Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Cancer Therapy: Clinical

Clinical Cancer Research

Long-Lasting Complete Responses in Patients with Metastatic Melanoma after Adoptive Cell Therapy with Tumor-Infiltrating Lymphocytes and an Attenuated IL2 Regimen

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Abstract

Purpose: Adoptive cell transfer therapy (ACT) based on autologous tumor-infiltrating lymphocytes (TIL) has achieved impressive clinical results in several phase I and II trials performed outside of Europe. Although transient, the toxicities associated with high-dose (HD) bolus IL2 classically administered together with TILs are severe. To further scrutinize whether similar results can be achieved with lower doses of IL2, we have carried out a phase I/II trial of TIL transfer after classical lymphodepleting chemotherapy followed by an attenuated IL2 regimen.

Experimental Design: Twenty-five patients with progressive treatment-refractory metastatic melanoma, good clinical performance, age < 70 years, and at least one resectable metastasis were eligible. TIL infusion was preceded by standard lymphodepleting chemotherapy and followed by attenuated doses of IL2 administered in an intravenous, continuous decrescendo regimen (ClinicalTrials.gov Identifier: NCT00937625).

Results: Classical IL2-related toxicities were observed but patients were manageable in a general oncology ward without the need for intervention from the intensive care unit. RECIST 1.0 evaluation displayed three complete responses and seven partial responses (ORR 42%). Median overall survival was 21.8 months. Tumor regression was associated with a higher absolute number of infused tumor-reactive T cells. Moreover, induction and persistence of antimelanoma T-cell responses in the peripheral blood was strongly correlated to clinical response to treatment.

Conclusions: TIL-ACT with a reduced IL2 decrescendo regimen results in long-lasting complete responses in patients with treatment-refractory melanoma. Larger randomized trials are needed to elucidate whether clinical efficacy is comparable with TIL-ACT followed by HD bolus IL2. *Clin Cancer Res; 22(15); 3734–45. ©2016 AACR.*

Introduction

Adoptive cell transfer therapy (ACT) based on infusion of autologous tumor-infiltrating lymphocytes (TIL) has shown impressive results in patients with metastatic melanoma. The efficacy of this personalized immunotherapy based on preconditioning chemotherapy followed by infusion of TILs and IL2 has been confirmed by several independent centers. Objective response rates of 40%–50% including complete tumor regression in 10%–20% of treated patients have consistently been reported (1–4). The major advantage of TIL-ACT over current standard treatments is the relative high frequency and long-term durability of complete responses, highlighting the curative potential of this treatment.

Despite these remarkable results, TIL-ACT has never been established as a standard treatment; only few centers around the world have applied TIL-ACT and mostly as a salvage treatment after failure of standard therapies, including monotherapy with standard high-dose (HD) IL2. The complexity of TIL production and severe treatment-associated toxicity are obvious boundaries for a more widespread application. The preparative lymphodepleting chemotherapy contributes markedly to treatment-related toxicity but is necessary for achievement of durable clinical responses (5–10). Previous studies showed that high persistence of the infused cells was achieved only after implementation of lymphodepletion prior to TIL transfer. Even though the cellular basis of the effect of lymphodepletion is complex and still not fully understood, it is now considered essential to achieve consistent efficacy (11, 12).

Major toxicities resulting from current ACT regimens are related to HD IL2 administered after cell transfer. IL2 is administered to support *in vivo* proliferation and persistence of the infused cells. Monotherapy with HD bolus IL2 of 600,000 to 720,000 IU/kg

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-15-1879

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Translational Relevance

Adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TIL) has the potential to cure a significant fraction of patients with treatment-refractory metastatic melanoma. However, the toxicities associated with high-dose (HD) bolus IL2 classically administered after TIL infusion are severe and represents a major limitation for a more widespread use of TIL-ACT. In this study, TIL-ACT with a continuous decrescendo IL2 regimen resulted in long-lasting clinical responses associated with induction and persistence of tumor-reactive T cells in the blood for over one year. Classical IL2–related toxicities were observed but manageable in a general oncology ward without the need for intervention from intensive care units. Although this trial included a limited number of patients, our results indicate that long-lasting complete tumor regression can be obtained with ACT also in the absence of HD bolus IL2 and the associated side effects.

every 8 hours up to a maximum of 14 doses was initially approved in 1998 by the FDA for the treatment of metastatic melanoma based on a low but consistent fraction of patients achieving durable complete responses (13). Because of the significant toxicities associated with HD bolus IL2 and a well-known dose–toxicity relationship (14), various doses and schedules of IL2 administration were evaluated in the previous decades in an attempt to reduce toxicity without compromising efficacy (15). These studies suggested a dose–response relationship for IL2 in favor of HD bolus IL2 for metastatic melanoma (15); however, larger randomized studies comparing different IL2 regimens were not performed. Accordingly, the HD bolus IL2 regimen was used in the early ACT trials (9, 16) where cells were infused without prior lymphodepletion. Since the implementation of lymphodepleting chemotherapy, most ACT-TIL trials have also used the HD bolus IL2 regimen and the efficacy of this three-step treatment has been proven in several phase II trials (1–4). However, different doses and schedules of IL2 administered in this context have only been sparsely explored. Thus, whether the HD bolus IL2 is necessary for achievement of durable complete responses remains currently unknown.

The decrescendo regimen described by Keilholz and colleagues (17), consists of continuous infusions of IL2 with a decreasing dose over time: 18 MIU/m^2 over 6, then 12, and then 24 hours followed by 4.5 MIU/m^2 over 24 hours for 3 consecutive days. This regimen was evaluated in a small randomized phase III trial that demonstrated improved clinical response and reduced cumulative IL2 toxicity compared with a continuous IL2 plus IFNα regimen (18). The initial high dose of IL2 is administered with the aim to saturate the α-chain of the IL2 receptor on lymphocytes for optimal signaling and subsequently taper to a lower maintenance dose to reduce the induction of TNF and associated toxicities (19, 20).

We recently published the results from a small pilot study in which 6 patients received classical lymphodepleting chemotherapy and TIL transfer followed by a low-dose subcutaneous (s.c.) IL2 regimen consisting of 2 MIU/day for 14 days (21). Two of these patients achieved complete, long-lasting responses suggesting that high-dose IL2 may not be required for obtaining durable clinical responses (21). Here we report the results from a clinical trial where 25 patients with metastatic melanoma were sequentially treated with TIL-ACT preceded by lymphodepleting chemotherapy and followed by an attenuated dose of IL2 administered in a continuous decrescendo regimen.

Materials and Methods

Patients

Patients ages 18–70 years with AJCC stage IV or unresectable stage III metastatic melanoma, good clinical performance, and a life expectancy of at least 3 months were eligible for the study. Main exclusion criteria were uveal melanoma, symptomatic brain metastases, severe comorbidities, active autoimmune disease, and chronic infections. All patients had a resectable metastatic lesion suitable for TIL production, and in addition at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 (22). The Ethical Committee of the Capital region of Denmark, the Danish Data Protection Agency, and the Danish Medical Agencies (EudraCT no. 2008-008141-20) approved the study and all patients signed a written consent form. The trial was conducted in accordance with the Helsinki declaration and Good Clinical Practice (GCP) and monitored by the GCP-unit, Copenhagen, Denmark (ClinicalTrials.gov ID NCT00937625). Patients were enrolled and treated at the Department of Oncology, Herlev Hospital, University of Copenhagen (Herlev, Denmark).

Study design

Primary endpoints were safety and feasibility; secondary endpoints were objective response rate (ORR), overall survival (OS), progression-free survival (PFS), and immunologic response.

All patients received lymphodepleting chemotherapy consisting of cyclophosphamide 60 mg/kg/day for 2 days (day -7 and -6) followed by fludarabine 25 mg/m^2/day for 5 days (day -5 to -1) as previously described (21). On day 0 all patients received a bolus infusion of TIL followed by a continuous IL2 infusion administered in a decrescendo regimen (18 MIU/m^2 over 6, 12, and 24 hours followed by 4.5 MIU/m^2 over 24 hours for 3 days) as previously described by Keilholz and colleagues (17). IL2 infusion was started within 12 hours after the TIL infusion. Maximum total dose of IL2 administered was limited to 135 MIU, corresponding to a body surface area of 2 m^2. All patients received prophylactic antibiotics and antiemetics as described previously (21). To reduce the neutropenic period and time in hospital, a single subcutaneous injection of G-CSF was administered on the day of TIL infusion. Patients were monitored daily during hospitalization and received platelet (PLT) or red blood cell (RBC) transfusions when clinically indicated. Blood samples for immunologic analyses were collected the day before chemotherapy, approximately one week and one month after TIL infusion, and subsequently at time points related to clinical assessments. Treatment and monitoring schedule is outlined in Supplementary Fig. S1.

Toxicity was assessed using Common Terminology Criteria for Adverse Events (CTCAE version 3.0). Clinical efficacy was assessed using Fluor-18-deoxyglucose-positron emission tomography/computed tomography (FDG-PET/CT) scans before treatment, after 8 and 16 weeks, and thereafter approximately every third month until disease progression.

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Objective responses (OR) were categorized into complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to RECIST version 1.0. Surgical resection of few residual lesions was considered in patients who achieved at least a PR.

Transient tumor regression followed by tumor progression has been previously reported in other ACT trials in melanoma and related to the mechanism of action of the infused T cells (23). This phenomenon may be related to the short-lived nature of some specific T-cell subsets infused, which may indeed induce initial (major or minor) regression while other subsets may be responsible for the propagation and maintenance of response (reviewed in ref. 24). Thus, to inform more in detail on mechanistic effects of ACT, in some analyses, patients were additionally stratified in two groups: best decrease in the sum of target lesions plus the sum of eventual new target lesions (as in the immune related response criteria; ref. 25) of at least 20% from baseline: this group is indicated as "best reduction in target lesion size from baseline >20%"; and patients not qualifying for this group: indicated as "no significant tumor regression."

Generation of TIL

Melanoma lesions of at least 1 cm^3 were surgically resected and cut into 1–2 mm^3 fragments as described previously (26). In brief, up to 48 fragments were placed in individual wells in 24-well culture plates (Nunc) with 2 mL culture medium [90% RPMI1640, (Invitrogen), 10% heat-inactivated human AB serum (Sigma-Aldrich), IL2 6,000 IU/mL (Aldesleukin, Novartis), penicillin, streptomycin, and fungizone (Bristol-Myers Squibb)].

TIL cultures were expanded *in vitro* in two major steps: the pre-Rapid Expansion Procedure (Pre-REP; ref. 26) and a dynamic expansion in REP (27). TIL microcultures from all fragments were pooled to one bulk culture, according to the "young TIL" method (28, 29) and the pooled pre-REP TIL culture was either cryopreserved or entered directly into the REP. Approximately, 20×10^6 cells were used to initiate the REP, during which the TILs were expanded to therapeutic levels in a large scale expansion procedure with the Wave Bioreactor 2/10 system (GE Healthcare) as described previously (27). At day 14, the cells were harvested and transferred to an infusion bag in a suspension of 250 mL sodium chloride with 2.5% albumin and 300 IU/mL IL2. Within 30 to 45 minutes, the cells were administered to the patient intravenously over 30 minutes. Sterility testing and microbiologic control was performed on all TIL cultures before REP or cryopreservation and before infusion.

Immunologic analyses

For phenotype characterization of TIL infusion products, the cells were stained with the following fluorochrome-conjugated antibodies: CD3, CD4, CD8, pan-γδ TCR (BD Biosciences), and acquired on a BD FACSCanto II.

TILs and peripheral blood lymphocytes (PBL) obtained before and after treatment were tested for reactivity against autologous melanoma cell lines or allogeneic HLA-A–matched melanoma cell lines in coculture (antitumor reactivity) assays. Typing at the HLA-A locus was performed at Herlev Hospital by standard PCR on selected HLA-A alleles.

All melanoma cell lines were established internally at our laboratory. Melanoma cell lines were identified from their morphology and *in vitro* growth patterns. In some cases, when morphology or growth patterns were not typical, the melanocyte origin of the continuously growing cell lines was confirmed by PCR for melanocyte antigens. The cell lines were not otherwise authenticated. Autologous cell lines were established from the same lesion where TILs were obtained from by serial passage of adherent cells in RPMI1640 supplemented with 10% FBS and 500 ng/mL of Solu-cortef, as described previously (26). In two cases, one patient with CR (M42) and one patient with no evidence of tumor regression (M40) where autologous melanoma cell lines were not available, single-cell suspensions obtained from enzymatically digested tumor fragments were used in antitumor reactivity assays (standard digestion protocol, as described previously; ref. 30). In all other cases where autologous tumor cell lines were not available, TILs were tested against multiple (5–12) HLA-A–matched allogeneic melanoma cell lines, and the highest level of reactivity to any of the cell lines was reported. PBLs were only tested against the cell line to which the corresponding TILs displayed the highest level of reactivity.

A fraction of TILs may be reactive to private antigens such as those derived from mutations of the individual patient's tumor. Thus, it may be postulated that testing against allogeneic melanoma cells carry a lower chance of seeing TIL reactivity. However, autologous tumor targets were available in 8 of 10 patients in the group "no significant tumor regression" and 7 of 14 in the group "best reduction in target lesion size from baseline >20%." Thus potential underestimation of tumor reactivity, if any, is likely to affect mainly the latter group.

Antitumor reactivity assays were performed and analyzed with flow cytometry as described previously (25). Tumor-reactive cells were defined as those staining double positive for any combination of IFNγ, TNF, or CD107a. The use of allogeneic HLA-matched melanoma cell lines may reduce the sensitivity and specificity of the assay for detection of tumor-specific T cells. To increase assay specificity, a positive response was defined as at least twice the background and at least a difference of 0.1% from the background. Background staining of functional markers was subtracted in all cases.

Statistical analysis

Survival curves were computed by GraphPad Prism software v. 5.0 according to the Kaplan–Meier method. Responders and nonresponders were compared using the log-rank test. Median time to follow-up was calculated by the reverse Kaplan–Meier method (31). Patient and TIL characteristics were compared for responders and nonresponders, using the Mann–Whitney *U* test and Fisher exact test (SPSS version 19). All *P* values were two-tailed and presented without adjustment for multiple comparisons.

Results

Patient characteristics

Twenty-five patients with progressive and treatment-refractory metastatic melanoma received TIL transfer between August 2011 and August 2014. Patients were enrolled in the protocol in a two-step enrolment process. Supplementary Figure S2 shows an outline of patient enrolment and number of dropouts. Patient characteristics are summarized in Table 1. Fifteen females and 10 males aged 25 to 68 years were treated. The patients had received 1 to 4 prior systemic therapies and the majority had received and progressed on IL2 treatment (96%) and ipilimumab

Table 1. Patient characteristics

							At treatment initiation						
Patient	**Sex**	**Primary tumor origin**	**HLA-A2 pos/neg**	**BRAF status**	**Previous treatments**	**Time from diagnosis to ACT (y)[a]**	**Age**	**PS**	**AJCC stage**	**Target lesion sum (cm)**	**CNS Mets**	**LDH level**	**Metastatic sites**
Responders, OR ($n = 10$)													
M15	F	Skin	neg	wt	IL2/IFNα	4.1	48	0	M1b	5.7	No	Normal	LN, lung
M17	M	Skin	neg	wt	IL2/IFNα, Ipi	15.3	49	0	M1c	3.7	No	Normal	LN, mesentery
M20	F	Skin	neg	wt	IL2/IFNα, Ipi, Tem	11.6	65	0	M1c	21.3	No	Normal	SC, IM, lung
M22	F	Skin	neg	wt	IL2/IFNα, Ipi	3.9	60	1	M1c	9.7	No	Elevated	SC, lung, IM, pleura, intestines, bone
M24	M	Skin	pos	V600E	IL2/IFNα, Vem	1.8	56	0	M1a	1.9	No	Normal	LN
M26	M	Skin	pos	BRAF del	Ipi	6.6	46	1	M1c	34.2	No	Elevated	SC, muscle, intestines, peritoneal carcinosis
M31	M	Mucosa	neg	wt	IL2/IFNα, Ipi	1.6	65	1	M1c	16	No	Elevated	SC, LN, lung, kidney, peritoneal carcinosis, bone
M36	F	Skin	neg	V600E	IL2/IFNα, Ipi, Vem	10.9	40	1	M1c	7.8	No	Elevated	LN, IA
M42	F	Skin	neg	V600E	IL2/IFNα, Ipi	2.8	68	0	M1c	15.3	No	Elevated	SC, LN, lung, bone
M45	M	Unknown	neg	V600K	IL2/IFNα, Ipi, Vem	1.6	56	2	M1c	34.2	Yes	Elevated	IM, adrenal, IA
Median (range)					*2 (1–4)*	*4 (1.6–15.3)*	*56 (40–68)*			*12.5 (1.9–34.2)*			*2 (1–6)*
n (%)	*5 (50)[b]*	*8 (80)[c]*	*2 (20)[d]*	*5 (50)[e]*					*8 (80)[f]*		*1(10)*	*6 (60)[g]*	
Nonresponders, NR ($n = 14$)													
M14	F	Unknown	neg	wt	IL2/IFNα, Tem, Ipi, DC	2.3	43	0	M1c	21.2	No	Elevated	LN, IM, lung, pleura, adrenal, mesentery, bone
M16	M	Skin	pos	V600E	IL2/IFNα, Ipi	8.5	60	0	M1c	20	No	Normal	SC, LN, pleura
M18	M	Skin	pos	wt	IL2/IFNα, Ipi	7.6	51	1	M1c	19.3	No	Elevated	SC, LN, IM, lung, IA, adrenal
M25	M	Skin	neg	V600E	IL2/IFNα, Ipi	4.5	25	0	M1b	11.2	No	Normal	LN, lung
M27	F	Skin	neg	V600E	IL2/IFNα, Vem	2.7	62	0	M1c	17.8	No	Elevated	SC, LN, IM, lung, kidney, mesentery, bone
M29	F	Skin	pos	V600E	IL2/IFNα, DC, Ipi, Vem	6.6	52	3	M1c	5.7	No	Normal	SC, LN, lung, bone
M34	F	Mucosa	neg	wt	IL2/IFNα, Ipi, Tem	2.8	46	1	M1c	12.1	No	Elevated	LN, lung, liver, pancreas
M35	F	Skin	neg	V600E	IL2/IFNα, Ipi, Vem	19.7	46	0	M1c	10.3	Yes	Normal	SC, IM, bone, brain
M37	F	Skin	pos	V600E	IL2/IFNα, Ipi	3.4	36	0	M1c	15.6	Yes	Elevated	LN, IA
M40	M	Skin	neg	V600E	IL2/IFNα, Ipi	4.0	53	0	M1c	9.5	Yes	Normal	LN, lung, peritoneal carcinosis, brain
M43	F	Unknown	pos	wt	IL2/IFNα, Tem	0.8	48	0	M1c	13.7	No	Elevated	LN, lung, bone
M46	F	Skin	neg	V600E	IL2/IFNα, Ipi	5.6	50	1	M1c	9.1	No	Elevated	LN
M47	F	Unknown	pos	wt	IL2/IFNα, Ipi	5.8	63	0	M1c	16.8	No	Elevated	LN, lung, pleura, liver
M51	M	Skin	pos	wt	IL2/IFNα, Ipi, Dac	12.4	55	0	M1b	5.5	No	Normal	LN, lung
Median (range)					*2 (2–4)*	*5.1 (0.8–19.7)*	*51 (25–63)*			*12.9 (5.5–21.2)*			*4 (1–7)*
n (%)	*5 (36)[b]*	*10 (71)[c]*	*7 (50)[d]*	*6 (43)[e]*					*12 (86)[f]*		*3 (21)*	*8 (67)[g]*	
P (OR vs. NR)	*0.68*	*1.00*	*0.21*	*1.00*	*0.63*	*0.78*	*0.20*		*1.00*	*0.88*	*0.62*	*1.00*	*0.30*
Not evaluable ($n = 1$)													
M50	F	Skin	pos	V600E	IL2/IFNα, BRAF inh.	4,8	49	1	M1c	8.8	Yes	Elevated	SC, LN, liver, brain

Abbreviations: BRAF del, BRAF deletion; BRAF inh., BRAF inhibitor (experimental treatment, RO5212054/PLX3603); Dac, Dacarbazine; DC, dendritic cell vaccination (experimental treatment); F, female; HLA, human leukocyte antigen; IA, intra-abdominal; IM, intramuscular; LDH; lactate dehydrogenase; LN, lymph node; M, male; PS, performance status; SC, subcutaneous; Tem, Temozolomide; Vem, Vemurafenib; wt, wild type.

[a]Time from diagnosis to TIL treatment in years.

[b]% Male.

[c]% Skin.

[d]% HLA-A2 positive.

[e]% BRAF wild-type.

[f]% Stage M1c disease.

[g]% with elevated LDH level.

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Table 2. TIL characteristics (A) and objective response (B)

A. TIL characteristics											B. Objective response		
						Infused cells							
Patient	Site of biopsy	TILs cryo before REP	Treatment after surgery[a]	Young-TIL days in culture	REP Fold expansion	Total ($\times 10^9$)	CD8%	CD4%	γδ%	CD8 ($\times 10^9$)	Best clinical response RECIST v 1.0	PFS months	OS months
Responders, OR ($n = 10$)													
M15	LN	No	None	16	4,284	86	31	55	10.7	27	CR	47+	47+
M17	LN	No	None	31	7,125	143	25	72	2.5	35	PR (NED)	45+	45+
M20	SC	Cryo	None	34	4,100	82	89	11	0.0	93	PR	12	22
M22	SC	Cryo	None	20	6,139	117	53	47	0.2	62	PR	28+	28+
M24	LN	No	None	24	5,515	110	67	20	0.1	74	PR (NED)	36+	36+
M26	IM	Cryo	None	17	6,560	131	93	5	0.1	122	CR	32+	32+
M31	SC	No	None	27	5,860	117	53	47	0.2	62	PR	11.3	20
M36	IA	Cryo	Vemurafenib	30	6,169	120	55	44	0.6	66	PR	8.2	25
M42	SC	No	None	20	4,148	83	8	91	0.0	7	CR	22+	22+
M45	LN	Cryo	None	14	5,118	102	53	44	0.3	54	PR	11.3+	17+
Median				*22*	*5,690*	*114*	*53*	*46*	*0.2*	*62*		*NR*	*NR*
Range				*14–34*	*4,100–7,125*	*82–143*	*8–93*	*5–91*	*0–11*	*7–122*			
n (%)		*5 (50)*	*1 (10)*										
Nonresponders, NR ($n = 14$)													
M14	LN	No	None	21	2,856	86	17	82	0.3	15	PD	2.5	3.2
M16	SC	Cryo	None	23	3,075	62	88	11	0.2	54	SD	3.9	45+
M18	SC	Cryo	None	17	9,975	200	86	9	3.6	172	SD	3.9	5.3
M25	LN	No	None	29	6,350	127	55	35	9.7	70	SD	3.8	5.5
M27	SC	Cryo	Vemurafenib	24	4,920	98	48	52	0.2	47	PD	2.0	3.5
M29	LN	No	Vemurafenib	13	4,080	82	37	10	51.0	30	SD	3.8	5.4
M34	LN	No	None	24	5,525	110	92	4	0.9	101	SD	2.8	5.0
M35	SC	Cryo	Vemurafenib	17	4,930	98	6	93	0.3	6	SD	3.1	17+
M37	SC	Cryo	None	13	6,283	125	50	29	16.9	62	SD	3.0	12
M40	IA	Cryo	None	19	3,968	78	28	58	9.5	22	SD	3.7	14
M43	SC	No	None	36	4,455	99	32	67	0.2	32	PD	1.9	21+
M46	LN	No	None	17	3,769	75	31	54	13.5	23	SD	5.8	20+
M47	Pleura	No	None	20	4,173	83	70	28	0.2	23	SD	3.3	19+
M51	LN	No	None	31	3,055	61	33	49	0.4	20	SD	3.2	14+
Median				*21*	*4,314*	*92*	*42*	*41*	*0.7*	*30*		*3.3*	*13.1*
Range				*13–36*	*2,856–9,975*	*61–200*	*6–92*	*4–93*	*0.2–51*	*6–172*			
n (%)		*6 (43)*	*3 (21)*										
P (OR vs. NR)		*1.00*	*0.62*	*0.60*	*0.1*	*0.14*	*0.56*	*0.98*	*0.08*	*0.18*		*0.0001*	*0.02*
Not evaluable ($n = 1$)													
M50	SC	No	None	26	3,205	64	16	82	0.9	10	Na	0.1	0.1
All patients (n = 25)													
Median				*21*	*4,920*	*98*	*50*	*47*	*0.3*	*47*		*3.9*	*24.6*
Range				*13–36*	*2,856–9,975*	*61–200*	*6–93*	*4–93*	*0–51*	*6–172*			
n (%)		*11 (44)*	*4 (16)*										

Abbreviations: cryo, cryopreserved; IA, intra-abdominal; IM, intramuscular; LN, lymph node; NED, no evidence of disease; NR, nonresponders; OR, objective responders; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; REP, rapid expansion protocol; SC, subcutaneous; SD, stable disease.

[a]Systemic treatment administered between tumor resection (surgery) and TIL-ACT.

(80%). The majority of patients had stage M1c disease (84%) according to American Joint Committee on Cancer (AJCC) classification, and 60% had elevated levels of plasma lactate dehydrogenase (LDH). Four patients underwent surgery and/or stereotactic radiosurgery due to central nervous system (CNS) involvement before entering the trial. One patient with three asymptomatic brain metastases (12, 8, and 4 mm) received no preceding treatment of the CNS metastases. No statistically significant differences in baseline patient characteristics between responding and nonresponding patients were found.

TIL characteristics

Characteristics of the cells administered to the patients are summarized in Table 2A. Young TIL cultures were generated after a median of 21 days (range 13–36) and success rate of TIL production was very high (32 of 33 patients). Within the weeks in REP, the TILs expanded by median 4,920-fold (2856–9975) with no significant difference in total number of cells administered to responding and nonresponding patients ($P = 0.14$). In addition, we found no significant difference in total number or percentage of $CD8^+$ or $CD4^+$ T cells infused in responding and nonresponding patients. We did however see a trend towards a higher number of γδ T cells infused in nonresponding the patients. The high interpatient variability however does not allow drawing conclusions from this observation.

Treatment and toxicity

One patient (M50) died 4 days after TIL infusion due to an intratumoral hemorrhage in a previously irradiated left temporal metastasis (13 mm) and therefore only 24 patients are included in clinical efficacy and immunologic analyses. Treatment characteristics and severe adverse events are summarized in Table 3.

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Table 3. Treatment characteristics and severe adverse events

	All patients ($n = 25$)	Responders OR ($n = 10$)	Nonresponders NR ($n = 14$)	*P* OR vs. NR
Treatment characteristics (median, range)				
Days in hospital[a]	19 (15–36)	17.5 (15–36)	20 (15–27)	*0.12*
Units RBC transfusion	5 (1–25)	5 (2–14)	5 (1–25)	*0.52*
Units PLT transfusion	7 (3–17)	7 (3–14)	7 (3–17)	*0.81*
Days with neutrophils $< 0.5 \times 10^9$/L	8 (4–13)	8 (5–12)	9 (4–13)	*0.79*
Dose IL2 administered, MIU[b]	112 (50–135)	107 (58–135)	112 (50–135)	*0.75*
% IL2 administered[c]	95 (60–100)	93 (60–100)	96 (60–100)	*0.57*
Severe adverse events[d] (n)				
Febrile neutropenia	24	10	14	
Infections, verified[e]				
Urinary tract	1	1		
Pneumonia[f]	2		2	
Aspiration pneumonia	1	1		
Central venous catheter	2	1	1	
Pulmonary edema	1		1	
Renal failure	1		1	
Atrial fibrillation	1		1	
Diarrhea	1	1		
Hemoperitonium	1		1	
Petechia	2	1	1	
Venous thromboembolism	1		1	
Delirium	2	2		
Mortality (grade 5)	1	NA	NA	NA
Autoimmune reactions, any grade (*n*)				
Vitiligo	2	2		
Uveitis	1	1		
Vasculitis	1	1		
Pernicious anemia	1	1		

NOTE: The table shows treatment characteristics and severe adverse events related to treatment ($\leq$8 weeks after treatment).
Abbreviations: NR, nonresponders; OR, objective responders; PLT, platelets; RBC, red blood cell.
[a]Measured from the first day of chemotherapy to discharge.
[b]Total dose of IL2 administered in MIU.
[c]Percent IL2 administered of prescheduled dose.
[d]According to the CTCAE v 3.0.
[e]Infection verified by microbiological tests.
[f]Pneumonia verified/likely by chest X-ray.

Median time in hospital was 19 days (range 15–36). All but two patients received the scheduled lymphodepleting chemotherapy without dose reductions. For two patients (M22 and M47) chemotherapy dose was reduced due to impaired kidney function [glomerular filtration rate (GFR) of 40% and 61%, respectively, of the expected value] and both patients achieved the intended deep lymphodepletion on day 0 with recovery of bone marrow function afterwards as expected.

Anticipated grade 3–4 hematologic toxicities related to the chemotherapy were observed in all patients. The decline in hematologic function was followed by subsequent recovery of total white blood cells, including neutrophils and lymphocytes, in all patients (data not shown). The patients received PLT and RBC transfusions as needed and their counts increased back to normal levels within a few weeks after treatment. Most patients (92%) received G-CSF on the day of TIL infusion and median number of days with neutrophils $< 0.5 \times 10^9$/L was 8 days (range 4–12). A considerable requirement for platelet transfusion compared with our previous experience with low-dose subcutaneous IL2 (21) was observed (Table 3). This was likely a result of the administration of G-CSF and the increased IL2 dosing.

In most patients, TIL infusion was followed by low-grade reversible adverse reactions such as fever and chills. Occasionally transient hypoxia, dyspnea, and nausea were also observed. We observed no grade 3 or 4 toxicities, except for fever, directly associated to TIL administration.

The prescheduled IL2 dose was reduced by 25%–50% in four patients due to either poor performance status at treatment initiation (M22 and M29) or preexisting comorbidity (patient M31 and M43 suffered from chronic obstructive lung disease and ulcerative colitis, respectively). The continuous IL2 infusion was associated with known IL2-related adverse effects such as flu-like symptoms, high fever, fluid retention, hypotension, respiratory, gastrointestinal, and neurologic symptoms, electrolyte derangement, coagulopathies, and abnormal kidney and liver function tests.

All patients experienced nearly constant high fever with temperatures often rising above 40°C (88%). Antipyretic drugs were generally not allowed, but acetaminophen (paracetamol) was administered if temperature rose above 41°C. Consequently, all patients fulfilled the criteria for neutropenic fever (32) and received prophylactic antibiotics according to local guidelines. Five patients had documented infection arising from central line catheter (M17, M29), urinary tract (M24) or lungs (M37, M51), but no positive blood cultures were found. All infections were resolved after appropriate antibiotic treatment and removal of foreign bodies (catheter).

The prescheduled IL2 dose was administered with a median dose of 112 MIU (93% of prescheduled dose) corresponding to

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

approximately four days of continuous IL2 infusion. The majority of patients (75%) discontinued IL2 therapy slightly premature. The main reasons for discontinuation included respiratory symptoms (M27, M34, M35, and M40), atrial fibrillation (M47), elevated levels of creatinine (M16, M45), low platelet counts (M18), low level of potassium (M14), neurologic toxicity (M36, M42), or accumulation of various IL2–related toxicities ($n = 6$). Patient M26 had peritoneal carcinomatosis prior to treatment, known from previous palliative abdominal surgery for intestinal bowel obstruction (4 weeks prior TIL infusion) and increased PET signal in the area at baseline scan (Supplementary Fig. S3A). This patient developed symptomatic bowel obstruction in relation to nonmeasurable, nontarget, non-CT–evident lesions during treatment (one day after IL2 discontinuation, 6 days after TIL infusion). The patient underwent acute palliative surgery with resection of 10-cm small bowel and recovered completely after a prolonged stay in hospital. This patient achieved a CR, still ongoing after 24 months. Another patient (M16) developed acute renal failure shortly after finishing IL2 therapy and hemodialysis was necessary until kidney function was normalized a few weeks later.

In general, IL2-related toxicities were transient and responded to standard interventions or resolved within 2 to 3 days of IL2 discontinuation. Importantly, no patients experienced prolonged hypotension necessitating vasopressor support and no patients needed pulmonary intubation.

Autoimmune reactions were observed exclusively in clinical responders. Vitiligo was seen in two responding patients, including one patient who also developed anterior uveitis a few weeks after TIL therapy (M20). The latter, resolved upon topical steroid eye drops for several months. Interestingly, 6 patients with preexisting vitiligo after prior IL2/IFNα therapy responded to TIL-ACT, two of whom experienced substantial aggravation of vitiligo. Petechiae and purpura of varying degree were observed in several patients most likely caused by low platelets and a decline in coagulation factors during IL2 therapy.

In summary, as expected, acute toxicities were considerable. However, toxicities were manageable in a regular oncology ward and essentially no long-term or late toxicities (occurring more than 1 month after treatment) were observed.

Clinical efficacy

Patients were followed for up to 47 months with a median follow-up time of 28.1 months. OS and PFS were calculated from TIL infusion to death or progression, respectively, or to the date of last follow-up (October 16, 2015). Median OS and PFS of individual patients and objective clinical response according to RECIST 1.0 are summarized in Table 2B.

The median PFS of all 25 patients was 3.9 months and the median OS 21.8 months, at the time of follow-up 12 patients were still alive. Kaplan–Meier curves of OS for all patients are shown in Fig. 1A. The 1- and 3-year survival rates were 72% and 40.8%, respectively. Kaplan–Meier curves of OS and PFS for responders ($n = 10$) versus nonresponders ($n = 14$) are shown in Supplementary Fig. S4. Supplementary Fig. S5 shows OS by stratification for patients achieving best reduction in target lesions size from baseline of >20% ($n = 14$) or no evidence of tumor regression ($n = 10$). Responders ($n = 10$) did not reach the median OS time by the end of the study and nonresponders ($n = 14$) had a median OS of 13.1 months ($P = 0.008$). Seventy percent of responding patients were alive at the time of follow-up as opposed to 36% of the nonresponders indicating that objective response was significantly associated with survival benefit. Median PFS of nonresponders 3.3 months and for responders the median PFS time was not reached.

Objective clinical responses according to RECIST 1.0 were observed in 10 of 24 (42%) evaluated patients and tumor regression was observed at multiple sites, including subcutaneous, lymph node, intramuscular, lung, pleura, intraabdominal, bowel, bone, and visceral metastases. Responses included 3 CR (13%) and 7 PR (29%). Eleven patients achieved disease stabilization lasting 4–6 months after treatment and 3 patients progressed at first evaluation after treatment. Progression after an initial PR was observed in three patients after 8, 11 and 12 months, respectively. All CR and 4 PR are ongoing with the duration of response ranging from 6 to 45 months. We observed no impact from previous treatments or response to previous immunotherapy on clinical efficacy. Previous responses to immunotherapy are shown in Supplementary Table S1.

The swimmer plot in Fig. 1B shows the best overall response according to RECIST 1.0 of the individual patients and the waterfall plot in Fig. 1C illustrates the best percentage change in target lesions compared with baseline. In general, the greatest tumor shrinkage occurred within the first few months after TIL therapy as seen in the spaghetti plot in Fig. 1D. Patient M26 achieved a PR shortly after treatment and probably due to very slow disappearance of scaring tissue, remaining lesions were visible on FDG-PET/CT scan for several months after treatment. FDG-PET/CT scan 13 months after treatment showed that this patient finally achieved a CR according to RECIST 1.0. FDG-PET/CT scans from baseline and after 2, 13, and 24 months are shown in Supplementary Fig. S3A.

Three patients (M17, M24, and M36) with unresectable lesions before TIL therapy underwent surgical resection of residual lesions after obtaining a PR from TIL therapy. Resection of residual lesions was performed approximately 7 months after TIL therapy and malignant cells were found on histologic examination of the resected lesions from all three patients. Patient M17 and M24 both have ongoing response without evidence of disease 44 and 34 months after TIL therapy whereas patient M36 had a local recurrence four months after surgery. This illustrates that surgical resection of remaining tumor lesions after initial response to TIL-ACT can be beneficial. FDG-PET/CT scans of patient M17 is shown in Supplementary Fig. S3B.

Evidence of clinical activity in terms of transient regression of target lesions was observed in four patients (M18, M25, M43, and M47). Transient regression was defined as regression of target lesions of more than 20% in at least one scan but not qualifying as an objective response according to RECIST 1.0. For example, patient M43 had 77% regression of all known tumor lesions but simultaneously one new lesion appeared on the first evaluation scan 8 weeks after treatment and, according to RECIST criteria, the patient had PD. The new lesion was resected surgically since all other lesions showed significant regression, but four months after surgery (8 months after TIL therapy) the patient progressed in the lungs. In general, patients with transient tumor regression appeared to have initial clinical benefit (reduction of disease-related symptoms) alongside objective tumor regression but no apparent long-term benefit from the treatment.

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

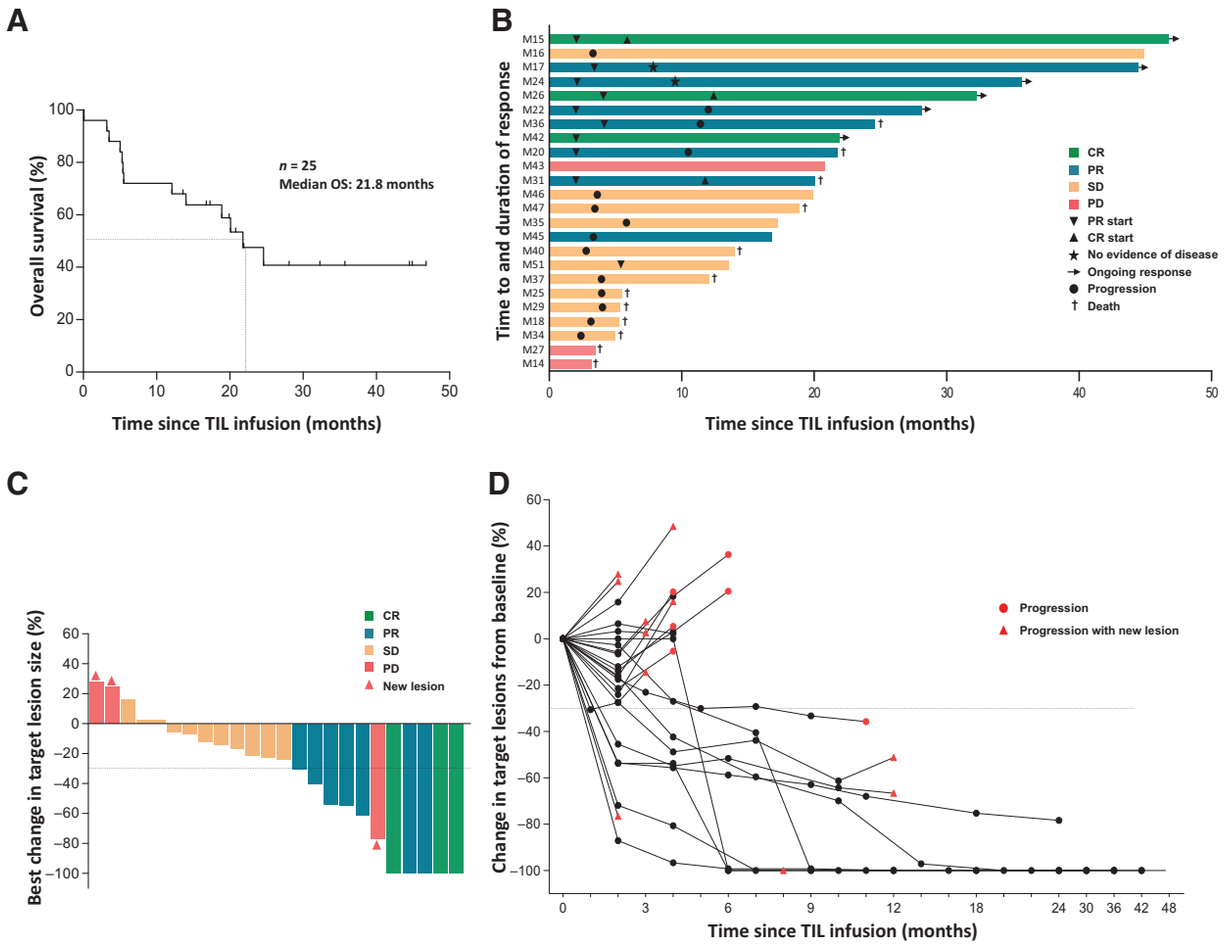

Figure 1.
Characteristics of tumor regression and response duration in patients receiving TIL therapy. **A,** Kaplan–Meier curve of overall survival in all 25 treated patients. **B,** time to response and duration of response according to RECIST 1.0 of individual patients treated with TIL-ACT. The triangles indicate first evidence of complete response (CR) and the reverse triangles indicate first evidence of partial response (PR). The stars indicate first evidence of "no evidence of disease after surgical removal of remaining tumor lesions" and the arrows indicate ongoing response at time of analysis. Closed circles indicate progression and the cross indicates time of death. **C,** maximum reduction or minimum increase in the sum of target lesion measurements compared with baseline in patients with at least one follow-up PET/CT scan ($n = 24$). Horizontal line at -30 indicates the threshold for defining objective response in the absence of new lesions or nontarget disease progression according to RECIST 1.0. Patients with a partial response with 100% reduction in target lesion size have nontarget lesions present. **D,** response kinetics in patients with at least one follow-up PET/CT scan ($n = 24$). Triangles indicates first occurrence of a new lesion. Horizontal line at -30 indicates the threshold for defining objective response according to RECIST 1.0.

Antitumor reactivity of TIL infusion products and PBLs

Transient tumor regression after infusion of tumor-reactive T cells is related to the mechanism of action of ACT (23). In this trial, four patients achieved transient tumor regressions which did not qualify as confirmed objective response by RECIST 1.0. However, these transient tumor regressions were consistent with the initial activity of the infused TILs, and accordingly a nonclassical stratification (see Materials and Methods, Study Design) is included in the following analyses.

Our results show that the total number of infused tumor-reactive T cells was significantly correlated to tumor regression, suggesting a specific antitumor activity of the infused TILs at tumor sites (Fig. 2A). However, when patients were stratified as responders and nonresponders according to the clinical response criteria RECIST 1.0 this correlation was not statistically significant (Supplementary Fig. S6A). To understand whether a measurable antitumor response could be reflected in the periphery, PBLs were tested for reactivity against tumor cells before treatment (pre) and at several time points after TIL transfer. Simultaneous assessment of multiple $CD8^+$ T-cell functions after exposure to autologous or, when these were not available, allogeneic melanoma antigens was used (Fig. 2B and C). Figure 2B show that percentage of tumor-reactive T cells of $CD8^+$ PBLs one months after treatment was significantly correlated to tumor regression ($P = 0.002$).

In a few patients, measurable but low magnitude antitumor responses could be detected in the peripheral blood already before treatment (Fig. 2C). Notably, while the majority of patients with no significant tumor regression did not display any measurable or very low antitumor responses after treatment, we detected tumor-reactive T cells in the majority of patients with evidence of tumor regression (best reduction in target lesion size from baseline >20%; Fig. 2C). In patients with no significant tumor

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

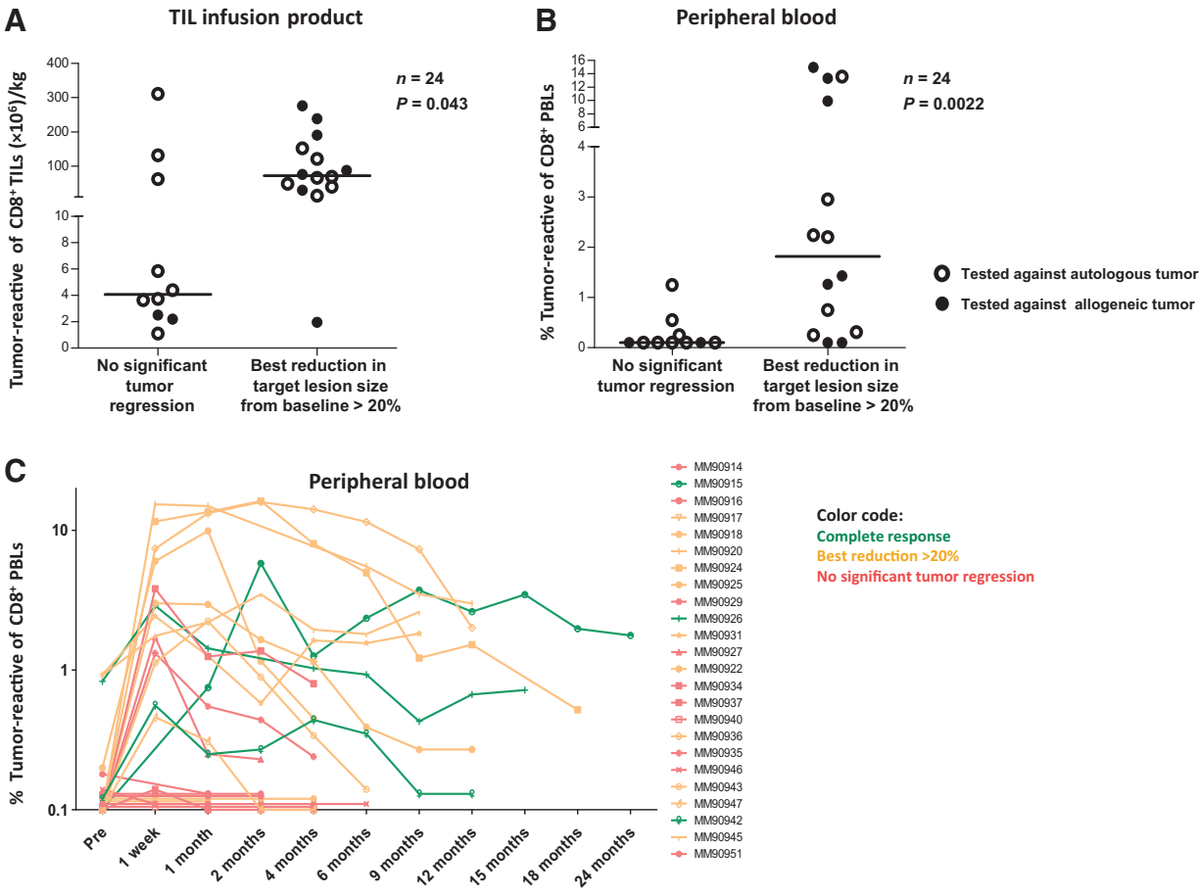

Figure 2.
Antitumor responses of infusion products and peripheral blood. TILs or PBLs were cocultured with autologous short-term cultured melanoma cell lines or HLA-semimatched allogeneic melanoma cell lines, and tumor reactivity was evaluated by assessing the amount of $CD8^+$ T cells staining double positive for any combination of IFNγ, TNF, or CD107a. **A,** the figure shows the absolute number per kg of body weight of *in vitro* tumor-reactive TILs contained in the infusion products, and its association with tumor regression in 24 patients. Patients are stratified in two groups "best reduction in target lesion size from baseline >20% ($n = 14$)" and "no significant tumor regression ($n = 10$)." **B,** the figure shows the percentage tumor-reactive of $CD8^+$ ($CD3^+$) PBLs one month after treatment and its association with tumor regression in 24 patients. Patients are stratified in two groups "best reduction in target lesion size from baseline >20% ($n = 14$)" and "no significant tumor regression ($n = 10$)." **C,** the figure shows induction and persistence of antitumor responses in the blood of the treated patients ($n = 24$). Patients are stratified into "complete responders (CR)" in green ($n = 3$), patients with "best reduction in target lesion size from baseline > 20%" in yellow ($n = 11$) and patients with "no significant tumor regression" in red ($n = 10$).

regression, but with measurable antitumor responses *in vitro* demonstrated shortly after TIL infusion, the tumor-reactive TILs disappeared very quickly from the circulation (Fig. 2C). These patients all had high numbers of tumor reactive $CD8^+$ TILs in the infusion products (Fig. 2A). Importantly, in most patients achieving tumor regression, antitumor immune responses persisted at high levels for up to 2 years after treatment (Fig. 2C). Supplementary Figure S6 shows the antitumor responses in the infusion product and in peripheral blood with patients stratified by RECIST 1.0. An example of T-cell responses from a patient achieving a CR are shown in Fig. 3.

Discussion

HD bolus IL2 is associated with transient but severe toxicities and it is therefore recommended that this treatment is administered only when acute intensive care support is available. HD bolus IL2 has until now been used in most clinical trials investigating TIL-ACT as it is believed to be important for sustained T-cell activity after cell transfer. In this study, we show that TIL-ACT followed by a reduced dose IL2 administered in a continuous decrescendo regimen can induce long-lasting complete responses. Overall response rates appears to be comparable with results from trials where HD bolus IL2 was administered after TIL transfer (1–4, 33), but this needs to be confirmed in larger, preferably randomized clinical trials.

As expected, the reduced dose of IL2 gave rise to the well-known toxicities associated with IL2 infusion, but the toxicities were readily managed in a regular oncology ward.

The HD bolus IL2 can induce fast developing life-threatening toxicities such as hypotension, pulmonary, or cardiac toxicity, that can be challenging to manage once the bolus infusion has already been administered (34, 35). When IL2 is administered in a continuous decrescendo regimen there is a slow gradual accumulation of IL2-related toxicities, which allows for early and appropriate adjustment of symptomatic and supportive care, including

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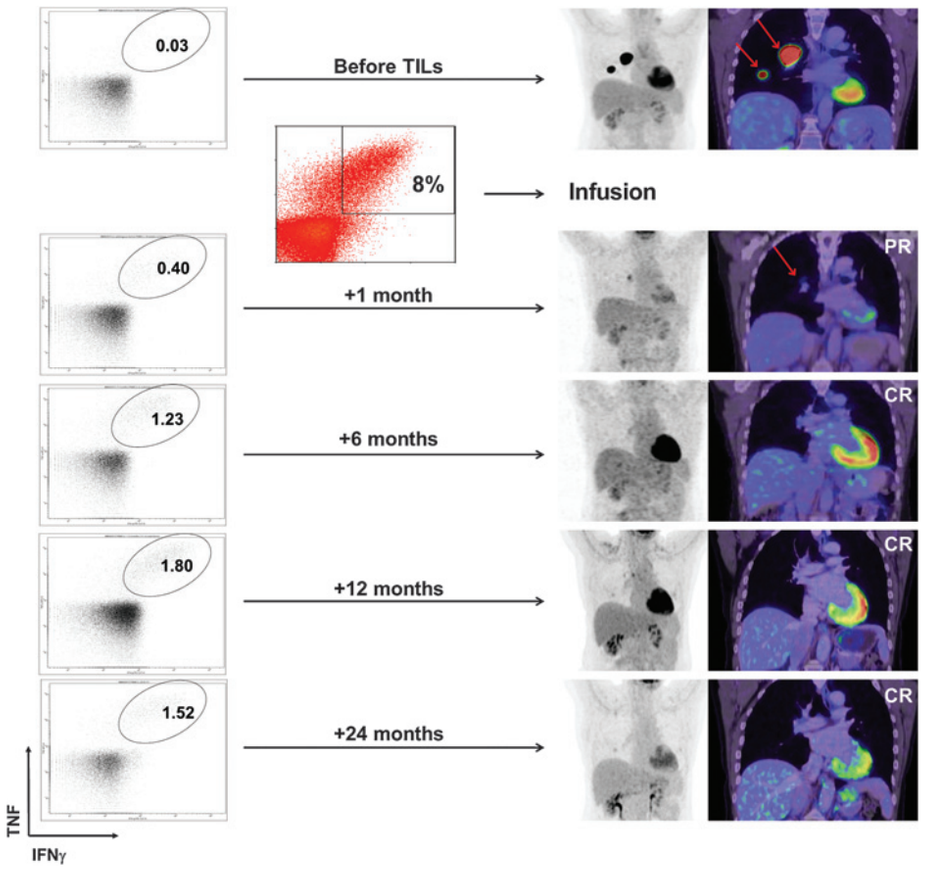

Figure 3.
Induction of anticancer responses in blood associated with a complete response. The figure shows the induction and long-term persistence of anticancer responses in the peripheral blood of a patient (M15) treated with a high number of tumor-reactive TILs. TILs or PBLs were cocultured with autologous short-term cultured melanoma cell line and tumor-reactive cells are shown as T cells simultaneously expressing TNF and IFNγ. All plots shown are gated on $CD3^+CD8^+$ cells.

early and immediate discontinuation or pausing of IL2 infusion well in time to prevent the occurrence of more severe toxicity. Indeed, according to our previous experience with treatment of more than 100 melanoma patients with IL2 administered in the continuous decrescendo regimen, virtually all IL2-related toxicities quickly remit when IL2 infusion is paused or discontinued (our internal observation, data not shown). The clinical observations in this study, where standardized discontinuation protocols for IL2 were used, confirmed this pattern of toxicity resolution when IL2 was paused or discontinued in patients after lymphodepleting chemotherapy plus TIL infusion. Thus, we observed no life threatening hemodynamic changes requiring vasopressor support and no patients needed pulmonary intubation. Treatment-related toxicity was manageable in a regular oncology ward without the need for intervention from intensive care unit and more than 90% of the prescheduled IL2 was administered. However, although our data indicate that toxicity can be reduced by use of the decrescendo regimen IL2 this needs to be confirmed in a randomized trial comparing the decrescendo regimen directly to the HD bolus infusions.

Few other studies have investigated the use of lower doses of IL2 in the context of TIL-ACT in its current form and none of them made direct comparisons to ACT trials using HD bolus IL2. We previously published a small phase I feasibility study in which 6 patients with progressive metastatic melanoma received lymphodepleting chemotherapy and TIL infusion followed by low-dose (2 MIU) subcutaneous IL2 injections daily for 14 days (21). Two complete responses were observed suggesting clinical efficacy even with very low dose of IL2. The two responding patients are still alive and disease free 73 and 51 months after TIL-ACT. However, both patients recurred 13 and 48 months after TIL-ACT, respectively, with solitary metastatic lesions which were resected with no subsequent systemic therapy administered. Whether long-term durability of complete responses after TIL-ACT depends on higher doses of IL2 following cell transfer will need to be explored further in randomized trials comparing the HD bolus regimen with reduced doses and schedules of IL2. In another study carried out in Sweden between 2005 and 2010, 24 patients with stage IV metastatic melanoma were treated with classical lymphodepleting chemotherapy followed by infusion of TILs and low-dose s.c. IL2 (36). An objective response rate of only 21% was reported in this study, but the cell numbers infused (median 5.4×10^9 in total) were well below the amount of cells infused in our and most other TIL-ACT studies (1–4).

It was recently reported that an increasing number of IL2 doses administered after TIL transfer may promote reconstitution of peripheral $CD4^+$ regulatory T cells after lymphodepletion, and the latter was inversely associated with clinical responses (37). In addition, patients with objective clinical response after ACT received fewer doses of IL2 compared with nonresponders (1). Our results showed no difference in total dose of IL2 administered to responding or nonresponding patients. But interestingly, the

Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

prescheduled dose of IL2 was reduced by 25%–50% in 4 patients due to either poor performance status or preexisting comorbidities and all four patients experienced at least 20% regression of target lesions and two had a partial response according to RECIST. Clinical ACT trials without IL2 administration after cell transfer are ongoing (ClinicalTrials.gov Identifier: NCT01468818) and may bring further insight to the importance of IL2 dosing and schedule to achieve durable clinical responses after transfer of TILs.

Efficacy after failure from previous immunotherapies was observed in this study. This is consistent with previous reports (2) and indeed we found that heavily pretreated patients, refractory to prior immunotherapies such as monotherapy with IL2 and anti-CTL-associated protein 4 (CTLA-4) antibodies could benefit from TIL-ACT. A recent review from the group of Steve Rosenberg states that in their hands comparable response rates have been reported regardless of prior therapy, including prior anti-PD1 antibodies (38). Further studies are needed to clarify whether TIL-ACT can indeed be used as salvage treatment after treatment with new immune checkpoint inhibitors.

Previous data from Dudley and colleagues showed an association between IFN-γ release from TILs after coculture with tumor digests (magnitude of T-cell antitumor reactivity), and other studies showed an association between the total number of infused $CD8^+$ TILs and clinical responses (2, 3, 39). We analyzed these factors (level of *in vitro* reactivity and absolute number of cells) simultaneously by calculating the total number of reactive $CD8^+$ TILs, which was shown to be associated with tumor regression. Notably, as previously described (40), only a fraction of $CD8^+$ TILs from any individual patient displayed any tumor reactivity.

When autologous tumor targets were not available, we used coculture assays with multiple allogeneic HLA-A–matched melanoma cell lines. Although the use of multiple cell lines increase the likelihood to detect TILs reactive to melanoma antigens, this is not an optimal assay as it does not detect T cells reactive to patient-specific tumor antigens such as mutant "neo"-antigens, which may be pivotal to achieve and maintain clinical responses (41). Nevertheless, a similar trend with increased likelihood of response following infusion of higher number of $CD8^+$ TILs reactive to these allogeneic melanoma cells was observed. This suggests that recognition of shared melanoma antigens may either *per se* be mediating tumor regression or it may be a biomarker of more potent responses to mutant neoantigens within the infused TILs.

Our results show that tracking tumor-reactive $CD8^+$ T cells from the peripheral blood of individual patients after infusion of TILs is feasible and consistent. A significant association was found between tumor reactivity at 1 month after infusion and tumor regression, and tumor-reactive $CD8^+$ T cells could be detected in several patients after months to years after infusion.

In conclusion, these results suggest that the classical toxicities of TIL-ACT can be reduced with modified protocols without compromising clinical efficacy. These results warrant further testing of TIL-ACT with attenuated doses of IL2 in larger randomized studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The funding body had no role in the design, collection, analyses or interpretation of the data, or in the writing of the manuscript.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Andersen, M. Donia, P. Kongsted, T.Z. Iversen, M.H. Andersen, P.T. Straten, I.M. Svane
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Study supervision: R. Andersen, I.M. Svane

Acknowledgments

The authors thank the technicians from the Laboratory of Hematology and the staff at the Department of Oncology, including all nurses and ward doctors for taking care of the patients under admission. The authors also thank the project nurse Anne Kuld-Nielsen for taking excellent care of the patients and Tobias Wirenfeldt Klausen for statistical assistance.

Grant Support

The study was supported by grants from Aase and Ejner Danielsens Foundation, the Danish Cancer Society, the Lundbeck Foundation, and the Capital Region of Denmark Research Foundation.

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Received August 25, 2015; revised January 15, 2016; accepted February 20, 2016; published OnlineFirst March 22, 2016.

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Published OnlineFirst March 22, 2016; DOI: 10.1158/1078-0432.CCR-15-1879

Clinical Cancer Research

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Clin Cancer Res 2016;22:3734-3745. Published OnlineFirst March 22, 2016.