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Low-dose interferon-alpha preconditioning and adoptive cell therapy in patients with metastatic melanoma refractory to standard (immune) therapies: a phase I/II study

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ABSTRACT

Background Adoptive cell therapy (ACT) with tumor-reactive T cells has shown consistent clinical efficacy. We evaluated the response to ACT in combination with interferon alpha (IFNa) preconditioning in patients with stage IV metastatic melanoma, most of which were progressive on cytotoxic T-lymphocyte-associated protein 4 and/or programmed cell death protein 1 checkpoint blockade therapy.

Methods Thirty-four patients were treated with ex vivo expanded tumor reactive T cells, derived from mixed lymphocyte autologous tumor cultures, or with autologous tumor-infiltrating lymphocytes and evaluated for clinical response. Clinical and immunological parameters associated with response were also evaluated.

Results Best overall response defined as clinical benefit, comprising either complete response, partial response or stable disease >6 months, was observed in 29% of the patients. Forty-three per cent of the 14 immunotherapy-naïve patients and 20% of the 20 patients progressive on prior immunotherapy benefited from ACT. The overall survival (OS) was 90% versus 28.6% at 1 year and 46.7% versus 0% at 3 years follow-up, of responder and non-responder patients, respectively. Median OS was 36 versus 7 months, respectively. IFNa pretreatment resulted in leukopenia, neutropenia and lymphopenia, which was sustained during the treatment in clinical responders and associated with response. Differences in antigen specificity, but not in phenotype, cytokine profile or CD8+ T cell number of the ACT products correlated with clinical response. Cross-reactivity of the ACT products to one or more allogeneic human leukocyte antigen-matched melanoma cell lines was associated with short OS after treatment while the ACT products of very long-term survivors showed no cross-reactivity but recognized patient-specific neoantigens.

Conclusion This study demonstrates that ACT in combination with a mild IFNa preconditioning regimen can induce clinical benefit even in immunotherapy pretreated patients, although with lower success than in immunotherapy-naïve patients. ACT products comprising neoantigen reactivity may be more effective.

BACKGROUND

The emergence of several new treatment options including targeted and checkpoint-blocking therapy for melanoma has dramatically improved the response rate from a very poor median survival time of 6–9 months to almost 2 years.[1] Nevertheless, almost half of the patients do not respond or eventually become refractory to these therapies.[2–6] Adoptive cell therapy (ACT) offers an additional treatment option for patients presenting with standard treatment refractory progressive disease (PD). ACT involves the reinfusion of ex vivo expanded autologous tumor-reactive T cells (TRT) or tumor infiltrating T cells (TIL) and is proven to be a very effective treatment modality for solid tumors resulting in an objective response rate of up to 50% in melanoma when administered after non-myeloablative conditioning by chemo-depletion and additional postinfusion of interleukin-2 (IL-2) in immunotherapy-naïve patients.[7–10] However, the response rate and overall survival (OS) considerably drop when patients are progressive on anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and/or programmed cell death protein 1 (PD-1) blockade prior to ACT treatment.[11]

T cells used for infusion, that is, the ACT product, are generally obtained by ex vivo expansion of TIL, of which it is known that their abundance correlates with better survival in melanoma.[12–14] Alternatively, the

ACT product can be formed by TRT, expanded from peripheral blood mononuclear cells (PBMC) by mixed lymphocyte tumor cell culture (MLTC),[15 16] requiring an established tumor cell line for repeated ex vivo stimulation, which is not feasible for most patients.

Previous ACT trials demonstrated the need for chemotherapy-driven lymphodepletion prior to T cell infusion and concomitant administration of high-dose IL-2 to obtain clinical success.[17–19] A considerable reduction of the toxicity associated with these protocols could be obtained by reduction of the postinfusion IL-2 dose.[10 20–22] In an interim analysis, we showed that clinical benefit can also be obtained when low-dose interferon alpha (IFNa) is used as a very mild and safe preconditioning and T cell supporting regimen.[15]

Here, we report the data of the complete trial in which we investigated the safety and feasibility to treat patients with metastatic melanoma with adoptively transferred T cells in combination with IFNa. We dissected the effect of pretreatment clinical parameters, IFNa conditioning and the phenotypical as well as antigen-specificity characteristics of the ACT product in order to determine their association with clinical response.

PATIENTS, MATERIALS AND METHODS

Patient selection

Patients were eligible if 18 years or older with histologically proven stage IV or irresectable stage III cutaneous melanoma, with a WHO performance status 0–2 and a life expectancy of at least 6 months. Patients had PD at the start of treatment and systemic treatment had to be discontinued for 4 weeks in case of chemotherapy, radiotherapy or immunotherapy and 2 weeks in case of targeted therapy (BRAF/MEK inhibitors). At least one resectable or bioptable lesion was required for establishment of a tumor cell line and/or TIL culture and at least one additional measurable target lesion was required for response evaluation. 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 3 years prior to entry into the study, a known allergy to penicillin or streptomycin or seropositivity for hepatitis B/C, HIV, HTLV or *Treponema pallidum*.

Study design

All patients were treated with autologous T cells in combination with IFNa. Low-dose IFNa injections (3 million units subcutaneous daily) were started 1 week (wk -1) before the first T cell infusion (wk 0) and continued for a total period of 12 weeks. T cell infusions were given intravenously with a 3-week interval. Patients were treated in three increasing dose cohorts of $1–2.5\times10^8$, $2.5–5\times10^8$ or $7.5–10\times10^8$ T cells per infusion for cohort I, II and III, respectively. Cryopreserved T cells were thawed and administered intravenously over a time period of 30–60 min.

Before and at several time points after infusions heparinized venous blood was collected and isolated PBMC as well as serum/plasma samples were cryopreserved until further analysis.

Before start of treatment and after three T cell infusions, the tumor response was evaluated by physical examination and imaging studies (CT and/or MRI) according to the Response Evaluation Criteria In Solid Tumors (RECIST) V.1.0 and V.1.1. Patients were admitted to the hospital for only 24 hours after the T cell infusions for observation. The primary objective was to evaluate the safety of the combination of T cells with low-dose IFNa, which was assessed using the NIH Common Terminology Criteria for Adverse Events (CTCAE) V.2.0 and V.4.0. Secondary objectives were clinical response evaluation and analysis of immunological parameters.

Generation of T cell products for infusion

Patients were treated with PBMC-derived TRT obtained by MLTC, as previously described.[15] These cultures required the use of an established autologous tumor cell line, which was not available for all patients. Alternatively, patients received TIL, which were readily available for each patient and cultured from a small resected tumor sample essentially using a previously described protocol[23] (online supplementary figure 1). TIL were cultured in T cell medium (Iscoves Modified Dulbecco's Medium (IMDM) with penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM) (all from Life Technologies, Breda, The Netherlands), and 7.5% heat inactivated pooled human serum (Sanquin, Bloodbank, Amsterdam, The Netherlands) supplemented with IL-2 1000 IU/mL (Proleukin/Aldesleukin, Novartis, Arnhem, The Netherlands) for a total period of 14–21 days. Next, the TIL were expanded according to the described Rapid Expansion Protocol[23] for another 14 days before harvesting and cryopreservation, until further use. The production and batch release were performed under full Good Manufacturing Practices compliance (GMP).

Cell line generation and culture

Autologous melanoma cell lines were established in our GMP facility from resected tumor tissue as described previously.[15] All other melanoma cell lines were established in the laboratory of Medical Oncology (LUMC, Leiden, The Netherlands) except for melanoma cell lines FM3 and FM6 which were provided by P. Thor Straten, Copenhagen, Denmark. BLM was obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands), and MZ7.4-mel obtained from J. Gutenberg University (Mainz, Germany). Authentication of the cell lines was performed by human leukocyte antigen (HLA)-genotyping at the Department of Immunohematology and Bloodbank of the LUMC and they were regularly tested to be mycoplasma negative. All melanoma cell lines were cultured in tumor cell medium (ie, Dulbecco's minimal

essential medium (Life Technologies) with 8% heat inactivated fetal calf serum (FCS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM) all from Life Technologies). Autologous EBV-LCL B cells and phytohemagglutinin (PHA)-stimulated T cell blasts (PHA-blasts) were established and cultured in B cell medium, that is, IMDM with 8% heat inactivated FCS, penicillin (100 IU/mL), streptomycin (100 µg/mL) and L-glutamine (4 mM). Epstein-Barr virus-transformed lymphoblastoid B cell lines (EBV-LCL) were used as APCs. These autologous EBV-LCL are known to process and present peptide both in HLA class I and II. The transformation was induced by incubation of patients' PBMC with supernatant of the marmoset B cell line containing infectious particles of EBV strain B95-8 for 1 hour at 37°C. Culture medium consisted of RPMI-1640, supplemented with 5 µg/mL PHA (Thermo Fisher Scientific), 10% FCS, L-glutamine (4 mM), penicillin (100 µg/mL) and streptomycin (100 µg/mL). Cells were refreshed every 5–6 days with B cell medium and cultured for 3 weeks before being used as target cells.

Phenotypical analysis of PBMC

PBMCs collected before and after 1 week of IFNa treatment were thawed and divided into multiple samples that were stained with separate antibody panels for myeloid-derived suppressor cell (MDSC), inhibitory/memory, regulatory T cell and dendritic cell (DC) markers, respectively (online supplementary table 1a). Dead cells were stained using Yellow ArC-Qdot585 (ThermoFisher, L34959).

Staining was carried out according to our standard protocols,[24] washed with Fluorescence Activated Cell Sorting (FACS) buffer, fixed in 1% paraformaldehyde and analyzed using a LSRFortessa X20 (BD Biosciences).

Staining of the regulatory T cell panel was conducted using the Transcription Buffer Set (BD Biosciences) as previously described.[25] FACS results were analyzed with BD FACSDiva software (V.8.02).

Cytokine analysis in serum/plasma

The serum/plasma concentration of homeostatic cytokines IL-7, IL-15 and IL-21 was analyzed using ELISA (R&D diagnostics; DY207, Biolegend; 435104, Mabtech; 3540–1 H-6), according to the manufacturer's instructions.

Phenotypical characterization of infused T cell

For detailed phenotypical characterization, reference vials of T cell batches used for infusion were thawed, counted and resuspended in FACS buffer consisting of phosphate-buffered saline+0.5% bovine serum albumin. Dead cells were stained using Yellow ArC-Qdot585 (ThermoFisher, L34959). Next, the T cells were divided into multiple samples and stained with separate antibody panels for inhibitory, homing, memory and regulatory T cell markers, respectively (online supplementary table 1b). The staining was carried out according to our standard operating procedures as previously described,[24] washed with FACS buffer, fixed in 1% paraformaldehyde and analyzed using a LSRFortessa X20 (BD Biosciences).

Functional characterization of the infused T cells

Tumor reactivity. The antigen specificity of the infusion product was tested against a broad panel of melanoma cell lines that were (partially) matched for at least one HLA class I allele with the corresponding patient. If available, autologous tumor cells were also tested. Briefly, 1.5×10^4 T cells (effector cells) were co-cultured with 3×10^4 target cells in a total volume of 150 µL B cell medium (ie, T cell medium with 8% FCS instead of human serum) in triplicate wells of a U-bottom 96-well plate. Medium alone and EBV-LCL B cells or PHA-blasts were used as negative controls and staphylococcal enterotoxin B (SEB, 0.5 µg/mL) or PHA (5 µg/mL) were used as positive controls. After overnight incubation at 37°C, the supernatant was harvested to determine the interferon-gamma (IFNg) secretion as a measure of reactivity by ELISA (Sanquin) according to manufacturer's recommendations.

Neoantigen reactivity. To identify recognition of neoantigens derived from non-synonymous somatic mutations within expressed genes whole exome and RNA sequencing was performed and either 31-mer synthetic long peptides (SLPs) or 8–12-mer synthetic short peptides (SSPs) covering the mutation were manufactured as previously described.[26] Of note, in contrast to the SLP, the SSP were selected based on in silico prediction using the ISABELLA algorithm (ISA Pharmaceuticals, Leiden, The Netherlands). Next, T cells were incubated as described in the previous paragraph with target cells, that is, tumor cells or autologous B cells either unloaded or preloaded overnight with SLP pools or single peptides (10 µg/mL per peptide). Recognition of SSP was analyzed by direct addition of SSP (1 µg/mL per peptide) to the T cells. Medium alone or unloaded autologous B cells were included as negative controls and SEB (0.5 µg/mL) or PHA (5 µg/mL) as positive controls. Reactivity of T cells was measured after 24 hours co-incubation with target cells/peptides by IFNg secretion using ELISA (Sanquin).

Cytokine profile. To characterize the cytokine profile potentially released on activation of the infused T cells, T cells were stimulated with SEB (0.5 µg/mL) or PHA (5 µg/mL) and if available with the autologous melanoma cell line as positive control and autologous EBV-LCL B cells, PHA-blasts or medium alone as negative controls. After incubation for 24 hours supernatant was harvested and used to analyze the cytokine production using the human Th1/Th2 cytometric bead array (BD Pharmingen). Specific cytokine production was defined by a cytokine concentration above the cut-off value (IFNg 50 pg/mL; other cytokines 10 pg/mL) and >2× the concentration of the medium control.[27]

Statistical analysis

Descriptive statistics were used to summarize patient baseline characteristics at start of treatment. Survival from start of treatment to progression and death was estimated

according to the method by Kaplan-Meier using SPSS (V.25, IBM, released 2017).

Paired analyses between FACS data from PBMC samples of patients before and after 1 week of IFNa use were compared using Cytosplore V.2.1.5, R V.3.4.4, R studio V.1.1.442 and using the R-package cytofast.[28]

Furthermore, paired and independent analyses were performed on the data generated by FACS analysis on both the T cell products and the PBMCs by GraphPad Prism V.7.00 for Windows (GraphPad Software, La Jolla, California, USA) and SPSS. A D'Agostino and Pearson omnibus K2 test was performed to determine whether data were normally distributed within groups. To compare paired data following a normal distribution a paired t-test was used, when the assumption of normality was violated a Wilcoxon signed rank test was performed. For unpaired data following a normal distribution a unpaired t-test was used, when the assumption of normality was violated a Mann-Whitney U test was performed.

RESULTS

Patient characteristics at baseline

Forty-one patients with progressive stage IV metastatic melanoma were included for treatment with ACT in combination with low-dose IFNa (ACT+IFNa) in our phase I/II trial between 2006 and 2018. All patients had PD before treatment and seven patients did not complete their full cycle of three infusions due to rapid disease progression. Thirty-four patients completed one full cycle of T cell infusions and were evaluated for safety/toxicity, clinical response and immunological parameters (online supplementary figure 1). The patients were treated in three dose cohorts and received either TRT or TIL. The baseline characteristics of all patients are shown in table 1. Details of start of treatment, (pre)-treatment regimens and response to treatment of individual patients are given in online supplementary table 2. Comparison of previously described prognostic factors of worse OS did not differ at baseline between the different dose cohorts (table 1) nor between patients treated with TRT versus TIL (online supplementary table 3). Since several lines of systemic treatments are currently available for patients with stage IV metastatic melanoma, the majority of the evaluated patients (65%) received two or more lines of prior systemic therapies. Notably, the majority of TIL-treated patients (83%) was pretreated with checkpoint therapy (online supplementary table 3). The percentage of patients with a confirmed brain metastasis was higher

Table 1 Patient characteristics at baseline

		Cohort I (n=7)	Cohort II (n=21)	Cohort III (n=6)	P value*
Age mean (min-max)		50.43 (33-67)	54.24 (41-77)	46.50 (36-56)	0.24
Gender, n (%)	Male	7 (100)	14 (66.7)	5 (83.3)	
	Female	0 (0)	7 (33.3)	1 (16.7)	
LDH mean (min-max)		234.3 (144–477)	341.6 (136–973)	279 (166–446)	0.46
LDH level, n (%)	<250	5 (71.4)	11 (52.4)	2 (33.3)	
	250–500	2 (28.6)	7 (33.3)	4 (66.7)	
	>500	0 (0)	3 (14.3)	0 (0)	
WHO, n (%)	0	4 (57.1)	12 (57.1)	4 (66.7)	
	1	2 (28.6)	7 (33.3)	2 (33.3)	
	2	0 (0)	1 (4.8)	0 (0)	
	Missing	1 (14.3)	1 (4.8)	0 (0)	
Brain metastasis (confirmed), n (%)		5 (71.4)	6 (28.6)	1 (16.7)	0.07
Pretreatment, n (%)	BRAFi/MEKi	2 (28.6)	8 (38.1)	4 (66.7)	0.34
	Anti-CTLA-4 only	1 (14.3)	1 (4.8)	0 (0)	n.e.
	Anti-PD-1±anti-CTLA-4	(0)	12 (57.1)	6 (100)	**0.001**
Prior lines of systemic therapies, n (%)	0–2	7 (100)	14 (66.7)	1 (16.7)	**0.007**
	≥3	0 (0)	7 (33.3)	5 (83.3)	
TRT or TIL, n (%)	TRT	6 (85.7)	4 (19)	0 (0)	**0.001**
	TIL	1 (14.3)	17 (81)	6 (100)	
Responders†, n (%)		1 (14.3)	7 (33.3)	2 (33.3)	
		CR	CR, PR, 5×SD	2×SD	

*Statistically significant p values are indicated in bold, n.e.=not evaluable because n=2.
†Responders are defined by patients having CR, PR or SD.
CR, complete response; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; LDH, lactate dehydrogenase; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

in the group of patients treated with TRT when compared with TIL-treated patients, 50% vs 29.2%, respectively (online supplementary table 3). Univariate analyses of all baseline characteristics, including blood parameters previously reported to be important for immunuotherapy, such as absolute leukocyte, lymphocyte, neutrophil counts and ratios thereof[29–33] showed that the WHO status as well as the leukocyte, monocyte and neutrophil counts as well as their ratios to lymphocytes were correlated with OS. In the multivariate analyses, only the WHO status, immunotherapy pretreatment and the monocyte-to-lymphocyte ratio (MLR) were associated with OS (online supplementary figure 2a, online supplementary table 4). Interestingly, except for MLR none of the other parameters was associated with time till progression after ACT in the multivariate analyses (online supplementary figure 2b, online supplementary table 5).

Clinical responses to ACT in combination with IFNa

T cell treatment was safe and well tolerated since no treatment-related events >3 grading according to CTCAE were observed (online supplementary table 6). The adverse events were predominantly associated with the IFNa-treatment. A transient grade 3 leukopenia was observed in 4 out of 34 (11.8%) patients, grade 3 neutropenia in 5 of 34 (14.7%) patients and grade 3 lymphopenia in 7 of 34 (20.6%) patients, whereas most other patients experienced a mild leukopenia, neutropenia and lymphopenia.

Thirty-four of the patients with progressive stage IV metastatic melanoma could be evaluated for treatment response according to RECIST. Responder patients who obtained clinical benefit (CB) of treatment were defined as patients with complete response (CR), partial response (PR) or durable ≥6 months stable disease (SD) according to RECIST. From the 34 evaluable patients, 2 showed a CR, 1 PR and 7 displayed a prolonged SD. Thus, 10 out of 34 (29,4%) of the treated patients were defined as responder patients. The overall 3-year OS was 14.1% (95% CI 1.9 to 26.3) and 3-year overall progression-free survival was 8.8% (95% CI 0 to 18.4). Patients were treated in different dose cohorts but the responses were distributed among all doses. The two patients who obtained a CR both had elevated lactate dehydrogenase (LDH) (between 250 and 500 U/mL). One patient had a confirmed brain metastasis, was treated in cohort I with TRT and did not receive prior immunotherapy. The other patient who obtained a CR did not have a brain metastasis, was treated with TIL in cohort II and was progressive after prior immunotherapy including anti-PD-1. The patient who obtained a PR had normal LDH, a confirmed brain metastasis and was immunotherapy naïve before treatment with TRT in cohort II.

Interestingly, 6 out of 14 (42.8%) patients who were not pretreated with checkpoint therapy showed clinical benefit, whereas 4 out of 20 (20%) patients who received prior checkpoint therapy still responded to ACT+IFNa (figure 1A). In addition, we compared patients who were pretreated with less than three lines of treatment before the start of ACT and IFNa with patients who received three or more prior systemic therapies with respect to their response to ACT and IFNa. Overall, non-responding patients to ACT and IFNa were more frequently pretreated with three or more systemic therapies, when compared with patients who responded, 40% vs 14%, respectively. The 24 non-responder patients showed PD prior, at or after the first evaluation time-point. Interestingly, three out of the seven patients with SD and two patients with an SD <6 months showed a mixed response since some of their lesions clearly showed regression after ACT+IFNa suggesting that the infused TIL did have the capacity to kill tumor cells in vivo but that other factors hampered their efficacy in the other lesions (online supplementary figure 3).

The responder patients showed a significantly longer time-to-progression when compared with non-responders (figure 1B). This indicates that the clinical benefit was durable as reflected by the significantly improved 1-year (90.0% versus 28.6%) and 3-year (46.7% versus 0%) OS in the responder and non-responder patients, respectively ($p<0.0001$, figure 1C). Importantly, interaction analyses between CB and the baseline MLR showed that the difference in time-to-progression after ACT+IFNa between responders and non-responders was not influenced by this baseline characteristic (online supplementary figure 4a). Similarly, the baseline MLR did not influence the OS in responder patients but the effect of the pretreatment MLR on OS was retained in the group of non-responders (online supplementary figure 4b) indicating that ACT+IFNa treatment successfully changed the clinical course of patients, even when they previously had progressed on checkpoint therapy.

IFNa pretreatment induces leukopenia via the reduction of distinct subsets of immune cells

IFNa pretreatment resulted in a mild leukopenia detectable after 1 week of IFNa and characterized by a decrease in total leukocyte, neutrophil, monocyte and lymphocyte counts (figure 2A–E, pre start of IFNa treatment versus infusion 1). The numbers of leukocytes, in particular neutrophils and monocytes, rapidly bounced back in non-responders. In contrast, in responding patients IFNa pretreatment caused a reduction in leukocytes and neutrophils which was retained during the whole treatment period. No difference was observed in this respect between patients who obtained CR or PR versus SD. The number of these cells were significantly lower than in the non-responding patients at the time of TIL infusions. Monocytes were already lower at baseline and were not altered in responder patients (figure 2A–E). Consequently, the MLR was always lower in the group of responding patients when compared with the group of non-responders and also did not overtly change over time (figure 2H). All IFNa-induced changes were transient and recovered within several weeks after cessation of IFNa injections (not shown).

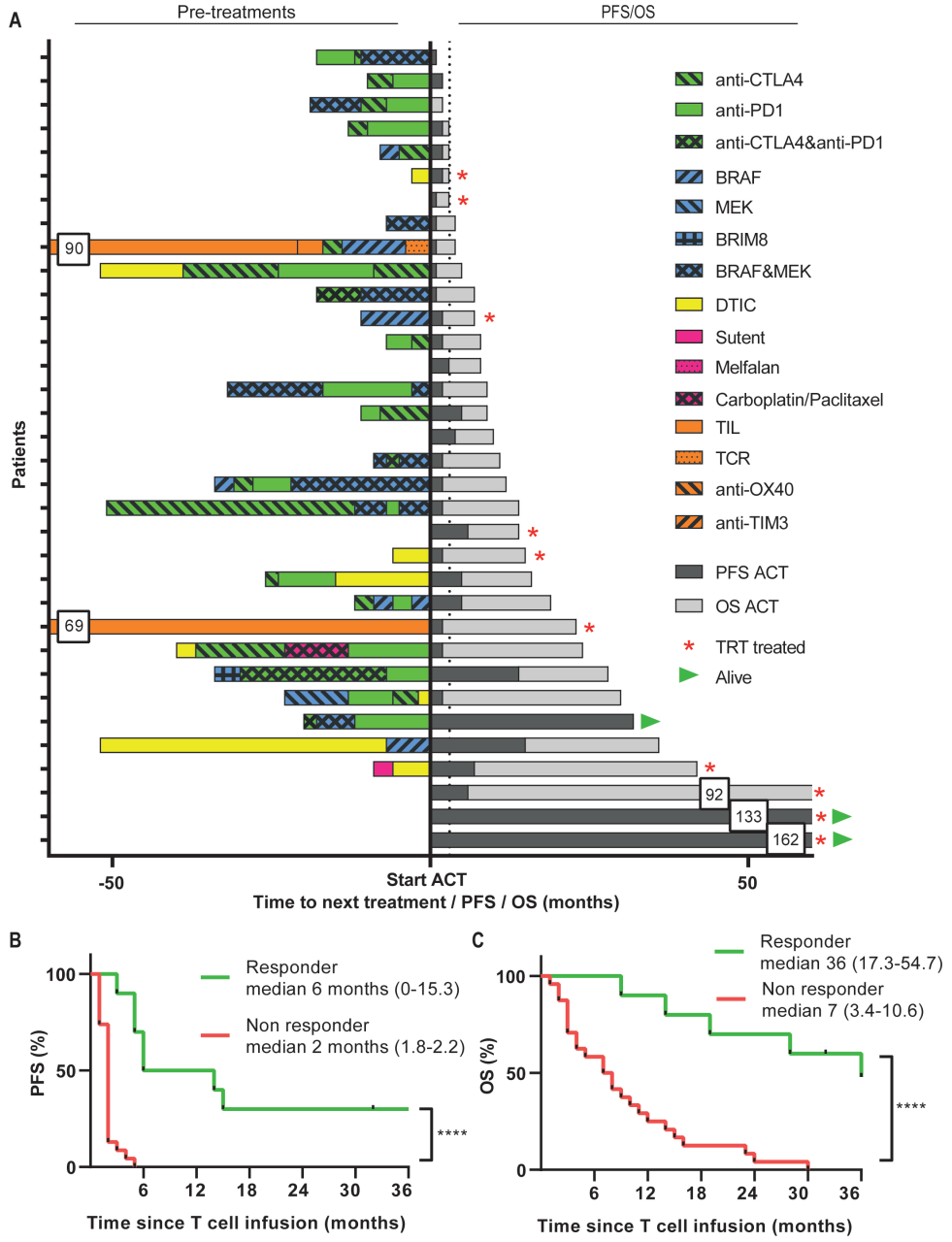

Figure 1 Pre-treatment and survival after start of ACT treatment. (A) Treatments received before start of ACT are depicted for every individual patient in the left part, followed by their PFS and OS in months in the right part. Kaplan-Meier curves for PFS (B) and OS (C) as measured from the start of therapy for responding (R, green lines, n=10, defined as CR, PR or SD >6 months) and non-responding (NR, red lines, n=24). Differences were calculated using the log rank test, ****p<0.0001. ACT, adoptive cell therapy; CR, complete response; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease.

Leukopenia induced by more intense preconditioning regimens for ACT may result in increased levels of circulating homeostatic cytokines.[7] Therefore, we measured the serum levels of IL-7, IL-15 or IL-21 but no effect of IFNa on these cytokines was observed (online supplementary figure 5).

To study the effects of IFNa on immune cells, the PBMC of 18 patients were analyzed with different sets of antibodies to analyze T cell subsets, MDSC, macrophages and DCs. In general, there were no effects on the percentages of CD3, CD8 and CD4 T cells relative to the total percentage of viable cells (online supplementary figure 6a-c). We used combined Hierarchical Stochastic Neighbor Embedding to analyze the complex set of different T cell populations detected by the antibody mix to inhibitory and memory markers. This revealed three distinct immune populations (clusters), comprising CD8+PD-1+CD45RO+CD62L+CD28+central memory T cells (#1), CD4+PD-1-CTLA-4+TIM-3+CD45RO+CD62L+CD28+central memory T cells (#5) and CD45RO-CD62L+CD28+CD8+PD-1+effector/central memory T cells (#10), which significantly decreased after IFNa pretreatment (figure 3A–D). Regulatory T cells were gated according to the consensus strategy,[25] but no changes

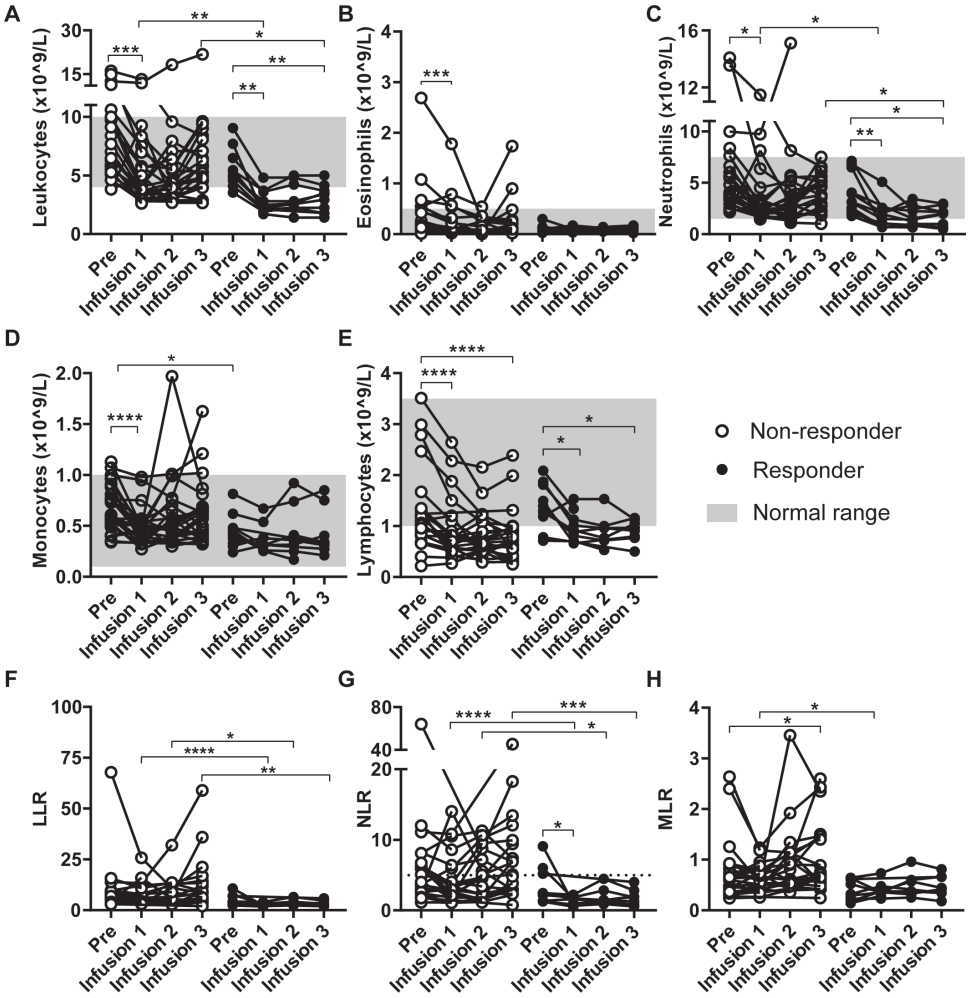

Figure 2 Treatment effect on peripheral blood counts. Absolute blood counts were performed on peripheral blood collected at different time points: before start of IFNa treatment (Pre) and at the time of T cell infusions (Infusion 1–3) just prior to the T cell infusion. Data from non-responding patients (n=24) are compared with data from responding patients (n=10, defined as CR, PR or SD >6 months) in each panel. The absolute leukocyte (A), eosinophil (B), neutrophil (C), monocyte (D) and lymphocyte count (E) are shown. In addition, the leukocyte-tolymphocyte (LLR) (F), neutrophil-to-lymphocyte (NLR) (G) and monocyte-to-lymphocyte (MLR) (H) ratios are shown. Differences within patients were calculated using the Wilcoxon signed rank test, data between response groups were calculated using a Mann-Whitney U test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CR, complete response; IFN, interferon; PR, partial response; SD, stable disease.

were observed (online supplementary figure 6d). Analysis of the different populations of myeloid cells revealed no changes in monocytic MDSC (CD14+HLA-DR-), M1 (CD14+HLA-DR+CD33-CD163-) or M2 (CD14+HLA-DR+CD33-CD163+) blood macrophages or on NK cells (CD3-CD56+) following IFNa treatment (online supplementary figure 6e-h). The percentage of CD14-CD11b-CD11c+DCs, however, decreased (figure 3E). Identification of the different subsets according to Villani *et al*[34] showed a decrease in CD32B+DC2, CD141-CD1c-DC4, whereas the CD36+CD163+DC3 and CD123+pDC increased (online supplementary figure 6i-m). Based on the earlier observation that CD14+CD16-HLA-DRhi classical monocytes predicted time-to-progression and OS on PD-1 blockade in metastatic melanoma,[35] we analyzed non-classical (CD14±CD16++), CD14+CD16+intermediate and CD14+CD16- classical monocytes.[35 36] Although significant shifts were observed after IFNa pretreatment in the non-classical and intermediate monocytes, this was not the case for the population of classical monocytes (figure 3F, online supplementary figure 6n,o).

In summary, IFNa pretreatment had distinct effects on different immune cells. Most notably, a sustained reduction in leukocytes and neutrophils was observed during the treatment period in responder patients. This may explain why the number of pre-existent neutrophils was not associated with the time-to-progression after treatment with ACT+IFNa.

ACT products comprise high percentages of CTLA-4 and PD-1 expressing T cells

Ten evaluable patients were treated with TRT and 24 patients received TIL. We previously showed that the TRT in the MLTC cultures of responder patients proliferated stronger than in non-responders[15] and a similar trend was observed here with respect to the TRT and TIL

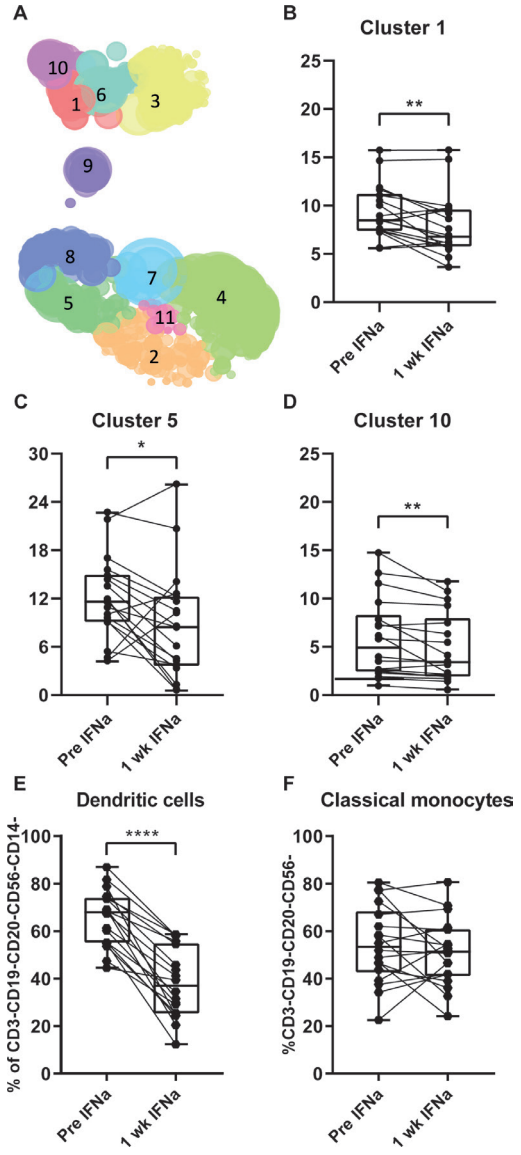

Figure 3 Effect of IFNa conditioning on phenotype of PBMC. Blood was collected before and 1 week after start of IFNa treatment, PBMCs were isolated and phenotypically characterized by flow cytometry. (A) The obtained data were analyzed by Hierarchical Stochastic Neighbor Embedding. Paired testing revealed three distinct immune clusters that were significantly decreased in percentage after 1 week of IFNa (B–D). Cluster 1 comprises CD8+PD-1+CTLA-4-TIM-3-central memory T cells (B), cluster 5 comprises CD4+PD-1-CTLA-4+TIM-3+central memory T cells (C) and cluster 10 comprises CD8+PD-1+CTLA-4-TIM-3-effector/central memory T cells (D). Significantly decreased percentages of dendritic cells (CD3-CD19-CD20-CD56-CD14-CD11b-CD11c+) (E), but not in classical monocytes (CD3-CD19-CD20-CD56-CD14+CD16-) (F) are shown. PBMCs from 18 patients were analyzed. Differences within patients were calculated using paired t-test, data between response groups were calculated using an unpaired t-test. Responding patients are defined as having a CR, PR or SD >6 months. *P<0.05, **p<0.01, ****p<0.0001. CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CR, complete response; IFN, interferon; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PR, partial response; SD, stable disease.

of responders (figure 4A). The TRT and TIL cultures comprised mainly CD3+ T cells (median and range: 99%, 74%–100%), but varied enormously in the ratio of CD3+CD8+ (median and range: 56.7%, 4%–95%) vs CD3+CD4+ (median and range: 42%, 5–96%) T cells. Based on the composition of the ACT product, the total number of CD8+ T cells that was infused could be calculated and was shown not to correlate with clinical outcome (online supplementary figure 7a) (). The majority of the ACT products (MLTC 9 out of 10; TIL 17 out of 24) produced predominantly IFNg when stimulated with the super-antigen SEB (figure 4B). The ACT product used for treatment of patients who obtained a CR or PR did not differ from other ACT products with respect to proliferation rate, ratio of CD3+CD8+ vs CD3+CD4+ cells or cytokines production.

The expression of the inhibitory markers CTLA-4, PD-1 and TIM-3 was analyzed on 14 ACT products. This revealed that a substantial percentage of the infused T cells express one or more of the checkpoint inhibitory markers (figure 4C). However, no overt differences were observed between the ACT products given to responder (n=6) and non-responder (n=8) patients (online supplementary figure 7b-i) online supplementary figure 7. These data suggest that the full capacity of the transfused T cells to control tumor cell growth may have been hampered due to checkpoint inhibition.

T cell reactivity to private tumor antigens is associated with longer overall survival

An important parameter for ACT is the recognition of tumor cells. As a first screen for T cell reactivity of the ACT products, we stimulated them with an extended panel of 37 different melanoma cell lines and scored the reactivity against all cell lines, matched for at least one HLA class I allele, as already published for a number of the TRT.[15] In 8 of the 21 ACT products tested one or more of the matched cell lines were recognized (figure 5A–C). Plotting the level of cross-reactivity against OS suggested that treatment with a low (<7%) cross-reactive ACT product often results in longer OS (figure 5D). The absence of cross-reactivity may also indicate lack of tumor cell-reactivity. In order to elucidate if the correlation between OS and low cross-reactivity reflects the recognition of neoantigens, we set out to identify neoantigen reactivity for the four patients with the longest OS including one CR and one PR patient, of whom also an autologous cell line was available. The presence of neoantigen-specific T cells in the ACT products was previously reported for two of the four patients[26 37] and using the same approach now also in the ACT products of the two other patients with a relatively long survival after therapy. Whole exome sequencing revealed 306 and 605 non-synonymous mutations. and based on RNA expression level 207 and 106 potential neoantigens were detected, respectively, in these two patients. Analyses of the peptides harboring the mutated sequences that were recognized showed neo-epitope-specific T cell reactivity against one and seven

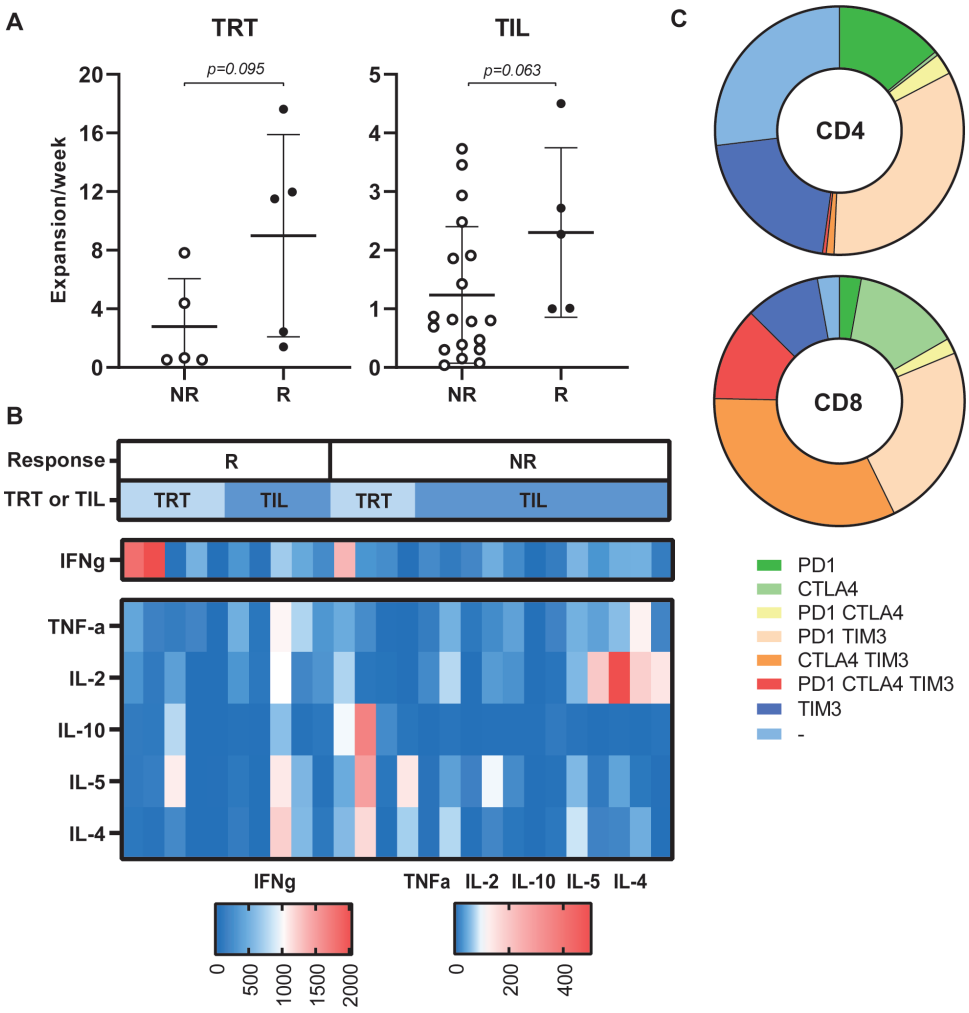

Figure 4 Characteristics of the ACT product used for treatment. The expansion rate of TRT (n=10) and TIL (n=24) used for infusions are depicted for responding (R, defined as CR, PR or SD >6 months) and non-responding (NR) patients (A). The expansion rate was calculated as the total number of cells after the initial expansion phase divided by the number of cells (for TRT) (or initiated wells (for TIL) at the start of the culture and the duration of the culture period in weeks. Differences were calculated using a Mann-Whitney U test. (B) The cytokine profile of the infused ACT products was analyzed after stimulation with staphylococcal enterotoxin B (24 hours) and cytokine production was measured by cytometric bead array assay. The concentration of the indicated cytokines produced by ACT products administered to responding patients (R, left side) and non-responding patients (NR, right side) are shown in the heatmap (n=28) . Concentrations of cytokines are shown according to the legend boxes below the figure with low concentrations indicated in blue and high concentrations in red. Whether patients were treated with TRT or TIL is indicated in the bar above the figure in light and dark blue, respectively. (C) The expression of checkpoint molecules/activation markers was analyzed by flow cytometry on infused T cells. The fraction of negative and single, double or triple positive CD4+ (upper) and CD8+ T cells are depicted in the pie plots (n=15). ACT, adoptive cell therapy; CR, complete response; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

epitopes, respectively, in each patient (table 2). These data show that the lack of cross-reactivity in the ACT products of long-living patients more likely is associated with the specific recognition of private antigens.

DISCUSSION

Adoptive transfer of both TIL and TRT in combination with IFNa is safe, feasible and results in clinical benefit in 10 of 34 (29%) patients with stage IV metastatic melanoma.

The ACT product infused in responders and non-responders did not overtly differ in composition, cytokine production or expression of CTLA-4, PD-1 and TIM-3 co-inhibitory molecules. However, we observed in a number of cases cross-reactivity to melanoma cell lines which were HLA-matched for at least one allele. In those cases, the patients displayed short OS after treatment, while a longer OS was observed for the patients of which the ACT product showed no to low cross-reactivity to allogeneic HLA-matched melanoma cell lines. Four of the very long survivors were treated with

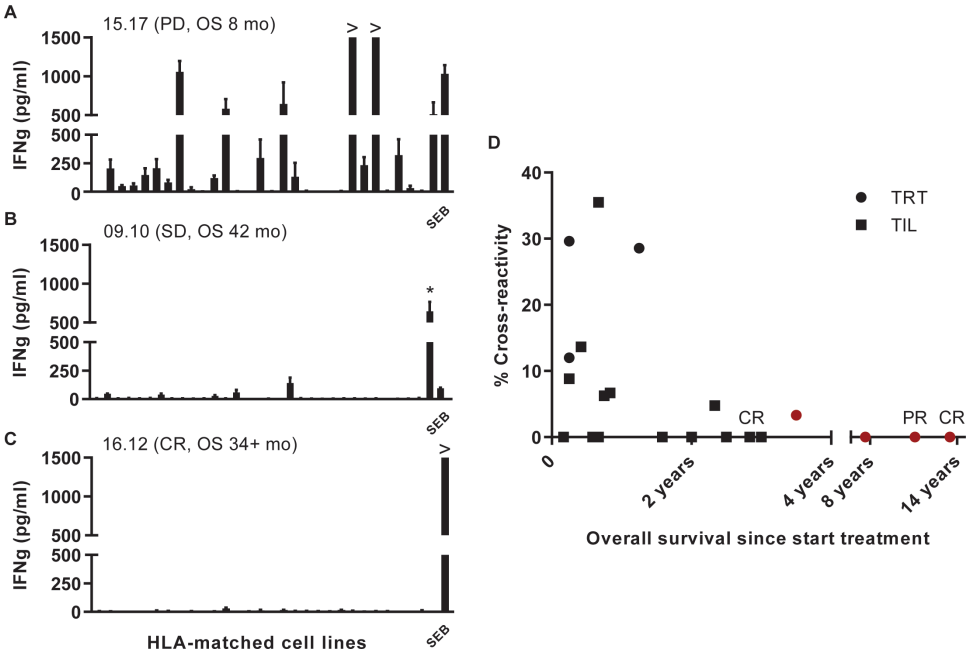

Figure 5 Cross-reactivity of ACT products. The recognition of shared antigens was investigated using a panel of melanoma cell lines that share at least one HLA class I allele. Recognition of allogeneic cell lines is defined as cross-reactivity. The percentage cross-reactivity is depicted and calculated by division of number of cell lines recognized by the number of cell lines tested×100%. Results for TRT (n=7) and TIL (n=14) are depicted. Representative examples of three ACT products with a relatively high or low percentage cross-reactivity are depicted (A–C). (A) A high percentage cross-reactivity was observed for TIL of patient 15.17, who was progressive on treatment and had an OS of 8 months. (B) Shows a rather restricted recognition pattern for TRT of patient 09.10 who obtained stabilization of disease and a relatively long OS, while (C) shows the recognition pattern of TIL from a complete responder 16.12, who only recognizes the positive control (SEB). Asterisk (*) indicates the autologous cell line of patient 09.10. ACT, adoptive cell therapy; CR, complete response; HLA, human leukocyte antigen; IFN, interferon; OS, overall survival; PD, progressive disease; PR, partial response; SD, stable disease; SEB, SEB, staphylococcal enterotoxin B; TIL, tumor infiltrating T cells; TRT, tumor-reactive T cells.

an ACT product that displayed no cross-reactivity, but recognized somatically mutated antigens identified in the autologous melanomas. This suggests that treatment with neoantigen-specific T cells may increase clinical benefit. This is supported by the finding that mutational load predicts clinical outcome after ACT in patients with melanoma[38] and that response to checkpoint inhibitors mediated by reinvigoration of tumor-specific T cell reactivity is also correlated with mutational load in melanoma and other malignancies.[39–41]

Similar to what has been reported for other immunotherapy trials,[30–33] the MLR was associated with shorter OS of the whole group by multivariate analyses. Elevated neutrophil-to-lymphocyte ratio (NLR) has been shown to predict poor response to nivolumab in melanoma.[42] There was no apparent association between NLR and OS in our trial, which may be explained by the fact that the NLR is normalized by IFNa conditioning thus abrogating impact on survival. In contrast to the NLR, the MLR was associated with shorter survival and shorter time-to-progression. Interestingly, the MLR displayed an impact on OS only in those patients who did not respond to therapy, as shown in the interaction analyses. Apparently, the MLR normalization by IFNa, which was most pronounced in the non-responding patients displaying higher pretreatment MLR levels, was not strong enough to revert the impact of baseline levels on OS and progression. The relatively mild leukopenia obtained by IFNa may also explain why we do not observe an increase in homeostatic cytokine levels. Elevation of serum IL-7 and IL-15 levels after lymphodepletion are suggested to be of critical importance for clinical response after ACT,[7] although elevated levels were not directly compared with clinical response and especially the role of IL-7 seems less important.[22 43] Nevertheless, if these cytokines and induction of leukopenia are of major importance for treatment outcome it is advised to choose a more intense conditioning regimen for patients with a relatively good condition, whereas the mild conditioning using IFNa may be more appropriate for the remaining patients otherwise not eligible for ACT.

Overall, IFNa conditioning induces leukopenia and neutropenia and favorable blood count ratios that, if persistent during therapy, correlate with clinical response. Leukocytosis has been suggested to be driven by the increased production of homeostatic cytokines, in particular granulocyte-colony stimulating factor and IL-6, by tumor cells or other cells in the tumor microenvironment,[44 45] which augments hematopoiesis and migration of myeloid progenitor cells from the bone marrow to the blood. Potentially, the infused T cells of responder patients effectively reduced the tumor load,

Table 2 Mutation load, putative and identified immunogenic neoantigens in melanoma cell lines

Cell line code (response)	InDELs	Substitutions (total)	Non-synonymous substitutions	Tested peptides	Recognized T cell epitopes	Reference
MEL 04.01 (SD > 6 months)	3	487	320	226 SLP	EML1(R64W) SEPT2 (R300C) CAD(R1854Q)3	26
MEL 05.18 (CR)	1	1243	811	501 SLP	RPS12(V104I) ZC3H18(G269R) TNIK(S502F) KIAA0020(P451L) ribosomal protein RPL28(S76F)	39 39 39 26 26
MEL 08.11 (PR)	0	442	306	207 SLP	TP53 (L194F)	This article
MEL 09.10 (SD > 6 months)	2	952	635	106 SSP	CLPTM1 (P485L) ETV5 (P465S) NIPAL2 (L95P) TNFRSF12A (I197N) MPDU1 (P213L) ERRFI1 (L338F) ZNF532 (S263L)	This article This article This article This article This article This article This article

The mutation load, defined by number of insertions and deletions (InDELs) and the total number of substitutions is depicted. The number of synthetic long (SLP) or short (SSP) peptides comprise all the non-synonymous substitutions which have a detectable RNA expression (>0) in the tumor sample, excluding those that introduce a premature stop codon.
CR, complete response; PR, partial response; SD, stable disease.

thereby decreasing the production of homeostatic cytokines and consequently the induction of leukocytosis. Hence, a failure of the ACT product to control tumor growth may explain the leukocyte rebound as observed in non-responders.

We observed that the percentage of immunotherapy-naïve patients responding to therapy is twice that of the group of patients who were progressive on prior immunotherapy, confirming the results of a recently published study in which patients progressive on CTLA-4 blockade responded worse to ACT than CTLA-4-naïve patients.[11] More importantly, our data show that patients with resistance to PD-1 blockade may still respond to ACT using a mild conditioning and support regimen, confirming other recent studies reporting a 22%–38% response rate after ACT in patients resistant to anti-PD-1 immunotherapy.[22 46] Interestingly, Sarnaik *et al* reported that TIL therapy was not effective in patients who developed secondary resistance to PD-1 blockade.[46] These findings underscore the hypothesis that patients who acquire immune escaped tumor variants after checkpoint blocking therapy may include modifications that also affect TIL-mediated tumor eradication, for example, antigen loss or HLA loss or other defects in the antigen processing pathway.[47] However, some of the patients in our study developing SD after ACT included a patient who initially had responded to anti-PD-1, indicating that secondary resistance to checkpoint therapy does not exclude patients to benefit from ACT therapy per se. This latter also applies to patients who develop (severe) autoimmune side effects leading to permanent discontinuation of checkpoint blockade, which occurs in approximately 15% of the cases.[48] Patients achieving CB in our trial displayed a lower objective response rate when compared with a recently reported ACT trial in patients with melanoma.[11] This may partially be due to the fact that a higher number of patients in our trial had unfavorable staging and LDH levels, and also received more lines of prior therapy. The fraction of patients in our trial that were pretreated with anti-CTLA-4 (with or without anti-PD-1) was twice as high as that in the study by Forget *et al*.[11] In their study, this was shown to result in reduced response to therapy and shorter OS compared with that obtained in treatment-naïve patients (24.6 versus 8.6 months; HR, 2.3; 95% CI, 1.3 to 4.1, p=0.003). However, the median OS in the CTLA-4-pretreated group in their trial (8.6 months) was similar to what was observed in our trial (9 months).

A substantial percentage of the infused T cells express one or more of the inhibitory checkpoint molecules CTLA-4, PD-1 or TIM-3. Whereas the transient expression of PD-1 and other checkpoint molecules is induced after normal T cell activation, the sustained expression and gradual accumulation of multiple checkpoint molecules is associated with T cell exhaustion due to continued antigenic stimulation in the tumor environment comparable to what is observed during chronic viral infection. Continued expression of multiple checkpoint molecules is associated with gradual loss of effector function and proliferative capacity.[49] The

association between impaired proliferation of infused T cells with worse clinical response observed in our trial, thus may reflect an increased exhausted phenotype, although there is no significant difference in the frequency of inhibitory marker positive T cells between infusion products administered to responding and non-responding patients. However, the simultaneous expression of multiple inhibitory checkpoint molecules may reflect true exhausted T cells.[50–52] To overcome this, ACT in combination with anti-PD-1 is proposed and implemented in our recently initiated and currently ongoing trial (NCT03638375).

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Contributors EV, EK and SvdB designed the clinical study. GL performed surgery and provided the resected tumor. PM and ATvS performed QP and QC checks of the production process and of the infusion product. IR and EK treated the patients. EV, MvdK, EK and SvdB analyzed and interpreted the patient data. EV, MvdK and SvdB designed and interpreted the monitoring experiments. EV, MvdK, MV, CvdM, LdB produced the infusion product and performed all analysis. MW and SS designed and validated the Ab panels for flow cytometry. EV, NdM, MV and SvdB designed, executed and interpreted the neoantigen identification experiments. EV, MvdK and SvdB wrote the manuscript and all authors read and approved the final manuscript.

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Patient consent for publication All patients gave written informed consent before inclusion in this study.

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REFERENCES

1 Vosoughi E, Lee JM, Miller JR, *et al*. Survival and clinical outcomes of patients with melanoma brain metastasis in the era of checkpoint inhibitors and targeted therapies. *BMC Cancer* 2018;18:490.
2 Hodi FS, Chesney J, Pavlick AC, *et al*. Combined nivolumab and ipilimumab versus ipilimumab alone in patients with advanced melanoma: 2-year overall survival outcomes in a multicentre, randomised, controlled, phase 2 trial. *Lancet Oncol* 2016.
3 Robert C, Karaszewska B, Schachter J, *et al*. Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* 2015;372:30–9.
4 Flaherty KT, Infante JR, Daud A, *et al*. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 2012;367:1694–703.
5 Long GV, Weber JS, Infante JR, *et al*. Overall survival and durable responses in patients with BRAF V600-mutant metastatic melanoma receiving dabrafenib combined with trametinib. *J Clin Oncol* 2016;34:871–8.
6 Larkin J, Chiarion-Sileni V, Gonzalez R, *et al*. Five-Year survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med* 2019;381:1535–46.
7 Dudley ME, Yang JC, Sherry R, *et al*. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *JCO* 2008;26:5233–9.
8 Besser MJ, Shapira-Frommer R, Treves AJ, *et al*. Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 2010;16:2646–55.
9 Radvanyi LG, Bernatchez C, Zhang M, *et al*. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 2012;18:6758–70.
10 Andersen R, Donia M, Ellebaek E, *et al*. Long-Lasting complete responses in patients with metastatic melanoma after adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated IL2 regimen. *Clin Cancer Res* 2016;22:3734–45.
11 Forget M-A, Haymaker C, Hess KR, *et al*. Prospective analysis of adoptive TIL therapy in patients with metastatic melanoma: response, impact of anti-CTLA4, and biomarkers to predict clinical outcome. *Clin Cancer Res* 2018;24:4416–28.
12 Clemente CG, Mihm MC, Bufalino R, *et al*. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 1996;77:1303–10.
13 Oble DA, Loewe R, Yu P, *et al*. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma. *Cancer Immun* 2009;9:3.
14 Erdag G, Schaefer JT, Smolkin ME, *et al*. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res* 2012;72:1070–80.
15 Verdegaal EME, Visser M, Ramwadhdoebé TH, *et al*. Successful treatment of metastatic melanoma by adoptive transfer of blood-derived polyclonal tumor-specific CD4+ and CD8+ T cells in combination with low-dose interferon-alpha. *Cancer Immunol Immunother* 2011;60:953–63.
16 Khammari A, Labarrière N, Vignard V, *et al*. Treatment of metastatic melanoma with autologous Melan-A/MART-1-specific cytotoxic T lymphocyte clones. *J Invest Dermatol* 2009;129:2835–42.
17 Rosenberg SA, Packard BS, Aebersold PM, *et al*. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. *N Engl J Med* 1988;319:1676–80.
18 Dudley ME, Wunderlich JR, Yang JC, *et al*. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* 2005;23:2346–57.
19 Dudley ME, Yang JC, Sherry R, *et al*. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008;26:5233–9.
20 Ellebaek E, Iversen TZ, Junker N, *et al*. Adoptive cell therapy with autologous tumor infiltrating lymphocytes and low-dose interleukin-2 in metastatic melanoma patients. *J Transl Med* 2012;10:169.
21 Ullenhag GJ, Sadeghi AM, Carlsson B, *et al*. Adoptive T-cell therapy for malignant melanoma patients with TILs obtained by ultrasound-guided needle biopsy. *Cancer Immunol Immunother* 2012;61:725–32.
22 Nguyen LT, Saibil SD, Sotov V, *et al*. Phase II clinical trial of adoptive cell therapy for patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and low-dose interleukin-2. *Cancer Immunol Immunother* 2019;68:773–85.

23 Dudley ME, Wunderlich JR, Robbins PF, *et al*. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–4.
24 van der Burg SH, Kalos M, Gouttefangeas C, *et al*. Harmonization of immune biomarker assays for clinical studies. *Sci Transl Med* 2011;3:108ps44.
25 Santegoets SJAM, Dijkgraaf EM, Battaglia A, *et al*. Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunol Immunother* 2015;64:1271–86.
26 Verdegaal EME, de Miranda NFCC, Visser M, *et al*. Neoantigen landscape dynamics during human melanoma-T cell interactions. *Nature* 2016;536:91–5.
27 de Jong A, van Poelgeest MIE, van der Hulst JM, *et al*. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. *Cancer Res* 2004;64:5449–55.
28 Beyrend G, Stam K, Höllt T, *et al*. *Cytofast*: A workflow for visual and quantitative analysis of flow and mass cytometry data to discover immune signatures and correlations. *Comput Struct Biotechnol J* 2018;16:435–42.
29 Diehl A, Yarchoan M, Hopkins A, *et al*. Relationships between lymphocyte counts and treatment-related toxicities and clinical responses in patients with solid tumors treated with PD-1 checkpoint inhibitors. *Oncotarget* 2017;8:114268–80.
30 Khoja L, Atenafu EG, Templeton A, *et al*. The full blood count as a biomarker of outcome and toxicity in ipilimumab-treated cutaneous metastatic melanoma. *Cancer Med* 2016;5:2792–9.
31 Machet L, Zaragoza J, Finon A, *et al*. Baseline neutrophil-to-lymphocyte ratio in patients with advanced melanoma treated with immune checkpoint inhibitors. *Br J Dermatol* 2018;179:224.
32 Rosner S, Kwong E, Shoushtari AN, *et al*. Peripheral blood clinical laboratory variables associated with outcomes following combination nivolumab and ipilimumab immunotherapy in melanoma. *Cancer Med* 2018;7:690–7.
33 Capone M, Giannarelli D, Mallardo D, *et al*. Baseline neutrophil-to-lymphocyte ratio (NLR) and derived NLR could predict overall survival in patients with advanced melanoma treated with nivolumab. *J Immunother Cancer* 2018;6:74.
34 Villani A-C, Satija R, Reynolds G, *et al*. Single-Cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* 2017;356. doi:10.1126/science.aah4573
35 Krieg C, Nowicka M, Guglietta S, *et al*. High-Dimensional single-cell analysis predicts response to anti-PD-1 immunotherapy. *Nat Med* 2018;24:144–53.
36 Stansfield BK, Ingram DA. Clinical significance of monocyte heterogeneity. *Clin Transl Med* 2015;4:5.
37 Linnemann C, van Buuren MM, Bies L, *et al*. High-Throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* 2015;21:81–5.
38 Lauss M, Donia M, Harbst K, *et al*. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nat Commun* 2017;8:1738.
39 Snyder A, Makarov V, Merghoub T, *et al*. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 2014;371:2189–99.
40 Hugo W, Zaretsky JM, Sun L, *et al*. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. *Cell* 2016;165:35–44.
41 Rizvi NA, Hellmann MD, Snyder A, *et al*. Cancer immunology. mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015;348:124–8.
42 Fujisawa Y, Yoshino K, Otsuka A, *et al*. Baseline neutrophil to lymphocyte ratio combined with serum lactate dehydrogenase level associated with outcome of nivolumab immunotherapy in a Japanese advanced melanoma population. *Br J Dermatol* 2018;179:213–5.
43 Heczey A, Louis CU, Savoldo B, *et al*. Car T cells administered in combination with Lymphodepletion and PD-1 inhibition to patients with neuroblastoma. *Mol Ther* 2017;25:2214–24.
44 Kasuga I, Makino S, Kiyokawa H, *et al*. Tumor-Related leukocytosis is linked with poor prognosis in patients with lung carcinoma. *Cancer* 2001;92:2399–405.
45 Tavakkoli M, Wilkins C, Mones JV, *et al*. The significance of leukocytosis in malignancies: a novel paradigm between leukocytosis, G-CSF, myeloid-derived suppressor cells and prognosis 2018;132:4944.
46 Sarnaik A, Khushalani NI, Chesney JA, *et al*. Safety and efficacy of cryopreserved autologous tumor infiltrating lymphocyte therapy (LN-144, lifileucel) in advanced metastatic melanoma patients who progressed on multiple prior therapies including anti-PD-1. *JCO* 2019;37:2518.
47 Dafni U, Michielin O, Lluesma SM, *et al*. Efficacy of adoptive therapy with tumor-infiltrating lymphocytes and recombinant interleukin-2 in advanced cutaneous melanoma: a systematic review and meta-analysis. *Ann Oncol* 2019;30:1902–13.
48 Larkin J, Minor D, D'Angelo S, *et al*. Overall survival in patients with advanced melanoma who received nivolumab versus investigator's choice chemotherapy in CheckMate 037: a randomized, controlled, open-label phase III trial. *J Clin Oncol* 2018;36:383–90.
49 Thommen DS, Schumacher TN. T cell dysfunction in cancer. *Cancer Cell* 2018;33:547–62.
50 Fourcade J, Sun Z, Benallaoua M, *et al*. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* 2010;207:2175–86.
51 Kuchroo VK, Anderson AC, Petrovas C. Coinhibitory receptors and CD8 T cell exhaustion in chronic infections. *Curr Opin HIV AIDS* 2014;9:439–45.
52 Thommen DS, Schreiner J, Müller P, *et al*. Progression of lung cancer is associated with increased dysfunction of T cells defined by coexpression of multiple inhibitory receptors. *Cancer Immunol Res* 2015;3:1344–55.