Clinical Cancer Research

Larger Size of Donor Alloreactive NK Cell Repertoire Correlates with Better Response to NK Cell Immunotherapy in Elderly Acute Myeloid Leukemia Patients

Antonio Curti[1], Loredana Ruggeri[2], Sarah Parisi[1], Andrea Bontadini[3], Elisa Dan[1], Maria Rosa Motta[1], Simonetta Rizzi[1], Sara Trabanelli[1], Darina Ocadlikova, Mariangela Lecciso[1], Valeria Giudice[3], Fiorenza Fruet[3], Elena Urbani[2], Cristina Papayannidis[1], Giovanni Martinelli[1], Giuseppe Bandini[1], Francesca Bonifazi[1], Russell E. Lewis[4], Michele Cavo[1], Andrea Velardi[2], and Roberto M. Lemoli[5]

Abstract

Purpose: In acute myeloid leukemia (AML), alloreactive natural killer (NK) cells are crucial mediators of immune responses after haploidentical stem cell transplantation. Allogeneic NK cell infusions have been adoptively transferred with promising clinical results. We aimed at determining whether the composition of NK graft in terms of frequency of alloreactive NK cells influence the clinical response in a group of elderly AML patients undergoing NK immunotherapy.

Experimental Design: Seventeen AML patients, in first complete remission (CR; median age 64 years, range 53–73) received NK cells from haploidentical KIR-ligand–mismatched donors after fludarabine/cyclophosphamide chemotherapy, followed by IL2. To correlate donor NK cell activity with clinical response, donor NK cells were assessed before and after infusion.

Results: Toxicity was moderate, although 1 patient died due to bacterial pneumonia and was censored for clinical follow-up. With a median follow-up of 22.5 months (range, 6–68 months), 9 of 16 evaluable patients (0.56) are alive disease-free, whereas 7 of 16 (0.44) relapsed with a median time to relapse of 9 months (range, 3–51 months). All patients treated with molecular disease achieved molecular CR. A significantly higher number of donor alloreactive NK cell clones was observed in responders over nonresponders. The infusion of higher number of alloreactive NK cells was associated with prolonged disease-free survival (0.81 vs. 0.14, respectively; $P = 0.03$).

Conclusions: Infusion of purified NK cells is feasible in elderly AML patients as post-CR consolidation strategy. The clinical efficacy of adoptively transferred haploidentical NK cells may be improved by infusing high numbers of alloreactive NK cells. *Clin Cancer Res; 22(8); 1914–21. ©2016 AACR.*

See related commentary by Muntasell and López-Botet, p. 1831

Introduction

Treatment of acute myeloid leukemia (AML) in adult patients is based on intensive chemotherapy, followed by allogeneic stem cell transplantation (SCT) in selected cases. Complete remission (CR) rates following chemotherapy in this patient population range from 60% to 85% in patients younger than 60 years, although 5 year-overall survival (OS) rates average 40%, mainly still due to high rate of subsequent relapse. In elderly patients, OS falls to 10%, due to the higher prevalence of high-risk biologic factors, such as poor prognosis cytogenetics and underlying comorbidities (1).

Many advances in the use of broad-spectrum chemotherapeutic regimens and targeted therapy have improved the CR rate in elderly AML patients (2). However, a significant number of patients who achieve CR after induction/consolidation chemotherapy still harbor a minimal residual disease (MRD), which is often resistant to further chemotherapeutic treatments and eventually leads to relapse and disease progression. These data indicate the importance of preventing relapse by addressing MRD with novel strategies other than chemotherapy. Among these, immunologic therapies, which have mechanism(s) of action different than cytotoxic drugs, are a promising strategy with the potential to significantly impact on MRD eradication.

Natural killer (NK) cells, which are defined by CD56 or CD16 expression and absence of CD3 (3), have been shown

[1]Department of Experimental, Diagnostic and Specialty Medicine, Institute of Hematology "L. and A. Seràgnoli", University of Bologna, S.Orsola-Malpighi Hospital, Bologna, Italy. [2]Division of Hematology and Clinical Immunology, Department of Medicine, University of Perugia, Ospedale Santa Maria della Misericordia, Perugia, Italy. [3]Immunohematology Service and Blood Bank, S.Orsola-Malpighi Hospital, Bologna, Italy. [4]Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy. [5]Clinic of Hematology, Department of Internal Medicine (DiMI), University of Genoa, IRCCS Azienda Ospedaliera Universitaria S. Martino-IST, Genoa, Italy.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding author: Antonio Curti, Department of Experimental, Diagnostic and Specialty Medicine, Institute of Hematology "L. and A. Seràgnoli", University of Bologna, Via Massarenti, 9, 40138, Bologna, Italy. Phone: 3905-1636-4074; Fax: 3905-1636-4037; E-mail: antonio.curti2@unibo.it

doi: 10.1158/1078-0432.CCR-15-1604

AACR

Translational Relevance

In acute myeloid leukemia (AML), alloreactive natural killer (NK) cells are crucial mediators of immune responses after haploidentical stem cell transplantation. Allogeneic NK cell infusions have been adoptively transferred with promising clinical results. The current study extends our previous investigation by reporting biologic and clinical results of NK cell infusion from haploidentical KIR-L–mismatched donors in elderly AML patients, who had achieved CR after induction/consolidation chemotherapy. Our results indicate that the size of alloreactive donor NK cell repertoire is correlated with a reduced relapse rate after NK cell immunotherapy.

to play a role in immune control of tumor development and growth (4). NK cells express activating and, more importantly, inhibitory receptors which recognize MHC class I alleles, termed "Killer cell Immunoglobulin-like Receptors" (KIRs) (5–7). In the transplantation setting, preclinical and clinical data from haploidentical T-cell–depleted SCT have demonstrated that alloreactive KIR-Ligand (L)–mismatched NK cells play a major role as antileukemia effector cells and protect AML patients against leukemia relapse (8–12). The data from allogeneic unrelated T-cell–replete SCT are more controversial and important differences among the studies have been reported with regard to the impact of KIR-L mismatching on clinical outcome (13–18). Outside the transplant setting, Miller and colleagues. first reported the safety and feasibility of adoptive immunotherapy with haploidentical NK cells in cancer patients, including AML (19). In particular, 19 poor risk AML patients with active disease received enriched NK cells. Five of 19 patients achieved CR after NK cell immunotherapy, which was well tolerated by patients. Rubnitz and colleagues reported their experience with haploidentical NK cell infusion in a cohort of 10 childhood AML patients. In this pediatric cohort, the 2-year event-free survival was 100% (20). Our group has published the results of a similar phase I study of adoptive immunotherapy with highly purified from KIR-L–mismatched, haploidentical donors in adult high-risk AML patients, mostly with relapsed or resistant disease (21). No NK cell–related toxicity, including graft-versus-host disease (GVHD), was observed. Importantly, donor-versus-recipient alloreactive NK cells were demonstrated *in vivo* by the detection of donor-derived NK clones and adoptively transferred NK cells were alloreactive against recipient's leukemic cells (15). The current study extends our previous investigation by reporting biologic and clinical results of NK cell infusion from haploidentical KIR-L–mismatched donors in elderly AML patients, who had achieved CR after induction/consolidation chemotherapy. Our results indicate that the size of alloreactive donor NK cell repertoire is correlated with reduced relapse rate after NK cell immunotherapy.

Patients and Methods

This prospective clinical trial was approved by the local Ethics Committee. Written informed consent was obtained before donor and patient enrollment. The trial was registered at www.clinicaltrial.gov. (NCT00799799).

Inclusion and exclusion criteria

The trial was first designed for refractory/resistant patients as a phase I feasibility/safety study 21) and subsequently amended to include patients in first CR. In particular, adult (age $\geq$ 18 years) patients with AML in morphologic or better CR after induction/consolidation chemotherapy, who were not eligible for SCT and had an haploidentical KIR-L–mismatched donor, were included in the study. Donors and patients with active and uncontrolled infections, abnormal renal (serum creatinine > 2 mg/dL), pulmonary (Sat $O_2 \leq 96\%$), and hepatic (ALT/AST > 2.5 x N) function and poor Karnofsky performance score (<70) were excluded. Seventeen patients who had achieved CR after two or three courses of chemotherapy were enrolled.

Donor selection and NK cell purification

Relatives were screened in the search for one haploidentical KIR-L–mismatched donor. HLA typing of recipient and donor(s) tested class I alleles belonging to the three class I groups recognized by KIRs (HLA-C group 1, HLA-C group 2, and HLA-Bw4 alleles) was performed as described previously (21). Briefly, HLA typing of recipient and donor(s) tested class I alleles belonging to the three class I groups recognized by KIRs (HLA-C group 1, HLA-C group 2, and HLA-Bw4 alleles). In NK alloreactive donors, HLA-C and HLA-B typing showed KIR ligand mismatches in the graft-versus-host (GVH) direction, that is, the recipient did not possess either one HLA-C allele group (C1 or C2) or the HLA-Bw4 group which were present in the donor, or both. The presence of inhibitory *KIR* gene and functional protein was confirmed by molecular genotyping, and FACS and functional analyses were performed (see below).

KIR-L mismatch of infused patients is reported in Table 1. Donors underwent mononuclear cell collection, which was then processed by NK-cell separation CliniMACS system (CliniMACS, Miltenyi Biotec). A cell product sample was evaluated by flow cytometry to count T, B, NK cells, and monocytes before and after CliniMACS selection. Cytotoxicity was tested against NK-sensitive K562 cells. Nonmanipulated highly purified $CD56^{+}CD3^{-}$ NK cells were cryopreserved.

Treatment

To favor haploidentical NK cell engraftment, all patients received immunosuppressive chemotherapy, [fludarabine 25 mg/mq/ from day 7 to 3 and cyclophosphamide 4 g/mq on day 2 (fludarabine/cyclophosphamide)]. Two days after cyclophosphamide administration, patients received the NK cell infusion (day 0), which was followed by subcutaneous administration of IL2 (10×10^6 IU/day, 3 times weekly; Novartis) for 2 weeks (6 doses total). No GVHD prophylaxis was used. Following our institutional guidelines for AML, granulocyte colony-stimulating factor (G-CSF, filgrastim) was not routinely used, albeit in case of neutropenic infections accordingly to clinical decision. We considered those patients as responders who maintained CR for at least 6 months after NK cell infusion.

End points

Primary endpoints were: donor/patient safety; the feasibility of the selection and reinfusion of 5×10^6 haploidentical NK cells/kg of body weight (target cell dose) in at least 40% of adult patients with AML entering the study, and the feasibility of the reinfusion of the minimum accepted cell dose (1×10^6 haploidentical NK cells/Kg) in all patients enrolled into the protocol. Secondary

Table 1. Demographic, hematologic, and graft features of enrolled AML patients

UPN	Age	Sex	WBC (10^9/L)	Karyotype	Genotype	FAB	HLA Typing (mismatch)	DonorKIR-L (mismatch)	Disease status at NK infusion	Infused NK Cells ($\times 10^6$/K)
1	63	M	7.3	complex	CBFMYH11	M4	-Bw4	3DL1	CR	3.10
2	72	F	1.17	+4 +8	FLT3 WT	M1	-C group 2	2DL1	CR	4.14
3	70	F	58.6	46 XX	FLT3 WT	M5	-Bw4	3DL1	CR	3.28
4	72	M	3.0	46 XY	FLT3 WT NPM WT	NE	-C group 1	2DL2/3	CR	5.53
5	68	F	59.0	46 XX	FLT3 ITD NPM WT	NE	-C group 1	2DL2	CR	5.00
6	59	F	4.32	46 XX	FLT3 WT NPM WT	M1	-C group 2	2DL1	CR	4.00
7	73	M	75.0	46 XY	FLT3 TKD	M5	-C group 1-Bw4	2DL2/3 3DL1	CR	4.75
8	58	M	74.8	46 XY	FLT3 WT NPM WT	M4	-Bw4	3DL1	CR	1.81
9	64	M	25.0	46 XY	FLT3 WT NPM WT	M1	-C group 1	2DL2/3	CR	2.05
10	53	F	4.1	46 XX +8 -7	NE	M1	-C group 1-Bw4	2DL2	Molecular Relapse	3.89
11	67	M	2.7	NE	FLT3 WT NPM WT	M0	-C group 1	2DL2	CR	2.74
12	61	M	2.9	46 XX	FLT3 WT NPM WT	sec	-C group 1	2DL2/3	CR	2.,51
13	58	F	5.8	inv(16)	CBFMYH11	NE	-C group 2	2DL1	Molecular relapse	1.29
14	61	M	2.5	del(12)	FLT3 WT NPM WT	sec	-C group 2	2DL1	CR	5.00
15	62	F	1.27	t(11)	FLT3 WT NPM WT	M1	-C group 1	2DL2	CR	5.1
16	64	F	27.4	inv(16)	CBFMYH11	M4	-C group 2	2DL1	MRD+	5.00
17	65	M	189.5	46 XY	FLT3 WT NPM MUT	M0	-C group 2	2DL1	CR	5.00

Abbreviations: CBF, core binding factor; CR, complete remission; FAB, French-American-British; FLT3, FMS-like tyrosine kinase 3; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; NE, not evaluated; NK, natural killer; NPM, nucleophosmin; Pt, patient; TKD, tyrosinekinase domain; WBC, white blood count; WT, wild type.

endpoints included disease-free survival (DFS) of AML patients infused with haploidentical NK cells and, biologically, the correlation between donor alloreactive NK repertoires and DFS.

NK cell KIR phenotyping, chimerism, cell cloning, and functional assays

NK cell KIR phenotyping, chimerism, cell cloning, and functional assays were performed on days 3, 9, 12, 18, and 20 after NK cell infusion, as described previously (22). Donor and patient alloreactive NK cell repertoires were assessed after generating large numbers of NK clones by limiting dilution (21). Briefly, donor alloreactive NK cell repertoires were assessed once at the time of leukapheresis. Patient NK cell repertoires were assessed at days +3, +9, +12, +18, and +20 after NK cell infusion. Large numbers of NK clones were generated by limiting dilution from donors and patients after infusions and tested in cytotoxicity assays against K562 to select functional clones and then, against patient cryopreserved PHA blasts. As negative control, they were tested against autologous donor PHA blasts. In one case, we also tested donor and postinfusion NK cell clones against the patient's leukemia cells.

Peripheral blood mononuclear cells (PBMC) depleted of T cells by negative anti-CD3 immunomagnetic selection (Miltenyi Biotec) were plated under limiting-dilution conditions, activated with phytohemagglutinin (PHA; Biochrom KG), and cultured with IL2 (Chiron BV) and irradiated feeder cells. Feeder cells were obtained by pooling buffy coats from 5 to 9 healthy donors. Cloning efficiencies ranged from 1 in 5 to 1 in 10 plated NK cells. Clones were assessed against K562 to assess function. Approximately 100 NK clones that killed K562 from each patient and donor were screened for alloreactivity by standard ^{51}Cr-release cytotoxicity at an effector-to-target ratio of 10:1 against patient KIR ligand–mismatched PHA lymphoblasts, autologous donor PHA blasts, and leukemic cells from one recipient. Clones exhibiting greater than 30% lysis were scored as alloreactive. Peripheral blood and bone marrow chimerism was determined by variable number tandem repeat (VNTR) assay (23).

Statistical considerations

This is a prospective, monocentric, phase I study. To expose the minimum number of patients to the risk of an ineffective therapy, we planned a two-step study according to Gehan. We define the minimum desired efficacy (MDE) as the lowest proportion of successes under which the treatment is stopped. Success was defined as the infusion of the target cell dose of haploidentical NK cells (i.e., 5×10^6/kg). The MDE is 40%, with α: 0.05 two sided and 1-β: 80%. Time of neutrophil engraftment was considered the first of 3 successive days with an absolute neutrophil count (ANC) $\geq$ 500/μL post-NK infusion nadir. Time of platelet engraftment was considered the first of 3 consecutive days with platelet count $\geq$ 20,000/μL, in the absence of platelet transfusions. OS and DFS were estimated using the Kaplan–Meier method followed by the Mantel–Cox log-rank test. OS was defined as time from infusion to death resulting from any cause, and surviving patients were censored at the last follow-up. DFS from infusion was calculated using death and disease relapse/progression as events. Mean $\pm$ SD of donor NK cell alloreactive clones was analyzed by Student *t* test. Comparisons of donor NK profiles between pre- and postinfusion samples were made using a Wilcoxon signed rank test. The relationship of number of infused NK cells and donor alloreactive NK cell clone number and relapse status was examined using logistic regression. Optimal NK cell criterion cut-off values were defined using the Youden Index. Two-sided statistical tests were used for all reported *P* values. The results are presented as median values and ranges or, when indicated, as mean $\pm$ SD. All analysis was performed using Stata version 13.1 (StataCorp) and MedCalc Statistical Software version 13.3 (MedCalc Software).

Results

Patients

Clinical characteristics of study patients at diagnosis are reported in Table 1. The median age was 64 (range 53–73). In addition to older age, 5 patients had a leukocyte counts over 30 $\times$ 10^9/L; 3 showed leukemic evolution from previous

myelodysplastic syndrome, 2 had a favorable karyotype, but showed persistent or relapsing molecular disease, 2 exhibited high-risk cytogenetics with the remaining 13 exhibiting intermediate-risk cytogenetics. As for the molecular profile, the majority of patients were nucleophosmin 1(*NPM1*) and Fms-like tyrosine kinase 3 (*FLT-3*) wild-type and 2 patients harbored *NPM1*$^-$*FLT3-ITD*$^+$ leukemia. One patient had *NPM1*$^+$*FLT3*$^-$ leukemia, but concomitantly showed high-risk hyperleucocytosis at diagnosis. At the time of NK cell infusion, all patients were in morphologic CR. Three of them showed MRD positivity, as evaluated by increasing levels of leukemia-associated (Wilms' tumor 1 gene, *WT1*) and leukemia-specific (*CBF-MYH11*) transcripts.

Toxicity

After administration of fludarabine/cyclophosphamide chemotherapy and infusion of highly purified NK cells, hematopoietic cell recovery was similar to what is observed after a standard chemotherapy cycle (Supplementary Fig. S1). In particular, median times to ANC >0.5 $\times$ 10^9/L and to >20 $\times$ 10^9 plt/L were 20.1 (range 16.1–24.0) and 21.6 (range 18.1–25.2) days, respectively. Ten cases of fever were documented; most were of unknown origin (FUO). Four patients developed bacterial infections, which were complicated by pneumonia ($n = 2$) and sepsis ($n = 2$). One patient died during the neutropenic phase due to *Escherichia coli* pneumonia, while the remaining patients recovered successfully. No cases of fungal infections, including pulmonary aspergillosis, occurred. To shorten the neutropenic phase, 4 patients with infections received filgrastim for a mean of 10 days (range 4–21). No clinical and/or laboratory signs of GVHD were observed. All patients received all IL2 injections as scheduled. In some cases, local side effects, such as mild erythema, were observed at the injection site.

NK cell purification

A median of 19.56 $\times$ 10^6 CD56$^+$CD3$^-$ cells/kg (range 3.8–42.5) was collected from peripheral blood in one single collection. The total number of collected CD3$^+$ T cells was 1,387.4 $\times$ 10^5/kg (range 580.2–2167.9). Immunomagnetic selection provided a median of 9.83 $\times$ 10^6 CD3$^-$CD56$^+$ cells/kg (range 2.48–18.61) with a median purity of 95.8% (range 79.7–99.2) and a median recovery of 54.41% (range 31.82–65.4) for infusion (Table 1). Positive NK cell selection resulted in a median of 3.11 log T-cell depletion (range 2.15–6.0). After the whole procedure, the number of CD3$^+$ T cells in the cellular product was 2.32 $\times$ 10^5/kg (range 0.2–12.1). The median number of infused NK cells/kg was 4.0 $\times$ 10^6 (range 1.29–5.53) and that of infused CD3$^+$ T cells was 0.65 $\times$ 10^5/kg (range 0.1–3.1). NK cell viability after purification was 95% (range 92–98). Purified NK cells killed NK-sensitive K562 cells in a flow cytometry–based cytotoxicity assay (data not shown). No significant difference was observed on the capacity of NK cells to kill K562 cells before and after thawing (data not shown).

Clinical outcome

Seventeen patients were infused with NK cells. The patient who died during aplasia was considered not evaluable for the assessment of clinical response to NK therapy. With a median follow-up of 22.5 months (range, 6–68 months), 9 of 16 (0.56) are disease-free, whereas 7 of 16 (0.44) relapsed with a median time to relapse of 9 months (range, 3–51 months). Interestingly, 2 of the relapsed patients, showed a very prolonged CR phase of 24 and 51 months, respectively, in absence of any concomitant antileukemia treatments. The patient who relapsed 51 months after NK cell infusion underwent a second NK cell infusion after CR reinduction. Again, with a follow-up of 6 months, the patient maintained CR. According to response criteria (see above), 11 of 16 (69%) patients were responders, whereas 5 of 16 (31%) were nonresponders. The 3 MRD$^+$ patients at the time of NK infusion (2 with molecular relapse, 1 with persistent MRD$^+$ leukemia after induction/consolidation chemotherapy) achieved molecular CR, which lasted 9 and 4 months in 2 cases and 8+ months in the third case.

Correlation between clinical outcome and donor NK cell repertoire

We previously reported that after NK cell infusion donor-derived cells may be detected in peripheral blood and, in some cases, in the bone marrow of AML patients with active disease (21). Similar results were obtained in the current group of patients, who underwent NK cell infusion after achieving CR. In particular, of 17 evaluable patients, donor NK cells were detected in peripheral blood and bone marrow of 11 and 6 patients, respectively. Donor and donor-derived NK cells were alloreactive against recipient's cells, including PHA blast and, in one case, leukemic blasts. As previously reported for patients with active disease 21), alloreactive NK cell clones were detected, albeit at different levels, in all patients who were treated in CR. Flow cytometry analyses of 2 representative alloreactive NK cell clones are shown in Supplementary Fig. S2.

To determine whether donor NK alloreactivity is associated with better clinical outcome and response, donor NK cell clones were assessed in donor peripheral blood and after NK cell infusion. Chimerism analyses performed in randomly selected alloreactive NK clones demonstrated they were of donor origin (21). More alloreactive NK cell clones were found in the donor repertoire of responders than in nonresponders (Supplementary Table S1) and, as expected on day 3 after NK cell infusion, more alloreactive NK cell clones were found in responders (Fig. 1).

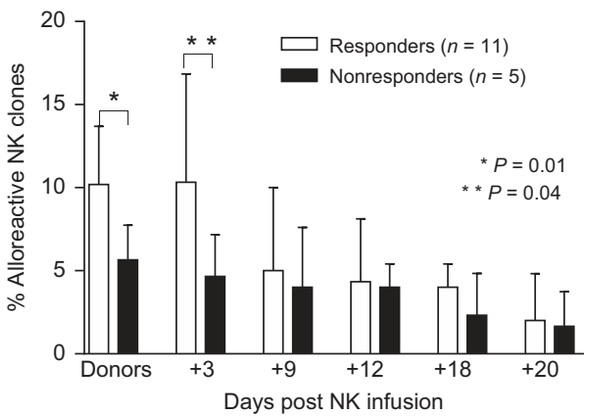

Figure 1.
Analysis of donor-versus-recipient alloreactive NK cell repertoires. Time kinetics of alloreactive NK cell clones from 16 evaluable patients according to clinical response to NK cell infusion. Analysis was performed before and after NK infusion. After the NK cell infusion, NK cell clones were obtained from NK cell–infused patients (see Materials and Methods) and their alloreactivity against recipient PHA blasts was determined in a ^{51}Cr-release assay. Only alloreactive clones, exhibiting $\geq$ 30% specific lysis, were included. Data are reported as the median of the number of alloreactive NK cell clones for each patient at the different time points.

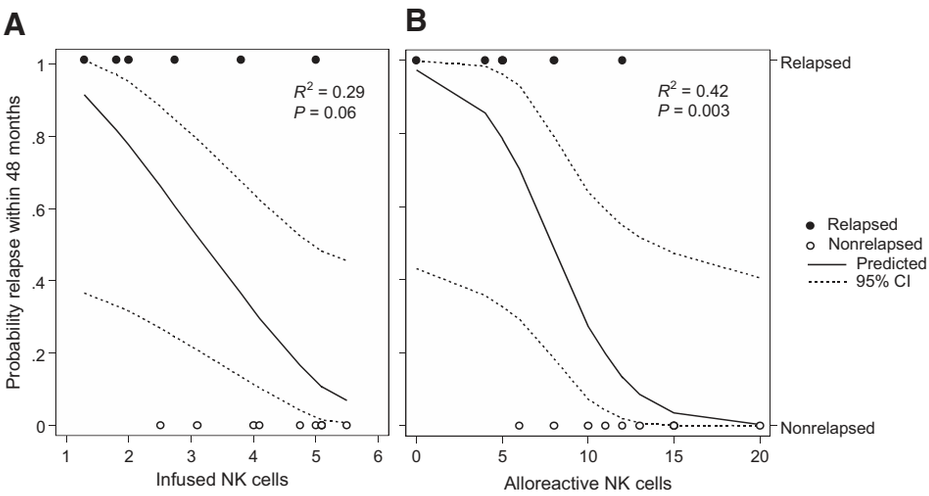

Figure 2.
Correlation between relapse rate and composition of NK cell graft. Logistic regression analysis of relapse status (inclusive of relapse out to 72 months) for total NK cells/kg (A) and number of alloreactive NK cells/100 clones (B). Open circles are nonrelapsed patients, closed circles are relapsed patients. Analysis was performed on donor cells before NK infusion.

When analyzed by logistic regression, the total number of infused NK cells was associated with a downward trend in the relapse rate ($P = 0.06$) (Fig. 2A) and the percentage of donor-derived alloreactive NK cell clones before NK cell infusion correlated significantly with relapse status (Fig. 2B, $P = 0.003$). Also, the percentage of NK cell clones obtained from day 3 after infusion correlated with the probability of clinical response by logistic regression, (Supplementary Fig. S3; $P = 0.03$), although fewer patient data were available to precisely estimate the odds of response [aROC, 1.93; 95% confidence interval (CI), 0.96–3.87].

To better characterize the absolute size of donor NK cell repertoire, on the basis of previously reported percentages of alloreactive NK cell clones, we calculated the absolute number of infused alloreactive NK cells for each patient. Interestingly, the absolute number of alloreactive NK cell clones in the responder group of patients was increased over that in nonresponders (30.1 and 9.6×10^6, respectively) and a statistically significant correlation with the probability to achieve clinical response was established (Supplementary Fig. S4; $P = 0.008$). At the functional level in terms of ability to kill target cells, no differences between clones in the responders and nonresponders were observed. In particular, all clones were fully functional and they did kill K562 at the same percentage and entity of lysis (data not shown). The clinical relevance of the contaminating T-cells was also evaluated. A slight, but not statistically significant increase in the number of infused T cells was observed in the responders over nonresponder patients (0.71 and 0.53×10^5/kg, respectively; $P =$ ns). Accordingly, no correlation was found between the absolute number of infused T cells and clinical response. A comprehensive description of cell processing results in both responders and nonresponders is detailed in Supplementary Table S2.

Identification of a threshold of alloreactive NK cell clones predictive for clinical response

On the basis of ROC analysis, a threshold of >8 of 100 alloreactive NK clones were identified a group of patients with reduced relapse risk (aROC 0.97; 95% CI, 0.75–1.0, Fig. 3). To compare the clinical outcome of NK patients, a control group (Table 2) of AML patients who achieved CR after induction/consolidation chemotherapy, but did not undergo NK cell immunotherapy due to the absence of an appropriate NK cell donor, was analyzed. Controls were matched to cases on biologic (i.e., age, gender, karyotype, hyperleukocytosis, genotype) and other features at diagnosis. Despite a trend toward an increase of DFS for total NK-treated patients compared with the control group, such difference was not statistically significant (Fig. 4A). However, when analyzed up to 48 months after treatment, the NK patients who received more than 8 of 100 alloreactive NK clones showed a significantly prolonged relapse-free duration versus NK patients with $\leq$ 8 of 100 alloreactive NK clones (mean 43 versus 21.6 months, respectively, $P = 0.03$; Fig. 4B). Interestingly, DFS of the latter group was similar to the historical control group (mean 20.1 months; Fig. 4B). Compared with historical controls, the estimated HR for relapse was 1.02 (0.29–3.67) for patients receiving ≤ 8 of 100 alloreactive NK clones, and 0.13 (0.04–0.41) for patients receiving >8 alloreactive NK clones ($P = 0.03$). This finding suggests that the therapeutic effect of the whole procedure, which

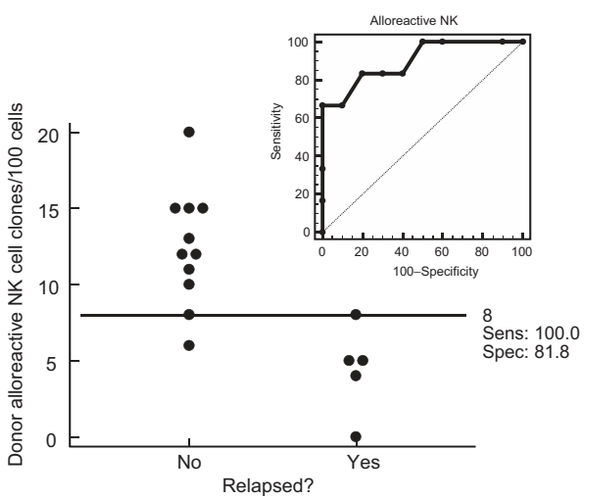

Figure 3.
Impact of donor-reactive NK clones on the probability of remaining relapse-free. Criterion threshold plot of nonrelapsing versus relapsing patients at alloreactive NK cell dose of >8 alloreactive clones/100 cells. Insert graph shows the receiver operator curve. Analysis was performed on donor cells before NK infusion.

Table 2. Clinical and biologic characteristics of historical control group

UPN	Age	Sex	WBC (10^9/L)	Karyotype	Genotype	FAB
1	64	M	2,23	Complex	FLT3 WT, NPM WT	M1
2	64	M	0,6	46 XY	FLT3 WT	M1
3	66	M	165	49, XY	FLT3 WT, NPM WT	M4
4	67	F	1,6	46,XX	FLT3 WT, NPM WT	Secondary
5	71	F	38	46,XX	FLT3 WT, NPM WT	M4
6	62	M	2,08	46 XY	FLT3 WT, NPM WT	M0
7	60	F	5,6	Complex	AML-ETO	Secondary
8	64	F	34,7	Complex	FLT3 ITD, NPM MUT	M1
9	65	F	96,4	46,XX	NE	Sec
10	62	F	13,3	t(4)	FLT3 WT, NPM WT	Secondary
11	67	F	71,7	46,XX	FLT3 WT, NPM MUT	M2
12	70	F	3,8	del5q	FLT3 WT, NPM WT	M1
13	70	M	9,7	46 XY	FLT3 ITD, NPM MUT	M4
14	63	M	8	46 XY	FLT3 WT, NPM WT	M1
15	71	F	0,6	46,XX	FLT3 WT	M1

Abbreviations: CBF, core binding factor; CR, complete remission; FAB, French-American-British; FLT3, FMS-like tyrosine kinase 3; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; NE, not evaluated; NK, natural killer; NPM, nucleophosmin; Pt, patient; TKD, tyrosinekinase domain; WBC, white blood count; WT, wild type.

comprises chemotherapy (fludarabine/cyclophosphamide) plus immunotherapy, may rely on the antileukemia activity of alloreactive NK cells. The greater antileukemia effect in patients receiving more donor alloreactive NK cells was not associated with increased myelosuppression, as hematologic recovery was similar whether patients received $\leq$8 of 100 or >8 of 100 alloreactive NK clones (Fig. 5; P = ns). Taken together, these data support the positive correlation between the alloreactive potential of donor NK cells in the graft and the clinical response to NK therapy.

Discussion

The current study reports the clinical and the correlative biologic results of KIR-L–mismatched NK cell adoptive immunotherapy in elderly AML patients in CR. The purpose of the study was to determine whether the composition of NK graft in terms of frequency of alloreactive NK cells influenced clinical response to NK immunotherapy. Miller and colleagues were the first to infuse NK cells as a means of adoptive immunotherapy in adult relapsed or resistant AML patients (19). The biologic and clinical results of this pioneering trial were remarkable. In particular, a retrospective analysis of responders versus nonresponders revealed that a better response was associated with KIR-L mismatch between donor and recipient. Given that KIR-L incompatibility in the setting of haploSCT is the major factor influencing NK cell–mediated disease control through NK cell alloreactivity, these results suggest that donor alloreactivity may impact on the efficacy of adoptively transferred NK cells. Moreover, at the clinical level, these data offered the rationale to exploit alloreactive NK-cell based adoptive immunotherapy as a means of consolidation therapy to prevent disease relapse in adult AML patients who achieved CR. To address these biologic and clinical points, the ideal setting is that of elderly AML patients with poor prognosis, whose OS is particularly dismal and who are mostly not eligible to allogeneic SCT (1).

Several biologic factors, both of recipient and donor origin, may be implicated in the therapeutic effect of NK cells after infusion into AML patients. Miller and collaborators have recently reported about the critical impact of some components of the recipient immune response on the antileukemia activity of infused NK cells. In particular, they reported that NK cell expansion correlates with the postchemotherapy serum concentrations of some cytokines, such as IL15 and IL35. In particular, the number of circulating T regulatory cells (T_{regs}) after NK cell infusion critically influences the capacity of infused NK cells to expand and to kill AML cells (24). The clinical relevance of these findings is supported by a better DFS in patients undergoing NK

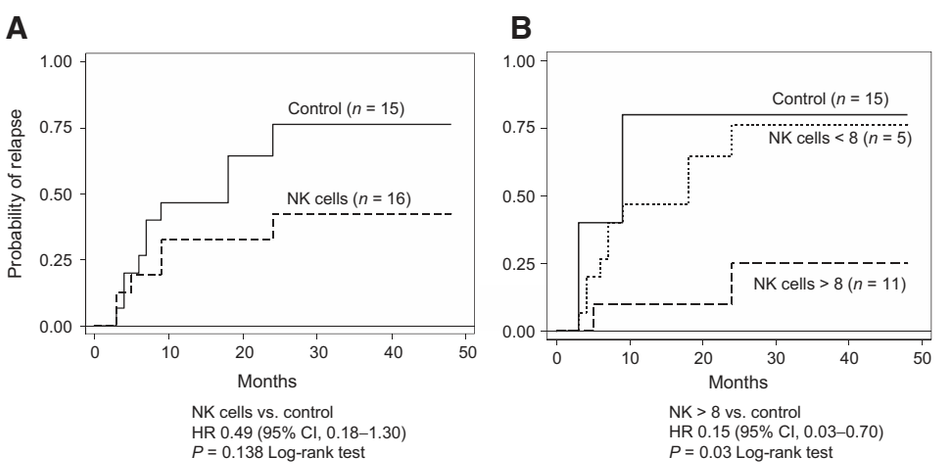

Figure 4.
Curves of relapse rate in treated and not treated patients. A, Kaplan–Meier plot of total NK cell patients (n = 16) and historical non-NK cell–treated controls (n = 15; dotted line). B, Kaplan–Meier plot of NK cell patients with >8 alloreactive clones/100 cells (dashed line), $\leq$8 alloreactive clones/100 (solid line), and historical non-NK cell–treated controls (dotted line). Analysis was performed on donor cells before NK infusion.

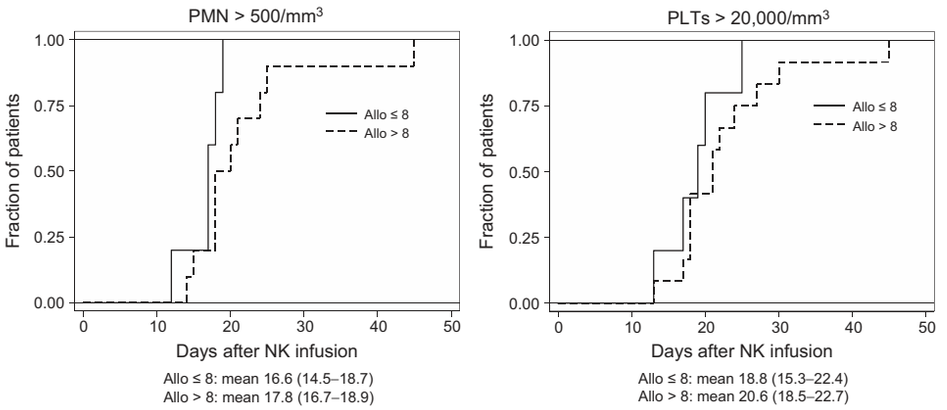

Figure 5.
Impact of donor-reactive NK clones and hematologic recovery after NK cell infusion. NK cell patients with >8 alloreactive clones/100 cells (dashed line) and ≤8 alloreactive clones/100 (solid line). Analysis was performed on donor cells before NK infusion.

immunotherapy depleted of T_{regs}. On the basis of these data, we retrospectively analyzed the number of circulating T_{regs} at different time points after NK cell infusion in 5 over 16 evaluable patients. With the limitation of the low number of cases, the frequency of T_{regs} was below the threshold indicated by Miller and colleagues as predictive of different clinical response to NK immunotherapy (data not shown). Then, our analysis has been focused on the donor and, in particular. on the composition of donor NK cell population. Our findings demonstrate that the functional composition of donor NK cell population has a crucial role in determining antileukemia effects. In particular, the number of donor alloreactive NK cells may discriminate patients undergoing NK immunotherapy in two groups with significantly different clinical outcome. However, our data, referring to a study population of previously selected KIR-L–mismatched donor–recipient pairs, pointed out that in the setting of adoptive NK immunotherapy, only KIR-L mismatch between recipient and donor may not be enough to translate into a significant clinical benefit. Indeed, a group of KIR-L–mismatched NK patients had the same clinical outcome as a historical control group, not treated with NK cells. As no other evaluated factors than the frequency of donor alloreactive NK cells might explain such a different outcome, the main message of our study is that the frequency of alloreactive NK cell clones in the donor may have a crucial effect on the antileukemia potential of infused NK cells. These data are in line with previous reports in the mouse model, where escalated doses of human alloreactive NK cells are associated with increased antileukemia effect, leading to leukemia eradication (9). However, a better definition of the cell composition of NK cell product in terms of NK cell subsets together with a more extensive phenotypical and functional characterization of infused cells may be useful in the identification of other relevant parameters to be correlated with clinical response.

Clinically, albeit with the limitations due to the low number of patients, our results expand the field by demonstrating that the whole procedure is feasible in a population of over 60-year old AML patients. As already reported 21), NK cells engrafted and were detectable both in the peripheral blood and in the bone marrow of infused patients. Noteworthy, no signs of GvHD were registered. Hematologic toxicity was tolerable, although bone marrow aplasia was complicated in some cases by infections, including one death due to bacterial pneumonia. Although hematologic recovery was not significantly different from what was observed after conventional chemotherapy, these adverse effects suggest the need to carefully evaluate the eligibility to NK therapy, which should be proposed only to chemotherapy-fit patients. Our results suggest that NK cell–based therapy has a potential clinical benefit for elderly patients with poor prognosis AML. Indeed, with a median follow-up of 22.5 months (range, 6–68 months), 9 of 16 evaluable patients are disease-free. Interestingly, among 8 relapsed patients, 2 of them showed a very prolonged CR phase, without concomitant treatments. The disappearance of the leukemia-associated transcript in molecular MRD^{+} patients suggests that immunosuppressive chemotherapy followed by NK cells exerted an antileukemic effect. In particular, the positive correlation between the frequency of donor alloreactive NK cell repertoires and the clinical outcome strongly indicates the therapeutic effect of the whole procedure relies on the immunologic activity of alloreactive NK cells included in the graft rather than on the chemotherapy.

Some important concerns may arise about the methods we used to investigate the alloreactive NK cell repertoire. Indeed, these are based on NK cell limiting dilution cloning and ^{51}Cr-release assay, which may be considered complex, time-consuming, and expensive. For these reasons, one major challenge is to develop alternative methods, while maintaining the same diagnostic potential. When mAbs were not able to discriminate between activating and inhibitory KIRs, some studies showed the alloreactive NK cell repertoire could be analyzed only in individuals who were homozygous for the group A *KIR* haplotype. (25, 26) Subsequently, new anti-KIR antibodies were developed that could distinguish inhibitory versus activating KIRs when used in appropriate combinations, thus allowing to identify alloreactive NK subpopulations in all individuals. One persistent limitation is that no mAb can currently distinguish KIR2DS2 from KIR2DL2 cells when both are expressed in the KIR genotype.(11) Because of these limitations, in the current study, we preferred to use our standard procedures to detect NK alloreactive repertoires. However, one of the endpoints of our future study will be to compare our methods with the phenotypic identification of alloreactive NK cell repertoire/CD107 assay, as also proposed by other authors (11, 25, 26).

In conclusion, our data demonstrate that a better outcome after infusion of haploidentical KIR-L–mismatched NK cells is correlated with high number of donor alloreactive NK cells. Clinical results suggest a potential effect in preventing disease relapse in a subset of elderly patients with AML. To validate these promising data, further clinical studies are highly warranted.

Disclosure of Potential Conflicts of Interest

G. Martinelli is a consultant/advisory board member for Ariad, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Curti, L. Ruggeri, A. Velardi, R.M. Lemoli
Development of methodology: A. Curti, A. Bontadini, E. Dan, M.R. Motta, S. Rizzi, E. Urbani, A. Velardi, R.M. Lemoli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Curti, L. Ruggeri, S. Parisi, E. Dan, M.R. Motta, S. Rizzi, S. Trabanelli, D. Ocadlikova, M. Lecciso, V. Giudice, E. Urbani, C. Papayannidis, F. Bonifazi, A. Velardi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Curti, L. Ruggeri, S. Parisi, E. Dan, S. Trabanelli, G. Martinelli, R.E. Lewis, A. Velardi, R.M. Lemoli
Writing, review, and/or revision of the manuscript: A. Curti, L. Ruggeri, S. Parisi, E. Dan, M.R. Motta, S. Rizzi, G. Bandini, R.E. Lewis, M. Cavo, A. Velardi, R.M. Lemoli
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Dan, F. Fruet
Study supervision: A. Curti, L. Ruggeri, A. Velardi

Acknowledgments

The authors thank Mrs. Valeria Martelli for phenotypic analysis of NK cells. Miltenyi Biotec is gratefully acknowledged for providing the separation kits for NK cells.

Grant Support

This research was supported by Italian Ministry of Health (Progetto Strategico Oncologia, 2006), Regione Emilia-Romagna (Progetto di Ricerca Università-Regione Emilia-Romagna, 2007-2009, N°1412), Italian Association Against Leukemia, Section of Bologna (BolognAIL), and Cassa di Risparmio in Bologna, a Translational Research Grant from the Leukemia and Lymphoma Society, Italian Association for Cancer Research. L. Ruggeri is a Leukemia and Lymphoma Society Special Scholar in Clinical Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 6, 2015; revised December 17, 2015; accepted December 17, 2015; published OnlineFirst January 19, 2016.

References

1. Löwenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med 1999;341:1051–62.
2. Ofran Y, Rowe JM. Genetic profiling in acute myeloid leukaemia–where are we and what is its role in patient management. Br J Haematol 2013; 160:303–20.
3. Farag SS, Caligiuri MA. Human natural killer cell development and biology. Blood 2006;20:123–37.
4. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene 2008;27:5932–43.
5. Höglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. Nat Rev Immunol 2010;10:724–34.
6. Moretta L, Moretta A. Killer immunoglobulin-like receptors. Curr Opin Immunol 2004;16:626–33.
7. Moretta A, Locatelli F, Moretta L. Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. Immunol Rev 2008;224:58–69.
8. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood 1999;94:333–9.
9. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002;295:2097–100.
10. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood 2007;110:433–40.
11. Pende D, Marcenaro S, Falco M, Martini S, Bernardo ME, Montagna D, et al. Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and re-definition of inhibitory KIR specificity. Blood 2008;113:3119–29.
12. Willemze R, Rodrigues CA, Labopin M, Sanz G, Michel G, Socié G, et al. KIR-ligand incompatibility in the graft-versus-host direction improves outcomes after umbilical cord blood transplantation for acute leukemia. Leukemia 2009;23:492–500.
13. Giebel S, Locatelli F, Lamparelli T, Velardi A, Davies S, Frumento G, et al.et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. Blood 2003;102:814–9.
14. Davies SM, Ruggieri L, DeFor T, Wagner JE, Weisdorf DJ, Miller JS, et al.et al. Evaluation of KIR ligand incompatibility in mismatched unrelated donor hematopoietic transplants. Killer immunoglobulin-like receptor. Blood 2002;100:3825–7.
15. Bornhäuser M, Schwerdtfeger R, Martin H, Frank KH, Theuser C, Ehninger G. Role of KIR ligand incompatibility in hematopoietic stem cell transplantation using unrelated donors. Blood 2004;103:2860–1.
16. Hsu KC, Gooley T, Malkki M, Pinto-Agnello C, Dupont B, Bignon JD, et al. et al. KIR ligands and prediction of relapse after unrelated donor hematopoietic cell transplantation for hematologic malignancy. Biol Blood Marrow Transplant 2006;12:828–36.
17. Malmberg KJ, Schaffer M, Ringdén O, Remberger M, Ljunggren HG. KIR-ligand mismatch in allogeneic hematopoietic stem cell transplantation. Mol Immunol 2005;42:531–4.
18. Cooley S, Weisdorf DJ, Guethlein LA, Klein JP, Wang T, Le CT, et al.et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood 2010;116:2411–9.
19. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and *in vivo* expansion of human haploidentical NK cells in patients with cancer. Blood 2005;105: 3051–7.
20. Rubnitz JE, Inaba H, Ribeiro RC, Pounds S, Rooney B, Bell T, et al. NKAML: a pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. J Clin Oncol 2010;28:955–9.
21. Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, et al. Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood 2011;118:3273–9.
22. Bontadini A, Testi M, Cuccia MC, Martinetti M, Carcassi C, Chiesa A, et al. Distribution of killer cell immunoglobulin-like receptors genes in the Italian Caucasian population. J Transl Med 2006;4:44–52.
23. Kristt D, Gesundheit B, Stein J, Shapira MY, Or R, Amar A, et al. Quantitative monitoring of multi-donor chimerism: A systematic, validated framework for routine analysis. Bone Marrow Transplant 2010;45:137–47.
24. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, et al. Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. Blood 2014;123:3855–63.
25. De Santis D, Foley BA, John E, Senitzer D, Christiansen FT, Witt CS. Rapid, flow cytometric assay for NK alloreactivity reveals exceptions to rules governing alloreactivity. Biol Blood Marrow Transplant 2010;16:179–91
26. Fauriat C, Andersson S, Bjorklund AT, Carlsten M, Schaffer M, Bjorkstrom NK, et al. Estimation of the size of the alloreactive NK cell repertoire: studies in individuals homozygous for the group A KIR haplotype. J Immunol 2008;181:6010–9.