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Verdegaal, E.M.E.; Verpoorte, A.L.C.; Kooij, M.K. van der; Bruin, L. de; Visser, M.; Minne, C.E. van der; ... ; Kapiteijn, E.H.W.

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Effective TIL Therapy for Patients with Checkpoint-Resistant Melanoma without Lymphodepleting Regimens Requires IFNα

Els M.E. Verdegaal[1], Anique L.C. Verpoorte[1], Monique K. van der Kooij[1], Linda de Bruin[1], Marten Visser[1], Caroline E. van der Minne[1], Vera Weeda[1], Inge C.F.M. Roozen[2], Mare A. Jonker[2], Inge M. Westra[3], Pauline Meij[3], Frank M. Speetjens[2], Stephanie M. Zunder[2], Gerrit-Jan Liefers[4], Saskia J. Santegoets[1], Sjoerd H. van der Burg[1], and Ellen H.W. Kapiteijn[2]

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ABSTRACT

Purpose: Patients with melanoma progressing on immune checkpoint blockade may benefit from adoptive transfer of tumor-infiltrating lymphocytes (TIL).

Patients and Methods: We investigated the impact of a pegylated IFNα conditioning and support regimen on the safety and efficacy of TIL plus nivolumab (NCT03638375). Patients with immune checkpoint blockade–resistant stage III/IV melanoma were treated with TIL plus nivolumab without ($n = 9$) or with ($n = 25$) IFNα.

Results: The treatment was safe, and side effects included IFNα-induced lymphopenia (16%) and neutropenia (12%). No febrile neutropenia or >grade 4 adverse events were observed. Disease control was obtained in 11.1% (95% confidence interval, −14.5%–36.7%) of the patients treated without and in 41.7% (95% confidence interval, 20.4%–62.9%) of the patients treated with IFNα, clearly suggesting the need for IFNα support. IFNα treatment strongly reduced the numbers of circulating leukocytes and neutrophils, more consistently in therapy responders. No differences were observed in the phenotype and dose of TIL administered.

Conclusions: Taken together, our low-toxicity therapy comprising TIL, nivolumab, and IFNα is safe, shows evidence of clinical activity, and may be particularly suitable for more frail patients who are less able to tolerate lymphodepletion and high-dose IL-2 regimens.

Introduction

The advent of targeted therapies (e.g., BRAF and MEK inhibitors) and immune checkpoint blockade (ICB, e.g., anti–CTLA-4, anti–PD-1, and anti–PDL1/2) has improved the treatment options and prognosis of patients with metastatic melanoma. However, more than half of the patients do not respond or experience disease relapse after such treatments (1). Salvage therapy for the large subset of patients with melanoma refractory to ICB poses a strong unmet need and formidable clinical challenge with current existing interventions.

The efficacy of anti–PD-1, blocking a critical inhibitory checkpoint for T cells, relies on the presence of tumor-specific T cells, and one of the reasons of unresponsiveness to anti–PD-1 may be the lack of sufficient frequencies of such tumor-specific T cells. Adoptive cell transfer (ACT), i.e., the *ex vivo* expansion and reinfusion of autologous tumor-infiltrating lymphocytes (TIL), can be used to overcome this problem. TILs consist of polyclonal $CD8^+$ and $CD4^+$ T cells that are reactive to diverse tumor antigens, and ACT of TILs is clinically successful in several solid tumor types (2–6). TIL therapy for melanoma was recently approved by the FDA (7) and in the Netherlands (8). With its unique personalized approach, TIL therapy may overcome insufficiency of tumor-specific T cells. We previously reported that approximately half of the TILs used for ACT express PD-1, but only a fraction of these TILs co-expresses the other checkpoint molecules CTLA-4 and Tim-3 (9). Hence, these TILs are not terminally exhausted, and their *in vivo* reactivity may be retained by treatment with anti–PD-1.

Previous ACT trials demonstrated the need for chemotherapy-driven lymphodepletion prior to T-cell infusion and concomitant administration of T cell–supportive high-dose IL-2 to obtain clinical success (10). This approach comes with substantial treatment-related toxicity, especially attributable to IL-2, potentially curtailing the effective use of this form of immunotherapy, particularly in more frail patients. Several other approaches, including reduced IL-2 dosing (11) or the use of pegylated IL-2 (12), have been investigated aiming to reduce these adverse events (AE) but with modest effect. In a note on preliminary outcomes, NCT01468818 reported direct clinical effects of TILs without IL-2 co-treatment in patients with melanoma, indicating that IL-2 support is not an absolute requirement (13).

Allogeneic stem cell transplantation with IFNα to sustain effector donor lymphocyte infusion shows superior efficacy over donor lymphocyte infusion alone (14). In addition, IFNα increases the survival and effector function of antigen-specific T cells (15, 16). Therefore, we have pioneered an alternative approach of TIL

[1]Department of Medical Oncology, Oncode Institute, Leiden University Medical Center, Leiden, the Netherlands. [2]Department of Medical Oncology, Leiden University Medical Center, Leiden, the Netherlands. [3]GMP facility Leiden, Center of Cell and Gene Therapy, Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, the Netherlands. [4]Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands.

E.M.E. Verdegaal and A.L.C. Verpoorte share first authorship.

S.H. van der Burg and E.H.W. Kapiteijn share last authorship.

Corresponding Author: Sjoerd H. van der Burg, Department of Medical Oncology C7-141, Leiden University Medical Center, P.O. Box 9600, Leiden 2300 RC, the Netherlands. E-mail: s.h.van_der_burg@lumc.nl

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Translational Relevance

There is a strong unmet need for salvage therapy for the large subset of patients with melanoma that progresses after immune checkpoint blockade. We evaluated the safety and efficacy of adoptively transferred tumor-infiltrating lymphocytes (TIL) plus anti–PD-1 and the impact of a pegylated IFNα–mediated conditioning and support regimen in patients with immune checkpoint blockade–refractory stage III/IV melanoma. The treatment was safe and resulted in disease control in 42% of the patients treated with the full regimen, whereas this was only 11% when TIL plus nivolumab without IFNα was given, clearly illustrating the supportive effect of IFNα. This novel, clinically effective treatment is associated with low toxicity and burdening and may provide an additional treatment option, particularly for more vulnerable patients who are less capable of enduring the considerable toxicity associated with lymphodepleting chemotherapy and high-dose IL-2 regimens.

infusion plus low-dose IFNα that was considerably less toxic than lymphodepleting chemotherapy plus IL-2 and showed evidence of clinical benefit (17). Patients were treated with TIL plus IFNα without anti–PD-1. Although 43% of the treatment-naïve patients obtained clinical benefit, this was only 20% of the patients that relapsed after ICB therapy. The majority of infused TILs were shown to be PD-1 positive, suggesting that anti–PD-1 therapy may improve treatment outcomes, especially in the latter group.

In the current trial, anti–PD-1 was added to TIL treatment to further improve treatment outcomes in patients progressive after ICB. Here, we demonstrate the requirement for IFNα during combined TILs with anti–PD-1 treatment for disease control in patients with stage III/IV melanoma progressive after ICB.

Patients and Methods

Study participants

Patients with histologically proven stage IV or irresectable stage III cutaneous melanoma were eligible who were 18 years of age or older, with a serum lactate dehydrogenase (LDH) level less than or equal to twice the upper limit of normal, a World Health Organization performance status of 0 to 2, and a life expectancy of at least 6 months. All patients had progressive disease (PD) following anti–PD-1 mono– or anti–PD-1/CTLA–4 combination therapy and all other standard-of care-therapies. Systemic treatment had to be discontinued for 4 weeks in case of chemo-, radio-, or immunotherapy and 2 weeks in case of targeted therapy (BRAF/MEK inhibitors). Patients with asymptomatic or neurologically stable brain metastases were eligible for this study. Exclusion criteria were clinically significant heart disease (New York Heart Association class III or IV), active immunodeficiency or autoimmune disease, other malignancy within 2 years prior to entry into the study except for treated nonmelanoma skin cancer, and *in situ* cervical carcinoma, a known allergy to penicillin or streptomycin or seropositivity for hepatitis B/C, human immunodeficiency virus, human T-cell lymphotrophic virus, or *Treponema Pallidum.* At least one lesion that could be resected or biopsied was required for the establishment of a TIL culture, and at least one additional measurable target lesion was required for response evaluation. All patients gave written informed consent. The study was approved by the Central Committee on Research Involving Human Subjects and the Medical Ethics Committee of the Leiden University Medical Center (L18-039). The study was registered at ClinicalTrials.gov (NCT03638375) and conducted in accordance with the Declaration of Helsinki. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for reporting were used.

Definition of primary and secondary PD-1 ICB resistance

Based on the definition of primary resistance in the recently published Society for Immunotherapy of Cancer recommendations (18) and allowing first-line anti–CTLA-4/PD-1 combination ICB therapy in the evaluation, patients are considered to have a primary resistance to PD-1 ICB when they have PD or stable disease (SD) < 6 months after ≥6 weeks of drug exposure to PD-1–based immunotherapy. Also, patients with PD or SD within 6 months from the start of or within 12 weeks after stopping (neo)adjuvant anti–PD-1–based immunotherapy are defined as having a primary resistance to PD-1 ICB.

Study design

The study design of this investigator-initiated, single-center, nonrandomized, nonblinded phase I/II trial was published in detail (19). Briefly, patients were treated in two cohorts with autologous TILs with anti–PD-1 (cohort I) alone or in combination with pegylated IFNα (cohort II). After the safety evaluation of patients treated in cohort I, the study proceeded with the treatment of patients in cohort II. Nivolumab (anti–PD-1) was initially given at the standard dose of 3 mg/kg as 2 weekly infusions and was already started 2 to 3 weeks after biopsy/resection of the tumor for TIL expansion, i.e., 4 weeks prior to the first TIL infusion (**Fig. 1A**). The frequency was adjusted to 4 weekly infusions according to standard-of-care recommendation. This adjusted regimen is used for the treatment of patients #25 through #47. One week prior to the first TIL infusion, patients in cohort II started with weekly subcutaneous injections of IFNα, 1 μg/kg/week (maximum 90 μg/week) for 11 weeks in total. This is a comparable effective dose with the daily nonpegylated IFNα used in our previous protocol (17), which is no longer available. Based on our previous study (9), TIL infusions were given intravenously with a 3-week interval at a dose range of 2.5 to 7.5×10^8 TILs per infusion during daycare. At several timepoints, heparinized venous blood was collected for the isolation of serum/plasma and peripheral blood mononuclear cells, which were cryopreserved until further analysis. Where relevant, blood was drawn before nivolumab or TIL infusion. The absolute numbers of leukocytes, neutrophils, monocytes, lymphocytes, and thrombocytes were determined by differential blood counts performed as routine diagnostic tests by the Central Clinical and Hematological Laboratory of the Leiden University Medical Center.

Study endpoints/objectives

The primary objective was to evaluate the safety of the combination of TILs and anti–PD-1 with or without IFNα conditioning using the NIH Common Terminology Criteria for Adverse Events versions 4.0 and 5.0. The secondary objective was to evaluate the disease control rate (DCR) at the first evaluation (3 months, i.e., 2–4 weeks after the last TIL infusion). The DCR was defined as stabilization of disease (SD), partial response (PR), or complete response (CR) according to RECIST version 1.1 (20). PD was defined as a more than 20% increase in the sum of target lesions or appearance of any new lesion. For response evaluation, target lesions

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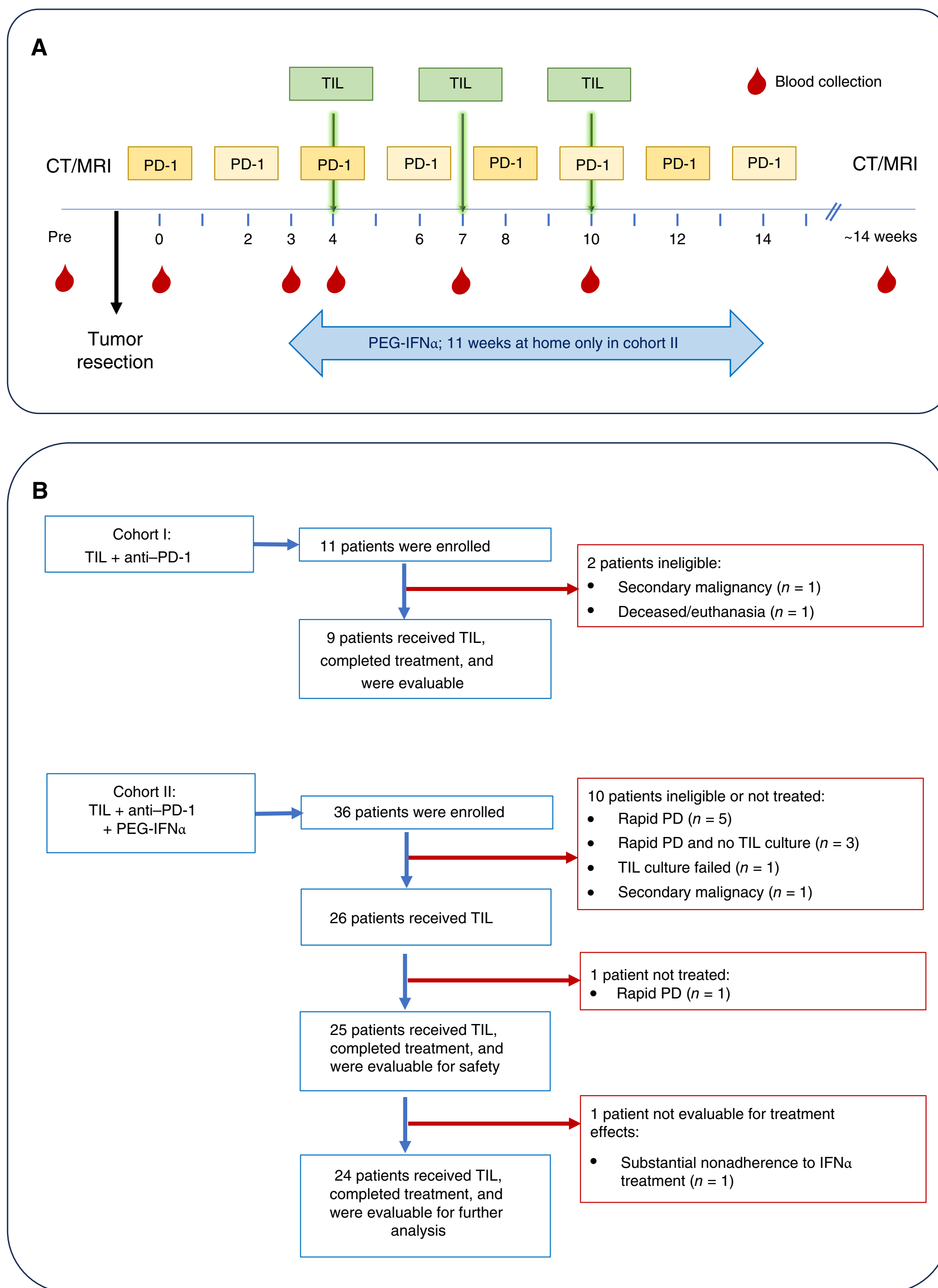

Figure 1.

Study design and patient disposition. **A,** Treatment schedule given in weeks. Baseline imaging and tumor resection/biopsies were performed prior to treatment. Anti–PD-1 nivolumab was given biweekly and from patient #25 onward 4 weekly according to standard recommendations. Pegylated IFNα (PEG-IFNα) was given as a weekly injection for 11 weeks in total, starting 1 week before the first TIL infusion. In total, three TIL infusions were given at a 3-week interval at weeks 4, 7, and 10. Blood was drawn at the indicated timepoints, and MRI/CT imaging was performed every 3 months to evaluate the clinical response. **B,** Patient disposition: 11 patients were enrolled in cohort I (TIL + anti–PD-1), and 9 patients were evaluable after the completion of the treatment. Thirty-six patients were enrolled in cohort II (TIL + anti–PD-1 plus IFNα). Twenty-five patients were evaluable for the safety of the treatment. All but one, i.e., 24 patients, completed and adhered to the full treatment and were evaluable for further clinical and immunologic evaluation.

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were identified at baseline, and the change in the sum of target lesions was evaluated by physical examination and imaging studies (CT and/or MRI) at 3 months and at follow-up every 3 months. The study was defined to have a positive clinical outcome if more than 30% DCR was obtained in cohort II at the first evaluation (3 months). This endpoint was considered a meaningful and valuable clinical effect in patients progressing after all other lines of treatment including ICB/anti–PD-1.

Additional secondary objectives comprise overall survival (OS) and progression-free survival (PFS) analyses, characterization of the infusion product, and potential correlations between the clinical response and hypothesis-related immune parameters. Survival is calculated from the start of the treatment to either progression, i.e., PFS, or the date of death or date of final analysis at study closure for OS.

Sample size calculation

First, the feasibility and safety of TILs in combination with anti–PD-1 without IFNα were assessed ($n = 9$, cohort I). Next, to assess the efficacy of the combination treatment, $n = 25$ patients were treated in the second cohort with TILs, anti–PD-1, and IFNα. The sample size is calculated based on Fleming's design for single-stage phase II trials and A'Hern's adaptation of Fleming's design (21, 22). Patients eligible for this phase I/II clinical trial are progressive on the standard treatment lines. Therefore, a response rate of less than 10% (P0) would not be sufficiently large enough to warrant further investigation. A response rate of 30% (P1) or more would indicate that the combination of anti–PD-1, TILs, and IFNα justifies subsequent testing in a phase III setting. Using a one-sided α of 5% and 80% power (β), this requires a total of 25 patients in our study ($\alpha = 0.05$; $\beta = 0.20$; P0 = 10%; P1 = 30%). If more than 30% of the patients in cohort II experience disease control, there is evidence to proceed to phase III at the end of the study.

Generation of TILs for infusion

TILs for infusion were cultured from a small resected tumor sample or biopsy, as previously described (9). In brief, TILs were cultured in T-cell medium [Iscove's modified Dulbecco's medium with penicillin (100 IU/mL), streptomycin (100 μg/mL), and L-glutamine (4 mmol/L); all from Life Technologies] and 7.5% heat-inactivated pooled human serum (Sanquin Bloodbank) supplemented with IL-2 (1,000 IU/mL, aldesleukin, Clinigen Healthcare B.V.) for a total period of 14 to 21 days. Next, the TILs were expanded according to the so-called rapid expansion protocol (REP) for another 14 days before harvesting and cryopreservation until further use. TILs were released for infusion when they met the release criteria with respect to phenotype (>80% T or NK cells), viability (>70% viable cells), and if negative for microbial contamination.

TIL characteristics

TILs were phenotyped as required for Qualified Person/Qualified Control release by flow cytometry after staining with CD3-FITC (clone SK7, BD Biosciences, RRID: AB_2811220), CD4-PE (clone RPA-T4, BD Biosciences, RRID: AB_395752), CD8-APC H7 (clone SK1, BD Biosciences, RRID: AB_1645482), CD56-PE (clone MY31, BD Biosciences, RRID: AB_2868831), and CD16-APC (clone 3G8, BioLegend, RRID: AB_314211) antibodies. Next, the samples were analyzed using a BD Scientific Canto II flow cytometer (RRID: SCR_018056) and FlowJo software (RRID: SCR_008520). For in-depth phenotyping of TILs, multispectral flow cytometry was performed using a 37-marker panel [see Supplementary Table S1; as described previously, samples were stained with LIVE/DEAD Zombie UV-fixable amine-reactive dye (BioLegend, cat no. 423107)] at room temperature for 20 minutes, after which the cells were washed and incubated with 50 μL PBS/0.5% BSA containing 2.5 μL Human TruStain FcX blocking solution (BioLegend, cat. number. 422302) for 10 minutes on ice to block Fc receptors. Next, the cells were stained for 30 minutes at room temperature in the dark with the cell surface antibodies in two consecutive rounds with sufficient washing in between. Intracytoplasmic/nuclear staining was performed using the True-Nuclear Transcription Factor Buffer Set (BioLegend, cat. number. 424401) according to the manufacturers' instruction. Finally, the cells were washed twice and stored in FACS buffer (PBS/0.5% BSA). Acquisition of the samples was done within 24 hours on a 5-laser Aurora Cytek spectral analyzer (Cytek Biosciences, RRID: SCR_019826) using SpectroFlo acquisition software (version 3, RRID: SCR_025494). The different cell populations were visualized and quantified after high-dimensional single-cell data analysis with opt-distributed stochastic neighbor embedding dimensionality reduction followed by FlowSOM consensus metaclustering and evaluation using the cloud-based OMIQ/edgeR data analysis software (RRID: SCR_012802). The cytokine secretion potency of TIL batches was assessed in response to CD3/CD28 activation beads (Dynabeads, Thermo Fisher Scientific; ratio of beads to T cells = 1:4). After 24 hours of stimulation, the supernatant was harvested, and secretion of 13 different cytokines was determined using the 13-plex human Th1/Th2 fixed panel Luminex assay (R&D Systems, cat. number. LKTM008B) according to the manufacturer's instructions.

The proliferation during the pre-REP phase and during REP was calculated by dividing the number of wells/cells harvested at the end of the culture period by the number of wells/cells at the start of the culture and corrected for a difference in total culture period, if applicable.

Statistical analysis

Descriptive statistics and univariate Cox regression were used to summarize patient baseline characteristics at the start of the treatment. OS and PFS were estimated according to the Kaplan–Meier method using IBM SPSS Statistics version 28 (RRID: SCR_002865) and/or GraphPad Prism version 10.2.3 (RRID: SCR_002798). Nonparametric tests were used for the analysis of absolute white blood cell counts and TIL characteristics.

Data availability

The data generated in this study are not publicly available because that could compromise patient privacy or consent but are available upon reasonable request to the corresponding author.

Results

Patients and treatment

Between November 2018 and March 2024, 47 patients with metastatic melanoma were enrolled in two different cohorts, with a time lag during the COVID-19 pandemic. Patients in cohort I were treated with autologous TILs and nivolumab, whereas patients in cohort II additionally received pegylated IFNα (**Fig. 1A**). Thirteen patients did not receive or complete treatment because of a secondary malignancy ($n = 2$) or rapid disease progression ($n = 11$) precluding (further) treatment. One patient (#22) was not evaluated for efficacy or immune parameters because of a substantial deviation from the standard IFNα treatment (**Fig. 1B**). In total, 9 patients treated in cohort I and

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24 patients treated in cohort II were evaluable for safety, efficacy, and immunologic effects.

The baseline characteristics of all evaluable patients are given in **Table 1**. The study representativeness is given in Supplementary Table S2. All patients had progressed on ICB and all other standard-of-care therapies. A similar fraction (67%) of patients with primary resistance to anti–PD-1 was included in each cohort. Baseline characteristics associated with worse prognosis, including LDH, brain metastasis, World Health Organization status, and neutrophil-to-lymphocyte ratio (NLR), are slightly more frequent in cohort II, albeit not significantly (**Table 1**). None of the other known prognostic parameters differed between treatment groups (**Table 1**). Although there is no statistical difference in gender, a bias toward men was observed in both cohorts at baseline. This may be explained by the fact that men, compared with women, are less likely to self-

Table 1. Baseline characteristics.

	All patients (*n* = 34)	Nivolumab + TIL (*n* = 9)	Nivolumab + TIL + IFNα (*n* = 25)	*P* value
Age (years)				0.53
Median (range)	54 (26–80)	53 (40–75)	55 (26–80)	
Gender, *n* (%)				0.45
Male	23 (67.6%)	7 (77.8%)	16 (64.0%)	
Female	11 (32.4%)	2 (22.2%)	9 (36.0%)	
WHO status, *n* (%)				0.55
0	18 (52.9%)	4 (44.4%)	14 (56.0%)	
1	16 (47.1%)	5 (55.6%)	11 (44.0%)	
Brain metastasis, *n* (%)				0.27
No	19 (55.9%)	7 (77.8%)	12 (48.0%)	
Yes	13 (38.2%)	2 (22.2%)	11 (44.0%)	
Unknown	2 (5.9%)	0 (0.0%)	2 (8.0%)	
LDH, *n* (%)				0.51
≤ULN	18 (52.9%)	6 (66.7%)	12 (48.0%)	
1x–2x ULN	14 (41.2%)	3 (33.3%)	11 (44.0%)	
>2x ULN	2 (5.9%)	0 (0.0%)	2 (8.0%)	
LDH				0.55
Mean (range)	274 (87–742)	244 (185–381)	285 (87–742)	
BRAF mutation, *n* (%)				0.42
No	19 (55.9%)	4 (44.4%)	15 (60.0%)	
Yes	15 (44.1%)	5 (55.6%)	10 (40.0%)	
Prior lines of treatment				0.74
Median (range)	2 (1–6)	3 (1–4)	2 (1–6)	
1–3 lines (%)	23 (67.6%)	7 (77.8%)	16 (64.0%)	
>3 lines (%)	11 (32.4%)	2 (22.2%)	9 (36.0%)	
Anti–CTLA-4 + anti–PD-1 combination (%)	19 (55.9%)	3 (33.3%)	16 (61.5%)	
Absolute leukocyte count				0.63
Mean (range)	7.10 (3.97–17.62)	6.69 (4.35–10.51)	7.24 (3.97–17.62)	
Absolute neutrophil count[a]				0.47
Mean (range)	4.83 (2.04–14.33)	4.47 (3.00–9.07)	4.96 (2.04–14.33)	
Absolute lymphocyte count				0.32
Mean (range)	1.36 (0.47–2.55)	1.24 (0.58–2.00)	1.40 (0.47–2.55)	
Absolute monocyte count				0.85
Mean (range)	0.60 (0.09–1.29)	0.61 (0.29–1.23)	0.60 (0.09–1.29)	
MLR				0.52
Mean (range)	0.48 (0.19–1.31)	0.53 (0.31–1.31)	0.46 (0.19–0.96)	
NLR[a]				0.97
Mean (range)	3.99 (1.40–15.64)	4.46 (2.31–15.64)	3.82 (1.40–9.90)	
LLR				0.94
Mean (range)	5.94 (2.92–18.12)	6.25 (3.86–18.12)	5.83 (2.92–14.23)	
CRP				0.92
Mean (range)	16.84 (0.3–87.60)	19.42 (0.3–82.5)	15.91 (0.4–87.60)	
Primary resistance, *n* (%)				0.94
No	12 (32.4%)	3 (33.3%)	8 (32.0%)	
Yes	22 (67.6%)	6 (66.7%)	17 (68.0%)	

Patient characteristics for all patients and per cohort, i.e., nivolumab and TILs without (cohort I) or in combination with IFNα (cohort II), respectively.
Abbreviations: CRP, C-reactive protein; LLR, leukocyte-to-lymphocyte ratio; MLR: monocyte-to-lymphocyte ratio; ULN, upper limit of normal; WHO, World Health Organization.
[a]Data of one patient are missing.

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detect their melanomas and make fewer visits to healthcare providers, resulting in diagnostic delay (23). The HRs for PFS and OS for all baseline characteristics were determined per cohort. None of the characteristics led to significant HRs for PFS or OS, except for cohort II, in which the leukocyte-to-lymphocyte ratio and NLR were shown to be prognostic for OS and NLR for PFS using univariate Cox regression (Supplementary Fig. S1A–S1D).

Safety

The treatment was generally well tolerated; all (serious) AEs [(S) AE] are given in Supplementary Table S3, and all (S)AEs ≥ grade 3 and their relation to treatment are shown in **Table 2**. Evaluation according to Common Terminology Criteria for Adverse Events version 5.0 gave similar results as version 4.0. The majority of the treatment-related (S)AEs were consistent with the known AEs of nivolumab and/or IFNα and could be adequately managed. Of the patients treated in cohort I, one patient experienced a grade 3 lymphopenia and another patient experienced a grade 3 diarrhea related to nivolumab treatment. Of the patients treated in cohort II, 7 of 25 (28%) patients experienced (S)AEs ≥ grade 3, including leukopenia, neutropenia or lymphopenia related to treatment with IFNα. These events were transient with a median total duration of 23 days (range, 12–65 days) and resolved in all cases when followed up after treatment. No febrile neutropenia–related infections were observed. In one patient (#22), the probably IFNα-related (S)AEs led to cessation of IFNα treatment during TIL infusion (week 4–6), followed by dose reduction to 50%. This substantial protocol deviation clearly altered the required leukodepleting effect of IFNα (Supplementary Fig. S2), which was only retained during the first TIL infusion. Therefore, it was justified to exclude this patient from further evaluations (**Fig. 1B**). Three other patients also deviated from the treatment schedule because of (S)AEs and/or disease progression. In patient #43, IFNα was not administered from week 10 onward. In patient #20, IFNα was not administered at week 10 and was given at 75% of the dose thereafter. Patients #45 and #47 received IFNα every other week from weeks 6 and 7 onward, respectively. However, as these less substantial modifications of the IFNα treatment still resulted in the desired and more prolonged leukodepletion supporting all or most TIL infusions (Supplementary Fig. S2), we considered it justified to include these patients in further evaluations.

Table 2. Safety evaluation.

	All patients (n = 34)	Nivolumab + TIL (n = 9)	Nivolumab + TIL + IFNα (n = 25)
(S)AEs ≥ grade 3, n (%)			
Leukopenia	1	0 (0.0%)	1 (4.0%)
Neutropenia	3	0 (0.0%)	3 (12.0%)
Lymphopenia	5	1 (11.1%)	4 (16.0%)
Anemia	1	0 (0.0%)	1 (4.0%)
Diarrhea	1	1 (11.1%)	0 (0.0%)

Safety evaluation for all patients and per cohort, i.e., nivolumab and TILs without (cohort I) or in combination with IFNα (cohort II), respectively. All (S) AEs ≥ grade 3 according to Common Terminology Criteria for Adverse Events version 5.0 possibly related to therapy are given.

Clinical efficacy

At the first evaluation of the treated per protocol population, only one of the nine patients [11.1%; 95% confidence interval (CI), 14.5%–36.7%] treated in cohort I obtained disease control, whereas significantly more patients (10/24, i.e., 41.7%; 95% CI, 20.4%–62.9%) obtained disease control when co-treated with IFNα (cohort II; **Fig. 2A**). The obtained DCR of 41.7% is higher than the preset >30% required for a positive outcome of the trial. Moreover, the mean response duration was longer in cohort II, 27.8 weeks (95% CI, 18.4–37.3), than in cohort I [13.8 (95% CI, 11.5–16.1; **Fig. 2B**; Supplementary Table S4)], whereas the median response duration was similar. The best overall response and percentage change in the sum of target lesions at any evaluation timepoint show that a reduction in the sum of the target lesions was only observed in 2 patients in cohort I compared with 14 of 24 patients treated with IFNα in cohort II (**Fig. 2C**). However, in 6 of these 14 patients displaying shrinking target lesions, new lesions developed. This defines them as having PD, whereas the results indicate that they experienced a mixed response. According to RECIST version 1.1, the objective response rate (CR plus partial response) was 0% in cohort I and 13% in cohort II, and no CR was obtained. Based on the intention-to-treat population, disease control was obtained in 9% (1/11) of patients treated in cohort I and 28% (10 of 36) of patients in cohort II.

At data closure (November 2024), the mean follow-up was 41 weeks. Kaplan–Meier analysis showed that the PFS and OS did not differ between both cohorts (Supplementary Fig. S3). Nevertheless, the percentage of patients with PFS at 6 months was 38% in cohort II (the IFNα group) versus 0% in cohort I. The median PFS in cohort II was 51 weeks (95% CI, 25.9–76.1) for patients with disease control compared with 12 weeks (95% CI, 11.3–12.7) for patients without disease control ($P < 0.0001$, **Fig. 2D**), with an HR for progression of 0.235 (95% CI, 0.094–0.589) for responders. The median OS in cohort II was 94 weeks (95% CI, 52.5–135.5) for patients with disease control, which was also longer than that for patients without disease control, i.e., 40 weeks [95% CI, 28.3–51.7; $P = 0.0019$; HR = 0.281 (95% CI, 0.108–0.735); **Fig. 2E**]. Interestingly, within cohort II, the 1-year survival of the patients without disease control was 14% compared with 89% of the evaluable patients with disease control ($n = 9$; 1 patient did not yet reach 1 year follow-up).

None of the well-known baseline prognostic factors for immunotherapy response [e.g., LDH, brain metastases, monocyte-to-lymphocyte ratio, NLR, or C-reactive protein; Supplementary Table S5] differed between the disease control and no disease control groups. Interestingly, 8 of 11 (73%) patients with disease control after treatment displayed a primary resistance to PD-1 ICB.

Translational studies

The response to TIL therapy plus IFNα in metastatic melanoma was previously shown to be associated with a more sustained leukopenia and neutropenia (9). To investigate an association in the current trial, various white blood cell counts were evaluated for both cohorts (**Fig. 3A–L**). Treatment with TILs and anti–PD-1 (cohort I) did not result in the reduction of circulating leukocyte or neutrophil counts (**Fig. 3A** and **D**, respectively). In cohort II, leukopenia and neutropenia was observed after the start of IFNα treatment, and this effect was most prominent in patients with disease control compared with patients without disease control (**Fig. 3B**, **C**, **E**, and **F**, respectively), supporting our earlier observations (9). Lymphocyte and monocyte counts were not overtly affected (**Fig. 3G–L**).

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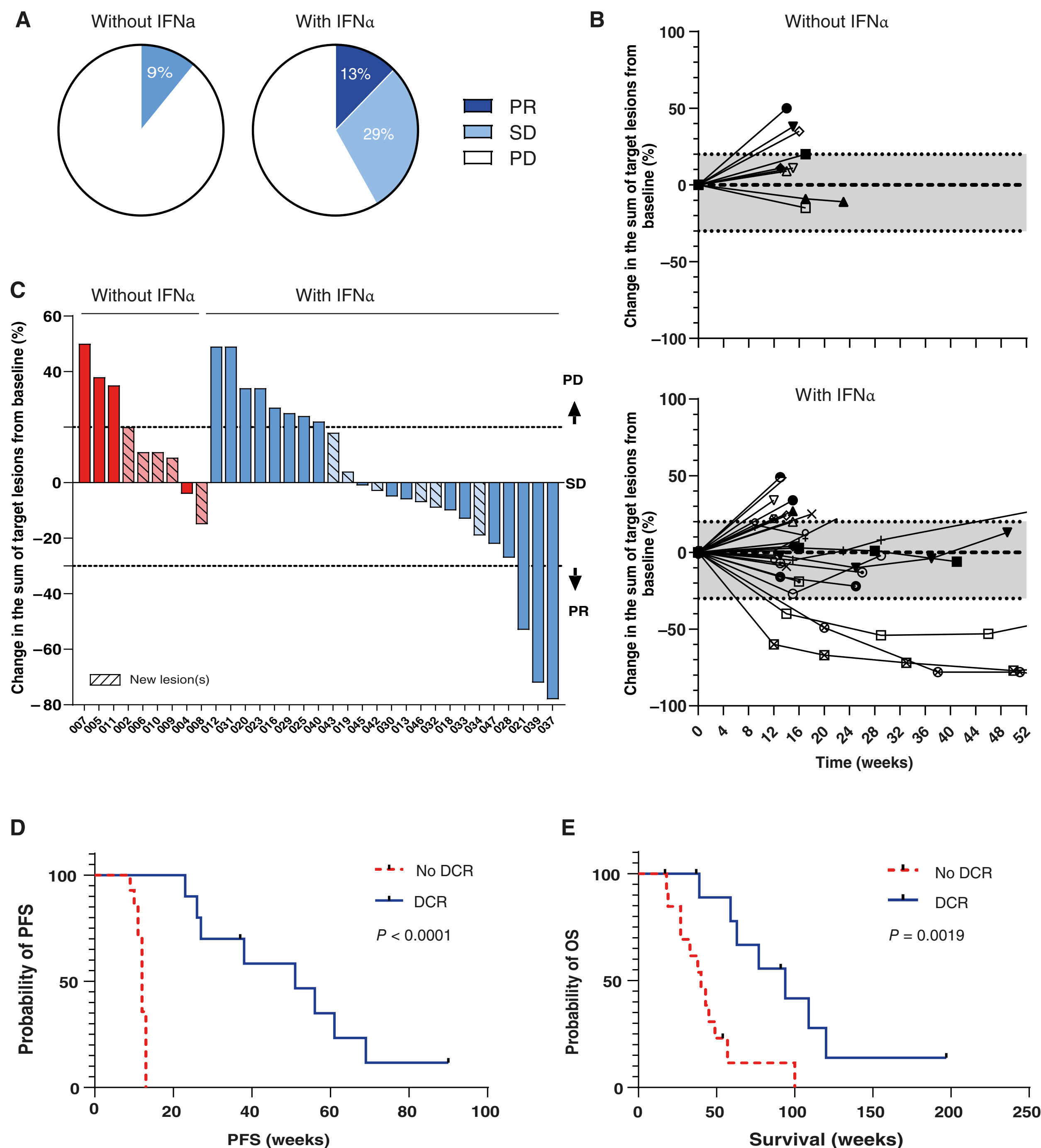

Figure 2.

Clinical response evaluation. The clinical response was evaluated according to RECIST version 1.1. **A,** Pie plots showing the percentage of patients who had a partial response (PR), stable disease (SD), or progressive disease (PD) at the first evaluation timepoint (3 months) after treatment with TILs + anti–PD-1 in cohort I (without IFNα, $n = 9$, left plot) or cohort II (with IFNα, $n = 24$, right plot). **B,** Spider plot showing the percentage change in the sum of target lesion size from baseline and duration thereof (in weeks) for cohort I (without IFNα, top) and cohort II (with IFNα, bottom). **C,** Waterfall plot showing the percentage change in the sum of target lesion size from baseline in patients who completed treatment in cohort I (without IFNα, in red) and cohort II (with IFNα, in blue). Hatched bars indicate patients who developed new lesions, defined as PD. A decrease of 30% or more in the sum of target lesion size is defined as a PR, with less than 30% decrease or 20% increase defined as SD and 20% or more increase in the sum of target lesions or appearance of new lesion(s) as PD, indicated by the gray area and dashed lines in **B** and **C**, respectively. **D,** PFS and (**E**) OS curves of patients who obtained DCR (blue lines) or no DCR (red lines) after treatment in cohort II (i.e., with IFNα) and significance according to Kaplan–Meier are shown.

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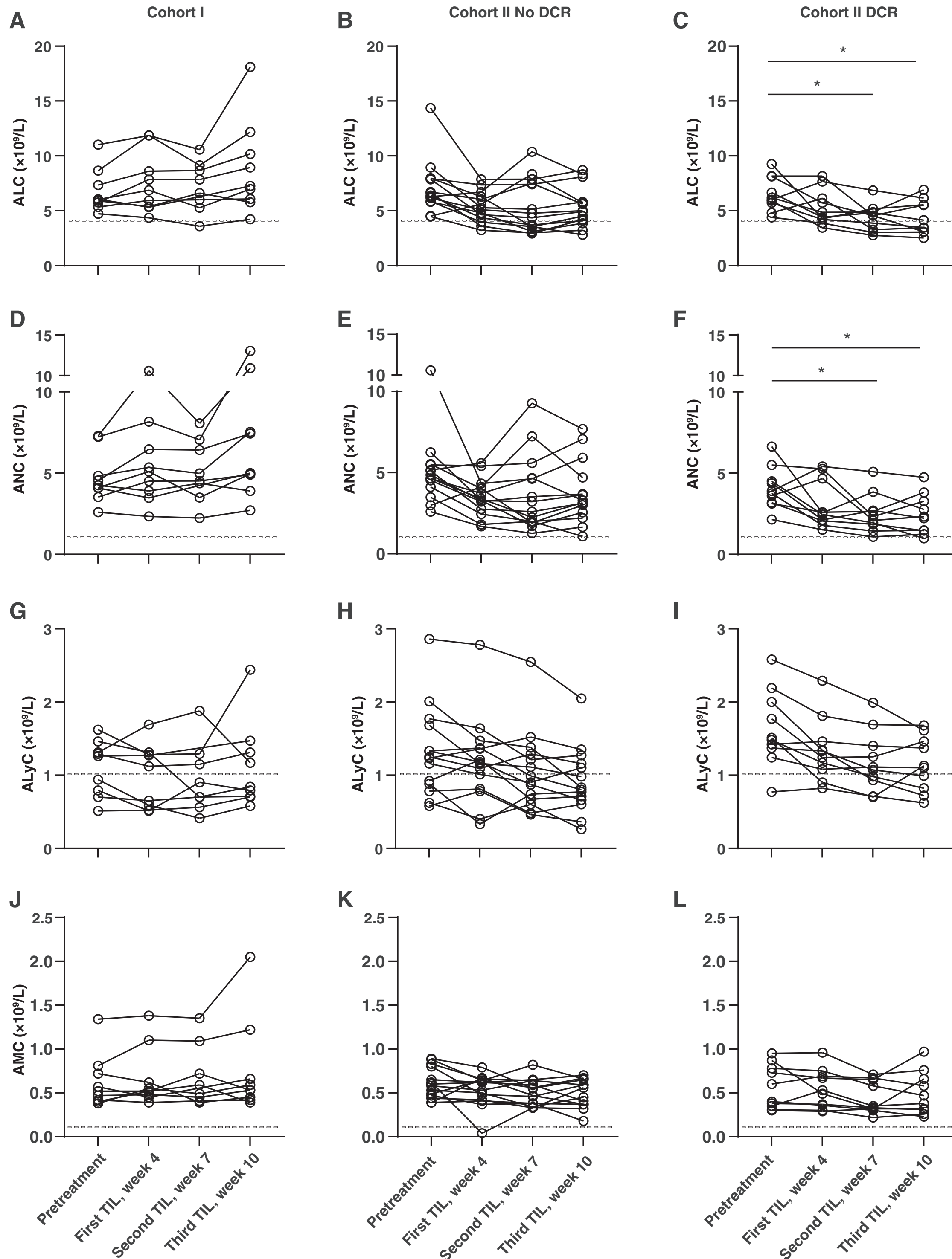

Figure 3.

Therapy-induced changes in the circulation. White blood cell counts were performed on blood samples obtained at the indicated timepoints at baseline (i.e. pretreatment) and during treatment. Absolute leukocyte cell count (ALC; **A–C**), absolute neutrophil cell counts (ANC; **D–F**), absolute lymphocyte cell counts (ALyC; **G–I**), and absolute monocyte cell counts (AMC; **J–L**) are shown. Results for patients treated in cohort I (without IFNα, left, $n = 9$) and patients without disease control (middle, $n = 14$) or with disease control (right, $n = 10$) after treatment in cohort II (with IFNα) are shown. The gray dashed lines indicate the lower limit of normal absolute cell counts for each cell type. *, $P < 0.05$ indicates significant changes from baseline using the Kruskal–Wallis test with Dunn correction for multiple comparisons.

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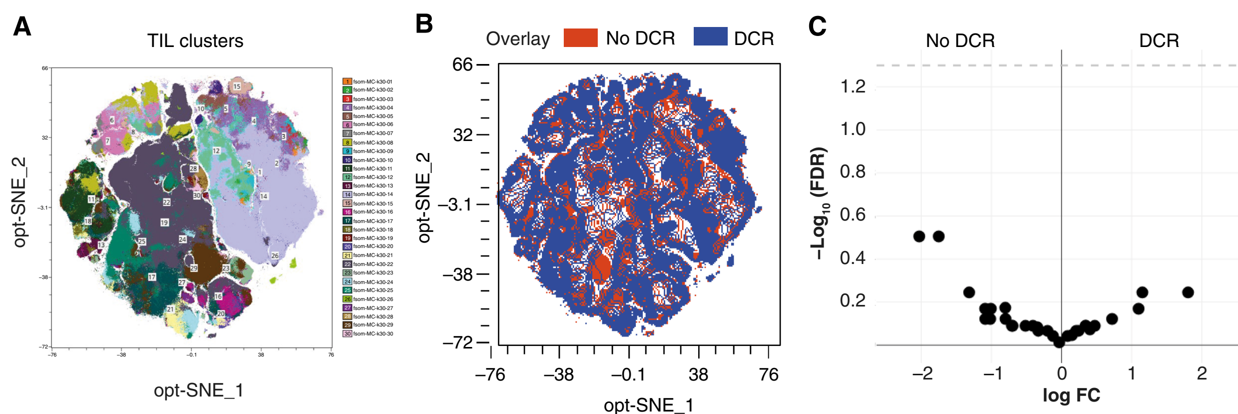

Figure 4.

Phenotyping of infused TIL batches. TILs were stained with a 37-marker panel (see Supplementary Table S1). High-dimensional single-cell data analysis of the stained TIL was performed by opt-distributed stochastic neighbor embedding (opt-SNE) and FlowSOM using OMIQ software. **A,** Opt-SNE plots visualizing cluster partitions by FlowSOM for all patient samples. **B,** Overlay plot of the clusters for patients who did (DCR, in blue) or did not (no DCR, in red) obtain disease control after treatment in cohort II (with IFNα). **C,** Volcano plot showing the magnitude (*x*-axis) and significance (*y*-axis) of the relative difference in frequency distribution of all identified clusters in TIL administered to patients with no DCR versus DCR after treatment in cohort II. The dashed line indicates threshold for significance ($P < 0.05$) calculated in OMIQ/edgeR. FC, fold change.

TIL characteristics

TILs were obtained from lymph node metastases (36.4%), subcutaneous lesions (18.2%), liver (15.2%), or other sites (30.3%; Supplementary Table S4). TIL cultures were not successful for five patients, and disease progression precluded a second attempt to timely obtain sufficient TILs for treatment. For three patients (#08, #39, and #47), a new biopsy for TIL generation was required. On average, TILs were cultured for 30 days in total and expanded >1,500 times. The time from biopsy/resection to TIL infusion was <7 weeks. These parameters did not differ between treatment or response groups. A median dose of 5.7×10^8 TILs per infusion was administered. On average, TILs consisted of 55% $CD3^+CD8^+$ cells, 41% $CD3^+CD4^+$ cells, and less than 0.5% $CD3^-CD16^+$ NK cells. The frequencies of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells and NK cells as well as the total dose of administered TILs did not differ between cohorts, nor between patients with or without disease control after treatment in cohort II (**Fig. 4**; Supplementary Fig. S4A–S4E). Thirty different TIL clusters were identified based on their specific expression profiles after using a 37-marker flow cytometry panel (**Fig. 4A**; Supplementary Fig. S5A–S5C). Frequencies of classical regulatory T cells expressing CD25 and FoxP3 were very low in all TIL batches, and the frequencies of the 30 identified clusters did not significantly differ between TILs administered to patients with DCR or no DCR after treatment with IFNα in cohort II, as shown in the overlay plot (**Fig. 4B**) and volcano plot (**Fig. 4C**). Upon stimulation via CD3/CD28 signaling, TIL batches used for the treatment secreted variable amounts of the 13 different Th1/Th2 cytokines tested. All TILs produced substantial amounts of IFNγ (all above the upper detection limit of the assay), TNFα, and GM-CSF, but no differences were observed between TILs administered to patients that did or did not obtain disease control (Supplementary Fig. S6).

Discussion

This study demonstrates that TIL plus nivolumab in combination with IFNα is safe and induces disease control in patients with late-stage metastatic melanoma progressive on prior ICB. IFNα contributed to the clinical effect as only 1 of 9 (11%) patients treated with TIL plus anti–PD-1 displayed disease control, whereas 10 of 24 (42%) patients also receiving IFNα obtained disease control. The obtained disease control is higher than what we observed in ICB-refractory patients after treatment with TIL plus IFNα but without anti–PD-1 in our previous trial (9). This suggests that the addition of anti–PD-1 to TIL plus IFNα, in order to prevent PD-1 checkpoint–mediated inhibition of transferred TILs, contributed to the improved outcome observed in the study reported here. A similar approach is investigated in the TILVANCE-301 trial (NCT05727904) evaluating the efficacy and safety of TIL therapy (lifileucel) plus pembrolizumab in patients with untreated advanced melanoma.

Importantly, no differences existed between the TIL product or prognostic baseline characteristics between the two treatment groups. In fact, the fractions of patients with a normal LDH and the absence of brain metastasis, both associated with better prognosis, were slightly higher in cohort I. The leukocyte-to-lymphocyte ratio, NLR, and monocyte-to-lymphocyte ratio associate with shorter survival in cohort II only in univariate analysis but not in multivariate analysis. All other known prognostic factors also do not associate with PFS or OS in our trial. This may be explained by the fact that all patients included in our study are progressive after all standard therapies and therefore comprise a population already selected for worse prognosis, where otherwise prognostic factors lose impact.

Interestingly, 8 of 11 (73%) treated patients who obtained disease control exhibited primary resistance to PD-1. This suggests that primary resistance to PD-1–based ICB could be overcome by providing sufficient tumor-reactive T cells and implies that in these patients, a lack of response to PD-1 ICB did not result from other extrinsic or intrinsic immune resistance mechanisms, except for those that can be overcome by IFNα treatment in cohort II. Indeed, responses to both anti–PD-1 and ACT rely on T cells in the tumor microenvironment (TME), and lack of such T cells may hamper responses to both types of therapies (24, 25). Although loss of HLA class I by tumor cells at the genetic level would preclude any subsequent contribution of T cells to tumor control, the loss or reduced expression of HLA class I due to epigenetic factors (26) is likely to be restored by the IFNα treatment (27). In total, 16 patients,

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receiving the full treatment, displayed primary PD-1 ICB resistance, but half of these patients did not respond to therapy, suggesting that the presence of sufficient tumor-reactive T cells may not be their major or only problem. Here, other mechanisms may be at play, including hardwired HLA loss or disrupted IFNγ signaling, also described as another frequent mechanism of acquired resistance to ICB (28, 29). A recent study demonstrated that the response rate to TIL therapy is lower in anti–PD-1–experienced patients with melanoma, with one possible reason being a reduced tumor mutational burden (TMB) in the ICB-resistant patients (30). Further in-depth translational studies are required to dissect other reasons for nonresponsiveness among our patients.

The treatment was well tolerated. Although possible side effects that are related to the TIL infusions cannot be fully ruled out, no clear TIL-related side effects, such as cytokine release syndrome, were observed. Most side effects were related to anti–PD-1 or IFNα. Importantly, the IFNα-induced effects on white blood cell composition were anticipated and desired, as we previously reported its association with response to ACT in patients with melanoma (9, 17). This association of IFNα-induced leukopenia and neutropenia with disease control was validated in the current study. Our mild conditioning protocol allowed administration of multiple TIL infusions, thereby increasing the presence of TILs over a longer period of time which may improve efficacy.

The clinical activity observed in our study is less than the reported response rates of 49% and 31% obtained in two other studies involving predominantly patients with PD-1–refractory melanoma (8, 31). However, in these trials, lymphodepleting chemotherapy and high-dose IL-2 were used at the cost of substantial toxicity. Recently, several approaches to minimize lymphodepleting conditioning and/or IL-2–related toxicity have been initiated (32), including trials using next-generation TIL products engineered to express membrane-bound IL-15 (33) or IL-12, to replace the need for high-dose IL-2. Preliminary results show promising and higher response rates than those observed in our study, but also at the cost of hematologic grade 4 AEs. In combination with reduced-dose lymphodepleting conditioning or our mild IFNα conditioning, these TIL products could achieve high response rates without severe toxicities and as such form promising alternatives for a larger patient population. The current study has several limitations. First, we did not perform a randomized trial to assess the impact of IFNα. Fortunately, baseline characteristics known to be associated with outcomes were comparable between both groups and, if anything, less favorable for cohort II. Second, the individual contribution of IFNα as well as the combination of IFNα and anti–PD-1 to tumor control cannot be assessed. However, historical data showed that IFNα monotherapy, given in the low dose applied here, does not confer any tumor response in metastatic melanoma (34). Moreover, the KEYNOTE-029 study investigating the effect of anti–PD-1 (pembrolizumab) combined with the same low-dose pegylated IFNα as used in our trial for advanced melanoma showed limited activity and was prematurely stopped (35). Third, we did not include a cohort that was treated with IL-2 instead of IFNα, and therefore a direct comparison between the supportive effect of IL-2 and IFNα is lacking. Finally, we manufactured TILs from ICB-resistant patients. Thus, our product may comprise less stem cell–like T cells thought to be required for optimal antitumor efficacy (24, 25).

In conclusion, this novel approach provides a feasible therapy with low toxicity, minimal burden, and evidence of clinical activity for patients with metastatic melanoma. It addresses three major issues that limit the application of TILs in general: (i) the inhibition of T-cell effector function via PD-1 signaling through administration of ICB; (ii) avoidance of high-dose IL-2–associated toxicity, and (iii) reduction of prolonged hospitalization of patients required for chemotherapy-mediated lymphodepletion used in most ACT protocols. As such, the results of our study warrant further evaluation of TIL plus anti–PD-1 and IFNα as a treatment for ICB-resistant melanoma, and it may be of particular interest for the treatment of more frail patients who are less capable of enduring the considerable toxicity associated with lymphodepleting chemotherapy and high-dose IL-2 regimens.

Authors' Disclosures

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Authors' Contributions

E.M.E. Verdegaal: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **A.L.C. Verpoorte:** Data curation, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **M.K. van der Kooij:** Conceptualization, formal analysis, investigation. **L. de Bruin:** Investigation, methodology. **M. Visser:** Investigation, methodology. **C.E. van der Minne:** Investigation, methodology, project administration. **V. Weeda:** Investigation, methodology. **I.C.F.M. Roozen:** Resources. **M.A. Jonker:** Resources. **I.M. Westra:** Resources, methodology. **P. Meij:** Resources, methodology. **F.M. Speetjens:** Resources. **S.M. Zunder:** Resources. **G.-J. Liefers:** Resources. **S.J. Santegoets:** Methodology. **S.H. van der Burg:** Resources, supervision, funding acquisition, methodology, writing–original draft, writing–review and editing. **E.H.W. Kapiteijn:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, writing–review and editing.

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Note

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