated patients achieve a durable complete response (CR), whereas treatment with dacarbazine has no effect on overall survival (OS; ref. 3). In addition cancer vaccine trials have shown disappointing results (4).

Adoptive cell transfer (ACT) using autologous tumor-infiltrating lymphocytes (TIL) has proven itself as one of the most effective treatments to date (5–7). Objective response (OR) rates between 51% to 72% have been reported in heavily pretreated advanced melanoma patients (8, 9).

Current ACT with TIL requires preconditioning of the patients with nonmyeloablative chemotherapy with or without the addition of total body irradiation and infusion of large numbers of autologous TIL followed by administration of high-dose bolus IL-2 (8, 9).

All reported studies use T cells isolated from the patient's own tumor mass. TILs are generated from multiple, independently grown tumor fragments, which results in several individual TIL cultures from one patient. The

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Translational Relevance

This article explores the efficacy and toxicity of adoptively transferred minimally cultured tumor infiltrating lymphoctes (TIL) in lympho-depleted metastatic melanoma patients.

Adoptive TIL transfer has been previously described. Dudley used TIL that were selected *ex vivo* for reactivity against tumor cells. We implemented this technology, but used nonselected, short-term cultured Young-TIL.

This modification considerably simplifies the production process of TIL, making it significantly more accessible to many cancer centers worldwide. Patient drop outs are rare compared with previous TIL protocols.

Of 20 patients, 10 achieved an objective clinical response including 2 ongoing complete and 8 partial responses. Four patients showed disease stabilization. Side effects were transient and manageable.

In summary, we show that Young-TIL adoptive cell transfer can mediate tumor regression in 50% of refractory melanoma patients. The convincing clinical results combined with the simplification of the process may thus have a major effect on cell therapy to cancer.

establishment of multiple TIL cultures requires between 21 to 36 days. In a screening process, only individual TIL cultures secreting IFNγ upon coincubation with autologous or human leukocyte antigen (HLA)-matched melanoma lines are eligible for treatment. This selection is very problematic, as it results in an exclusion of about half of all enrolled patients (10). The main reasons for this high drop out rate are (*a*) the necessity of autologous melanoma lines, often difficult to establish, to perform the IFNγ screening and (*b*) the existence of IFNγ-secreting TIL cultures in only about half of the enrolled patients (10).

Interestingly, previous studies investigating an *in vitro* predictor of therapeutic response and TIL characteristics could not correlate IFNγ secretion to clinical outcome (10, 11). On the other hand, two factors that were repeatedly reported to have a significant positive association to clinical response were short culture duration and telomere length (11–15).

In an *in vitro* study comparing short-term cultured TIL directly to older IFNγ-selected TIL, it was shown that younger TIL have longer telomeres and high levels of the costimulatory molecules CD27 and CD28, which can lead to persistence *in vivo* as well as OR (13–17).

Young-TIL are established from one single bulk T-cell culture. Those cells spend minimal time in culture and are usually generated after 10 to 18 days only. The establishment of a single TIL culture enormously simplifies the laboratory procedure.

A pilot study conducted on eight metastatic melanoma patients using ACT with minimally cultured Young-TIL in combination with nonmyeloablative chemotherapy

and high-dose bolus IL-2 showed encouraging clinical effectiveness, as three of eight patients experienced a clinical OR, including one complete remission (10).

These *in vitro* data and preliminary clinical findings led us to perform a phase II study with Young-TIL. Compared with previous TIL studies, Young-TIL have major advantages; they are easier to generate and do not have to undergo IFN-γ screening. This allows the treatment of most enrolled patients. The aim of the current trial is to evaluate the effectiveness and toxicity of adoptively transferred nonselected Young-TIL.

Patients and Methods

Patient treatment and clinical evaluation. Patient treatment was conducted as previously described (8, 10). Stage IV melanoma patients, older than 18 years, negative for HIV, Hepatitis B and C infection with a good performance status [Eastern Cooperative Oncology Group (ECOG) scale 0 or 1], no central nervous system involvement, and a life expectancy of at least 2 months were eligible for the study. Patients had to sign an informed consent approved by the Israeli Ministry of Health Approval no. 3518/2004 (ClinicalTrials.gov Identifier NCT00287131). Amendment for the use of short-term cultured bulk TIL was approved in October 2007. All patients had measurable disease by computed tomography scan or physical examination and were previously treated unsuccessfully, or exhibited relapse with IL-2–based therapies, including high–dose bolus IL-2 or chemobiotherapy. Four (20%) of the 20 treated patients responded to IL-2–based therapy alone, but then relapsed and entered the Young-TIL study.

Before nonmyeloablative chemotherapy, granulocyte colony-stimulating factor–mobilized stem cells were obtained by leukopheresis, as a back-up in case patients did not reconstitute their hematopoietic system after treatment.

Patients received the nonmyeloablative lympho-depleting regimen starting 7 d before the TIL infusion (day 0) at our institution's bone marrow transplantation department. Patients were treated on days −7 and −6 with cyclophosphamide (60 mg/kg) and on days −5 to −1 with fludarabine (25 mg/m^2). None of the patients received total body irradiation or peptide vaccinations. On day 0, Young-TIL were i.v. administrated to the patient within 30 minutes, followed by bolus high-dose IL-2 (720 000 IU/kg) every 8 hours to tolerance. A maximum of 15 doses was given to the patients.

After TIL infusion, all patients started preventive therapy with granulocyte colony-stimulating factor (10 μg/kg daily), as well as Fluconzole and Acyclovir until absolute neutrophil count reached 1.0 K/μL. Whenever platelet counts dropped below 20 K/μL, patients were given platelet transfusion. Additionally, patients received Sulfametoxazole/Trimetoprim twice weekly for at least 6 months and until CD4 counts reached 0.2 K/μL.

Hematologic and biochemistry parameters were monitored daily and hemodynamic parameters were measured

Table 1. Patients' characteristics before TIL therapy

Patient	Gender	At diagnosis			Diagnosis to ACT (y)*	Prior treatments for stage IV disease			
		Age	Stage	Site of metastasis		Treatment (not incl. IL-2)	IL-2–based treatment	Response to IL-2 treatment	TTP (mo)
Responders									
05-LA	M	39	M1a	SC	2	Surgery	CB	PD	Na
19-NS	M	50	IIb	Na	16	Taxol	CB	PD	Na
03-MG	M	26	Ib	Na	10		CB	PD	Na
06-TS	M	55	III	Na	5		HD bolus	SD	6
09-SD	M	44	IIb	Na	1		CB	PD	Na
13-BS	M	57	IIb	Na	4		CB	SD	7
14-SV	M	69	III	Na	2.3	Surgery	CB	PD	Na
16-SH	M	38	III	Na	2.8	Taxol	CB	PD	Na
18-WR	F	68	III	Na	2.2	Surgery	CB	PR	5
20-TY	M	55	III	Na	3.3	Surgery	CB	CR	5
Median					3.1				5.5
Nonresponders									
01-AY	M	51	M1a	SC	5.3		CB	SD	14
07-ZR	M	20	III	Na	1.8		CB	PD	Na
08-RM	F	31	Ia	Na	2.8		CB	PR	2
12-VS	F	36	III	Na	5.5	Surgery	CB	SD	2
02-PE	M	33	III	Na	3	Dacarbacine	CB	PD	Na
04-BA	M	41	III	Na	16.3	Surgery	CB	PR	16
10-BE	F	48	III	Na	4.8		CB	PD	Na
11-KB	M	55	III	Na	2.4		CB	PD	Na
15-SM	M	52	M1c	Liver, bone	Na	Taxol	CB	PD	Na
17-ZD	F	46	Ib	Na	22		CB	PD	Na
Median					4.8				8.0
					$P = 0.58$				$P = 0.47$

Abbreviations: TTP, time to progression; CB, chemobiotherapy consisting of IL-2, dacarbazine, and cisplatin; HD, high-dose; Na, not applicable.

*Years from diagnosis to adoptive cell transfer.

during IL-2 administration. Response rate was assessed using the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines (18) 4 weeks following TIL administration and every 3 months thereafter or as clinically needed. A CR or partial response (PR) was considered an OR and duration was measured from initiation of the treatment to time of relapse.

Primary end points of this study were the evaluation of overall response rate and toxicity; secondary end points included the assessment of progression-free survival (PFS) and OS.

TIL generation and expansion. After surgical resection of metastatic lesions and pathologic examination, Young-TIL cultures were established as previously described (10, 16). The tumor was sliced with a scalpel into small pieces, about 2 to 3 mm^3 of size. Enzymatic digestion of the pieces was done in most cases for 2 to 3 hours at 37°C or overnight at room temperature with media containing collagenase, hyaluronidase Type V, and DNase I Type IV (Sigma-Aldrich) as previously described (19). The obtained single-cell suspension was passed through a cell strainer, washed twice with

PBS, and transferred to a 24-well plate or culture flask at a concentration of 1.0×10^6 TIL/mL in complete medium (19) with 3,000 IU/mL rhIL-2 (Proleukin, Chiron B.V.). The next day, when melanoma cells had already adhered to the plastic surface, the cell suspension, including TIL, was in some cases removed and further purified with ficoll gradient. The purified bulk TIL culture was returned to the melanoma-containing plates and maintained at a concentration of $0.5\text{-}2 \times 10^6$ cells/mL in complete medium with 3,000 IU/mL rhIL-2 until all melanoma cells were eliminated and a cell number of at least 50×10^6 TIL was achieved. This process required about 10 to18 d. Short-term cultured TIL were then immediately cryopreserved or used directly for further large-scale expansion.

A total of 30 to 60×10^6 Young-TIL were expanded to treatment levels in a rapid expansion procedure (REP) by using anti-CD3 antibody (Orthoclone OKT-3, Cilag), 3,000 IU/mL rhIL-2, and irradiated feeder cells, as previously described (19–21). Within 2 wk, cultures expanded by ~1,000-fold to a final volume of 20 to 60 liters of medium. On day 14 of the large-scale expansion, culture

medium was reduced drastically by using a COBE Spectra apheresis machine (Gambro BCT; ref. 22) and the cells were infused into the lympho-depleted patient by i.v. administration.

Immunologic assays. TIL phenotype was determined by fluorescence-activated cell sorting analysis using antibodies against CD4 (FITC conjugated), CD8 (APC), CD27 (PE), and CD28 (Per-Cy5; eBioscience). Cells were sampled directly from the infusion bag. CD27 surface expression was furthermore tested after incubating TIL in complete medium without rhIL-2 for 48 hours (17). IFNγ release was examined after an overnight coincubation with autologous or HLA-A2–matched melanoma lines as previously described (10, 19). The concentration of secreted IFNγ was determined by ELISA according to the manufacturer's instructions (Pierce/Endogen).

Statistical analysis. Significance of variation between groups was evaluated using a nonparametric two-tailed Student's t test. Test for differences between proportions was done using two-sided Fisher's exact test with $P \leq 0.05$ considered significant and $P \leq 0.001$ highly significant. To strengthen the trend of dependence between two kinds of variables, we used the Spearman rank correlation coefficient.

Results

Patient characteristics. Between November 2007 and August 2009, 27 patients with assessable metastatic melanoma were enrolled to the study. Six patients (20.1%) were HLA-A*0201 positive. From 24 of 27 patients (89%), Young-TIL cultures were successfully established. The clinical performance status of three patients deteriorated drastically during TIL preparation and they were excluded from the trial. One more patient decided not to enter the study, resulting in a total drop out of 25.9% (7 of 27 patients). In comparison, ACT studies using exclusively TIL cultures that secrete IFN-γ after the coincubation with autologous

Table 2. Treatment characteristics during TIL therapy and clinical outcome

Patient	Age/sex	PS	Lactate dehydrogenase	Stage	Site of biopsy*	Evaluable metastasis	IL-2 doses	Resp.	PFS (mo)	OS (mo)
Responders ($n = 10$)										
05-LA	41/M	0	Normal	M1a	SC	SC nodules	10	CR	20[†]	20[†]
19-NS	66/M	1	Normal	M1c	Perito.	Peritoneum	3	CR	4[†]	4[†]
03-MG	36/M	0	Normal	M1c	LN	Soft tissue, lung, bone	15	PR	9	21[†]
06-TS	60/M	0	Normal	M1b	Lung	Lung	5	PR	18[†]	18[†]
09-SD[‡]	45/M	0	Normal	M1b	LN	Lung	7	PR	13[†]	13[†]
13-BS	61/M	0	Normal	M1b	Lung	Lung	9	PR	10[†]	10[†]
14-SV[‡]	71/M	0	Above	M1a	SC	SC, LN	9	PR	3	9[†]
16-SH	41/M	1	Normal	M1c	SC	Liver, adrenal, lung, LN	8	PR	6[†]	6[†]
18-WR	70/F	0	Normal	M1a	LN	SC, LN	8	PR	4	4[†]
20-TY	58/M	0	Normal	M1a	SC	SC, LN	7	PR	3[†]	3[†]
Median							8.1 ± 3.2		(7.3)	(9.3)
Nonresponders ($n = 10$)										
01-AY	56/M	0	Normal	M1c	Lung	Lung, SC, bone	9	SD	11	17
07-ZR	22/M	0	Normal	M1b	Lung	Lung	7	SD	3	15
08-RM	34/F	0	Normal	M1c	Liver	Liver	14	SD	5	6
12-VS[‡]	41/F	0	Normal	M1a	SC	SC, LN	11	SD	11[†]	11[†]
02-PE	36/M	0	Above	M1c	LN	LN, adrenal, periton.	9	PD	2	3
04-BA[‡]	57/M	0	Normal	M1c	Lung	LN, lung, adrenal,	13	PD	3	20[†]
10-BE	53/F	0	Normal	M1c	LN	SC, LN, adrenal	6	PD	1	5
11-KB	57/M	1	Above	M1c	LN	Lung, LN	6	PD	1	5
15-SM[‡]	52/M	1	Above	M1c	Liver	Bone, liver	10	PD	1	3
17-ZD[‡]	68/F	1	Above	M1c	Pleura	Lung, pleura, bone	5	PD	1	2
Median							9.0 ± 3.1		2.7	5.7
							$P = 0.53$			

Abbreviations: PS, performance status according to ECOG scale; Resp., best objective clinical response according to the RECIST criteria; PFS, PFS measured from day of TIL infusion; OS, OS measured from day of TIL infusion; LN, lymph node.
*Site of tumor sample for TIL harvest.
[†]Ongoing.
[‡]Patients with HLA-A*0201.

or HLA-matched melanoma lines have, in general, a drop out rate of over 60% (10).

All 20 treated patients had a good performance status according to the ECOG scale (ECOG 0 or 1) and 15 patients (75%) had stage M1b or M1c disease. Patients possessed multiple metastatic lesions, of which at least one was resected for the generation of Young-TIL cultures.

Clinical results and treatment characteristics. Ten (50%) of the 20 treated patients experienced an OR, including two complete and eight partial remissions. Four patients showed disease stabilization (SD); three of them had a minor response, although not qualifying them to be defined as partial responders. Six patients progressed after treatment (PD).

The characteristics of the responding (CR + PR) and nonresponding (SD + PD) patients previous to TIL therapy are summarized in Table 1. The median time from discovery of the primary lesion to ACT was 3.0 years in the responding group of patients and 4.8 years for nonresponders ($P = 0.58$). Nineteen of the treated patients received chemobiotherapy alone or chemobiotherapy in addition to surgery or further chemotherapy before TIL ACT (Table 1). Chemobiotherapy consisting of IL-2, dacarbazine, and cisplatin is the standard of care treatment in most Israeli cancer centers. One patient received prior high-dose bolus IL-2 therapy. In both groups, two patients achieved an OR to prior IL-2–based therapy and there wasno significant difference in their time to progression ($P = 0.47$), showing that there is no correlation between prior IL-2–based therapy and the clinical outcome to TIL ACT. All patients had disease progression or disease recurrence after the IL-2–based therapy (Table 1) and were then enrolled to the TIL ACT study.

Table 2 shows the treatment characteristics during TIL therapy. Young-TIL ACT is not restricted to any HLA genotype and the number of HLA-A*0201 patients was insignificantly different between responders and nonresponders ($P = 0.63$; Table 2).

There is a trend toward less favorable responses in M1c patients or patients with abnormally high lactate dehydrogenase levels, as 8 of 11 M1c patients and 4 of 5 patients with high lactate dehydrogenase did not respond. This trend was not statistically significant ($P = 0.07$ and $P = 0.21$, respectively). It should be emphasized that three M1c patients achieved an objective clinical response; one of them even achieved complete remission. Therefore, M1c patients can definitely benefit from Young-TIL ACT.

Tumor samples for TIL preparation were harvested from various anatomic sites (Table 2). If patients had numerous metastatic lesions within one organ, usually a single tumor lesion was removed for the purpose of TIL establishment. There was no correlation between biopsy sites and successful establishment of TIL cultures or clinical outcome (data not shown). The average number of IL-2 doses following TIL administration was 8.55 ± 3.0, with no difference between responders (8.1 ± 3.2 doses) and nonresponders (9.0 ± 3.1 doses, $P = 0.53$). Thus, the clinical outcome is not the reflection of IL-2 supportive treatment.

Fifty percent of our refractory melanoma patients objectively responded to Young-TIL therapy. Regression of metastatic lesions was observed in various anatomic sites, including lung, lymph node, soft tissue, gastrointestinal tract, and liver (Supplementary Fig. S1). Two patients (19-NS and 05-LA) achieved complete remission. CR patient 19-NS was staged M1c before Young-TIL administration and suffered from metastasis in the large bowel, which completely disappeared after treatment (Supplementary Fig. S1F-G). The second CR patient had M1a disease involving multiple s.c. lesions. Both patients have still ongoing responses after 4 and 20 months, respectively.

Eight patients, including five M1b/M1c patients, experienced partial remission. The PFS was 18+, 13+, 10+, 9, 6+, 4, 3+, and 3 months (Table 2). All patients were treated with a single course of Young-TIL ACT, except for patient 03-MG. Patient 03-MG experienced after the first course a PR in the lung, axillary lymph nodes, and s.c. tissue of the left chest wall (Supplementary Fig. S1A-B). The patient relapsed 9 months later (Table 2). A new tumor sample was resected; a fresh Young-TIL culture was generated; and he received a second full course of ACT with 10 doses of IL-2. The patient achieved again a PR in his lung and soft tissue masses (data not shown).

A total of seven patients have ongoing responses (PFS, 20+, 18+, 13+, 10+, 6+, 4+, and 3+ months; Table 2). To date, all clinical responders are alive. The median PFS and OS in the responding group of patients is currently 7.3 and 9.3 months, respectively (Table 2). Those numbers are not final, as most responses are continuing and all responders are still alive.

Among the nonresponders, four patients experienced SD. Three of them (patient 01-AY, 08-RM, and 12-VS) had minor responses, including one patient with multiple liver metastases (08-RM). The remaining tumor mass of patient 12-VS was surgically resected 2.5 months after TIL therapy and this patient has currently no evidence of disease. The other three SD patients progressed in the meantime (PFS, 11, 3, and 5 months) and died of disease (OS, 17, 15, and 6 months; Table 2).
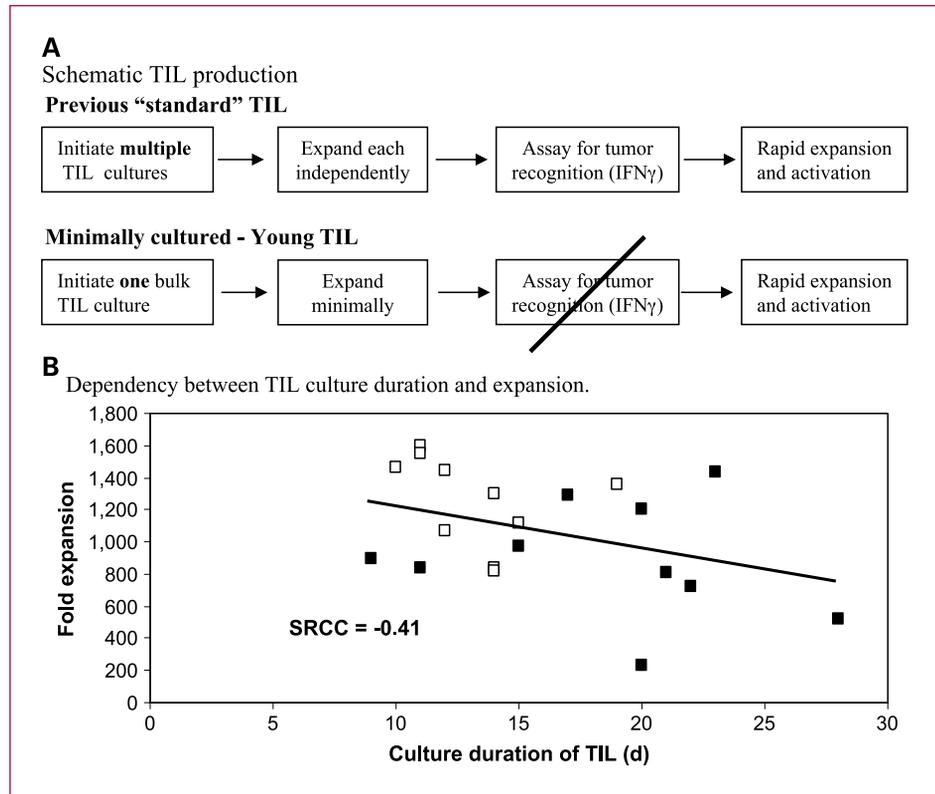
Of six patients who progressed immediately after treatment, five died of disease within 3.6 months (range, 2-5 months). Only one PD patient (04-BA) is still alive after 20 months (Table 2). The median PFS and OS in nonresponding patients were 2.7 and 5.7 months, respectively (Table 2).

As the median follow-up is comparable ($P = 0.24$) between the responding (9.3 months) and nonresponding group of patients (11.4 months), it is clear that responders have a benefit in PFS and OS. All 10 responders are still alive, whereas 8 of the nonresponders have died by now.

TIL characteristics. The major differences between the previous Specific-TIL generation and our modified Young-TIL protocol are schematically presented in Fig. 1A. Young-TIL cultures were established by slicing the resected tumor tissue into small pieces of 2 to 3 mm^3 (10, 16). Enzymatic

Fig. 1. TIL Production.
A, schematic TIL production.
Comparison of the previous
selected TIL production process
and the modified Young-TIL
protocol. B, dependency between
TIL culture duration and expansion.
X-axis, age (days) of Young-TIL
culture on the day of REP initiation;
Y-axis, fold expansion during
the 14-d rapid expansion process.
□, responder; ■, nonresponder;
SRCC, Spearman rank correlation
coefficient.

A
Schematic TIL production
Previous "standard" TIL

| Initiate **multiple** TIL cultures | → | Expand each independently | → | Assay for tumor recognition (IFNγ) | → | Rapid expansion and activation |

Minimally cultured – Young TIL

| Initiate **one** bulk TIL culture | → | Expand minimally | → | Assay for tumor recognition (IFNγ) | → | Rapid expansion and activation |

B Dependency between TIL culture duration and expansion.

SRCC = -0.41

digestion of the pieces was usually done within 1 to 3 hours after surgery for ~2 hours at 37°C. The washed single-cell suspension was plated at a concentration of $1 \times 10e6$ TIL/mL in IL-2–containing complete medium (18). If cell cultures obtained >80% melanoma cells, the TIL-containing cell suspension was simply separated from adherent melanoma cells by transfer to new wells. In case that erythrocyte counts were at least 10 times more than TIL counts, or in the presence of massive cell debris, ficoll separation was done. TILs $(0.5-2.0 \times 10e10)$ were maintained in IL-2–containing complete medium until all melanoma cells were eliminated and a minimal TIL number of $50 \times 10e10$ was achieved.

Tumor samples from 27 metastatic melanoma patients were harvested. By using the mentioned method, Young-TIL from 89% (24 of 27) of the patients were established within 14.2 ± 4.6 days (range, 9-28 days). Young-TIL cultures were either cryopreserved or used directly for further large-scale expansion. Twenty of those cultures were expanded to treatment levels. TIL were administered at an average of 68 days after surgery, with no difference ($P = 0.550$) between responding (74.4 ± 63.6 days) and nonresponding patients (61.4 ± 22.5 days; Table 3). The age of the Young-TIL cultures on the day of REP initiation are listed in Table 3. Interestingly, there was a significant difference ($P = 0.013$) between the TIL age of responding (13.2 ± 2.6 days) and nonresponding (18.6 ± 5.7 days) patients. TIL cultures from all responding patients entered the rapid expansion phase before day 20, compared with six nonresponding patients whose TILs were at least 20 days

old ($P = 0.01$), verifying that a shorter culture duration is beneficial to achieve a clinical response. Furthermore, Young-TIL of responders showed a significantly higher expansion rate during REP [1,255 ± 282 (OR) versus 892 ± 361 (nonresponder); $P \leq 0.022$]. Consequently, more cells were administered to responders (57.0 ± 18.1 × 10e9) compared with nonresponders (36.5 ± 17.5 × 10e9 cells; $P \leq 0.025$; Table 3). Although there is only a weak inverse correlation between TIL-youth and its fold expansion (Spearman rank correlation coefficient, −0.41; $P = 0.09$; Fig. 1B), both parameters are important to achieve an OR.

To evaluate other basic TIL characteristics, we performed fluorescence-activated cell sorting analysis and IFNγ ELISA after antigenic stimulation. CD8 frequency strongly varied between different patients (range, 2.1-94.0%; Table 3). There was no difference between the responding and nonresponding group in their CD27 and CD28 frequencies measured from cells sampled directly from the infusion bag or after 2 days of IL-2 withdrawal (data not shown). The final infusion product consisted exclusively of CD3 T cells (data not shown). T cells were either CD8 or CD4 positive. Although the average CD8 frequency was comparable in responding and nonresponding patients ($P = 0.370$), the total number of infused CD8 cells was significantly higher in the responding group ($P = 0.047$; Table 3).

IFNγ secretion after an overnight coincubation of Young-TIL and autologous melanoma cells was conducted retrospectively, as specific IFN-γ production over

200 pg/mL is the essential acceptance criteria in previous TIL protocols (Fig. 1A; refs. 8–10). If the TIL cultures were generated from HLA-A*0201–positive patients, HLA-A2–matched melanoma lines could be used instead of autologous melanoma for coculture. As shown in Table 3, we were unable to perform coculture from four of our responders, as those patients were HLA-A2 negative and no autologous melanoma line was available. Young-TIL cultures of one additional responder secreted only 36 pg/mL IFNγ. In other words, had we applied the IFNγ secretion criteria, half of our responders would not have been treated.

Although not statistically significant ($P = 0.44$), IFNγ secretion seemed to be lower in the nonresponding patient group. TIL cultures not secreting any IFNγ were mostly among nonresponders, although TIL from the second ACT treatment of partial responder 03-MG did not secret IFNγ as well (data not shown; $P = 0.26$).

Our data suggest that IFNγ secretion upon antigenic stimulation can serve as a vague predictor of response, but should certainly be excluded as an acceptance criterion.

Toxicity to treatment. All patients received nonmyeloablative chemotherapy with cyclophosphamide and fludarabine before Young-TIL infusion and high-dose bolus IL-2 after cell administration. None of the patients underwent total body irradiation. Patients were hospitalized 1 day prior chemotherapy and stayed for an average of 19.8 days (OR, 19.0 ± 1.2 days; nonresponder, 20.7 ± 3.5 days; Table 4). Patients were released from the hospital, upon recovering from toxicity and showing increasing blood counts.

Hematologic toxicity associated with nonmyeloablative chemotherapy was as expected and transient (Table 4). Hematologic toxicities were similar in responders and nonresponders, and comparable with previous TIL ACT studies. Patient conditioning with chemotherapy before TIL transfer results in a drop of neutrophil and lymphocyte

Table 3. TIL characteristics

Patients	Resp.	Surgery to ACT (d)*	TIL age at REP (d)	Fold expans.	Cells ×10⁹	% CD8[†]	Total CD8 (×10⁹)	IFN-γ (pg/mL)[‡]
Responders								
05-LA	CR	44	19	1,360	68	90.6	62	36
19-NS	CR	232	12	1,070	37	71.9	27	2,654
03-MG	PR	83	15	1,112	76	90.3	69	676
06-TS	PR	36	10	1,460	70	79.1	55	No target
09-SD[§]	PR	26	12	1,446	69	55.6	38	7,830
13-BS	PR	36	11	1,596	68	81.3	55	No target
14-SV[§]	PR	63	14	1,300	26	22.9	6	1,035
16-SH	PR	43	11	1,554	75	85.9	64	4,347
18-WR	PR	48	14	833	40	20	8	No target
20-TY	PR	133	14	822	38	72.3	27	No target
Average		74.4 ± 63.6	13.2 ± 2.6	1,255 ± 282	57.0 ± 18.1	67.0 ± 26.1	41.2 ± 23.2	
Nonresponders								
01-AY	SD	76	22	720	31	67.9	21	0
07-ZR	SD	56	20	1,200	60	77.8	47	No target
08-RM	SD	51	17	1,290	18	50.8	9	No target
12-VS[§]	SD	104	11	833	40	64.2	26	8,955
02-PE	PD	69	21	813	30	94	28	Nonspec.
04-BA[§]	PD	86	9	900	39	64.3	25	116
10-BE	PD	59	15	975	38	54	21	0
11-KB	PD	37	23	1,436	72	61.4	44	0
15-SM[§]	PD	34	20	230	11	26.6	3	412
17-ZD[§]	PD	42	28	521	24	2.1	1	0
Average		61.4 ± 22.5	18.6 ± 5.7	892 ± 361	36.5 ± 17.5	56.3 ± 25.9	22.4 ± 15.4	
		$P = 0.550$	$P = 0.013$	$P = 0.022$	$P = 0.025$	$P = 0.370$	$P = 0.047$	

Abbreviations: Resp., best objective response according to the RECIST criteria.

*Days from the tumor harvest to TIL infusion.

[†]% CD8⁺ cytotoxic T cells in the infusion bag, all the other cells are CD4 T helper cells.

[‡]IFNγ secretion (pg/mL) after coculture with melanoma cells as target.

[§]Patients with HLA-A*0201.

Table 4. Time in hospital and grade 3 and 4 toxicities

	Responders $n = 10$	Nonresponders $n = 10$
Days in hospital* (average)	19.0 ± 1.2 (17-21)	20.7 ± 3.5 (16-27)
Chemotherapy-related toxicity		
Units RBC transfusion (median)	5 (0-8)	5 (1-12)
Units platelet transfusion (median)	30 (6-30)	30 (0-114)
Febrile neutropenia	9	10
Opportunistic infection (H. Zoster)	0	1
IL-2–related toxicity		
Pulmonary congestion	4	3
Renal failure	4	1
Prolonged hypotension	2	1
Hyperbilirubinemia	2	2
Diarrhea	2	2
Confusional state	0	1
Autoimmunity	1	0

NOTE: Toxicities according to National Cancer Institute Common Toxicity criteria.

*Measured from the day of chemotherapy administration to discharge.

counts to nearly zero on the day of TIL and IL-2 administration. Figure 2 shows lymphocyte, neutrophil, and platelet counts before, during, and 1 month posttherapy. Day 0 represents the day of TIL administration. All patients recovered similarly from chemotherapy with no difference between responders and nonresponders. All except for one responder required RBC transfusion (median, 5; range, 0-12). Thrombocytopenia requiring platelet transfusion was seen in all but one patient (median, 5; range, 0-114). Only one nonresponder experienced an opportunistic infection with herpes zoster and recovered quickly. Neutropenia, lymphopenia, and extended depression of CD4 lymphocytes were observed in all patients. Febrile neutropenia, caused by chemotherapy, developed in 19 patients but was promptly resolved upon antibiotic therapy. Recovery of bone marrow was typically achieved 2 to 3 weeks after cell transfer. No patient required administration of the granulocyte colony-stimulating factor–mobilized stem cells banked before treatment. A single cycle of high-dose IL-2 was sufficient to achieve a clinical response, which was often observed already 4 weeks after TIL administration (data not shown). As the bone marrow, including lymphocytes, recovered after 2 weeks, additional cycles of IL-2 might be of disadvantage as IL-2 activates also nonspecific and regulatory T cells.

One or two nonhematologic grade 3 or 4 toxicities emerged in all but one (08-RM) patient. Nonhematologic toxicities were associated with high-dose bolus IL-2 administration and included pulmonary congestion ($n = 7$), renal failure ($n = 5$), prolonged hypotension ($n = 3$), hyperbilirubinemia ($n = 4$), diarrhea ($n = 4$), and confusional state ($n = 1$; Table 4). Frequencies were similar in both groups and toxicities were manageable. One responder developed autoimmune vitiligo. Due to the occurrence of IL-2–related

grade 3 or 4 toxicities in 19 of 20 patients, IL-2 administration was discontinued before achieving the maximum of 15 doses (Table 2). The average number of IL-2 doses was comparable between responders and nonresponders ($P = 0.53$; Table 2).

No grade 3 or 4 toxicities associated directly to the cell administration were observed and there was no treatment-related death.

On the whole, toxicities associated with Young-TIL ACT were similar to previous ACT therapies, in which TIL were administrated in combination with nonmyeloablative chemotherapy and high-dose bolus IL-2. All grade 3 and 4 toxicities were predictable and therefore well manageable.

Discussion

The treatment of metastatic melanoma patients with autologous TIL in combination with lympho-depleting chemotherapy and high-dose IL-2 has previously shown highly promising results (8, 9). Nevertheless, the major problem with previous TIL applications is that they are labor intensive and require high laboratory expertise. In previous TIL protocols, multiple TIL cultures were individually generated for each patient. Of those numerous cultures, only those secreting IFNγ upon coincubation with melanoma cells were eligible for treatment. This selection process is not only labor intensive but leads to a drop out of at least half of the enrolled patients, as often the appropriate tumor target is unavailable or TIL do not secret IFNγ.

We used a modified TIL production technique that is much less labor intensive and nullifies the IFNγ screening process, which has never been proven significant to

clinical response. Instead, we incorporated another important parameter into our new protocol: the culture duration. Short culture times and in-accordance telomere length were repeatedly reported to have a significant positive association with clinical response (11–15).

Young-TIL are established from one single bulk T-cell culture and spend minimal time in culture.

Additional advantages of this technique is that the TIL production of a single culture is obviously much cheaper than those of multiple cultures and that there is no HLA restriction, making it applicable for many patients.

We could show that Young-TIL cultures were generated for ~90% of our enrolled patients.

Twenty patients were finally treated with Young-TIL ACT. Fifty percent (10 of 20 patients) responded, including two complete and eight partial remissions. Responses were observed at various anatomic sites, including lung and visceral metastasis. Seven of our 10 responders have still ongoing responses (PFS, 20+, 18+, 13+, 10+, 6+, 4+, and 3+ months). To date, all clinical responders are alive with a median survival of 10 months.

We could verify the significant correlation between short culture duration and clinical outcome. All objective responders received Young-TIL cultured <20 days before entering the rapid expansion phase. Furthermore, responding patients received a significantly higher number of Young-TIL and CD8$^+$ Young-TIL in particular. Both TIL youth and high TIL number are critical parameters to achieve a clinical response.

All toxicities were anticipated and transient. Patients were hospitalized an average of 19.8 days, including 2 days of cyclophophamide and 5 days of fludarabine administration before TIL infusion. As fludarabine is given in most cancer centers ambulatory, we intend to adopt this approach and thereby reduce the days of hospitalization to 14.8 days.

There was no treatment-related mortality and the toxic effects were comparable with previous TIL studies.

ACT technology is a platform with high potential that can even further be improved. Future trials should explore the combination of Young-TIL ACT with low-dose s.c. IL-2 administration to simplify the clinical setting of this technology or the addition of whole body irradiation to Young-TIL ACT. Total body irradiation was shown to augment lympho depletion and thereby increases the response rate to TIL therapy (9). Most of all, this study should
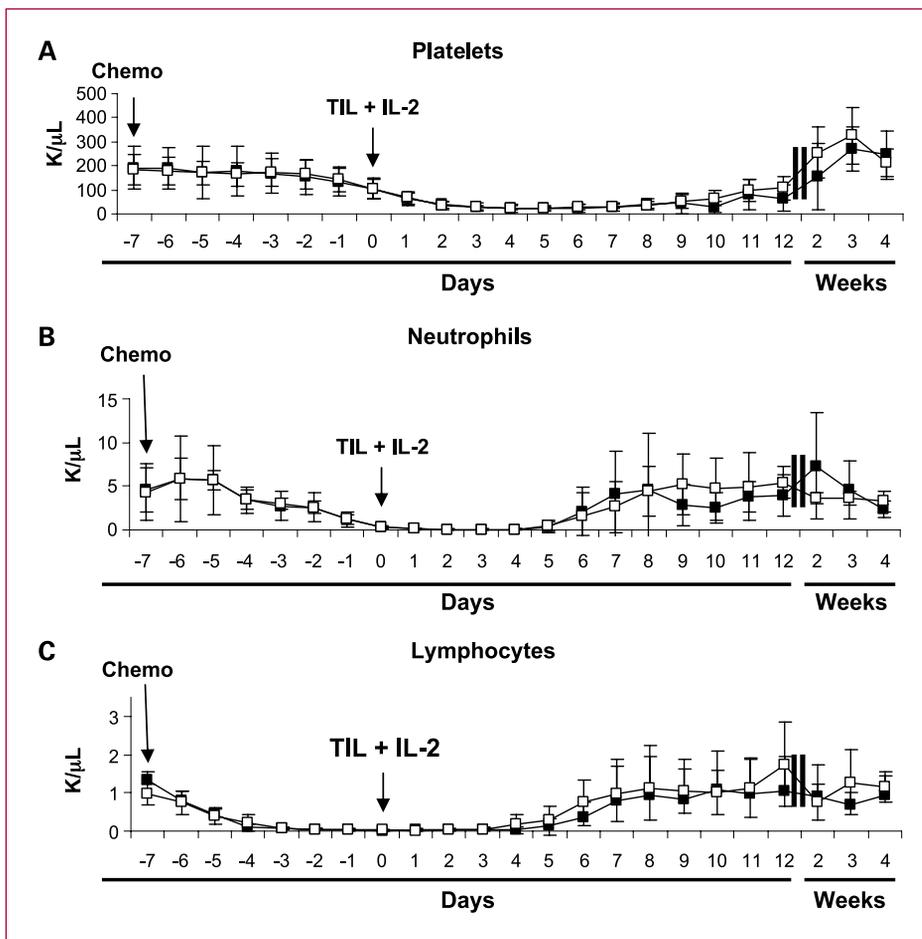
Fig. 2. Bone marrow recovery. Patients received nonmyeloablative lympho-depleting chemotherapy (chemo; days −7 to −1) before TIL infusion (day 0). A, platelet; B, neutrophil; C, lymphocyte counts. □, responder; ■, nonresponder.

encourage other cancer centers to participate in a multicenter randomized phase III study exploring the efficacy of Young-TIL ACT versus standard of care treatment. Such a study would finally reveal the true clinical contribution of TIL-ACT for the treatment of metastatic melanoma patients.

In summary, we could show that lympho-depleting chemotherapy followed by the transfer of nonselected, short-term cultured TIL can mediate tumor regression in 50% of refractory metastatic melanoma. Toxicities were well manageable. The convincing clinical results combined with the simplification of the process may thus have a major effect on cell therapy of melanoma patients.

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