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Cellular Therapy

Clearance of Hematologic Malignancies by Allogeneic Cytokine-Induced Killer Cell or Donor Lymphocyte Infusions

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Well-established donor lymphocyte infusion (DLI) and novel cytokine-induced killer (CIK) cell therapy for the treatment of relapsing hematologic malignancies after allogeneic hematopoietic stem cell transplantation (HSCT) were compared with respect to feasibility, safety, and efficacy. Altogether, a total of 221 infusions were given to 91 patients (DLI, n = 55; CIK, n = 36). T cell recovery was significantly improved after CIK cell therapy ($P < .0001$). Although patients with CIK cell treatment showed a significantly worse prognosis at the time of HSCT (risk score, 1.7 versus 2.1; $P < .0001$), DLI and CIK cell therapy induced complete remission (CR) in 29% and 53% patients, respectively, whereas relapse occurred in 71% and 47%. In both groups, all patients with overt hematologic relapse at the time of immunotherapy (DLI, n = 11; CIK, n = 8) succumbed to their disease, while 36% and 68% patients with DLI or CIK cell therapy applied due to molecular relapse or active disease at the time of transplantation achieved CR. The 6-month overall survival rate in the latter patients was 57% and 77%, respectively, with a median follow-up of 27.9 months (range, .9 to 149.2 months). The 6-month cumulative incidence of relapse was 55% and 22% in patients who received DLI and CIK cell therapy, respectively ($P = .012$). Acute graft-versus-host disease developed in 35% of the patients who received DLI and in 25% of those who received CIK. No transfusion-related deaths occurred. These data, while underscoring the therapeutic value of conventional DLI, suggest the improved safety and to a certain extent efficacy of CIK cell therapy for patients at high risk for post-transplantation relapse of various hematologic malignancies.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has become the standard of care for many high-risk hematologic malignancies. However, a high relapse rate, first indicated by molecular relapse, remains the leading cause of treatment failure. Cell-based immunotherapy offers promise for eradicating molecular disease and preventing overt relapse following allogeneic HSCT, especially when the leukemia burden is low. Donor immune cell populations can become activated on recognition of specific major histocompatibility complex antigens or minor histocompatibility antigens on host cells and thus can contain leukemia-reactive immune cells but can also induce graft-versus-host disease (GVHD). Separating graft-versus-leukemia (GVL) effects from GVHD is of special interest in nonspecific cell-based immunotherapy. Furthermore, owing to the short-lived and limited in vivo activity of these effectors, nonspecific immunotherapy is typically dependent on repeat administrations.

Examples of nonspecific adoptive immunotherapies include the well-established donor-derived lymphocyte infusion (DLI) therapy [1,2]. Highly innovative and advanced treatment strategies using CD19 leukemia-reactive chimeric antigen receptor-modified T cells have proven effective in patients with relapsed or refractory B cell precursor leukemia [3,4]. However, other

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novel cellular immunotherapies, such as CD3/CD28-activated DLI [5], lymphokine-activated killer cells [6], anti-CD3-activated killer cells [7], tumor-infiltrating lymphocytes [8], in vitro-generated or selected tumor cytotoxic T lymphocytes [9,10], γ/δ-T cells [11-14], and natural killer (NK) cells [15,16], have been found to have either low or target antigen-restricted antileukemic potential, cannot be generated in sufficient numbers, or cause severe GVHD [17-19], limiting their clinical applicability, especially in hematologic malignancies without target antigens.

In 1991, Schmidt-Wolf et al first described cytokine-induced killer (CIK) cells [20,21]. CIK cells have demonstrated cytotoxicity against a variety of malignant cells without previous exposure or priming [22-25] but not against acute lymphoblastic leukemia (ALL) blasts [26], with only minor effects on normal hematopoietic progenitor cells [20,27]. Previous studies have shown that CIK cells have a dual-functional capability of both T cells and NK cells, which transfer signals through T cell receptor/CD3 and NK group 2-member D (NKG2D), DNAX accessory molecule-1, NK cell p30-related protein, lymphocyte function-associated antigen 1, and Fas and Fas ligand, finally leading to the secretion of perforin and granzymes for the execution of cytotoxicity [28-34].

METHODS

Patients

All patients had undergone allogeneic HSCT for a high-risk hematologic malignancy. Consecutive post-transplantation patients who had received either conventional DLI or a novel CIK cell therapy at our center in Frankfurt am Main, Germany between July 1, 2001, and October 31, 2017, were included in this retrospective study. The institutional Ethics Committee approved this best practice approach at the center (no. 529/15). DLI and CIK cell infusions were generated in the Good Manufacturing Practices clean room facility of the German Red Cross Blood Donor Service, Frankfurt am Main, Germany.

Each patient provided written informed consent for cellular immunotherapy and retrospective studies before infusions. Between August 11, 2011, and June 2, 2014, CIK cell therapy was applied on a compassionate use basis approved by the respective regulatory authorities (Regierungspräsidium, Darmstadt and Paul Ehrlich Institute, Langen, Germany). CIK cell therapy was approved (national authorization Sec 4b, Abs. 3 AMG, "Hospital Exemption") by the Paul Ehrlich Institute on June 3, 2014 (PEI.A.11630.01.1) as an advanced therapy medicinal product in patients with hematologic malignancies at risk for relapse after allogeneic transplantation. The authors hold the manufacturing license and marketing authorization for CIK cell therapy.

Cellular Immunotherapy

Analyses of post-transplantation hematopoietic chimerism and/or minimal residual disease (MRD) in peripheral blood (PB) and/or bone marrow (BM) samples were performed at given post-transplantation time points (days +30, +60, and +90 and 6, 12, and 18 months) [35]. Patients with detectable MRD and/or mixed chimerism (MC), as well as patients with overt hematologic relapse despite withdrawal of immune suppression and no or a maximum of grade I acute GVHD (aGVHD), were immediately offered preemptive cellular immunotherapy. Patients with refractory disease at the time of HSCT due to the immense risk of relapse in the early post-transplantation period were treated prophylactically. No immunosuppressive or immunomodulatory medications or steroids were allowed during treatment with cellular immunotherapy.

Cellular immunotherapy included DLI only between July 1, 2001, and August 10, 2011, and either DLI or CIK cell therapy between August 11, 2011, and October 31, 2017. CIK cell infusions were given preferentially whenever a slot for in vitro generation was available.

The starting T cell dose of DLI was 1×10^6 cells/kg in patients with an HLA-matched related donor, $.5 \times 10^6$ cells/kg in patients with an HLA-matched unrelated donor, and $.1 \times 10^6$ cells/kg in patients with an HLA-haploidentical donor. The starting T cell dose for CIK cell therapy was 1×10^6 cells/kg regardless of donor type.

Prudent dose escalation was considered for subsequent infusion if no additional signs of aGVHD appeared. In DLI, a doubling of the infused $CD3^+$ T cell dose was contemplated only in the matched donor transplantation setting. In CIK cell therapy, the infused $CD3^+$ T cell dose was increased to 5×10^6 T cells/kg, 1×10^7 T cells/kg, and a maximum of 1×10^8 T cells/kg recipient body weight irrespective of donor type. The intervals between respective immune cell doses were 3 to 6 weeks. Cellular immunotherapy was stopped if complete molecular remission (CMR) was achieved and was withheld if the patient experienced aGVHD exceeding grade I.

Clinical-Scale Generation of CIK Cells

A protocol has been established using IL-15 to reproducibly and rapidly expand $CD3^+CD56^+CD25^+$ CIK cells [36]. CIK cells were generated from either PB or leukocytapheresis products of the original stem cell donors. The dose was adjusted to a cell concentration of 3×10^6 cells/mL and cultured in X-VIVO 10 medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated fresh-frozen plasma (German Red Cross Blood Donor Service, Frankfurt, Germany). CIK cells were activated and expanded by defined cytokines without any feeder cells. At day 0 of CIK cell generation, 1000 U/mL IFN-γ (IMUKIN; Boehringer Ingelheim, Ingelheim am Rhein, Germany) was added, followed by 100 ng/mL anti-CD3 monoclonal antibody (OKT-3, MACS GMP CD3 pure; Miltenyi Biotec, Bergisch-Gladbach, Germany) and 500 U/mL IL-2 (Proleukin; Novartis Pharma, Nuremberg, Germany) on day +1. CIK cell density was adjusted to 1×10^6/mL on days +4 and +8, and the cells were restimulated with 50 ng/mL IL-15 (PeproTech, Rocky Hill, NJ). CIK cells were harvested after 10 to 12 days of culture. After 10 to 12 days of cultivation (with a 10-fold expansion of $CD3^+CD56^+CD25^+$ cells compared with day 0), 1 dose of CIK cells was prepared for immediate use; the remaining cells were formulated at incremental doses for subsequent infusions and cryopreserved. Viability, phenotype, and efficacy of CIK cells were preserved after cryopreservation and thawing (data not shown).

Chimerism Analysis

Chimerism determination started at the time of leukocyte recovery post-transplantation [37]. The sensitivity of our assay for detecting autologous cells was 1%. If a patient showed 1% of recipient cells in a PB or BM sample, then another sample was obtained within 1 week. Patients with confirmed 1% autologous cells in 2 consecutive samples and patients with >1% of autologous cells in a single sample post-transplantation were considered to have MC.

MRD Analysis

Cell sample isolation and identification of the markers for MRD evaluation by polymerase chain reaction (PCR) for leukemia-specific Ig/T cell receptor rearrangements have been reported previously [38]. Analysis was performed in patients with ALL and lymphoma and interpreted according to the guidelines developed by the European Study Group for MRD Detection in ALL [39].

Immune Monitoring

Flow cytometry analyses for immune monitoring were performed monthly as described previously [40]. The patient's longitudinally determined measurements were calculated from the corresponding age-matched norms published by Huenecke et al [41].

Statistical Analysis

Single-arm comparisons were performed with respect to the feasibility, safety, and efficacy of the treatment. The median duration of follow-up for all patients was obtained using the reverse Kaplan-Meier estimator. Fisher's exact test or the Wilcoxon-Mann-Whitney test was used to compare patients' categorical data. Each patient's risk-adjusted outcome was compared by risk scores (risk score, 0 to 3: 1 point for complete remission (CR) 2, second HSCT, or nonremission; 1 point for non-matched sibling donor; 1 point for age >10 years) [42]. Kaplan-Meier estimates were performed to predict the overall survival (OS) probabilities. The log-rank test was used for comparisons. Treatment-related mortality (TRM) was defined as death in CR without previous relapse. A cumulative incidence curve was calculated for the cumulative incidence of relapse (CIR), considering TRM as a competing risk for relapse. Gray's test was used for comparisons of CIRs. Cox regressions were performed to identify associations between various patient and transplantation characteristics and OS and CIR. Only factors that attained significance in the univariable regressions were included in the multivariable analysis.

A mixed-effect regression with the linear spline model was fitted for longitudinal analysis of the T cell population after transplantation. The absolute cell values of T cells were age-adjusted and logarithmically transformed [41]. Furthermore, patients were classified according to the indication for cellular immunotherapy, type of therapy, and outcome by Sankey plots. Cumulative T cell doses $\times 10^6$/kg were shown in boxplots.

Statistical tests were 2-sided with a significance level of 5% and 95% confidence interval (CI). Data analysis was performed using R version 3.1.3 [43] with the survival package [44], cmprsk package [45], and nlme package [46].

RESULTS

Patients

Between January 2001 and February 2017, a total of 292 patients underwent allogeneic HSCT for hematologic malignancy at our center in Frankfurt am Main. Eighty-three (28%) of these patients received cellular immunotherapy, including 55 with DLI and 28 with CIK cell therapy. CIK cell therapy was also provided to 1 allogeneic HSCT recipient at the University Hospital Ulm, 3 recipients at the University Children's Hospital in Düsseldorf, and 4 recipients at the Medical Clinic II University Hospital in Frankfurt am Main.

DLI was given based on molecular (MC and/or MRD) hematologic relapse in 36 patients (66%; median risk score, 1.7) and on overt hematologic relapse in 10 patients (18%; median risk score, 1.5). Another 9 patients (16%) patients with active disease at the time of transplantation were treated prophylactically (risk score, 1.9).

Seventeen of 36 patients (47%) who received CIK cell therapy showed molecular relapse (MC and/or MRD) after transplantation (median risk score, 2.1), 11 (31%) had refractory disease at the time of transplantation (median risk score, 2.5), and another 8 (22%) experienced hematologic relapse (median risk score, 1.6) (Figure 1A and B).

Patient characteristics are reported in Table 1. Transplantation was performed after remission induction treatment as recommended by the AIEOP-BFM and COALL study groups. However, 15 DLI recipients (27%) and 14 CIK recipients (39%) were not in remission at the time of transplantation.

T Cell Doses

In all patients, the median T cell doses were 1×10^6/kg (range, .02 to 10×10^6/kg) for DLI and 5.27×10^6/kg (range, .1 to 200×10^6/kg) for CIK cell therapy ($P < .001$). By HSCT donor type, the median T cell doses infused were 1×10^6/kg (range, .02 to 10×10^6/kg) with DLI and 10×10^6/kg (range, .7 to 200×10^6/kg) with CIK cell therapy in the matched donor setting ($P < .001$), and $.1 \times 10^6$/kg (range, .025 to 5×10^6/kg) for DLI and 5×10^6/kg (range, .1 to 9.4×10^6/kg) for CIK cell therapy in the haploidentical donor setting ($P < .001$) (Table 2). The exact infused cell doses are shown in Table 3.

At least 1 infusion of either DLI or CIK cell therapy was given to every patient (DLI: median 1, maximum 11; CIK cell therapy: median 2, maximum 9). Altogether, a total of 221 infusions were provided, including 118 DLIs and 103 CIK cell infusions (Table 2). The total numbers of DLI and CIK cell infusions were not significantly different. However, the median cumulative T cell doses were 1.49×10^6/kg (range, .025 to 30.8×10^6/kg) for DLI and 16×10^6/kg (range, 1 to 721.4×10^6/kg) for CIK cell therapy in the matched donor setting ($P < .001$) and 1×10^6/kg (range, .04 to 5×10^6/kg) for DLI and 4.5×10^6/kg (range, 1 to 348.7×10^6/kg) for CIK cell therapy in the mismatched donor setting ($P < .001$) (Table 2). Differences in the cumulative T cell doses between DLI and CIK cell therapy were most pronounced in patients treated prophylactically or treated based on molecular relapse ($P < .001$) (Figure 3A).

Treatment Stratification and Clinical Responses

Overt hematologic or molecular relapse were criteria used to determine that the respective patient should receive DLI or CIK cell infusions. Molecular relapse was marked by MRD in patients with lymphoblastic malignancies and by MC in patients with both myeloid and lymphoblastic malignancies. Patients with lymphoblastic malignancies and MC were also MRD-positive at the same time (Supplementary Figure S7). All patients treated based on molecular relapse (MC and/or MRD positive) were in CR at the time of first DLI or CIK cell infusion.

All patients in the prophylactic group still had active disease at the time of transplantation and thus were considered at imminent risk for relapse in the early post-transplantation period with then-limited treatment options. Therefore, immune therapy was initiated early (between days +30 and +40) after allogeneic HSCT, even though all patients in this treatment arm were in CMR at the time of first infusion.

With either treatment, after the first infusion, chimerism monitoring was performed weekly in PB samples and monthly in parallel with MRD monitoring (if applicable) in BM samples. In case of reappearance or persistence of MRD or MC at 3 to 6 weeks after the previous immune cell infusion, subsequent infusions were considered. Immune cell infusions were stopped if MC converted to complete donor chimerism or in the event of clearance of MRD. Immune cell infusions were not provided to patients experiencing aGVHD exceeding grade I.

Thirty-five of 55 patients who received DLI treatment developed MC and/or MRD in post-transplantation follow-up analyses, 9 patients were treated owing to the high risk of relapse, and another 11 patients received DLI to treat overt hematologic relapse (Figure 1A). Sixteen of the 55 patients (29%) achieved CR, and 39 (71%) relapsed. One of the 16 patients in CR died due to Epstein-Barr virus encephalopathy and 1 of the 39 patients who relapsed died due to multiorgan failure after adenovirus (AdV) and human herpesvirus 6 infection, not associated with DLI in either case.

At post-transplantation follow-up analyses, 17 of the 36 patients who received CIK cell therapy had developed MC and/or MRD, 8 had experienced relapse, and 11 had been treated prophylactically (Figure 1B). Nineteen patients (53%) achieved CR, and 17 (47%) relapsed. Patients with transplantation-related toxicity sustained organ impairment due to AdV infection in 2 cases and due to cytomegalovirus, Epstein-Barr virus, and AdV infection in 1 case. One patient died from pneumocystis pneumonia, and 1 patient died from respiratory syncytial virus pneumonia. Altogether, CR was achieved in 16 of 44 patients (36%) who received preemptive DLI and in 19 of 28 (68%) who received preemptive CIK cell therapy. Patients with hematologic relapse without additional salvage therapies succumbed to their underlying disease. Here leukemic blast counts in BM samples ranged from 9% to 100% before DLI and from 13% to 62% before CIK cell therapy. Treatment stratification and patient outcomes are shown in a Sankey diagram in Supplementary Figure S4, and more details are provided in Supplementary Figures S6 and S7.

Survival and CIR

All patients treated during overt hematologic relapse died and thus were excluded from the survival analysis. Six-month OS estimates of the remaining patients with preemptive DLI and CIK cell therapy were 57% and 77%, respectively, including 50% and 81% in the prophylactic treatment group and 59% and 75% in the MRD-based treatment group (Figure 2A). The median follow-up in survivors ranged from .9 to 149.2 months (median, 27.9 months).

The 6-month CIR estimate was 55% in the patients who received preemptive DLI and 22% in those who received preemptive CIK cell therapy ($P = .012$; Figure 2B). CIR was significantly lower in the patients with CIK cell therapy compared with those who received conventional DLI. Prophylactic treatment was associated with significantly lower 6-month CIR estimates ($P = .006$). Furthermore, a trend was found between

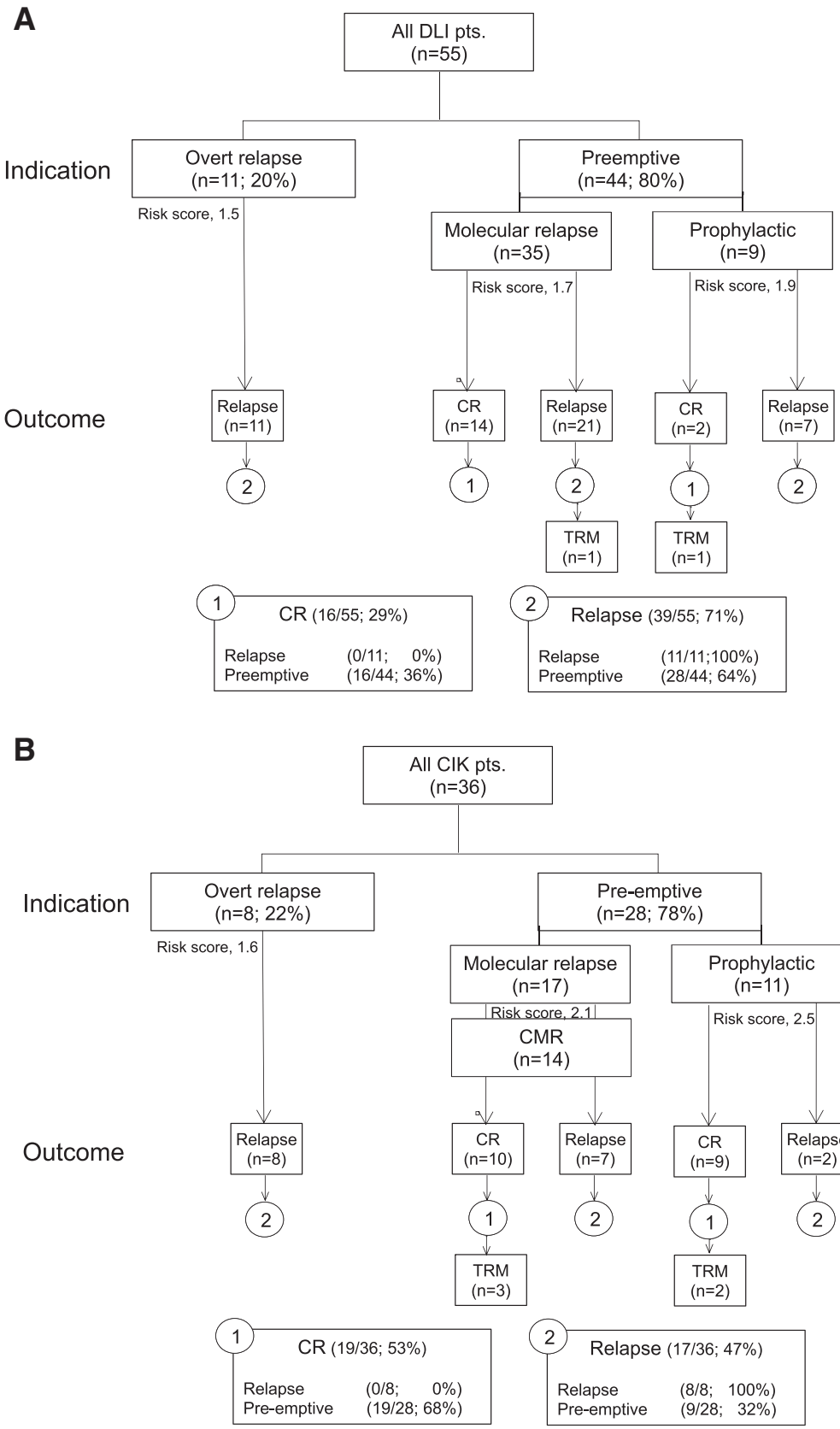

Figure 1. Molecular disease and outcomes. All patients were fully evaluable for stratification classified by chimerism and/or MRD. (A) Thirty-five of the 55 patients who received DLI developed molecular relapse in post-transplantation follow-up analyses, 9 patients were treated prophylactically, and another 11 patients were given DLI for the treatment of overt hematologic relapse. Sixteen of 55 patients (29%) achieved CR, and 39 of 55 patients (71%) relapsed. (B) Accordingly, 17 of the 36 patients with CIK cell therapy showed molecular relapse, 8 patients had experienced relapse at the time of first infusion, and 11 patients were treated prophylactically. Nineteen patients (53%) achieved CR, and 17 (47%) relapsed. Risk score, 0 to 3: 1 point for >CR2, second HSCT, or nonremission; 1 point for non-matched sibling donor; and 1 point for age >10 years.

Table 1
Patient Characteristics

	Immunotherapy			
Characteristic	All (n = 91; 100%)	DLI (n = 55; 60%)	CIK (n = 36; 40%)	*P* Value
Sex, n (%)				1.00
Female	32 (35)	19 (35)	13 (36)	
Male	59 (65)	36 (65)	23 (64)	
Age at SCT, yr				1.00
Median (range)	10.9 (.2-68.4)	11.6 (1.4-20.9)	9.6 (.2-68.4)	
≤18, n (%)	85 (93)	53 (96)	32 (89)	
>18, n (%)	6 (7)	2 (4)	4 (11)	
Disease, n (%)				.248
Acute myelogenous leukemia	31 (34)	16 (29)	15 (42)	
Chronic myelogenous leukemia	2 (2)	1 (1)	1 (3)	
ALL	53 (59)	35 (64)	18 (50)	
Biphenotypic	1 (1)	1 (2)	0 (0)	
T-NHL	4 (4)	2 (4)	2 (5)	
Remission at SCT, n (%)				.178
CR1	22 (24)	14 (26)	8 (22)	
CR2	30 (33)	17 (31)	13 (36)	
≥CR3	10 (11)	9 (16)	1 (3)	
NR	29 (32)	15 (27)	14 (39)	
Number of HSCTs, n (%)				<.001
1	69 (76)	51 (93)	18 (50)	
2	18 (20)	3 (5)	15 (42)	
≥3	4 (4)	1 (2)	3 (8)	
Donor, n (%)				<.001
MFD	20 (22)	17 (31)	3 (8)	
MUD	35 (38)	25 (45)	10 (28)	
MMFD	36 (40)	13 (24)	23 (64)	
Stem cell source, n (%)				.003
BM	46 (51)	35 (64)	11 (31)	
PBSCs	45 (49)	20 (36)	25 (69)	
T cell depletion of grafts, n (%)				.002
No	49 (54)	37 (68)	12 (33)	
Yes	42 (46)	18 (32)	24 (67)	
Serotherapy, n (%)				<.001
ATG	43 (47)	34 (62)	9 (25)	
OKT3	9 (10)	8 (15)	1 (3)	
Campath	16 (18)	0 (0)	16 (44)	
None	23 (25)	13 (23)	10 (28)	

T-NHL indicates T cell non-Hodgkin lymphoma; MFD, matched family donor; MUD, matched unrelated donor; MMFD, haploidentical donor; PBSCs, peripheral blood stem cells; ATG, antithymocyte globulin,

the median T cell dose and reduced CIR in univariate analysis (hazard ratio, 1.75; 95% CI, .94 to 3.23; $P = .076$).

GVHD

aGVHD occurred in patients who received prophylactic treatment, as well as in patients who received cellular therapy based on molecular or hematologic relapse. However, most patients (17 of 19 patients) with hematologic relapse did not develop aGVHD. Overall, 19 of 55 DLI recipients (35%) and 9 of 36 CIK cell recipients (25%) developed aGVHD, and grade III-IV aGVHD occurred in 5 DLI recipients (9%) and in 1 CIK cell recipient (3%). aGVHD occurred at a median of 31 days (range, 17 to 35 days) in patients who received DLI and at a median of 21 days (range, 7 to 81 days) in those who received CIK cell therapy and progressed to chronic GVHD (cGVHD) in 2 DLI recipients and in 1 CIK cell recipient (Table 4).

No differences in the cumulative incidence of aGVHD grade II-IV between CIK and DLI patients was found. Despite being applied in significantly higher T cell doses (Figure 3A), no association between cumulative T cell dose, median T cell dose, or maximum T cell dose provided by CIK cell infusions and aGVHD grade II-IV was found by multivariate analysis (data not shown).

T Cell Recovery

T cell recovery was significantly accelerated after CIK cell therapy, with the greatest impact on T cell reconstitution seen immediately after initiation of CIK cell treatment ($P < .0001$) (Figure 3B). In most cases, CIK cell therapy was applied earlier after transplantation compared with conventional DLI (Figure 3B). T cell recovery was augmented in patients with prophylactic, MRD-, and relapse-based CIK cell therapy.

Univariate and Multivariate Analyses

Univariate and multivariate analyses of survival showed benefits with CIK cell therapy given early after allogeneic

Table 2
T Cell Doses and Number of Infusions

Parameter	DLI (N =55)	CIK (N = 36)	Total (N = 91)	*P* Value
All patients				
Single T cell dose, n	118	103	221	
Median dose, $\times 10^6$/kg	1	5.27	1	<.001
IQR	.1-1.44	1-14.93	.60-5.18	
Range	.02-10	.10-200	.02-200	
Number of infusions				.064
Median	1	2	2	
IQR	1-2.5	1-4	1-3	
Range	1-11	1-9	1-11	
Mean ± SD	2.1 ± 1.9	2.9 ± 2.3	2.4 ± 2.1	
1	30	13	43	
2	11	9	20	
3	7	4	11	
4	3	4	7	
6	–	2	2	
7	2	2	4	
8	1	–	1	
9	–	2	2	
11	1	–	1	
Matched donor, n	42	13	55	
Single T cell dose, n	89	43	132	
Median dose, $\times 10^6$/kg	1	10	1.1	<.001
IQR	.5-2.0	4.95-69.66	1-5	
Range	.02-10	.70-200	.02-200	
Number of infusions				
Median	1.5	3	2	
IQR	1-2.75	2-4	1-3	
Range	1-11	1-9	1-11	
Cumulative T cell dose, $\times 10^6$/kg				<.001
Median	1.49	16	2	
IQR	1-3.94	5-72	1-8.5	
Range	.025-30.8	1-721.4	.025-721.4	
Mean ± SD	4.02 ± 6.17	117.9 ± 212.93	30.93 ± 11.7	
Mismatched donor, n	13	23	36	
Single T cell dose, n	29	60	89	
Median, dose, $\times 10^6$/kg	.1	5	1	<.001
IQR	.1-6.13	1-9.93	.2-6.13	
Range	.025-5	.1-9.4	.025-95.4	
Number of infusions				.291
Median	1	2	1	
IQR	1-2	1-3	1-3	
Range	1-7	1-9	1-9	
Cumulative T cell dose, $\times 10^6$/kg				<.001
Median	1	4.5	1.13	
IQR	.08-1	1-20.91	.92-6.5	
Range	.04-5	1-348.7	.04-348.7	
Mean ± SD	.87 ± 1.43	27.56 ± 72.38	17.92 ± 58.85	

IQR indicates interquartile range.

HSCT, but the results were not significant (Supplementary Figure S5). In contrast, multivariate analysis considering the cumulative T cell dose, CIK versus DLI, treatment indication (prophylactic, molecular relapse, hematologic relapse), and donor type (matched versus mismatched) showed that hematologic relapse at the time of first cell infusion was associated with a significantly worse OS (hazard ratio, 3.65; 95% CI, 1.62 to 8.21; $P = .002$) (data not shown).

DISCUSSION

The use of DLI has been a fundamental strategy for controlling relapse after allogeneic HSCT for hematologic malignancies. However, DLI is aggravated by concomitant GVHD and remains largely experimental in several neoplastic diseases with high proliferation indices, in which responses have been comparatively limited (<30%) [47]. In the present study, DLI achieved remission in 29% of patients, including CMR in 16

Table 3
T Cell Dose per Infusion

Parameter	DLI (N = 55)	CIK (N = 36)	Total (N = 91)	*P* Value
All patients, cumulative T cell dose × 10^6/kg				<.001
Median	1.10	6.50	2	
IQR	.55-3.15	1.75-31.55	1.0-7.50	
Range	.025-30.80	1.0-721.35	.025-721.35	
Mean ± SD	3.28 ± 5.59	60.17 ± 144.12	25.78 ± 94.23	
One T cell dose, N	30	13	43	.073
Median dose, x 10^6/kg	1	1	1	
IQR	.1-1.19	1.00-5.00	.10-1.36	
Range	.025-10.0	1.00-5.00	.025-10.0	
Mean ± SD	1.68 ± 2.67	2.23 ± 1.92	1.85 ± 2.46	
Two T cell doses, N	11	9	20	.016
Median dose, × 10^6/kg	2	6	2.40	
IQR	1.1-2.4	5.4-10.0	1.88-6.25	
Range	.6-12.88	2.0-27.32	.60-27.30	
Mean ± SD	2.99 ± 3.59	8.53 ± 7.68	5.49 ± 6.29	
Three T cell doses, N	7	4	11	.012
Median dose, × 10^6/kg	3.3	23.50	6.42	
IQR	2.28-6.15	14.00-90.76	3.15-13.50	
Range	.82-11.0	8.00-270.05	.82-270.05	
Mean ± SD	4.57 ± 3.51	90.76 ± 126.22	32.46 ± 79.27	
Four T cell doses, N	3	4	7	.057
Median dose, × 10^6/kg	1.9	35.84	13.4	
IQR	1.16-4.25	26.37-118.50	3.15-13.50	
Range	.43-6.60	13.40-351.00	.82-270.05	
Mean ± SD	2.98 ± 3.22	109.02 ± 161.72	63.57 ± 127.65	
Six T cell doses, N		2	2	
Median dose, × 10^6/kg		45.87	45.87	
IQR		39.53-52.20	39.53-52.20	
Range		33.19-58.54	33.19-58.54	
Mean ± SD		45.87 ± 17.93	45.87 ± 17.93	
Seven T cell doses, N	2	2	4	.333
Median dose, × 10^6/kg	.96	210.3	36.63	
IQR	.82-1.11	141.2-279.5	1.11-141.175	
Range	.68-1.25	72.0-348.7	.68-348.7	
Mean ± SD	.96 ± .41	210.3 ± 195.66	105.66 ± 165.45	
Eight T cell doses, N	1		1	
Median dose, × 10^6/kg	30.8		30.8	
IQR	30.8-30.8		30.8-30.8	
Range	30.8-30.8		30.8-30.8	
Mean ± SD	30.8		30.8	
Nine T cell doses, N		1	1	
Median dose, × 10^6/kg		393.3	393.30	
IQR		29.3-557.3	229.30-557.30	
Range		65.3-721.4	65.30-721.40	
Mean ± SD		393.3 ± 463.90	393.30 ± 463.90	
Eleven T cell doses, N	1		1	
Median dose, × 10^6/kg	23.8		23.8	
IQR	23.8-23.8		23.8-23.8	
Range	23.8-23.8		23.8-23.8	
Mean ± SD	23.8		23.8	

patients with molecular relapse of highly proliferative malignancies.

GVHD may imply an associated GVL effect. In our cohort, 6 of 14 patients with grade I-II aGVHD remained in CMR; however, long-lasting CMR was also achieved in 8 of 36 patients without signs of aGVHD, indicating that GVHD and GVL are not necessarily interrelated. Furthermore, 5 patients with grade III-IV aGVHD who received DLI experienced disease recurrence. Immunosuppressive therapy and dysregulated immune responses might have contributed to this finding. Because of the high risk of GVHD, which does not protect against relapse with certainty, DLI at full dose cannot be recommended in the haploidentical donor HSCT setting.

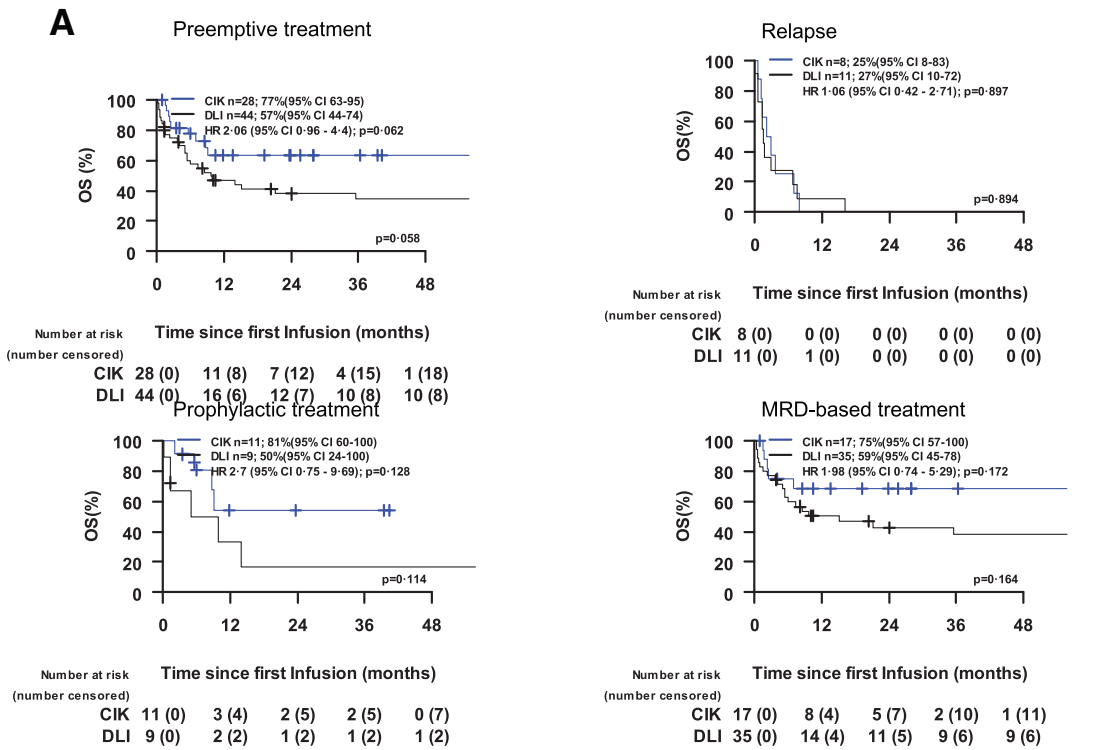

Figure 2. Survival and CIR. (A) All patients treated with DLI or CIK cells during overt hematologic relapse died and thus were excluded from the survival analysis. Six-month OS estimates of the remaining patients with preemptive DLI and CIK cell therapy (prophylactic and MRD-based treatment) were 57% and 77%, respectively. (B) The median survival follow-up ranged from .9 to 149.2 months (median, 27.9 months). The 6-month CIR was 55% and 22% in patients with DLI and CIK cell therapy, respectively (P = .012). The 6-month CIR was significantly lower in patients who received CIK cell therapy compared with patients who received conventional DLI, with the most pronounced effects seen in the prophylactic treatment setting. Follow-up ranged from .9 to 149.2 months (median, 27.9 months).

Table 4
GVHD

Type	DLI (N = 55)					CIK (N = 36)				
	n	%	Molecular relapse	Prophylaxis	Relapse	n	%	Molecular relapse	Prophylaxis	Relapse
aGVHD	19	35				9	25			
Grade I	12	22	11	1	-	3	8	1	2	-
Grade II	2	4	1	1	-	5	14	2	2	1
Grade III	4	7	1	3	-	1	3	-	1	-
Grade IV	1	2	-	-	1			-	-	-
cGVHD	2	4				2	6			
Limited	1	2	-	-	1	2	6	1	1	-
Extensive	1	2	1	-	-	-	-	-	-	-

The *P* values between the DLI and CIK groups were not significant for aGVHD ($P = .079$) or for cGVHD ($P = .646$).

Mechanisms responsible for the reduced alloreactive potential of CIK cells impute a limited lifespan of terminally differentiated $CD3^+CD56^+$ late effector cells following prolonged ex vivo expansion [48], targeted organs lacking NKG2D ligands, and the production of certain cytokines, such as IFN-y, known to be protective against GVHD [49]. Furthermore, CIK cells have demonstrated a significantly lower acquisition of homing molecules required for the entry into inflamed and GVHD targeted organs (a4b7, CCR9, E-selectin, CXCR3, and CCR5) [50]. Thus, the proliferation and spreading of CIK cells also may be driven by differences in minor histocompatibility antigens.

The first clinical application of CIK cells after allogeneic HSCT in a phase I trial was reported by Introna et al [51], demonstrating the feasibility and the low toxicity profile of this approach. These authors reported a 36% rate of aGVHD ($\leq$ grade II), with 2 patients progressing to extensive cGVHD. A similarly low incidence of GVHD was reported by the Stanford group [52] and by Linn et al [53], who recently analyzed CIK cell therapy in combination with salvage chemotherapy. Seventy-four patients were analyzed in another recent phase IIA study reported by Introna et al [54]. Patients were treated with 2 sequential DLIs to bridge 5 weeks of in vitro generation and quality control of CIK cells. Overall, 12 patients (16%) developed aGVHD (grade I-II, n = 7; grade III-IV, n = 5). In 8 of these 12 patients, aGVHD developed during DLI treatment. cGVHD was observed in 11 patients (15%). Therefore, the use of CIK cells has been suggested even in the context of HLA-mismatched haploidentical HSCT [55-57]. Indeed, owing to the low risk of aGVHD in our study, the median infused T cell dose could be significantly increased even up to 10×10^6/kg (range, .7 to 200×10^6/kg) in the matched donor HSCT setting and to 5×10^6/kg (range, .1 to 9.4×10^6/kg) in the haploidentical donor HSCT setting compared with the dose of conventional DLI (a 10-fold and 50-fold increase, respectively). Accordingly, the median cumulative T cell dose provided by CIK cell infusion was significantly increased compared with DLI in both the matched donor and mismatched donor HSCT settings ($P < .001$).

Despite increased T cell numbers, no association between T cell dose and the occurrence of aGVHD grade II-IV was found on multivariate analysis, and no transfusion-related deaths occurred, indicating the safety and low toxicity of the CIK cell therapy approach. Moreover, compared with DLI, CIK cell therapy significantly improved T cell recovery in the early post-transplantation period ($P < .0001$). Along with increased T cell doses, persistent lymphopenia might have facilitated T cell recovery in the early post-transplantation period, as has been reported for in vivo lymphodepletion administered to enhance immune cell expansion in several adoptive immunotherapy protocols.

As is often the case in settings where salvage therapies are used concurrently, assessing efficacy is difficult. Nevertheless, some patients achieved measurable responses in terms of improved donor chimerism or clearance of disease that could be solely attributable to donor CIK cell infusion [51-53,58]. In a recent phase IIA study of 74 patients treated with 2 sequential DLIs followed by CIK cell therapy in which 43 patients received the complete cell therapy as planned (58%), 19 patients (26%) achieved CR, 3 (4%) achieved a partial response, 8 (11%) had stable disease, 2 (3%) experienced early death, and the 41 patients (56%) experienced disease progression [54]. Progression-free survival was 31% at 1 year and 29% at 3 years, with respective OS of 51% and 40%. Disease control was achieved mostly after a cytogenetic or molecular relapse.

In our settings, no salvage therapies were used concurrently to better assess efficacy. Despite the unfavorable situation at the time of HSCT in patients who received CIK cell therapy (risk scores 2.1 to 2.6) compared with those who received DLI (risk scores 1.7 to 1.9) in our cohort, CR was achieved in 68% of the patients who received preemptive CIK cell therapy, resulting in a 6-month OS of 77% with a median survival of 27.9 months (range, .9 to 149.2 months). Of note, the 6-month CIR was significantly lower in the patients who received CIK cell therapy compared with those who received DLI (22% versus 55%; $P = .012$). Differences in T cell doses between the recipients of DLI and the recipients of CIK cell therapy were most pronounced in patients treated based on molecular relapse ($P < .001$; Figure 3A). Further subanalyses (univariate analyses) showed a trend but no clear association between median T cell dose and reduced CIR. In addition, Kaplan-Meier estimates showed a decreased CIR after CIK cell therapy compared with DLI in the mismatched donor HSCT setting ($P = .0520$; data not shown), suggesting that increased T cell doses facilitated by the NK-like nature of the CIK cell product may account for improved efficacy. However, a greater number of patients would have been desirable to prove efficacy. Greater numbers of high-risk patients also would have been desirable to interpret benefits in studies involving intervention as prophylaxis.

In contrast, our patients with hematologic relapse could not be rescued by CIK cell therapy without additional salvage or lymphodepletion chemotherapy. Salvage strategies aimed at reducing leukemic burden, providing lymphodepletion, and inducing an inflammatory milieu enabling expansion of CIK cells might have increased efficacy in relapsed patients. Thus, in our open-labeled multicenter phase II study with sequential administration of incremental doses of donor CIK cells to patients with hematologic malignancies initiated in March 2016 (Eudra-CT; 2013-005446-11), remission induction must be achieved before the first CIK cell infusion to enable efficacy.

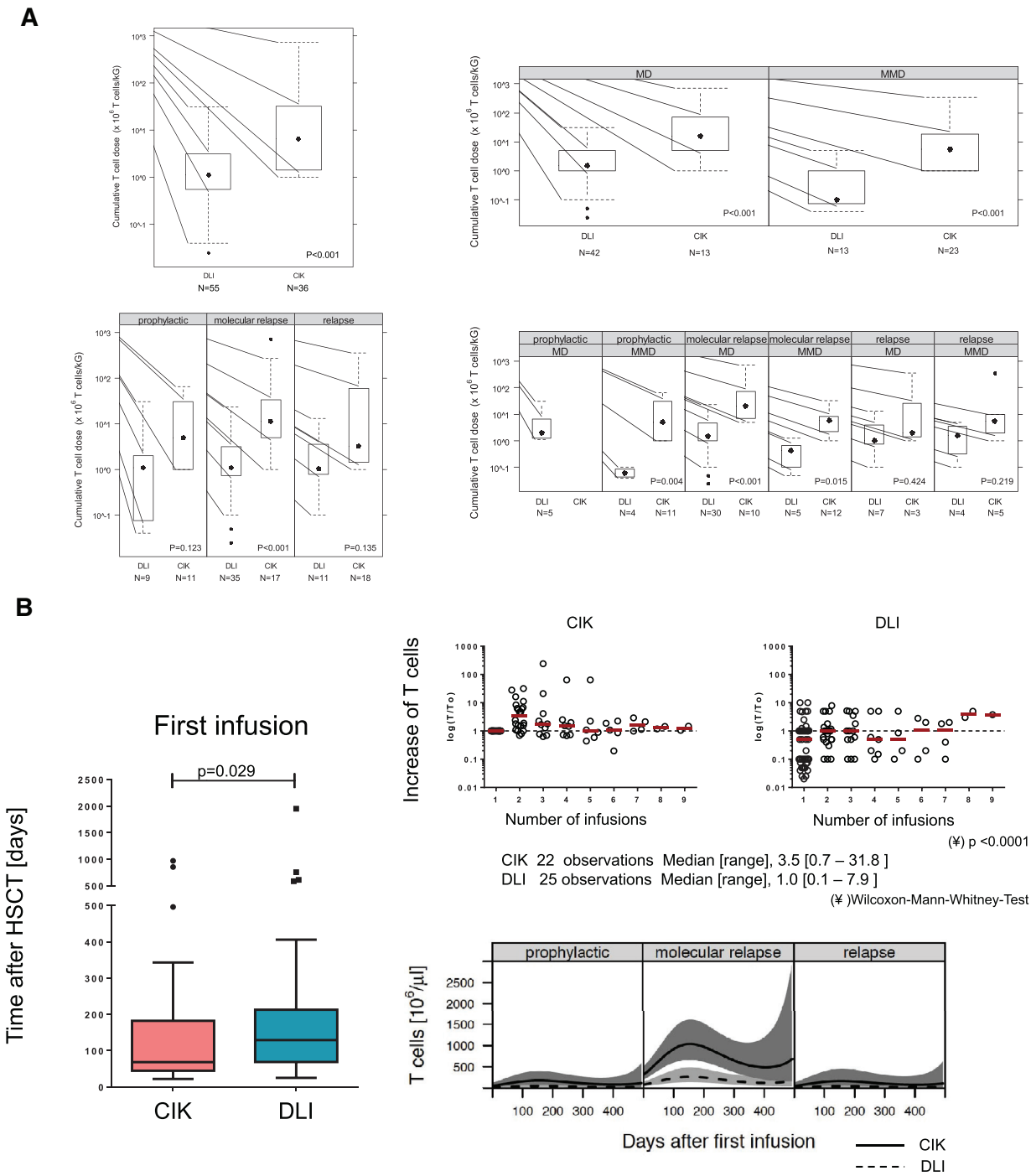

Figure 3. T cell doses and T cell recovery. (A) Cumulative T cell doses × 10^6/kg recipient body weight applied during DLI or CIK cell therapy were significantly increased in patients who received CIK cell therapy based on molecular relapse. CIK cell T cell dose was significantly increased in both the matched and mismatched donor settings, especially if patients were treated prophylactically or based on molecular relapse. (B) T cell recovery was significantly improved, showing the greatest impact on T cell reconstitution immediately after initiation of CIK cell therapy. Of note, discrimination of immune recovery after HSCT in general and T cell expansion due to CIK cell treatment was not possible. CIK cell therapy was applied earlier after transplantation than conventional DLI resulting in improved T cell recovery in patients with prophylactic, MRD-based, and relapse-based CIK cell treatment. Besides increased T cell doses, persisting lymphopenia in the early post-transplantation period may have facilitated T cell recovery after CIK cell transfusions.

This study includes retrospective comparisons of immune cell infusions given preemptively to patients at risk for relapse after allogeneic HSCT. The study has some inherent flaws owing to its retrospective design. The differences in terms of factors that could influence relapse risk in either direction of the 2 sequentially collected cohorts represents a limitation of these retrospective single-arm experiences. Conditioning and T cell depletion might increase the inherent relapse risk, but also increase the potential efficacy of infused cellular therapy. More patients in the CIK group were treated after 2 HSCTs. This might be taken to indicate increased risk of relapse and TRM, but, alternatively, the fact that patients responded to

salvage therapy and were considered for a second transplantation could also have been favorable. Of note, more patients in the CIK group with a second HSCT still had active disease and had not responded to salvage therapy at the time of transplantation, indicating an increased risk of relapse compared with the DLI group. However, more patients who received CIK cell therapy had a mismatched donor, which might increase the efficacy of cellular therapy. Overall, the lower toxicity and higher efficacy of CIK cell therapy compared with DLI is not so clearly demonstrated here. A better approach to performing the analyses presented here would be with a case-matched cohort; however, owing to its heterogeneity, our present cohort was not suitable for that kind of analysis, and thus a randomized controlled trial would be more convincing in this setting.

In conclusion, the results of DLI and CIK cell therapy in this study confirm the feasibility of adoptive cellular therapy. Especially with our short-term culture system, sufficient numbers of CIK cells were generated, meeting the requirements of a Good Manufacturing Practice-adapted generation protocol for pediatric and adult patients and the urgent clinical need for relapsing patients after allogeneic HSCT. Compared with DLI, CIK cell therapy showed similar or even improved GVL effects and significantly reduced 6-month CIR in patients with a low tumor burden of hematologic malignancies following allogenic HSCT, again confirming the importance of disease monitoring after HSCT. Finally, compared with conventional T cells, CIK cells appear to be endowed with a reduced propensity to cause GVHD, making them an appealing and promising alternative to classic DLI. These CIK cell results appear very promising and will hopefully lead to more large clinical trials in this setting.

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Authorship statement: S.B., A.W., A.J., J.S., A.S., R.M., G.B., T.K., P.B., and E.R. enrolled and cared for the patients. M.M., E.S.-M., V.K., S.H., M.B., S.B., A.W., A.J., J.S., A.S., R.M., G.B., H.B., T.K., P.B., and E.R. collected, analyzed, and interpreted the data. M.M., E.R., and P.B. wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2019.03.004.

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