

Comparison of non-myeloablative lymphodepleting preconditioning regimens in patients undergoing adoptive T cell therapy

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ABSTRACT

Background Adoptive cell therapy with T cells genetically engineered to express a chimeric antigen receptor (CAR-T) or tumor-infiltrating T lymphocytes (TIL) demonstrates impressive clinical results in patients with cancer. Lymphodepleting preconditioning prior to cell infusion is an integral part of all adoptive T cell therapies. However, to date, there is no standardization and no data comparing different non-myeloablative (NMA) regimens.

Methods In this study, we compared NMA therapies with different doses of cyclophosphamide or total body irradiation (TBI) in combination with fludarabine and evaluated bone marrow suppression and recovery, cytokine serum levels, clinical response and adverse events.

Results We demonstrate that a cumulative dose of 120 mg/kg cyclophosphamide and 125 mg/m² fludarabine (120Cy/125Flu) and 60Cy/125Flu preconditioning were equally efficient in achieving deep lymphopenia and neutropenia in patients with metastatic melanoma, whereas absolute lymphocyte counts (ALCs) and absolute neutrophil counts were significantly higher following 200 cGyTBI/75Flu-induced NMA. Thrombocytopenia was most profound in 120Cy/125Flu patients. 30Cy/75Flu-induced preconditioning in patients with acute lymphoblastic leukemia resulted in a minor ALC decrease, had no impact on platelet counts and did not yield deep neutropenia. Following cell infusion, 120Cy/125Flu patients with objective tumor response had significantly higher ALC and significant lower inflammatory indexes, such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR). Receiver-operating characteristics curve analysis 7 days after cell infusion was performed to determine the cut-offs, which distinguish between responding and non-responding patients in the 120Cy/125Flu cohort. NLR≤1.79 and PLR≤32.7 were associated with clinical response and overall survival. Cytokine serum levels did not associate with clinical response in patients with TIL. Patients in the 120Cy/125Flu cohort developed significantly more acute NMA-related adverse events, including thrombocytopenia, febrile neutropenia and cardiotoxicity, and stayed significantly longer in hospital compared with the 60Cy/125Flu and TBI/75Flu cohorts.

Conclusions Bone marrow depletion and recovery were equally affected by 120Cy/125Flu and 60Cy/125Flu

preconditioning; however, toxicity and consequently duration of hospitalization were significantly lower in the 60Cy/125Flu cohort. Patients in the 30Cy/75Flu and TBI/75Flu groups rarely developed NMA-induced adverse events; however, both regimens were not efficient in achieving deep bone marrow suppression. Among the regimens, 60Cy/125Flu preconditioning seems to achieve maximum effect with minimum toxicity.

INTRODUCTION

Adoptive cell therapy (ACT) has become a standard treatment option for patients with cancer. T cells genetically engineered to express a chimeric antigen receptor (CAR) against CD19 obtained FDA approval for acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma in 2017 and 2018.¹ In addition, phase 2, phase 3 and pivotal studies of ACT with tumor-infiltrating lymphocytes (TILs) are currently conducted in patients with metastatic melanoma²⁻⁵ and other solid tumor types.⁶⁻⁹ A common feature of all current T cell therapies, no matter if they use gene engineered T cells or TIL, is the non-myeloablative (NMA) lymphodepleting preconditioning of patients prior to cell infusion.

It has been demonstrated that NMA preconditioning of the host with cyclophosphamide (Cy) and fludarabine (Flu) or total body irradiation (TBI)¹⁰⁻¹¹ enhances the survival, persistence and antitumor activity of the infused cells¹²⁻¹³ by the elimination of regulatory T cells, increase of homeostatic cytokines like interleukin (IL)-7 and IL-15, and elimination of resident T cells competing for these trophic cytokines.¹⁻¹³⁻¹⁶ Removal of cellular sinks, such as natural killer cells which compete for IL-7 and myeloid cells competing for IL-15, is required for proliferation and long-term survival of T cells.¹⁵⁻¹⁷⁻¹⁸

Anthony *et al* demonstrated that IL-15 secretion, induced by inflammatory signals in response to lymphodepletion, drives CD8 T cell proliferation and promotes loss of tolerance against self-antigens, thereby enhancing the anti-tumor activity of T cells.¹⁹ In a small series, Kochenderfer *et al* reported that post lymphodepletion, high serum IL-15 levels are associated with remissions of patients with lymphoma receiving CAR-T cell therapy.¹⁸

NMA-induced bone marrow suppression and recovery can be monitored by measuring absolute lymphocyte, neutrophil and platelet counts in the blood of patients. Previously, we demonstrated that absolute lymphocyte counts (ALCs) 1 and 2 weeks post-TIL infusion were significantly correlated with objective response.² In addition, others could show that a combination of cell counts, defined as inflammatory indexes, can be used as prognostic factors for survival in different types of cancers.^{20–23} For example, several studies have reported that a high neutrophil-to-lymphocyte ratio (NLR) and a high platelet-to-lymphocyte ratio (PLR) predict poor survival in patients with stage I–III melanoma,^{24 25} in patients with diffuse large B cell lymphoma treated with the immunochemotherapy combination R-CHOP (rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine and prednisone)^{26 27} and in patients with metastatic melanoma treated with checkpoint inhibitors²⁸ or BRAF inhibitors.²⁹

Although there is a common consensus that NMA preconditioning is required to obtain a durable objective response in patients with cancer treated with adoptive T cell therapy, there is no study comparing the different NMA lymphodepleting regimens head-to-head.

Here, we systematically compare bone marrow suppression and recovery, the impact of NMA on inflammatory biomarkers, the effect on IL-15, IL-7 and IL-21 serum levels, and related adverse events following four different NMA therapies. In addition, we evaluate the impact of NMA on clinical response.

MATERIAL AND METHODS

Patients

Patients were enrolled to one of the three open-label, single arm, phase 2 trials. All patients were treated with T cell products manufactured in the same production facility at the Sheba Medical Center, as previously described.^{2 9 30} Patients signed an informed consent approved by the Israeli Ministry of Health.

Patients with stage IV melanoma were treated with TIL ACT as salvage therapy after failure of multiple treatments. The ‘120Cy/125Flu’ cohort (NCT00287131) received NMA conditioning regimen with cyclophosphamide (60 mg/kg for 2 days; days –7 and –6) and fludarabine (25 mg/m² for 5 days; days –5 to –1) before cell infusion (day 0) and the ‘60Cy/125Flu’ cohort cyclophosphamide (30 mg/kg for 2 days; days –5 and –4) and fludarabine (25 mg/m² for 5 days; days –5 to –1) (NCT03166397). The ‘TBI/75Flu’ cohort received TBI

(200 cGy, on day –4) and fludarabine (25 mg/m² for 3 days; days –3 to –1) (NCT03166397). TILs were administered intravenously, followed by high-dose bolus IL-2 with 720,000 IU/kg/dose, maximum of three times a day, for 5 days or up to tolerance. Patients received filgrastim for 7–10 days starting 1 day after cell infusion. Response was assessed using the Response Evaluation Criteria in Solid Tumors (RECIST V.1.1) guidelines 4 weeks following TIL administration and every 3 months thereafter or as clinically needed. Objective responders (ORs) were defined as complete and partial responders and non-responders (NRs) as patients with stable disease or progressive disease by determining the best overall response using RECIST.

Relapsed or refractory (r/r) pediatric and adult patients with ALL were treated with anti-CD19 CAR-T cells (NCT02772198). Lymphodepleting conditioning was induced by fludarabine 25 mg/m² for 3 days (days –2 to –4) and cyclophosphamide 900 mg/m² (which equals approximately 30 mg/kg Cy) for 1 day (day –2) (cohort ‘30Cy/75Flu’), followed by infusion of 1×10⁶ transduced CAR-T cells per kilogram weight.^{9 31} Patients did not receive IL-2. For clinical evaluation of response, patients with ALL underwent a bone marrow aspiration 28 and 60 days following CAR-T cell administration, and disease assessment was performed by morphology, flow cytometry and PCR minimal residual disease based on immunoglobulin or T-cell receptor rearrangement, with a lower level of detection of 5–10 leukemia cells in 10,000–100,000 healthy cells.

Cell counts and inflammatory indexes

Absolute neutrophil, platelet and lymphocyte counts were obtained before NMA initiation and up to 3 months after cell infusion. The following inflammatory indexes were calculated: NLR, defined as neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; and systemic immune-inflammatory index (SII), defined as absolute neutrophil count×absolute platelet count/absolute lymphocyte count. All cell counts were performed in a centralized, automated laboratory at the Sheba Medical Center.

Cytokine levels

Patients’ serum was collected on day 0, before cell infusion. IL-7, IL-15 and IL-21 serum levels were determined by ELISA (IL-7, Human IL-7 Quantikine HS ELISA kit, R&D Systems, Minneapolis, Minnesota, USA; IL-15 and IL-21, ELISA MAX Deluxe Set, BioLegend, San Diego, California, USA) according to the manufacturer’s instructions. Measurements were performed in duplicates.

Statistical analysis

Significance of variation between groups was evaluated using a parametric two-tailed Student’s t-test. The differences between proportions were tested using two-sided Fisher’s exact test. Analysis of covariance was performed to examine differences in the mean values of patients’ characteristics on variable production parameters. Cell

Table 1 Patient cohorts

Cohort	ClinicalTrials identifier	N	Cyclophosphamide		Fludarabine		TBI		Disease	T cells
			mg/kg	Days	mg/m ²	Days	cGy	Days		
120Cy/125Flu	NCT00287131	103	2×60	-7 to -6	5×25	-5 to -1	-	-	Melanoma	TIL
60Cy/125Flu	NCT03166397	8	2×30	-5 to -4	5×25	-5 to -1	-	-	Melanoma	TIL
TBI/75Flu	NCT03166397	9	-	-	3×25	-3 to -1	1×200	-4	Melanoma	TIL
30Cy/75Flu	NCT02772198	19	1×~30*	-2	3×25	-4 to -2	-	-	ALL	CAR-T

*900 mg/m².

ALL, acute lymphocytic leukemia; CAR-T, chimeric antigen receptor T cell; Cy, cyclophosphamide; Flu, fludarabine; TBI, total body irradiation; TIL, tumor-infiltrating T lymphocyte.

counts and inflammatory indexes were transformed to a logarithmic scale. Linear mixed models with Tukey's adjustment were used to compare absolute cell counts and inflammatory indexes between responder groups or the different regimens. Eastern Cooperative Oncology Group (ECOG) performance status was modeled as fixed effect. Effect sizes were calculated as Cohen's *d*. Linear mixed models were fit using the R statistical software (www.r-project.org). The receiver-operating characteristics (ROC) curve analysis was used to define sensitivity and specificity through the Youden's index, and the area under the ROC curve (AUC) to find the optimal cut-off values for NLR, PLR and SII using the MedCalc V.18.11.6 (MedCalc Software). Survival curves were plotted by the Kaplan-Meier method, and differences between groups were assessed by the log-rank test using the Prism GraphPad V.8.0.1 (GraphPad Software).

RESULTS

Cohorts

Between 2005 and 2016, 103 patients with metastatic melanoma were enrolled to a phase 2 ACT trial with TIL following preconditioning with an accumulating dose of 120 mg/kg cyclophosphamide and 125 mg/m² fludarabine² (120Cy/125Flu cohort; [table 1](#)). To analyze the impact of NMA on bone marrow suppression and recovery, absolute cell counts were collected, starting a few days before NMA initiation (baseline) and up to 3 months post therapy. Due to cyclophosphamide-induced toxicity (detailed later), a follow-up study was initiated. The cyclophosphamide dose was halved (60Cy/125Flu) or cyclophosphamide was replaced with 200 cGy TBI ([table 1](#)). In 2017 and 2018, nine patients with metastatic melanoma were enrolled to the TBI/75Flu group, and in 2019 and 2020, eight patients received 60Cy/125Flu-induced NMA. Patients in the 60Cy/125Flu cohort and those in the TBI/75Flu cohort did not respond to TIL therapy. The TIL manufacturing procedure was identical in all three cohorts. TILs were manufactured at the same Good Manufacturing Practice (GMP) facility and by the same staff.

The three melanoma cohorts were well matched for most baseline characteristics (eg, ECOG performance status) but were significantly different with respect to prior

treatments ([table 2](#)). Most patients in the 120Cy/125Flu cohort were treated in the pre-immune checkpoint inhibitor (pre-ICI) era, whereas patients in the 60Cy/125Flu and TBI/75Flu cohorts were all treated after treatment failure with ICIs.

Simultaneously, CD19 CAR-T therapy was conducted at our clinical center and both TIL and CAR-T were produced at the same GMP cell production facility. Between 2016 and 2019, patients with r/r ALL were treated with CD19 CAR-T cells following NMA with fludarabine and cyclophosphamide 900 mg/m² (which equals approximately 30 mg/kg Cy) ('30Cy/75Flu' cohort). Absolute cell counts were acquired from 19 patients, including 13 complete responders and 6 NRs. The patients received, on average, 4.0±1.2 prior treatments and 2 of 19 (11%) patients had brain metastasis. The 30Cy/125Flu ALL cohort included pediatric patients, thus the average age was low in this cohort. Since patients in the 30Cy/125Flu cohort had a different underlying disease and were treated with CAR-T cells instead of TIL, the results of this cohort will be demonstrated separately.

Bone marrow suppression

To evaluate the impact of the different preconditioning regimens on bone marrow suppression, absolute cell counts were monitored in the three melanoma groups ([figure 1](#)) and the ALL cohort (discussed later). Cell counts were performed in a centralized, automated laboratory. The number of patients with available cell counts per day is shown in online supplemental table 1. The day of cell infusion was defined as 'day 0'.

One hundred and one of 103 (98%) patients in the 120Cy/125Flu and 100% (8 of 8) patients in the 60Cy/125Flu cohort achieved severe neutropenia, defined as absolute neutrophil count (ANC)≤0.5 K/μL. The lowest values of ANC were found 3 days after cell infusion in the 120Cy/125Flu group (0.065±0.230 K/μL; median 0.025 K/μL) and 4 days after cell infusion in the 60Cy/125Flu cohort (0.050±0.044 K/μL; median 0.030 K/μL) (p=0.871) ([figure 1A](#), [table 3](#) and online supplemental figure 1). TBI/75Flu preconditioning did not result in severe neutropenia and the lowest value was 1.088±0.541 K/μL (median 1.100 K/μL) 1 month after cell infusion. In contrast, five of nine (56%) patients in the TBI/75Flu cohort demonstrated even a sharp

Table 2 Baseline and treatment characteristics of the three metastatic melanoma cohorts

	120Cy/125Flu patients N=103	60Cy/125Flu patients N=8	TBI/75Flu patients N=9	120Cy/125Flu vs 60cy/125Flu P value	120Cy/125Flu vs TBI/75Flu P value	60Cy/125Flu vs TBI/75Flu P value
Baseline characteristics						
Mean age, years	51±12	53±9	51±12	0.646	1	1
Male	66 (64%)	4 (50%)	6 (67%)	0.464	1	0.637
ECOG performance status*, n (%)						
0	58 (56%)	5 (62.5%)	8 (89%)	1	0.079	0.294
1	37 (36%)	3 (37.5%)	1 (11%)	1	0.164	0.294
2	8 (8%)	0	0	1	1	1
M stage†, n (%)						
IIIc	1 (1%)	0	0	1	1	1
M1a	7 (7%)	0	0	1	1	1
M1b	12 (12%)	1 (11%)	1 (13%)	1	1	1
M1c	83 (81%)	8 (89%)	7 (87%)	1	1	1
LDH level above normal, n (%)	42 (41%)	3 (37.5%)	6 (67%)	1	0.168	0.347
CNS metastases at baseline, n (%)	23 (22%)	2 (25%)	3 (33%)	1	0.431	1
More than 5 metastases, n (%)	78 (76%)	4 (50%)	8 (89%)	0.203	0.682	0.131
HLA-A0201 positive, n (%)	25 of 102 (25%)	0	3 of 8 (38%)	0.194	0.417	0.228
Previous therapy for metastatic disease, n (%)						
At least one prior therapy	103 (100%)	8 (100%)	9 (100%)	1	1	1
Chemotherapy	20 (19%)	4 (50%)	2 (22%)	0.065	1	0.335
Chemobiotherapy	55 (53%)	0	0	0.006	0.003	1
High-dose bolus IL-2 therapy	16 (16%)	0	0	0.599	0.354	1
Any other IL-2-based therapy	10 (10%)	0	0	1	1	1
Anti-CTLA4 antibody single therapy	34 (33%)	0	2 (22%)	0.104	0.716	0.471
Anti-PD1 antibody single therapy	15 (15%)	0	4 (44%)	0.595	0.044	0.082
Anti-PD1+CTLA4 combo therapy	2 (2%)	0	7 (78%)	1	≤0.001	0.002
Targeted therapy (BRAFI±MEKI)	15 (15%)	1 (13%)	3 (33%)	1	0.157	0.577
Other treatment	3 (3%)	7 (88%)	1 (11%)	≤0.001	0.288	0.003
Infusion product characteristics						
Total cell number (×10 ⁹)	49±24	40.5±19.7	44.9±22	0.332	0.622	0.672
CD8 frequency (%)	59±25	51.3±17.9	31.7±27.1	0.396	0.002	0.103
CD4 frequency (%)	41±25	48.2±17.4	66.3±28.3	0.427	0.005	0.139
NMA-related adverse events, days of hospitalization and transfusion units						
Days of hospitalization, median	20.8±5.1	15.4±1.5	13.7±1.4	0.004	≤0.001	0.029
Chemotherapy-related toxicity (grade 3–5)						
Febrile neutropenia	94 (91%)	4 (50%)	0	0.001	≤0.001	0.029

Continued

Table 2 Continued

	120Cy/125Flu patients	60Cy/125Flu patients	TBI/75Flu patients	120Cy/125Flu vs 60cy/125Flu	120Cy/125Flu vs TBI/75Flu	60Cy/125Flu vs TBI/75Flu
	N=103	N=8	N=9	P value	P value	P value
Thyroid cancer	1 (1%)	0	0	1	1	1
Fatal myelodysplastic syndrome	1 (1%)	0	0	1	1	1
Chemotherapy-related toxicity (grade 5)						
Cyclophosphamide-induced fatal cardiomyopathy	3 additional, unevaluated patients	0	0	1	1	1
Transfusion data						
No of RC units, average	4.7±5.8	0.63±0.92	0	0.055	0.019	0.057
No of PLT units, average	24.8±35.4	0	0	0.051	0.039	1

*ECOG Eastern Cooperative Oncology Group (ECOG) status ranges from 0 to 5, with higher scores indicating greater impairment (5 indicates death).

†The metastasis (M) stage was classified according to the tumor-node-metastasis (TNM) categorization for melanoma of the American Joint Committee on Cancer.

CNS, central nervous system; CTLA4, cytotoxic T-lymphocyte-associated protein 4; Cy, cyclophosphamide; Flu, fludarabine; HLA, human leukocyte antigen; IL, interleukin; LDH, lactate dehydrogenase; NMA, non-myeloablative; PD1, Programmed cell death protein 1; PLT, platelets; RC, red cells; TBI, total body irradiation.

increase of ANC, with values between 13.03 and 16.13 K/ μ L 1 day after cell infusion when the normal range for neutrophils is between 1.8 and 7.7 K/ μ L (figure 1A).

Similar results were observed for ALC (figure 1B and table 3). 120Cy/125Flu and 60Cy/125Flu preconditioning were equally effective in achieving deep lymphodepletion, with lowest average values of 0.029±0.036 K/ μ L (median 0.020 K/ μ L) and 0.024±0.017 K/ μ L (median 0.025 K/ μ L) on the day of cell infusion in the 120Cy/125Flu and 60Cy/125Flu cohorts, respectively ($p=0.389$), when normal values are between 1.0 and 4.8 K/ μ L. A direct comparison of the ALC in the 120Cy/125Flu and 60Cy/125Flu cohorts over time is shown in figure 1D. Administration of cyclophosphamide in the 120Cy/125Flu cohort was initiated on day -7 and in 60Cy/125Flu cohort on day -5, which explains the difference of ALC on days -4 and -3. In the TBI/Flu cohort, only a minor decrease in ALC was achieved (lowest value: 0.062±0.046 K/ μ L; median 0.050 K/ μ L; on day +1) (figure 1B). Lymphodepletion was significantly less efficient compared with the 120Cy/125Flu and 60Cy/125Flu cohorts (p values≤0.01; figure 1E and F).

Platelet counts at baseline were normal in the three melanoma cohorts (120Cy/125Flu cohort, 281±96 K/ μ L; 60Cy/125Flu cohort, 341±121 K/ μ L; TBI/75Flu cohort, 299±82 K/ μ L). In the 120Cy/125Flu and 60Cy/125Flu cohorts, an APC decrease was observed following NMA initiation; however, in the 120Cy/125Flu cohort, values were significantly lower (120Cy/125Flu, lowest value 34±26 K/ μ L, median 26 K/ μ L; 60Cy/125Flu cohort, lowest value 115±51 K/ μ L, median 103 K/ μ L; $p\leq 0.001$) (figure 1C and online supplemental figure 1D–F).

In conclusion, 120Cy/125Flu and 60Cy/125Flu preconditioning were equally efficient in achieving deep lymphopenia and neutropenia, which was not the case for TBI/75Flu NMA.

Bone marrow recovery

Both in the 120Cy/125Flu and 60Cy/125Flu cohorts, the patients recovered by day 6 and day 7 from their severe neutropenia, and counts within the norm (1.8–7.7 K/ μ L) were measured (120Cy/125Flu cohort, 3.63±5.22 K/ μ L, median 1.54 K/ μ L; 60Cy/125Flu cohort, 3.01±2.03 K/ μ L, median 2.73 K/ μ L) (figure 1A).

Interestingly, although TBI/75Flu-induced NMA caused a sharp increase of ANC 1 day after cell infusion followed by mild neutropenia, neutrophil recovery was delayed and baseline levels were only reached after 1 month.

Recovery of ALC was observed in all the cohorts 4–7 days after cell infusion and returned to the normal range of values (figure 1B). The duration of severe lymphopenia was 7 days in the 120Cy/125Flu and 60Cy/125Flu cohorts, but only 2 days in the TBI/75Flu cohort.

Platelet recovery was found 12–14 days after cell infusion in the 120Cy/125Flu cohort. In the TBI/75Flu cohort, APC steadily decreased over time and 50% (three out of six) of the patients had prolonged thrombocytopenia around 3 weeks after cell infusion (figure 1C).

Impact of NMA on clinical response

To evaluate if bone marrow suppression and recovery can serve as predictive markers of clinical response, we analyzed the cell counts in the 120Cy/125Flu melanoma patient cohort (ORs, N=29; NRs, N=74). The baseline

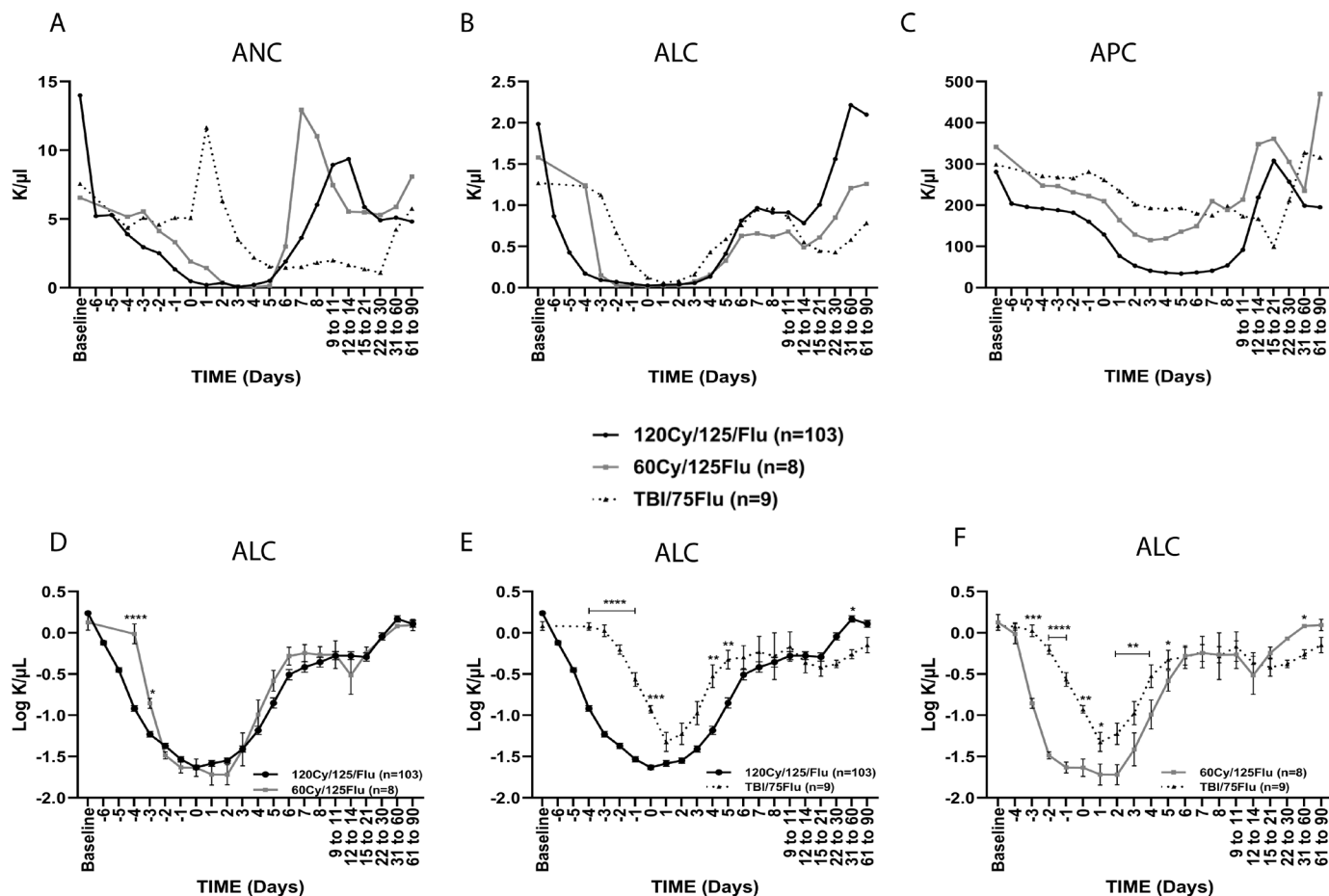


Figure 1 Cell counts following non-myeloablative lymphodepletion. (A) Absolute neutrophil count (ANC), (B) absolute lymphocyte count (ALC) and (C) absolute platelet count (APC). (D–F) Comparison of ALC between (D) the 120Cy/175Flu and 60Cy/175Flu cohorts, (E) the 120Cy/175Flu and TBI/75Flu cohorts and (F) the 60Cy/125Flu and TBI/75Flu cohorts. Day 0, day of cell infusion. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Cy, cyclophosphamide; Flu, fludarabine; TBI, total body irradiation.

characteristics of responders and non-responders were previously reported and multivariate logistic regression revealed that ECOG performance status was an independent factor of response in this cohort.²

Responding and non-responding patients had similar ANC, ALC and APC at baseline ($p = 0.953$, $p = 0.497$ and $p = 0.343$) (figure 2A–C). Following NMA initiation, there was no significant difference between responders and non-responders regarding ANC ($d = 0.083$, $p = 0.508$)

(figure 2A) and APC ($d = 0.007$, $p = 0.972$) (figure 2C). However, ALCs were significantly different ($d = 0.605$, $p \leq 0.001$) (figure 2B). From day 4 onwards, OR patients had significantly higher lymphocyte counts, with the highest significant difference between days 5 and 21 (p values ≤ 0.001). Absolute cell counts were further compared between ORs ($N = 29$) and NRs ($N = 29$), which were matched for baseline characteristics, including ECOG and prior treatments (online supplemental figure

Table 3 Lowest values of absolute cell counts

	ANC (K/ μ L)			ALC (K/ μ L)			APC (K/ μ L)		
Normal level	1.8–7.7			1.0–4.8			130–440		
Lowest value	Average \pm SD		Median	Average \pm SD		Median	Average \pm SD		Median
	Average \pm SD	Median	Day	Average \pm SD	Median	Day	Average \pm SD	Median	Day
120Cy/125Flu	0.065 \pm 0.230	0.025	3	0.029 \pm 0.036	0.020	0	34 \pm 26	26	5
60Cy/125Flu	0.050 \pm 0.044	0.030	4	0.024 \pm 0.017	0.025	0	115 \pm 51	103	3
TBI/75Flu	1.088 \pm 0.541	1.100	22–30	0.062 \pm 0.046	0.050	1	99 \pm 51	91	15–21

ALC, absolute lymphocyte count; ANC, absolute neutrophil count; APC, absolute platelet count; Cy, cyclophosphamide; Flu, fludarabine; TBI, total body irradiation.

120Cy/125Flu cohort

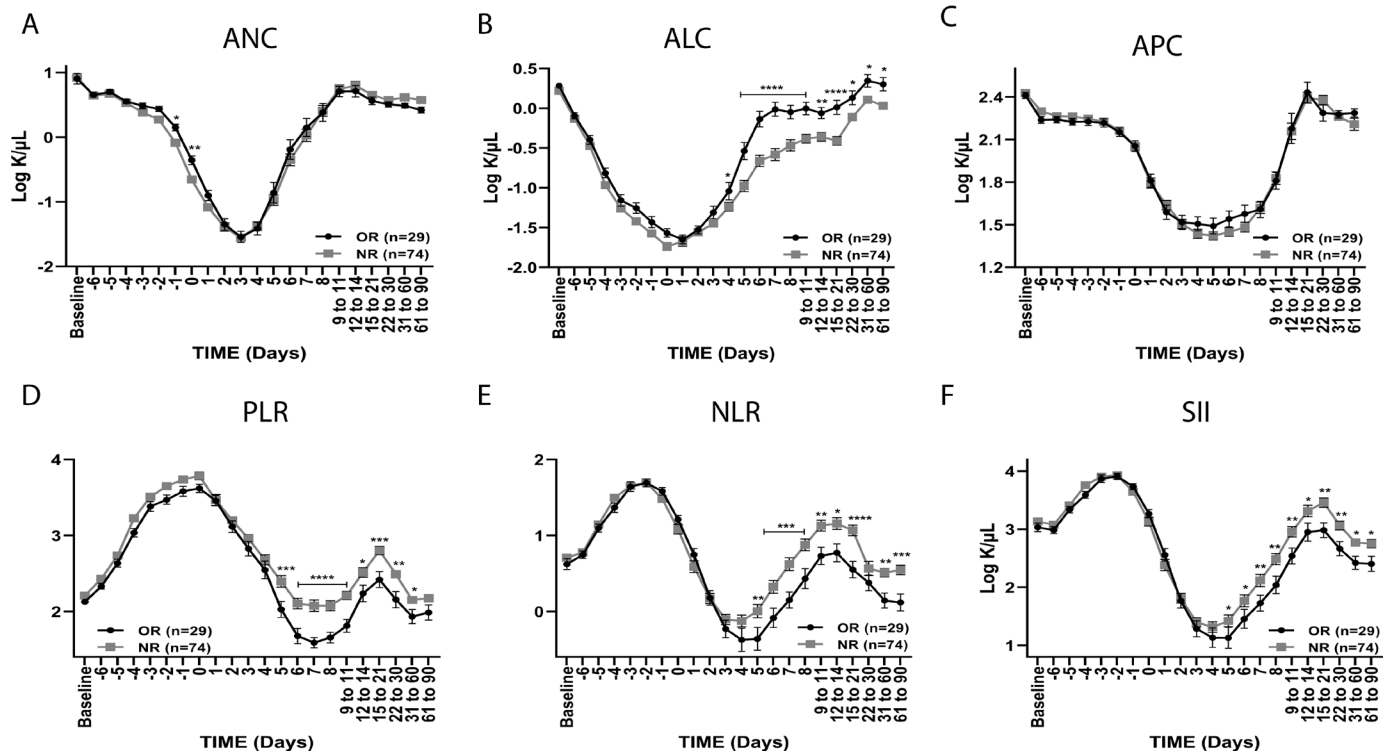


Figure 2 Absolute cell counts and inflammatory ratios of responders and non-responders in the 120Cy/125Flu cohort. (A) Absolute neutrophil count (ANC), (B) absolute lymphocyte count (ALC) and (C) absolute platelet count (APC), (D) platelet-to-neutrophil ratio (PLR), (E) neutrophil-to-lymphocyte ratio (NLR) and (F) systemic immune-inflammatory index (SII) of objective responders (ORs) and non-responders (NRs). Data are shown as mean±SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$. Cy, cyclophosphamide (Cy); Flu, fludarabine.

2A–C), as well as the entire cohort, excluding patients with ECOG 2 and prior anti-PD1 therapy (OR, $N=28$; NR, $N=53$) (online supplemental figure 2D–F) and demonstrated the same results.

Inflammatory indexes as prognostic factors for response and overall survival in the 120Cy/125Flu melanoma cohort

It was shown that the combination of absolute cell counts, defined as inflammatory indexes, can be used as a prognostic factor for response and survival in different types of cancer and may be more significant than individual counts. ORs in the 120Cy/125Flu melanoma cohort had significantly lower PLR, NLR and SII from day 5 onwards (figure 2D).

ROC curves were used to obtain the optimal inflammatory indexes cut-offs on day 7, which distinguish between ORs and NRs. The optimal cut-off for NLR was 1.79, for PLR 32.7 and for SII 368 $K/\mu L$.

We found that 61% (17 of 28) of OR patients compared with 24% (16 of 66) of NR patients had an $NLR \leq 1.79$ ($p=0.002$) and 61% (17 of 28) of the responders compared with 15% of the NRs had a $PLR \leq 32.7$ ($p \leq 0.001$). SII on day 7 could not be used to distinguish between OR and NR patients.

Kaplan-Meier analysis indicated that lower values of NLR and PLR were also significantly associated with

prolonged overall survival (OS) in the 120Cy/125Flu cohort. As shown in figure 3, patients with $NLR \leq 1.79$ had a median OS of 23 months compared with 13.5 months for patients with $NLR > 1.79$ months ($p=0.011$); patients with $PLR \leq 32.7$ had a median OS of 39 months compared with 12.5 months for patients with $PLR > 32.7$ ($p \leq 0.001$).

Impact of NMA on IL-7, IL-15 and IL-21 serum levels

To determine the impact of the different lymphodepleting regimens on cytokine levels, ELISA assays were performed with serum samples collected after completion of NMA and before cell infusion (day 0). Serum was available from patients with melanoma in the 120Cy/125Flu cohort ($N=24$, including 12 ORs and 12 NRs), 60Cy/125Flu cohort ($N=7$) and TBI/75Flu cohort ($N=8$).

IL-7 serum levels were significantly lower following TBI/75Flu-induced lymphodepletion compared with 120Cy/125Flu NMA (TBI/75Flu cohort, 12.0 ± 7.0 pg/mL ; 120Cy/125Flu cohort, 19.0 ± 5.7 pg/mL ; $p=0.008$) (figure 4A). IL-15 serum levels were insignificantly different in all three cohorts (figure 4B). Patients in the 60Cy/125Flu cohort had significantly higher IL-21 serum levels (240 ± 116 pg/mL) than patients from the TBI/75Flu cohort (122 ± 101 pg/mL ; $p=0.048$) and 120Cy/125Flu cohort (124 ± 71 pg/mL ; $p=0.002$) (figure 4C).

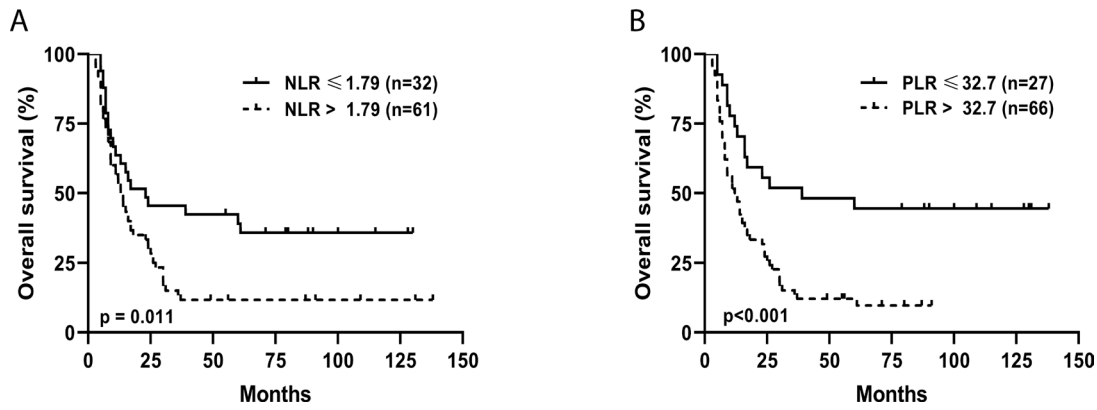


Figure 3 Kaplan-Meier curves for assessment of median overall survival. Kaplan-Meier curves for the inflammatory indexes (A) NLR and (B) PLR according to cut-off values received by ROC curve analysis. Survival analysis was performed by the log-rank test. ROC, Receiver operating characteristics; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; .

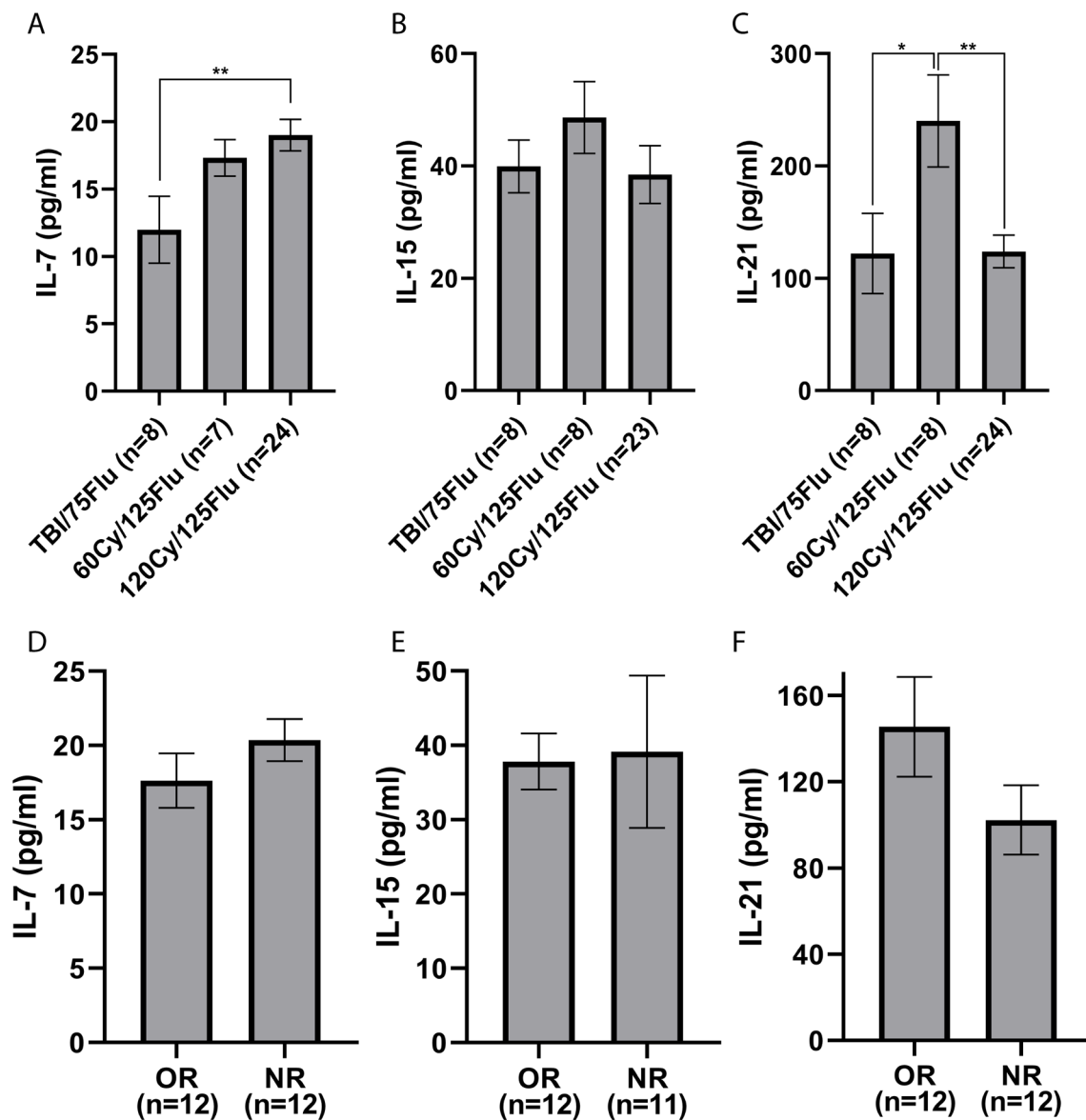


Figure 4 Cytokine serum levels on the day of cell infusion. (A) IL-7, (B) IL-15 and (C) IL-21. (D–F) Comparison of cytokine serum levels between objective responders (ORs) and non-responders (NRs) in the 120Cy/125Flu cohort. Data are shown as mean±SEM analyzed using a non-parametric two-tailed Student's t-test. * $p < 0.05$ and ** $p < 0.01$. Cy, cyclophosphamide; Flu, fludarabine; IL, interleukin; TBI, total body irradiation.

No significant differences between IL-7 ($p=0.252$), IL-15 ($p=0.902$) and IL-21 ($p=0.140$) serum levels were observed between ORs ($N=12$) and NRs ($N=12$) in the 120Cy/125Flu cohort (figure 4D).

NMA-related toxicity

120Cy/125Flu cohort

The NMA, TIL and high-dose IL-2 toxicity profiles in the 120Cy/125Flu cohort were recently published.² Table 2 shows NMA-related adverse events. In short, most 120Cy/125Flu-related toxicities were manageable, while the most common adverse effect was acute grade 3–4 neutropenic fever affecting 91% (94 of 103) of the patients. Administration of high-dose cyclophosphamide at a dose of 60 mg/kg for 2 days required antiemetic treatment adjusted for high emetogenic-risk chemotherapy, yet steroid treatment was avoided. The use of antiemetics during days –7 to –5 of hospitalization (starting prior to the cyclophosphamide infusion) increased the level of tolerability of the infusion of high-dose cyclophosphamide chemotherapy. Mild nausea, jaw discomfort or headaches during infusion were the most commonly reported immediate side effects. No hemorrhagic cystitis was noted. No acute drug-related side effects were found to be correlated with the infusion of fludarabine for 5 days. Patients reported lack of appetite, mild nausea and fatigue. Ninety-one of 103 (88%) and 79 of 102 (77%) patients required red cells (RCs) (4.7 ± 5.8 units) and/or platelet (PLT) transfusions (24.8 ± 35.4 units) to prevent complications from anemia or bleeding. The average duration of hospitalization from NMA admission to discharge was 20.8 ± 5.1 days.

Of note, there were three cases of early deaths, all considered to be related to cyclophosphamide induced cardiotoxicity. All cases presented with symptoms of acute heart failure before TIL infusion. High-dose cyclophosphamide is known to cause cardiotoxicity in 7%–28% of patients and cyclophosphamide-induced mortality has been described in 11%–43% of patients at a dose of 170–180 mg/kg.³²

As for late toxicity of chemotherapy, one patient developed papillary thyroid cancer 3.5 years after TIL therapy, which was successfully treated surgically, and one patient was diagnosed with myelodysplastic syndrome followed by acute leukemia 4.5 years after therapy.

60Cy/125Flu cohort

Due to the high-dose cyclophosphamide-induced toxicities, we investigated the possibility of reducing or replacing cyclophosphamide. 60Cy/125Flu-related toxicities are detailed in table 2. Decreasing the cyclophosphamide doses from 2×60 mg/kg (120Cy) to 2×30 mg/kg (60Cy) resulted in a significantly lower number of cases with grade 3–4 febrile neutropenia (60Cy/125Flu cohort, 50% vs 91%, 120Cy/125 Flu cohort; $p=0.001$) and shorter time of hospitalization (15.4 ± 1.5 days vs 20.8 ± 5.1 ; $p=0.004$). Three of eight patients developed anemia, which required RC transfusions (0.63 ± 0.92 units

vs 4.7 ± 5.8 units; $p=0.055$) and no patient experienced thrombocytopenia, which required transfusion of platelets ($p=0.039$). There was no fatality.

TBI/75Flu cohort

Acute toxicities were in general considerably mild in this cohort, which is reflected in a significantly shorter hospital stay (TBI/75Flu cohort 13.7 ± 1.4 , 120Cy/125Flu cohort 20.8 ± 5.1 days; $p\leq 0.001$) (table 2). No patient in the TBI/75Flu cohort experienced chemotherapy-related febrile neutropenia and no patient required RC and PLT transfusions.

30Cy/75Flu ALL cohort

The 30Cy/75Flu cohort differed in many aspects from the other three cohorts. Patients in the 30Cy/75Flu group suffered from ALL (instead of metastatic melanoma), were treated with CAR-T (instead of TIL), did not receive IL-2 administration following infusion (instead of high-dose bolus IL-2) and received prior lines of chemotherapies, which is uncommon for patients with metastatic melanoma. Therefore, this population is discussed here separately.

30Cy/75Flu preconditioning in the ALL cohort did not yield deep neutropenia (online supplemental figure 3A). The lowest ANC value was 0.835 ± 0.966 K/ μ L (median 0.537 K/ μ L) 9–11 days after cell infusion. In addition, only a minor decrease in ALC was achieved (lowest values: 0.164 ± 0.167 K/ μ L; median 0.367 , on day +1) (online supplemental figure 3B). Platelet counts were already low at baseline (119 ± 105 K/ μ L; median 95 K/ μ L) (online supplemental figure 3C). Baseline thrombocytopenia might be a result of leukemic infiltration of the bone marrow or extensive prior chemotherapy treatments. APCs were low, but unchanged from baseline and up to 3 months post therapy.

Comparing OR ($N=13$) and NR ($N=6$) patients of the 30Cy/75Flu cohort, there was no significant difference in ANC (OR vs NR; $d=1.380$, $p=0.053$) and ALC ($d=-0.246$, $p=0.678$) (online supplemental figure 2D–E). APCs were significantly lower in non-responding ALL patients 1 day after NMA initiation and throughout the entire time of monitoring (OR vs NR; $d=3.910$, $p=0.001$) (online supplemental figure 3F). In both groups, APC was not affected by 30Cy/75Flu lymphodepletion. Thus, the lower platelet counts in non-responding ALL patients seem disease dependent, rather than NMA related, which is in accordance with the existing literature.³³

Patients stayed, on average, 23 ± 8 days in the hospital and received 1.0 ± 1.4 units of RC and 1.3 ± 2.4 units of PLT. Febrile neutropenia was observed in 12 of 19 (63%) patients.

DISCUSSION

Lymphodepleting preconditioning is an integral part of all adoptive T cell therapies. However, to date, there is no

guideline or standardization and no data, which compare different NMA regimens.

In this study, we compared different NMA therapies in regard to bone marrow suppression and recovery, cytokine serum levels, clinical response and adverse events.

We could demonstrate that 120Cy/125Flu and 60Cy/125Flu preconditioning were equally efficient in achieving deep lymphopenia over a period of 1 week and deep neutropenia in patients with metastatic melanoma treated with TIL, whereas ALC and ANC were significantly higher following TBI/75Flu-induced NMA. In contrast, patients in the TBI/75Flu cohort had neutrophilia 1 day after cell infusion, followed by mild neutropenia. Acute thrombocytopenia was mainly observed in the 120Cy/125Flu cohort.

Despite the deep lymphopenia and neutropenia, patients in the 120Cy/125Flu and 60Cy/125Flu cohorts recovered within 6 days, whereas patients in the TBI/75Flu cohort recovered only after 1 month from their mild neutropenia.

The objective response rate in the 120Cy/125Flu cohort was 28%. Patients in the 60Cy/125Flu and TBI/Flu cohorts did not respond to TIL therapy. The lack of clinical efficacy in the 60Cy/125Flu and TBI/Flu groups may have multiple reasons. Of note, patients in the latter two cohorts were all refractory to anti-PD1 immunotherapy compared with only 17 of 103 patients (17%) in the 120Cy/125Flu group. Interestingly, among the 17 anti-PD1 refractory patients in the 120Cy/125Flu cohort, 16 patients did not respond to TIL therapy, which strengthens our hypothesis, that patients, refractory to anti-PD1 antibodies, are less likely to respond to TIL therapy compared with anti-PD1 naïve patients.

To investigate if bone marrow suppression and recovery can serve as prognostic marker of clinical response and survival, we analyzed the cell counts in the 120Cy/125Flu melanoma patient cohort. The analysis was performed on 29 responders and 74 non-responders. At baseline, there was no difference in absolute cell counts between responding and non-responding patients.

We could confirm that higher lymphocyte counts starting 4 days after cell infusion were significantly associated with clinical response. If the higher count reflects better engraftment and proliferation of infused T cells or a faster bone marrow recovery needs to be further investigated.

Previous studies have shown that the combination of cell counts, defined as inflammatory indexes, can serve as predictors of response.^{34–39} As anticipated, the PLR and NLR were lower in ORs from day 5 onwards.

ROC curve analysis 7 days after cell infusion was performed to determine the optimal cut-offs to distinguish between responding and non-responding patients and predict OS. Cut-offs were defined as $NLR \leq 1.79$ and $PLR \leq 32.7$. For example, 61% of patients with $PLR \leq 32.7$ responded to TIL therapy compared with 15% NRs ($p \leq 0.001$). The median OS was 39 months for patients with $PLR \leq 32.7$ and 12.5 months at PLR above 32.7.

Previous studies demonstrated that cytokine serum levels are associated with antitumor activity of the T cells and clinical response in patients undergoing adoptive T cell therapy.^{18,19} Therefore, we compared the cytokine serum levels following the different NMA regimens. We could show that IL-15 levels were insignificantly different, TBI/75Flu-induced lymphodepletion resulted in significantly lower IL-7 serum levels and 60Cy/125Flu-induced lymphodepletion resulted in significantly higher IL-21 serum levels compared with 120Cy/125Flu NMA. Since there were no differences between IL-7, IL-15 and IL-21 serum levels between overall responders and non-responders in the 120Cy/125Flu cohort, the clinical relevance of these findings is questionable.

We further analyzed the absolute cell counts of patients with relapsed or refractory ALL, who received a 30Cy/75Flu preparative regimen before CD19 CAR-T cell infusion. Only a minor decrease of ALC was observed. Although the contribution of leukemic blasts to ALC in patients with ALL cannot be absolutely excluded, it is very unlikely. Most patients were treated before had low or no circulating blasts at the time of lymphodepletion.³¹ Additionally, in the apheresis product, the starting material for CAR-T cell production, the total number of non-malignant and malignant B cells was less than 5% in most patients and 0% in the CAR-T cell product.⁹

Furthermore, we investigated the impact of 30Cy/75Flu-induced NMA on ANC and APC and could show that this regimen did not cause deep neutropenia and had no effect on APC.

Higher absolute platelet counts 1 day after NMA initiation (day -4) and throughout the entire time of monitoring were significantly correlated with clinical response. At baseline, there was a trend ($p=0.060$) of a higher platelet count in OR patients. Prolonged event-free survival following CAR-T cell therapy was previously reported in patients with ALL with higher pre-lymphodepletion platelet count (32). Thus, it should be further investigated if APC assessment could be a helpful predictive marker to make clinical decisions in this patient population.

A major aspect that must be taken into account when choosing chemotherapy for lymphodepletion is the toxicity related to it. Patients in our 120Cy/125Flu cohort developed significantly more acute adverse events, including thrombocytopenia, febrile neutropenia and cardiotoxicity. Thus, the time of hospitalization and the number of RC and PLT transfusions were significantly higher in this cohort. We reported three cases of acute cardiotoxicity following 120Cy/125Flu NMA. All three cases presented with symptoms of acute heart failure before TIL infusion and none of the patients suffered from cardiac problems before therapy. Of note, no other TIL trial reported cyclophosphamide-induced mortality, but, on the other hand, high-dose cyclophosphamide is known to cause cardiotoxicity in 7%–28% of patients.³² It was demonstrated that cyclophosphamide metabolites are responsible for cardiotoxicity due to depletion of antioxidants/ATP level, altered

contractility, damaged endothelium and enhanced proinflammatory/proapoptotic activities resulting in cardiomyopathy, myocardial infarction and heart failure.³² Mortality has been reported in 11%–43% of patients at a dose of 170–180 mg/kg. Halving the doses of cyclophosphamide from 60 mg/kg for 2 days (120Cy) to 30 mg/kg for 2 days (60Cy) might prevent this rare but severe adverse effect.

In summary, bone marrow suppression and recovery were equally effected by the 120Cy/125Flu and 60Cy/125Flu preconditioning in the melanoma cohorts; however, toxicity and consequently time of hospitalization were significantly lower in the 60Cy/125Flu cohort. Patients in the 30Cy/75Flu ALL cohort and TBI/75Flu melanoma cohort rarely developed NMA-induced adverse events; however, both regimens were not efficient in achieving deep bone marrow suppression.

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