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Allogeneic – Adult

Phase II Study of Sequential Infusion of Donor Lymphocyte Infusion and Cytokine-Induced Killer Cells for Patients Relapsed after Allogeneic Hematopoietic Stem Cell Transplantation

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A B S T R A C T

Seventy-four patients who relapsed after allogeneic stem cell transplantation were enrolled in a phase IIA study and treated with the sequential infusion of donor lymphocyte infusion (DLI) followed by cytokine-induced killer (CIK) cells. Seventy-three patients were available for the intention to treat analysis. At least 1 infusion of CIK cells was given to 59 patients, whereas 43 patients received the complete cell therapy planned (58%). Overall, 12 patients (16%) developed acute graft-versus-host disease (aGVHD) of grades I to II in 7 cases and grades III to IV in 5). In 8 of 12 cases, aGVHD developed during DLI treatment, leading to interruption of the cellular program in 3 patients, whereas in the remaining 5 cases aGVHD was controlled by steroids treatment, thus allowing the subsequent planned administration of CIK cells. Chronic GVHD (cGVHD) was observed in 11 patients (15%). A complete response was observed in 19 (26%), partial response in 3 (4%), stable disease in 8 (11%), early death in 2 (3%), and disease progression in 41 (56%). At 1 and 3 years, rates of progression-free survival were 31% and 29%, whereas rates of overall survival were 51% and 40%, respectively. By multivariate analysis, the type of relapse, the presence of cGVHD, and a short (<6 months) time from allogeneic hematopoietic stem cell transplantation to relapse were the significant predictors of survival. In conclusion, a low incidence of GVHD is observed after the sequential administration of DLI and CIK cells, and disease control can be achieved mostly after a cytogenetic or molecular relapse.

INTRODUCTION

Relapse after allogeneic hematopoietic stem cell transplantation (alloHSCT) has a very poor outcome. Donor lymphocyte infusion (DLI) and second transplant have been the best options offered, but survival data are largely unsatisfactory [1-5].

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Cytokine-induced killer (CIK) cells are obtained after in vitro stimulation of donor peripheral blood mononuclear cells with anti-CD3, IFN-γ, and recombinant human IL-2 to induce expansion of T cells and CD56 expression on CD3/CD8 T lymphocytes, as originally described for the first time by Schmidt-Wolf et al. [6] and more recently reviewed [7]. CIK cells thus acquire "double" T cell–natural killer (NK) cell function, retaining the T cell specificity, as shown in the case of anti-cytomegalovirus–specific CIK cells [8,9], and also acquiring nonspecific antitumoral NK cell–like cytotoxicity [8]. Indeed, lymphocyte function–associated antigen 1 and DNAX accessory molecule 1 have been described in both T and NK cell functions [8], whereas NKG2D, NKp30 [8], and CD56 [10] have been involved in NK cell–like cytotoxicity.

Several in vitro and in vivo experimental models suggest that CIK cells home to tumors and show antitumoral activity in vivo [7,11-16]. Moreover, they show very little graft-versus-host disease (GVHD) in several experimental models in the presence of either minor or major histocompatibility mismatches [17]. The molecular mechanism at the basis of such reduced alloreactivity have not been yet fully clarified, with contrasting results obtained by immune selection of different subpopulations present in the CIK cultures [18,19]. Indeed, several clinical phase I/II studies confirm very low GVHD in patients [16,17]. More specifically, in the context of tumor relapse after alloHSCT, CIK cells from matched and haploidentical donors have shown graft-versus-leukemia activity with little GVHD and therefore may represent an ideal candidate to treat post-transplant relapse [20-24]. We prospectively studied 74 relapsed patients after alloHSCT for hematologic malignancies in an open-label, multicenter, phase IIA study centered on the sequential administration of donor-derived unmanipulated DLI and CIK cells.

METHODS

Protocol and Patients Enrollment

This study was approved by Institutional Review Boards at Azienda Socio Sanitaria Territoriale (ASST) Papa Giovanni XXIII in Bergamo, San Gerardo Hospital in Monza and the Ospedale Centrale, Bolzano, Italy. This open-label, exploratory, phase IIA, multicenter study was authorized by Istituto Superiore di Sanità, as defined by the Advanced Therapeutic Medicinal Product regulations, and approved by the Agenzia Italiana del Farmaco (AIFAII/37533/P). The trial was registered as EudraCT 2008-003185-26 and as ClinicalTrial.gov NCT01186809. For each patient informed consent and subsequent authorization for the treatment by the appropriate commission was obtained (Commissione Ammissibilità Studi Clinici di Fase I). All patients have been registered in the Istituto Superiore di Sanità Data Bank for Somatic and Gene Therapy (study code 107). The database containing the case report forms (CRFs) has been established in Bergamo under the direct supervision of 1 of the authors (F.D.) and was available for scrutiny to all clinicians involved.

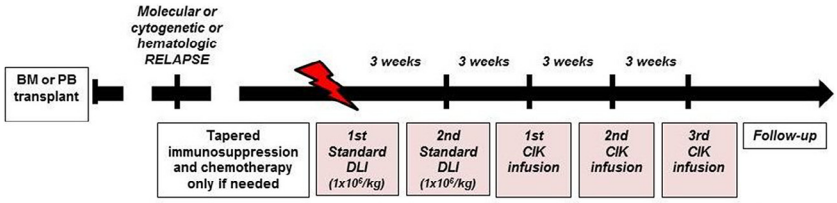
Patients could be enrolled for hematologic malignancies (excluding chronic myeloid leukemia) with a molecular, cytogenetic, or hematologic relapse after allogeneic transplantation. Inclusion criteria also included the availability of the donor's lymphocyte apheresis (the donor had to be the same who originally donated the hematopoietic stem cells for transplants) and withdrawal of the immunosuppression at the beginning of the program. Donors positive for HIV, hepatitis B virus, or hepatitis C virus were excluded to comply with the Good Manufacturing Practices (GMP) requirements of the cell factory. Patients with active acute or chronic GVHD (aGVHD and cGVHD, respectively) were excluded, as well as patients with rapidly progressive disease (ie, those with a disease not controlled by cytoreductive treatments including not only oral hydroxycarbamide but also systemic chemotherapy). The primary objectives of the phase IIA study were to define the safety profile and to evaluate the activity of the combined cellular therapy. Primary endpoints were the determination of the maximal tolerated dose and the estimation of cumulative incidence of molecular, karyotypic, or hematologic responses at day +100 after the end of the cell program. Secondary objectives were progression-free survival (PFS) and overall survival (OS) assessed by 1 year after the end of the cell therapy program.

Two infusions of unmanipulated donor lymphocytes (1×10^6/kg each) were given with a minimum interval of 3 weeks. Three infusions of donor CIK cells were administered according to a dose-escalating program, starting 3 weeks after second DLI. CIK cell administrations were separated by 3-weeks interval (Figure 1).

The safety phase was performed by 4 combinations of dose-escalating levels, each given to 3 patients: (1) 1×10^6/kg, 1×10^6/kg, and 5×10^6/kg for the first, second, and third dose, respectively; (2) 1×10^6/kg, 5×10^6/kg, and 5×10^6/kg; (3) 1×10^6/kg, 5×10^6/kg, and 10×10^6/kg; and (4) 5×10^6/kg, 5×10^6/kg, and 10×10^6/kg. The trial was conducted to identify the maximal tolerated dose. Grade IV aGVHD was considered as the dose-limiting toxicity. The maximal tolerated dose was the combination of doses where <1 in 6 patients experience dose-limiting toxicit. Apheresis material was used to perform the first and second DLI administrations (1×10^6/kg each).

CIK Cell Preparation

CIK cells were manufactured according to GMP-compliant standard operating procedures in Agenzia Italiana del Farmaco–authorized Cell Factories (USS Centro di Terapia Cellulare "G. Lanzani", USC Ematologia, located at the ASST Papa Giovanni XXIII at Bergamo, Italy for the Bergamo and Bolzano patients and at Laboratorio of Cell and Gene Therapy "StefanoVerri", Ospedale San Gerardo, Monza, Italy for the Monza patients) in a class A environment (sterile hood) placed in a class B area. The 2 cell factories received in 2007 the first approval for the manufacturing of sterile injectable biologic human medicinal products of small volumes and have received periodic inspections to assess the consistency with the GMP requirements [25]. This study was approved by Istituto Superiore di Sanità (authorization no.



Combination	1st CIK cells infusion	2nd CIK cells infusion	3rd CIK cells infusion
1	1x10⁶/Kg	1x10⁶/Kg	5x10⁶/kg
2	1x10⁶/Kg	5x10⁶/kg	5x10⁶/kg
3	1x10⁶/Kg	5x10⁶/kg	10x10⁶/kg
4	5x10⁶/kg	5x10⁶/kg	10x10⁶/kg

Figure 1. Treatment scheme and dosages. (Top) Scheme of DLI and CIK cell infusions is summarized. (Bottom) Escalating doses of CIK cells, corresponding to the 4 triplets of patients of the "safety trial," are shown.

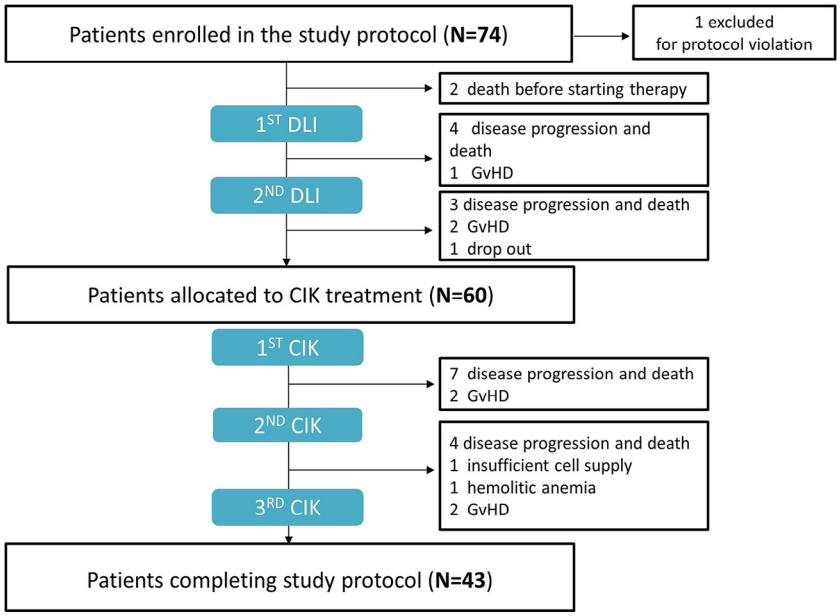


Figure 2. Treatment flow. The number of patients enrolled, allocated to CIK cell treatment, and who completed the 2 DLI and 3 CIK cell infusions are shown schematically. Patients who could not complete the therapeutic program are indicated on the right, with the main reasons for not receiving the cells.

64499(04)-PRE21-848 and subsequent amendment 21862(07)-PRE21-848). The production protocol, including reagents and excipients, has not changed with respect to those adopted in the production of CIK cells for the phase I study [20].

CIK cell production started from 30 to 50 mL of fresh lymphocytoapheresis material. Briefly, cells were washed, and 900×10^6 to 1.2×10^9 total cells were plated at 3×10^6/mL in X-VIVO 15 complete medium (Lonza, Basel, Switzerland) containing 1000 U/mL IFN-γ (Imukin; Boehringer-Ingelheim, Ingelheim-am-Rhein, Germany). At day +1 50 ng/mL monoclonal anti-CD3 antibody (OKT3, Orthoclone; Janssen Cilag, Schaffhausen, Switzerland) and 500 U/mL recombinant human IL-2 (Proleukin; Novartis, Basel, Switzerland) were added. Every 3 to 5 days fresh medium was added to maintain cells at .5 to 1.5×10^6/ mL and recombinant human IL-2 adjusted to 500 U/mL final concentration, for a total of 18 to 24 days of continuous culture. At the end of the expansion, CIK cells were frozen in human AB plasma and 10% DMSO (Wak Chemie, Steinbach, Germany) and quarantined until the results of quality controls were obtained: The release criteria were defined as ≥80% cell viability, ≥40% double positivity for CD3/CD56 (true CIK), sterility (anaerobic and aerobic), endotoxin levels <7 European Union (EU)/mL, and absence of mycoplasma (culture tests). All quality controls were validated and performed according to European Pharmacopeia guidelines.

A sufficient number of CIK cells could be prepared for all patients included in the protocol, although, as detailed below, not every patient could receive the planned cell program because of the aggressiveness of their disease and/or clinical complications (Figure 2). Sixty-five batches (157 infusion bags released for clinical use) were used to treat the 59 patients who could receive at least 1 infusion of CIK cells. The mean percentage of true CIK cells was 43.3% ± 10.7% and the median value 43% (varying from 7% to 61%), whereas the total CD3 positive T cells overall represented a mean of 95.3% ± 4.7% and median value of 97% (varying from 72% to 99%). For 14 batches (21%) the CD3/CD56 double positive cells were slightly below the 40% release criteria, but cells were administered in all cases to the critical clinical conditions, under the responsibility of the Qualified Persons and the responsible clinicians. In all cases the viability of the cells at the infusions always exceeded 80% (data not shown).

Outcomes

OS was defined as the probability of survival irrespective of disease state at any point in time from the first DLI. Patients living at their last follow-up were censored. PFS, defined as any evidence of molecular, cytogenetic, or hematologic disease progression, was calculated for all patients as time to relapse/progression or death from first DLI. Disease-free survival (DFS) was defined only for patients in complete remission (CR) as time to relapse or death from CR. GVHD was diagnosed according to the previously established criteria [3,26], and the cumulative incidence of aGVHD and cGVHD was defined as time from first DLI to aGVHD or cGVHD, considering death as a competing event.

Statistical Methods

Data are presented as medians with range for continuous variables and frequency with percentages for categorical variables. Time-to-event analysis was performed in terms of PFS, OS, and DFS. Survival estimates were calculated with the Kaplan-Meier method, and the log-rank test was applied to test differences between groups. The Cox proportional hazard model was used to assess hazard ratios with 95% confidence intervals (CIs) in univariate and multivariate analysis. Proportional hazard assumption was verified for all estimated models. aGVHD and cGVHD were considered as time-dependent variables; their unadjusted effect on OS was tested with the Mantel-Byar test [27]. Cumulative incidence of aGVHD and cGVHD was also assessed using cumulative incidence function, considering death as a competing event. All reported *P* values were 2-sided, and the conventional 5% significance level was fixed. Statistical analysis was performed using the SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA).

RESULTS

Patients' Characteristics

Table 1 shows the characteristics of the enrolled patients. A total of 74 patients were enrolled in the study from July 2009 to September 2016. These patients represent approximately the 50% of consecutive patients relapsed after alloHSCT. The 3 main causes of exclusion from the protocol were rapid progression of disease (34%), the presence of GVHD at relapse (22%), and physician's choice of different treatments, such as second alloHSCT (11%). Among them, 1 was excluded from the final analysis because of a major protocol violation: being an acute lymphoblastic leukemia (ALL) patient treated with blinatumomab for a molecular relapse before cellular therapy. Of the 73 patients, 39 were males and 34 females, with a median age of 46 years (rang, 2 to 68). Forty-one (56%) had acute myelogenous leukemia (AML), 19 (26%) ALL, 4 (5%) multiple myeloma, 3 (4%) Hodgkin disease, 2 (3%) non-Hodgkin lymphoma, and 4 (5%) myelodysplastic syndrome or myeloproliferative neoplasms. Fifteen patients had received previous autologous HSCT.

Table 1
Patient Characteristics at Registration

Characteristics	All (N = 73)	Pediatrics (n = 15)	Adults (n = 58)
Age, yr, median (range)	46 (2-68)	10 (2-21)	51.5 (19-68)
Sex			
Female	34 (46.6)	5 (33.3)	29 (50.0)
Male	39 (53.4)	10 (66.7)	29 (50.0)
Disease status at HSCT			
No CR	25 (34.3)	3 (20.0)	22 (37.9)
CR	46 (63.0)	12 (80.0)	34 (58.6)
nd	2 (2.7)	0	2 (3.5)
Diagnosis			
ALL	19 (26.0)	9 (60.0)	10 (17.2)
AML	41 (56.2)	4 (26.6)	37 (63.7)
Hodgkin disease	3 (4.1)	1 (6.7)	2 (3.5)
Multiple myeloma	4 (5.5)	0	4 (6.9)
Non-Hodgkin lymphoma	2 (2.7)	0	2 (3.5)
MDS	2 (2.7)	0	2 (3.5)
myeloproliferative neoplasms s	2 (2.7)	1 (6.7)	1 (1.7)
Previous HSCT			
No	58 (79.5)	10 (66.7)	48 (82.8)
Yes	15 (20.5)	5 (33.3)	10 (17.2)
Preparative regimen			
MAC	53 (72.6)	12 (80.0)	41 (70.7)
RIC	20 (27.4)	3 (20.0)	17 (29.3)
Stem cell source*			
Peripheral blood	42 (59.2)	2 (14.3)	40 (70.2)
Bone marrow	29 (40.8)	12 (85.7)	17 (29.8)
Donor			
SIB	37 (50.7)	5 (33.3)	32 (55.2)
MUD	31 (42.4)	8 (53.4)	23 (39.6)
HLA haploidentical	5 (6.9)	2 (13.3)	3 (5.2)
Relapse type			
Hematologic	44 (60.3)	6 (40.0)	38 (65.5)
Molecular + cytogenetic	29 (39.7)	9 (60.0)	20 (34.5)
Months from diagnosis to HSCT, median (range)	6 (2-102)	24 (5-62)	5 (2-102)
Months from HSCT to relapse, median (range)	6 (.5-124)	6 (1-26)	6 (.5-124)

Values are median numbers of cases with range in parentheses, unless otherwise defined. nd, not determined; MDS, myelodisplastic syndrome; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; SIB, sibling; MUD, matched unrelated donor.
* Two missing data.

All 73 patients relapsed after allogeneic transplants (31 unrelated, 37 sibling, and 5 haploidentical), and 53 had received ablative and 20 reduced-intensity conditioning regimens, respectively. The median time from HSCT to relapse was 6 months (range, 1 to 124). Importantly, 44 patients (60%) suffered from a hematologic and 29 (40%) from a cytogenetic or molecular relapse. In particular, cytogenetic relapse was observed in 4 patients with AML, whereas a molecular relapse was documented in 11 AML, 12 ALL, 1 atypical chronic myelogenous leukemia, and 1 AML with a loss of full donor chimerism (data not shown). Among the 11 AML with molecular relapse, 5 were *NPM1* mutated, 3 *AML-ETO* positive, whereas in the remaining 3 minimal residual disease was evaluated by *WT1*/*ABL* overexpression (data not shown). At relapse, due to an overt hematologic relapse, 24 patients received a cytoreductive treatment before starting the cell therapy program. According to the treatment protocol (Figure 2), independently of donor type, all patients should have received 2 unmanipulated DLIs of 1×10^6/kg separated by 3-week interval.

As detailed in Figure 1, 2 patients died before starting therapy due to disease progression, 7 patients had disease progression and died during the DLI administrations, 3 patients developed aGVHD and were not further treated with CIK cells, and 1 patient withdrew consent to participate in the study. Therefore, 60 patients received at least 1 CIK cell administration. Nine patients received only 1 CIK cell administration due to early disease progression and death (n = 7) and aGVHD development (n = 2). Eight patients received only 2 CIK cell administrations due to disease progression and death (n = 4), aGVHD development (n = 2), hemolytic anemia (n = 1), and insufficient cell supply (n = 1). Therefore, 43 of 74 enrolled patients (58%) received the complete planned cell therapy program with CIK cells (3 consecutive doses), of which 37 received the highest cell dosage (5×10^6/kg, 5×10^6/kg, and 10×10^6/kg).

Safety

The administration of CIK cells was generally well tolerated. In the first group of 12 cases (dose escalating "safety trial"), 2 patients developed grade II aGVHD, but no dose-limiting toxicity was found (grade IV aGVHD), even when the highest dose planned (5×10^6/kg, 5×10^6/kg, and 10×10^6/kg in succession) was reached. The toxicity data refer to the group of 73 patients (intention to treat analysis) and are summarized in Table 2. Overall, 12 patients (16%) developed aGVHD (of grades I to II in 7 cases and grades III to IV in 5). Of the 7 patients with grades I to II aGVHD, 5 had a skin involvement, 1 concomitant liver and skin, and 1 concomitant skin and gut. Among 5 patients with grades III to IV aGVHD, the organs involved were skin (n = 1), liver (n = 2), and concomitant liver and skin (n = 2). All patients except 1, who did not require therapy, received prednisolone at a dose ranging from .5 mg/kg to 2 mg/kg according to the severity of aGVHD, whereas only a minority (25%) restarted cyclosporine. In 8 of these 12 cases, aGVHD developed during DLI treatment, leading to interruption of the cellular program in 3 patients, whereas in the remaining 5 cases aGVHD was

Table 2
Toxicity

	Grade	No. of Patients (%)	No. of Insurgence during or after DLI Treatment	No. of Insurgence during or after CIK Cell Treatment
aGVHD	1-2	7 (9)	5	2
	3-4	5 (7)	3	2
cGVHD*	Mild	4 (5)	0	4
	Moderate	5 (7)	0	5
	Severe	2 (3)	1	1
Hemolitic Anemia	N.A.	1 (2)	0	1

* In 6 cases preceded by the insurgence of aGVHD. N.A. indicates not applicable.

controlled by steroid treatment, thus allowing the subsequent planned administration of CIK cells. Among 4 patients who developed aGVHD during CIK cell administration, 2 patients developed aGVHD after the first infusion of CIK cells and 2 after the third, respectively. In 1 patient the insurgence of acute hemolytic anemia (severe) occurred 15 days after the second infusion of CIK cells, which led to the interruption of the CIK cell administration. The treatment of this complication required 1 mg/kg prednisolone for 3 weeks and rituximab at a dose of 200 mg/m^2 once a week for 4 weeks, obtaining a long-lasting remission.

After exclusion of the 9 patients who died for progression during the first part of treatment and the 1 patient who dropped out (Figure 1), the incidence of aGVHD was of 19% (12/62). Moreover, 11 patients developed cGVHD (overall 15%) of mild (n = 4), moderate (n = 5), and severe (n = 2) grade. Of these, 5 patients had received the full CIK cell dosage, whereas because of the insurgence of aGVHD during treatment, 5 had received only 1 or 2 CIK cell administration and the remaining 1 only DLI. Oral mucosa and liver were the sites more frequent involved: oral mucosa (n = 4), concomitant oral mucosa and eyes (n = 2), concomitant oral mucosa and liver (n = 1), liver (n = 2), and concomitant skin and liver (n = 2). Most treatments used in this group of patients was 1 mg/kg prednisolone and calcineurin inhibitors. Extracorporeal photopheresis was used as second-line treatment in 3 patients (27%), and 1 patient (9%) received rituximab as third-line treatment. Figure 3A and B shows the cumulative incidence of aGVHD at 100 days (12%; 95% CI, 6% to 21%) and cGVHD at 3 years (17%; 95% CI, 9% to 28%). Among 12 patients who showed aGVHD, 5 were transplanted from siblings, 4 from matched unrelated donors, and 3 from haploidentical donors. In all 3 patients transplanted with a haploidentical donor, the complication of aGVHD occurred during DLI treatment. cGVHD developed in 3 patients with sibling donors, 7 patients with unrelated donors, and 1 patient with a haploidentical donor (data not shown).

Main Clinical Outcomes of Activity

We measured clinical response at 100 days after the completion of the cell therapy program and results are reported in Table 3. Two patients (3%) died before starting treatment due to disease progression and were considered as early deaths. Among the remaining 71 patients, 19 (26%) achieved CR, 3 (4%) partial remission (overall CR + partial remission, 22 patients [30%]), 8 (11%) had a stable disease, and the remaining 41 patients (55%) experienced disease progression.

For the whole patient cohort, the median follow-up was 12.4 months (range, .7 to 77.1); for alive patients the median

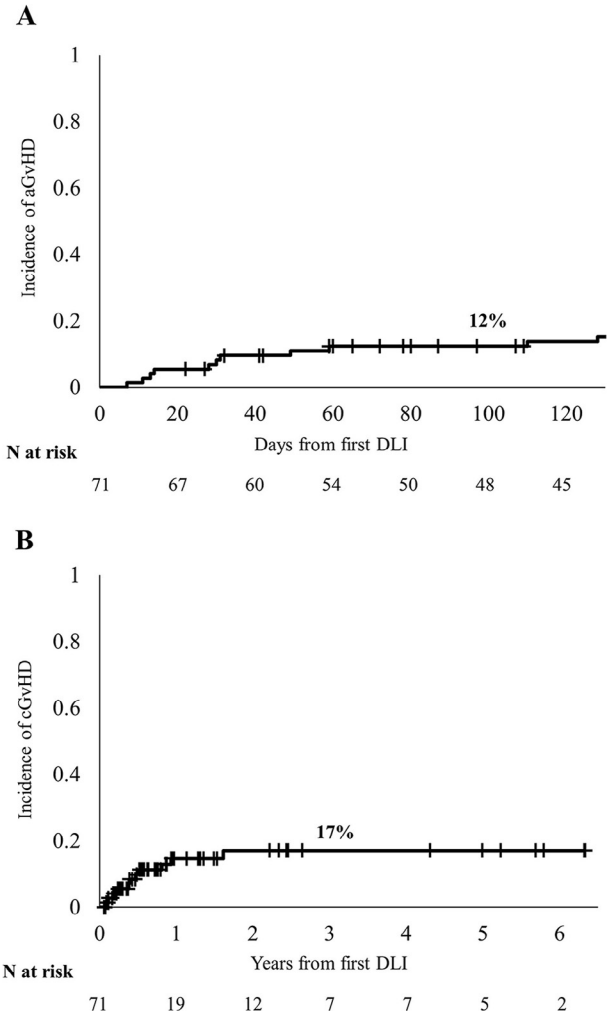


Figure 3. Cumulative incidence of GVHD. (A) aGVHD; (B) cGVHD.

follow-up was 28.3 months (range, 1.8 to 77.1). At 1 and 3 years, PFS of the 73 patients was 31% (95% CI, 20% to 41%) and 29% (95% CI, 19% to 40%) (Figure 4A), respectively, whereas for OS it was 51% (95% CI, 39% to 61%) and 40% (95% CI, 28% to 52%) (Figure 4B). For the small group of patients who achieved CR or partial remission (22 patients), DFS was 68% (95% CI, 45% to 83%) both at 1 and 3 years (data not shown).

Finally, we compared the outcomes of the 73 patients divided in 2 groups: those who entered the program with a cytogenetic or molecular relapse (29 patients) and those with an overt hematologic relapse (44 patients). The 1- and 3-year PFS was 57% (95% CI, 37% to 73%) and 54% (95% CI, 34% to 70%) for the cytogenetically or molecularly relapsed

Table 3
Clinical Response

	No. of Cases	Percent	
Total assessable patients	73		
CR	19	26	22 (30%) response
Partial remission	3	4	
Stable disease	8	11	
Progression of disease	41	56	
Early death	2	3	

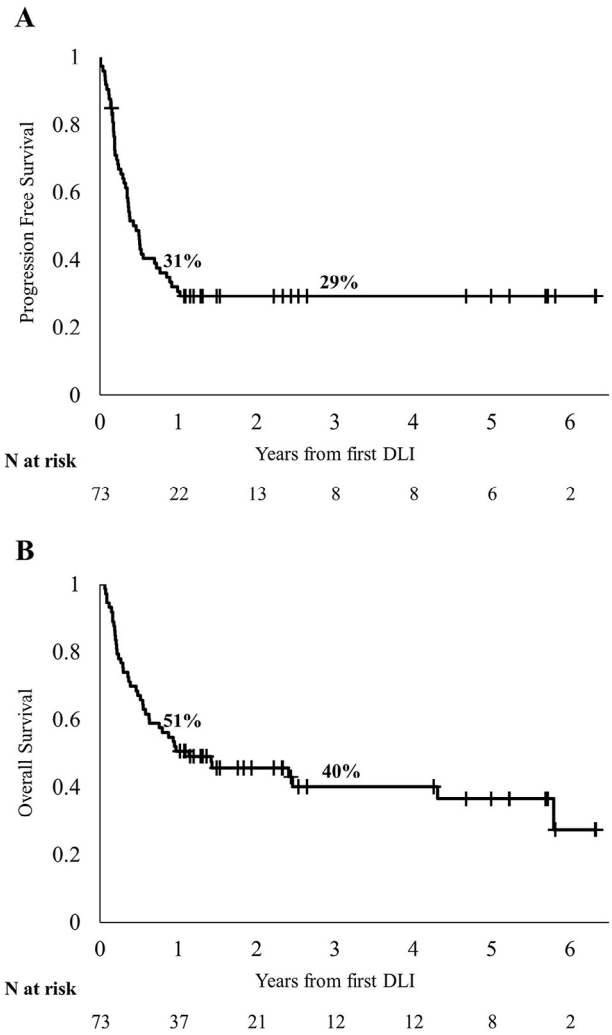


Figure 4. Clinical outcomes of patients. (A) PFS; (B) OS.

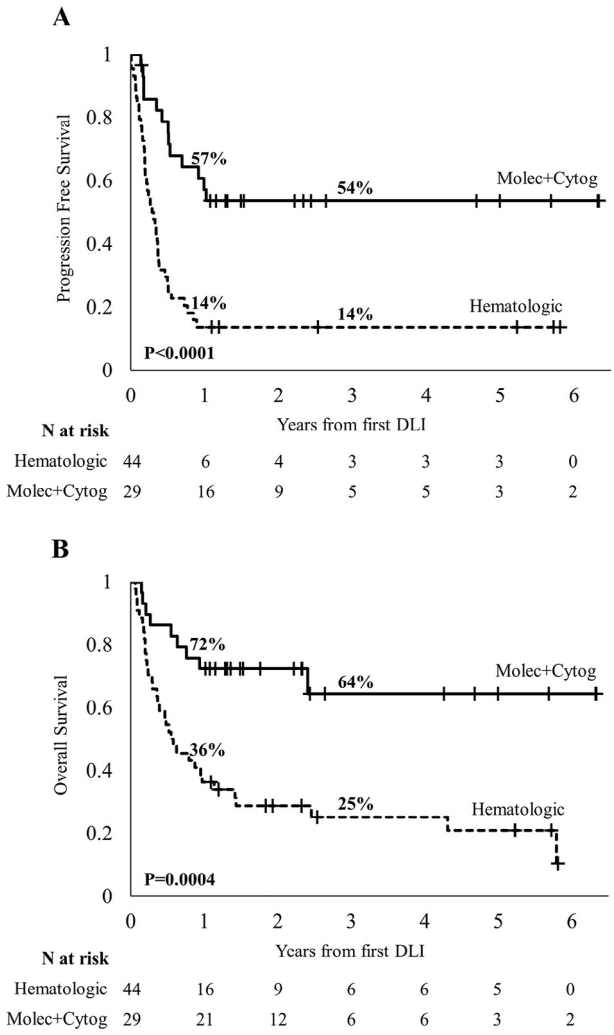


Figure 5. Clinical outcomes according to type of relapse. The outcome of patients with either hematologic (*dashed line*) or cytogenetic and molecular relapse (*continuous line*) are shown. (A) PFS; (B) OS.

patients, whereas it was 14% (95% CI, 6% to 25%) and 14% (95% CI, 6% to 25%) for the hematologic relapsed patients ($P < .0001$) (Figure 5A). OS was 72% (95% CI, 52% to 85%) and 64% (95% CI, 40% to 81%) at 1 and 3 years for the cytogenetically or molecularly relapsed patients, whereas it was 36% (95% CI, 23% to 50%) and 25% (95% CI, 13% to 39%) for the hematologic relapsed patients ($P = .0004$) (Figure 5B). Finally, DFS at 3 years for the 22 patients who had shown a response at 100 days after the end of the cell program was 75% (95% CI, 41% to 91%) for the cytogenetically or molecularly relapsed and 60% (95% CI, 25% to 83%) for patients with an overt hematologic relapse ($P = .41$) (data not shown). A subgroup analysis of 41 AML and 19 ALL patients showed that cytogenetic and molecular relapse were associated with a favorable outcome compared with overt hematologic relapse in both diseases: PFS at 3 years 50% (95% CI, 35% to 81%) versus 12% (95% CI, 10% to 42%; $P = .041$) in AML, and PFS at 2 years 60% (95% CI, 25% to 83%) versus 25% (95% CI, 4% to 56%; $P = .039$) in ALL, respectively (Figure 6A,B).

By univariate analysis we evaluated the relationship between age, sex, disease status at transplant, previous autologous transplant, type of conditioning regimen, type of donor, type of relapse, time from diagnosis to transplant, time from transplant to relapse, presence of aGVHD or cGVHD, and OS. The type of relapse (molecular or hematologic) and the presence of cGVHD were associated with improved survival: hazard ratio 3.49 (95% CI, 1.67 to 7.29), $P = .0009$, and hazard ratio 3.69 (95% CI, 1.10 to 12.41), $P = .0346$, respectively (Table 4). By multivariate analysis, relapse type, presence of cGVHD, and time from transplant to relapse remained independent significant predictors of survival (Table 5).

DISCUSSION

In this phase II A study we analyzed the safety and activity of sequential administration of donor-derived unmanipulated DLI and CIK cells in 73 patients with recurrent hematologic cancers after alloHSCT. Our results indicate that a small amount of donor lymphocyte DLI followed by donor CIK cells at the dosages used is a safe treatment schedule with a significant clinical activity in patients with low tumor burden. In particular, the only toxicity observed during or immediately after CIK cell administration was the insurgence of acute hemolytic anemia, whereas the incidence of severe aGVHD and cGVHD was remarkably low. These data therefore confirm and extend the observations that we and others have previously made in preliminary phase I clinical

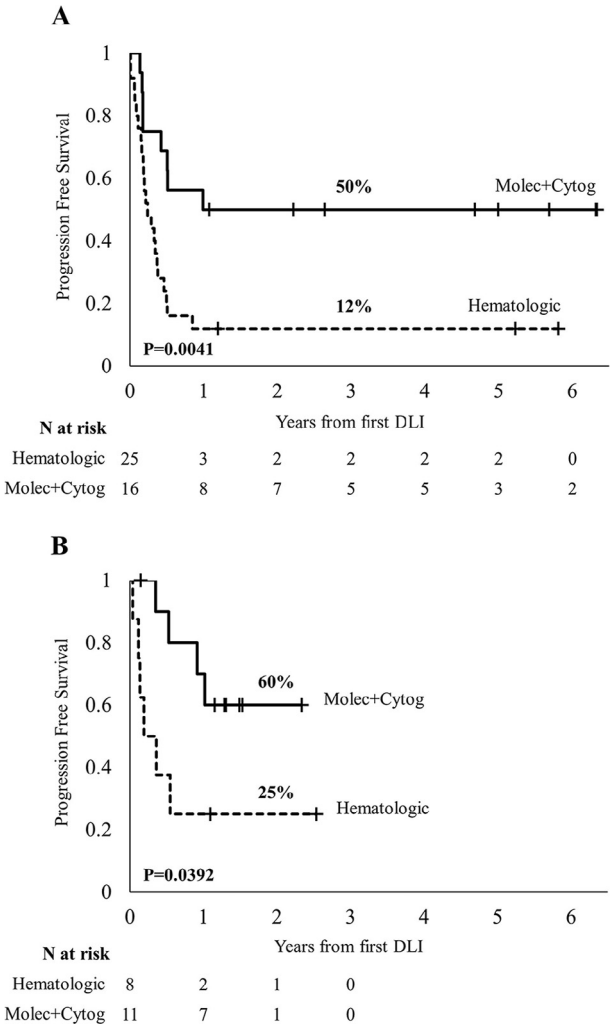


Figure 6. Clinical outcomes in AML and ALL patients according to type of relapse.
The outcome of patients with either hematologic (*dashed line*) or cytogenetic and molecular relapse (*continuous line*) are shown. (A) AML; (B) ALL.

studies and are furthermore in agreement with the observations made in several experimental models of the extremely reduced GVHD activity of CIK cells [16,17,20-24]. The limited incidence of severe GVHD represents the potential main advantage of this cellular therapy program compared with the use of standard DLI [5]. In a very recent retrospective study on 414 patients treated with DLI from matched unrelated donors, Miyamoto et al. [28] reported the achievement of a complete response in 57% of patients with cytogenetic/molecular relapse and in 20% patients with hematologic relapse. However, grades III to IV a GVHD was documented within 100 days post-unrelated DLI (UDLI) in 13% of the assessable patients, with a GVHD-related death occurring in 3% of cases.

Among the points of strength of this study are that it shows the feasibility of this cell therapy program in academic realities. In fact, 2 academic cell factories were involved that served 3 hematopoietic stem cell transplant units. Only in 1 case was the expansion of CIK cells insufficient (data not shown), resulting in only 2 CIK cell administrations instead of the programmed 3. In addition, we measured positive clinical activity results, particularly in the setting of the molecularly relapsed patients but also in some patients with a hematologic relapse, even in the subgroup of the 60 acute leukemias. In this regard, it is noteworthy that our cell therapy program showed a significant activity in a condition known to herald overt hematologic relapse, such as molecular relapse of ALL patients [29,30]. Moreover, no difference was observed between patients treated with cells from matched unrelated, sibling, or haploidentical donors in terms of incidence or severity of aGVHD and cGVHD. In addition, in most cases aGVHD developed after DLI infusion, and subsequent

Table 4
Risk Factors Associated with OS According to Univariate Analysis

Characteristics	HR (95% CI)	*P*
Age		
Pediatric	1.00	
Adult	1.86 (.78-4.43)	.1586
Sex		
Female	1.00	
Male	1.23 (.67-2.25)	.5038
Disease status at HSCT		
No CR	1.67 (.90-3.10)	.1061
CR	1.00	
Diagnosis		
ALL	1.00	
AML	1.47 (.68-3.16)	.3248
Previous HSCT		
No	1.30 (.62-2.74)	.4831
Yes	1.00	
Preparative regimen		
MAC	1.00	
RIC	1.11 (.57-2.14)	.7623
Stem cell source		
Peripheral blood	1.80 (.94-3.47)	.0775
Bone marrow	1.00	
Donor		
SIB	1.17 (.62-2.19)	.6329
MUD	1.00	
HLA haploidentical	1.54 (.45-5.33)	.4931
Relapse type		
Hematologic	3.49 (1.67-7.29)	.0009
Molecular + cytogenetic	1.00	
Months from diagnosis to HSCT	.99 (.97-1.01)	.4301
Months from HSCT to relapse		
≤6	1.66 (.91-3.03)	.0979
>6	1.00	
aGVHD*		
No	1.15 (.52-2.54)	.7249
Yes	1.00	
cGVHD*		
No	3.69 (1.10-12.41)	.0346
Yes	1.00	

HR indicates hazard ratio; CI, confidence interval.
* Time-dependent variables.

Table 5
Risk Factors Associated with OS According to Multivariate Analysis

	HR (95% CI)	*P*
Age		
Pediatric	1.00	
Adult	1.94 (.80-4.68)	.1404
Relapse type		
Hematologic	3.49 (1.66-7.35)	.0010
Molecular + cytogenetic	1.00	
Time from HSCT to relapse		
≤6 mo	1.80 (1.00-3.31)	.050
>6 mo	1.00	
cGVHD*		
No	4.51 (1.31-15.51)	.0169
Yes	1.00	

* Time-dependent variables.

administration of CIK cells did not induce further aGVHD. In this regard, a striking finding is that haploidentical CIK cells did not cause aGVHD also at higher doses (total 20×10^6 CIK cells/kg). This observation highlights a significant difference with respect to standard DLI treatment, in which a much higher incidence of GVHD is observed when matched unrelated versus sibling donors were used [31]. Moreover, even when more potent cytotoxic CIK cells, as produced by the addition of IL-15 [32], were tested in the haploidentical setting, very low aGVHD activity was observed [23,33]. Finally, we did not reach the dose-limiting toxicity, suggesting that higher CIK cell doses may actually be used, which would be technically possible in our cell factories, as shown by the yields of CIK cells in more than 200 consecutive batches (data not shown).

Nonetheless, we have to acknowledge that our study has some limitations. First, in the context of leukemia relapse, CIK cells have shown a limited activity, particularly in leukemias with a high proliferation index, as suggested by the significant correlation in multivariate analysis between survival and type of relapse and the time from transplant to relapse. For this group of patients it is now clear that a cytoreductive treatment should be given before the cell therapy program, with the aim to control the leukemic outgrowth and perhaps to induce in vivo lymphodepletion that may facilitate CIK cell engraftment as previously reported for several adoptive immunotherapy protocols [34]. Second, the protocol adopted in this study does not allow the defining of the relative activity of the CIK cells versus the unmanipulated DLI administered to the patients. DLI administration was, on the other hand, necessary for practical reasons, because 3 weeks are needed for CIK cell production and an additional 2 weeks for quality controls. To avoid a rapid progression of the relapsed hematologic malignancy, we reasoned that donor immunity should be granted immediately, while waiting for the CIK cells administration. On the other hand, we believe that the total amount of unmanipulated DLI infused (2×10^6/kg) is unlikely to explain most of the clinical effects so far observed. Third, a formal evaluation of disease status after cytoreductive therapy in patients with rapidly progressive disease was not performed, as well as after the DLI infusions. However, in this context it is unlikely that a limited use of cytoreductive treatment may have affected the long-term results. Fourth, in spite of the feasibility, the need to expand CIK cells in GMP conditions adds a significant burden in terms of costs and management complexity. Finally, we have not studied the in vivo kinetics of the administered cells, a particularly challenging task in an HLA-identical context, as were most patients included in the study. In addition, no data are available in the literature on the in vivo survival of these terminallly differentiated Effector Memory T cells re-expressing CD45 RA [35,36].

In conclusion, we believe this strategy may benefit from further improvements, such as the administration of higher doses of cells (given also at shorter intervals between each infusion), as well as the introduction of lymphoid-depleting regimens immediately before cell therapy. Similarly, the strategy may benefit from a pre-emptive administration of the cells in selected categories of patients at high risk of relapse [37,38]. Furthermore, CIK cells may represent an ideal platform to genetically modify the cells by chimeric antigen receptor (CART) transduction or by "arming" the cells by ex vivo or in vivo co-administration with bispecific antibodies, as already published by our group [39-41]. Indeed, CIK cells are mostly CD8 positive T memory lymphocytes, and this may represent an advantage with respect to unselected CART transduced cells populations used so far, which contain significant amounts of CD4 T cells, an element that may maximally contribute to the dramatic cytokine storms observed in vivo [36]. Finally, we emphasize that CIK cells have been used in very many oncologic clinical studies as documented in international registries [42], thus suggesting a possible generalized use of this new form of adoptive immunotherapy in cancer patients.

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Authorship statement: M.I. and F.L. contributed equally to this work. M.I. (Qualified Person) contributed to the planning of the trial, writing of the IMPD for approval, followed the database of the patients in GCP, and wrote the manuscript and takes the full responsibility for the CIK cell preparation and batch release. F.L. followed patients and contributed to control all the case report forms (CRF) produced and wrote the manuscript. A.A., C.M., A.G., E.T., I.C., S.D., E.B., A.B., A.R., M.P., S.N., followed patients. E.G. was in charge of the CIK preparations. R.V. was responsible for quality controls. Chiara Pavoni is a statistician in the Hematology at ASST Papa Giovanni XXIII, Bergamo and has analyzed all the data. M.L.F. updated the CRF of all patients for the database and kept full control of the database of the study. F.D. participated in data entry of all clinical data of the database and created the software. E.T. was part of the medical team who has followed patients in the Hematology at ASST Papa Giovanni XXIII, Bergamo. G.S. and E.M. managed data. P.P. was responsible for apheresis. D.B. was responsible for laboratory production. B.C. was responsible for quality controls. G.G. (Qualified Persons) takes full responsibility for the CIK cells preparation and batch release. J.G. was responsible for quality assurance and quality controls and contributed to the writing of and critically revising the manuscript. A.B. participated in the discussion of the clinical protocol. A.R. participated in the original planning of the study, preparation of the clinical protocol, and writing of the manuscript.

REFERENCES

1. Kongtim P, Lee DA, Cooper LJ, Kebriaei P, Champlin RE, Ciurea SO. Haploidentical hematopoietic stem cell transplantation as a platform for post-transplantation cellular therapy. *Biol Blood Marrow Transplant*. 2015;21:1714-1720.
2. Nikiforow S, Alyea EP. Maximizing GVL in allogeneic transplantation: role of donor lymphocyte infusions. *Hematol Am Soc Hematol Educ Progr*. 2014;2014:570-575.
3. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med*. 1980;69:204-217.
4. van den Brink MR, Porter DL, Giralt S, et al. Relapse after allogeneic hematopoietic cell therapy. *Biol Blood Marrow Transplant*. 2010;16(1 suppl):S138-S145.
5. Orti G, Barba P, Fox L, Salamero O, Bosch F, Valcarcel D. Donor lymphocyte infusions in AML and MDS: enhancing the graft-versus-leukemia effect. *Exp Hematol*. 2017;48:1-11.
6. Schmidt-Wolf IG, Negrin RS, Kiem HP, Blume KG, Weissman IL. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *J Exp Med*. 1991;174:139-149.

7. Introna M, Golay J, Rambaldi A. Cytokine induced killer (CIK) cells for the treatment of haematological neoplasms. *Immunol Lett*. 2013;155:27-30.
8. Pievani A, Borleri G, Pende D, et al. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood*. 2011;118:3301-3310.
9. Pfirrmann V, Oelsner S, Rettinger E, et al. Cytomegalovirus-specific cytokine-induced killer cells: concurrent targeting of leukemia and cytomegalovirus. *Cytotherapy*. 2015;17:1139-1151.
10. Valgardsdottir R, Capitanio C, Texido G, et al. Direct involvement of CD56 in cytokine-induced killer-mediated lysis of CD56+ hematopoietic target cells. *Exp Hematol*. 2014;42:1013-1021, e1.
11. Edinger M, Cao YA, Verneris MR, Bachmann MH, Contag CH, Negrin RS. Revealing lymphoma growth and the efficacy of immune cell therapies using in vivo bioluminescence imaging. *Blood*. 2003;101:640-648.
12. Hoyle C, Bangs CD, Chang P, Kamel O, Mehta B, Negrin RS. Expansion of Philadelphia chromosome-negative CD3(+)CD56(+) cytotoxic cells from chronic myeloid leukemia patients: in vitro and in vivo efficacy in severe combined immunodeficiency disease mice. *Blood*. 1998;92:3318-3327.
13. Lu PH, Negrin RS. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J Immunol*. 1994;153:1687-1696.
14. Sweeney TJ, Mailander V, Tucker AA, et al. Visualizing the kinetics of tumor-cell clearance in living animals. *Proc Natl Acad Sci USA*. 1999;96:12044-12049.
15. Thorne SH, Negrin RS, Contag CH. Synergistic antitumor effects of immune cell-viral biotherapy. *Science*. 2006;311:1780-1784.
16. Verneris MR, Ito M, Baker J, Arshi A, Negrin RS, Shizuru JA. Engineering hematopoietic grafts: purified allogeneic hematopoietic stem cells plus expanded CD8+ NK-T cells in the treatment of lymphoma. *Biol Blood Marrow Transplant*. 2001;7:532-542.
17. Nishimura R, Baker J, Beilhack A, et al. In vivo trafficking and survival of cytokine-induced killer cells resulting in minimal GVHD with retention of antitumor activity. *Blood*. 2008;112:2563-2574.
18. Sangiolo D, Martinuzzi E, Todorovic M, et al. Alloreactivity and anti-tumor activity segregate within two distinct subsets of cytokine-induced killer (CIK) cells: implications for their infusion across major HLA barriers. *Int Immunol*. 2008;20:841-848.
19. Rettinger E, Kreyenberg H, Merker M, et al. Immunomagnetic selection or irradiation eliminates alloreactive cells but also reduces anti-tumor potential of cytokine-induced killer cells: implications for unmanipulated cytokine-induced killer cell infusion. *Cytotherapy*. 2014;16:835-844.
20. Introna M, Borleri G, Conti E, et al. Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study. *Haematologica*. 2007;92:952-959.
21. Laport GG, Sheehan K, Baker J, et al. Adoptive immunotherapy with cytokine-induced killer cells for patients with relapsed hematologic malignancies after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:1679-1687.
22. Martin PJ. CIK: a path to GVL without GVHD? *Biol Blood Marrow Transplant*. 2011;17:1571-1572.
23. Rettinger E, Bonig H, Wehner S, et al. Feasibility of IL-15-activated cytokine-induced killer cell infusions after haploidentical stem cell transplantation. *Bone Marrow Transplant*. 2013;48:1141-1143.
24. Zhou X, Zhu J, Sun H, Shao L, Xu M, Guo H. Family haploidentical donor-derived cytokine-induced killer cell biotherapy combined with bortezomib in two patients with relapsed multiple myeloma in a non-allogeneic transplant setting. *Leuk Lymphoma*. 2013;54:209-211.
25. Gaipa G, Introna M, Golay J, et al. Development of advanced therapies in Italy: management models and sustainability in six Italian cell factories. *Cytotherapy*. 2016;18:481-486.
26. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation*. 1974;18:295-304.
27. Mantel N, Byar DP. Evaluation of response-time data involving transient states: an illustration using heart-transplant data. *J Am Stat Assoc*. 1974;69:81-86.
28. Miyamoto T, Fukuda T, Nakashima M, et al. Donor lymphocyte infusion for relapsed hematological malignancies after unrelated allogeneic bone marrow transplantation facilitated by the Japan marrow donor program. *Biol Blood Marrow Transplant*. 2017;23:938-944.
29. Bassan R, Hoelzer D. Modern therapy of acute lymphoblastic leukemia. *J Clin Oncol*. 2011;29:532-543.
30. Hoelzer D, Bassan R, Dombret H, et al. Acute lymphoblastic leukaemia in adult patients: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2016;27(suppl 5):v69-v82.
31. Chang YJ, Huang XJ. Donor lymphocyte infusions for relapse after allogeneic transplantation: when, if and for whom? *Blood Rev*. 2013;27:55-62.
32. Rettinger E, Kuci S, Naumann I, et al. The cytotoxic potential of interleukin-15-stimulated cytokine-induced killer cells against leukemia cells. *Cytotherapy*. 2012;14:91-103.
33. Rettinger E, Huenecke S, Bonig H, et al. Interleukin-15-activated cytokine-induced killer cells may sustain remission in leukemia patients after allogeneic stem cell transplantation: feasibility, safety and first insights on efficacy. *Haematologica*. 2016;101:e153-e156.
34. Wrzesinski C, Paulos CM, Kaiser A, et al. Increased intensity lymphodepletion enhances tumor treatment efficacy of adoptively transferred tumor-specific T cells. *J Immunother*. 2010;33:1-7.
35. Franceschetti M, Pievani A, Borleri G, et al. Cytokine-induced killer cells are terminally differentiated activated CD8 cytotoxic T-EMRA lymphocytes. *Exp Hematol*. 2009;37:616-628.
36. Rambaldi A, Biagi E, Bonini C, Biondi A, Introna M. Cell-based strategies to manage leukemia relapse: efficacy and feasibility of immunotherapy approaches. *Leukemia*. 2015;29:1-10.
37. Narayan R, Benjamin J, Laport G, et al. Donor-derived CIK cell infusion as consolidative therapy after non-myeloablative allogeneic transplant in patients with myeloid neoplasms. *Blood*. 2015;126:3232.
38. Todisco E, Ciceri F, Oldani E, et al. The CIBMTR score predicts survival of AML patients undergoing allogeneic transplantation with active disease after a myeloablative or reduced intensity conditioning: a retrospective analysis of the Gruppo Italiano Trapianto Di Midollo Osseo. *Leukemia*. 2013;27:2086-2091.
39. Golay J, D'Amico A, Borleri G, et al. A novel method using blinatumomab for efficient, clinical-grade expansion of polyclonal T cells for adoptive immunotherapy. *J Immunol*. 2014;193:4739-4747.
40. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. *Leukemia*. 2014;28:1596-1605.
41. Tettamanti S, Marin V, Pizzitola I, et al. Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. *Br J Haematol*. 2013;161:389-401.
42. Schmeel LC, Schmeel FC, Coch C, Schmidt-Wolf IG. Cytokine-induced killer (CIK) cells in cancer immunotherapy: report of the international registry on CIK cells (IRCC). *J Cancer Res Clin Oncol*. 2015;141:839-849.