SUCCESSFUL TRANSFER OF ALLOREACTIVE HAPLOIDENTICAL KIR LIGAND-
MISMATCHED NATURAL KILLER CELLS AFTER INFUSION IN ELDERLY HIGH RISK
ACUTE MYELOID LEUKEMIA PATIENTS

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Running Head: NK therapy for elderly AML patients

Scientific Section: Immunobiology

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ABSTRACT
Thirteen acute myeloid leukemia (AML) patients, 5 with active disease, 2 in molecular relapse and 6 in morphological complete remission (CR); (median age 62 years, range 53-73) received highly purified CD56⁺CD3⁻ natural killer (NK) cells from haploidentical KIR-ligand mismatched donors after fludarabine/cyclophosphamide immunosuppressive chemotherapy, followed by interleukin-2. The median number of infused NK cells was 2.74 x 10⁶/Kg. T cells were under 10⁵/Kg. No NK cell-related toxicity, including graft-versus-host disease, was observed. One of the 5 patients with active disease achieved transient CR, whereas 4/5 patients had no clinical benefit. Both patients in molecular relapse achieved CR which lasted for 9 and 4 months, respectively. Three/6 patients in CR are disease-free after 34, 32 and 18 months. After infusion, donor NK cells were found in the peripheral blood of all evaluable patients (peak value on day 10). They were also detected in bone marrow in some cases. Donor-versus-recipient alloreactive NK cells were demonstrated in vivo by the detection of donor-derived NK clones that killed recipient’s targets. Adoptively transferred NK cells were alloreactive against recipient’s cells, including leukemia. In conclusion, infusion of purified NK cells is feasible in elderly patients with high risk AML. The trial was registered at www.clinicaltrial.gov. (NCT00799799).
INTRODUCTION

Treatment of acute myeloid leukemia (AML) in adults is intensive. It is based on multiple cycles of cytosine arabinoside and anthracycline-containing chemotherapy regimens, followed by the option of allogeneic hematopoietic stem cell transplantation for eligible patients. Although complete remission (CR) rates in response to chemotherapy range from 60 to 85% in patients under 60 years of age, approximately 60% of patients subsequently relapse and the 5-year overall survival (OS) is 40%. In elderly patients, OS falls to 10%, due to the higher prevalence of unfavourable biological factors, such as poor risk cytogenetics.

The human lymphocyte subset of natural killer (NK) cells, which are defined by CD56 or CD16 expression and absence of CD3, plays a critical role in the innate immune response, particularly in immune control of tumor development and growth. NK cells express activating and inhibitory receptors which recognize MHC class I alleles. NK cells possess clonally distributed inhibitory receptors termed “Killer cell Immunoglobulin-like Receptors” (KIRs) that recognize allotypic determinants (“KIR ligands”) shared by certain groups of HLA class I alleles. KIR2DL1 recognizes HLA-C alleles with a Lys residue (HLA-Cw4 and related, “Group 2” alleles), KIR2DL2 and KIR2DL3 recognize HLA-C with an Asn residue (HLA-Cw3 and related, “Group 1” alleles), KIR3DL1 is the receptor for HLA-B alleles sharing the Bw4 specificity. NK cells which express, as their only inhibitory receptor for self, a KIR whose ligand is a HLA class I group which is absent on allogeneic targets, sense the missing expression of the self class I KIR ligand and mediate alloreactions.

Preclinical and clinical data from the haploidentical T-cell depleted transplantation setting demonstrated that haploidentical KIR-ligand mismatched NK cells play a major role as anti-leukemia effector cells. AML patients are significantly protected against leukemia relapse when transplanted from NK alloreactive donors. Furthermore, enriched NK cells can be infused safely as adoptive immunotherapy in leukemia and cancer patients following non-myeloablative and myeloablative immunosuppressive chemotherapy with some cases achieving significant clinical responses. Notably, these trials revealed that KIR-ligand mismatching between donor NK cells and recipient correlated with better response to NK therapy. A recent pilot study of haploidentical KIR-HLA mismatched NK
cell transplantation in childhood AML reported that NK cell therapy prolonged disease-free and overall survival.  

The present study investigated the feasibility and safety of infusing highly purified NK cells from haploidentical KIR-ligand mismatched donors into a cohort of elderly patients with high risk AML and demonstrated, for the first time, donor NK cell repertoires, trafficking, and function in the peripheral blood (PB) and/or bone marrow (BM) of recipients at different time-points. Given the critical role of NK alloreactivity in mediating the anti-leukemia effect after haploidentical stem cell transplant, adoptive immunotherapy with infusion of functionally active alloreactive NK may be of clinical benefit for patients with high-risk AML.
PATIENTS AND METHODS

Patients

Forty-two patients with high-risk AML, as indicated by poor prognostic features at diagnosis, advanced age or resistant/relapsed disease, were recruited to the study. Inclusion criteria were: AML, diagnosed according to WHO Classification; age \( \geq 18 \) years old, adequate renal, cardiac, and pulmonary function, a Karnofsky score \( \geq 70\% \), or a WHO score \( \leq 1 \), not eligibility for allogeneic stem cell transplantation. Relatives were screened in the search for one haploidentical KIR-ligand mismatched donor. A suitable donor was available for 18 patients (42.8%), 13 of whom received an infusion of donor NK cells. Tab.1 summarizes the characteristics of infused patients, all of whom had received chemotherapy according to institutional guidelines. All subjects gave written informed consent before entering the study in accordance with the Declaration of Helsinki, and all research was approved by the ethical committee of the University of Bologna. The trial was registered at www.clinicaltrial.gov. (NCT00799799).

Donor selection

Any consenting healthy family member who fulfilled donor criteria was eligible for leukapheresis. Donors who were classified as NK alloreactive against recipients, (NK alloreactive donors), possessed: 1) HLA class I KIR ligand(s) which were missing in the recipient, (2) KIR gene(s) for missing self recognition on recipient targets, and (3) alloreactive NK clones against recipient targets.

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, i.e. 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. Donor KIR genotyping was performed to ensure the donor possessed the relevant KIR gene. Donor NK cell KIR immunophenotyping and functional analyses were also performed (see below).

KIR genotyping
KIR typing was performed using a low resolution PCR-SSP assay (KIR Genotyping Kit, Invitrogen, USA) following the manufacturer’s instructions. The kit was designed to identify 14 KIR genes (2DL1, 2DL2, 3DL1, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1), 2 pseudogenes (2DP1 and 3DP1) and the common variants of KIR2DL5 (KIR2DL5A, KIR2DL5B), the KIR2DS4 allele (*001/002 and *003) and KIR3DP1 allele (*001/002 and *003).

PCR-SSP was performed following the manufacturer’s instructions. DNA was extracted from blood, NK cell clone or bone marrow samples and processed for multiplex PCR using the ABI AmpfSTR kit (ABI, Foster City, CA, USA) which has 10 polymorphic STR-PCR marker loci. STR-PCR was performed following the manufacturer’s instructions. Products were electrophoresed and fluorescence measured by ABI 310 Genetic Analyzer. Fluorescence intensity is an estimate of the quantity of amplificant in each electrophoretic peak (band) and single donor CHM (%CHM) was estimated from the peak area measurements.

**Immunophenotyping**

NK cell purification: fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and allophycocyanin (APC)-coupled control immunoglobulins or specific antibodies directed at CD56, CD3, CD14 or CD19 were used to evaluate NK purity and yield after immunomagnetic separation as well as the phenotype of circulating and BM cells in patients after the NK cell infusion.

KIR phenotype: four-color immunofluorescence analyses identified KIR\(^{-}/\)NKG2A\(^{+}\) versus KIR\(^{+}/\)NKG2A\(^{-}\), CD56\(^{-}/\)CD3\(^{-}\) NK cells. The following mouse monoclonal antibodies: APC–conjugated anti-CD56 (IgG1; Miltenyi Biotec, Bergisch Gladbach, Germany), PE-Cy7–conjugated anti-CD3 (IgG1; BD Bioscience, San Diego, CA), unconjugated anti-NKG2A (clone Z199, IgG2b, kindly donated by Alessandro Moretta, University of Genova) developed with FITC–conjugated goat anti–mouse IgG2b antibodies (Southern Biotech, Birmingham, AL), were used in combination with the following PE-conjugated anti-KIR antibodies (Bechman Coulter, Fullerton, CA): either anti-KIR2DL2/3/S2 (clone GL183, IgG1), or anti-KIR2DL1/S1 (clone EB6B, IgG1), or anti-KIR3DL1/S1 (clone Z27, IgG1). Two-color immunofluorescence analyses on NK cell clones were used to visualize NK cells expressing, as their only inhibitory receptor for self, a KIR for which there was no class I ligand in the recipient. KIR2DL2/3/S2 single-positive NK cells were identified with FITC-conjugated anti-KIR2DL2/3/S2 antibody (clone CH-L, IgG2b, BD Bioscience,
Franklin Lakes, NJ) in combination with a cocktail of PE-conjugated anti-KIR2DL1 (clone 143211, IgG1, R&D, Minneapolis, MI), anti-KIR3DL1 (clone DX9, IgG1, Miltenyi) and anti-NKG2A (clone Z199, IgG2b, Beckman Coulter, Brea, CA) mouse antibodies. KIR2DL1 single-positive NK cells were identified with PE-conjugated anti-KIR2DL1 (clone 143211, IgG1, R&D, Minneapolis, MI) in combination with a cocktail of FITC-conjugated anti-KIR3DL1 (clone DX9, IgG1, Miltenyi) and anti-KIR2DL2/3S2 (clone CH-L, IgG2b) and anti-NKG2A (clone Z199, IgG2b, Beckman Coulter, Brea, CA) mouse antibodies developed with FITC-conjugated goat anti–mouse IgG2b antibodies (Southern-Biotech).

**NK cell cloning and cytotoxicity assay**

Donor alloreactive NK cell repertoires were assessed once at the time of leukapheresis. Patient alloreactive 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 and cytotoxicity assays against recipient target cells were used to detect the frequency of alloreactive NK clones. Peripheral blood mononuclear cells (PBMCs) depleted of T cells by negative anti-CD3 immunomagnetic selection (Miltenyi) were plated under limiting-dilution conditions, activated with phytohemagglutinin (PHA; Biochrom KG, Berlin, Germany), and cultured with interleukin-2 (Chiron BV, Amsterdam, Netherlands) and irradiated feeder cells. Feeder cells were obtained by pooling buffy coats from 5 to 9 healthy donors. Such donors were not typed as PHA plus interleukin (IL)-2 activation allows efficient NK cell cloning regardless of feeder cell HLA type. Cloning efficiencies ranged from 1 in 5 to 1 in 10 plated NK cells. Cloned NK cells 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 and against one recipient’s leukemic cells. Approximately 100 NK clones from each person were screened. Clones exhibiting greater than 30% lysis were scored as alloreactive. The assay was considered positive when the frequency of lytic clones was more than 1 in 50.

**Leukapheresis and positive selection of CD3$^+$CD56$^+$ NK cells**

Standard volume leukaphereses were performed as already reported. A minimum of $5 \times 10^9$ total PBMCs were incubated with MACS colloidal superparamagnetic CD3 microbeads (Miltenyi Biotec), consisting of monoclonal mouse anti-human CD3 antibodies conjugated to microspheres. The CliniMACS® device was used in two steps: 1) CD3$^+$ T cell depletion
followed by 2) positive CD56⁺ NK cell selection under good manufacturing product (GMP) conditions. 5x10⁶ haploidentical NK cells/Kg of body weight was considered the target cell dose, with 1x10⁶ haploidentical NK cells/Kg as the minimum cell dose. A cell product sample was evaluated by flow-cytometry to count T, B, NK cells and monocytes before and after CliniMACS selection. Highly purified CD56⁺CD3⁻ NK cells were cryopreserved.

To reduce the risk of post-infusion GVH disease (GVHD), a maximum of 1x10⁵/kg CD3⁺ T cells was allowed in the NK graft. Fungal and bacterial sterility tests were performed on the final NK cell product, viability and the nucleated cell dose were assessed. Extensive phenotyping determined purity and residual B, T cells and monocytes. Cytotoxicity was tested against NK-sensitive K562 cells.

**Immunosuppressive chemotherapy and NK infusion**

To favor haploidentical NK cell engraftment, all patients received immunosuppressive chemotherapy, (fludarabine (Flu) 25 mg/mq/ from day –7 to -3 and cyclophosphamide (Cy) 4 g/mq on day -2 (Flu/Cy). Two days after Cy administration, patients received the NK cell infusion (day 0), which was followed by subcutaneous administration of IL-2 (10 x 10⁶ IU/day, 3 times weekly; Novartis; Basel, Switzerland) for 2 weeks (6 doses total). No GVHD prophylaxis was used as GVHD was not anticipated.

**Chimerism assay**

PB and BM chimerism was determined by variable number tandem repeat (VNTR) assay. Analysis was performed on whole blood. Nine loci were routinely analyzed (D3S1358, VWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317, D7S820) in addition to amelogenina which identifies XY chromosomes. Patient PBL and clones were studied before the NK cell infusion and at different time-points afterwards. Donor chimerism was expressed as the percentage of circulating donor cells.

**Serum cytokine concentrations**

“Human IL-15 ELISA Kit” (Tema Ricerca, Bologna, Italy) was used to test IL-15 concentration in serum. Briefly, 0.1 ml per well of standard solutions or patients’ serum were aliquotted in duplicate into a precoated 96-well plate. The plate was sealed and incubated at 37°C. After 90 min incubation, the plate content was discarded, 0.1 ml of biotinylated anti-human IL-15 antibody working solution was added to each well and the plate was incubated at 37°C for 60 min. The plate was then washed 3 times with 0.01 M
PBS. 0.1 ml of prepared ABC working solution was added to each well and the plate was incubated at 37°C for 30 min. After rinsing 5 times with 0.01 M PBS, 90 µL of prepared TBS color developing agent were added to each well and the plate was incubated at 37°C for 25 min. 0.1 ml of prepared TMB stop solution was added to each well and the plate was read at 450 nm in a microplate reader (Multiskan EX, M-Medical, Milan, Italy). The human IL-15 concentration was extrapolated from the standard curve.

**Statistical analysis**

The results are presented as median values and ranges or, when indicated, as mean ± SD. To investigate the relationship between serum IL-15 levels (day 3) and PB donor chimerism (peak value) after infusion, Pearson correlation coefficient was calculated.
RESULTS
Identification of donors able to mount donor versus recipient NK cell alloreactivity
Haploidentical KIR ligand mismatched donors were screened for 42 adults with AML, which was defined as high-risk because of poor prognostic features such as advanced age or resistant/relapsed disease at diagnosis. Eighteen patients had one suitable donor (42.8%). Thirteen patients entered a pilot study of adoptive NK cell immunotherapy (See Table 1 for patients' characteristics). Among donor-recipient pairs, 6/13 (46%) were mismatched for HLA-C group 1; 4/13 (30.9%) were mismatched for HLA-C group 2, 1/13 (7.7%) was mismatched for HLA Bw4; 1/13 (7.7%) was mismatched for both HLA-C1 and Bw4; 1/13 (7.7%) was mismatched for both HLA-C2 and Bw4.
The 13 mismatched donors all possessed the inhibitory KIR gene that recognized missing expression of its HLA class I ligand on recipient cells. The 7 HLA-C group 1 mismatched donors possessed KIR2DL2 and/or KIR2DL3, the 5 HLA-C group 2 mismatched donors possessed KIR2DL1, and the 1 HLA-Bw4 mismatched had KIR3DL1 (Table 1). Functional analyses showed the presence of alloreactive NK clones in all donors whose NK clones were tested against allogeneic targets which did not express their HLA-C group. Frequencies of donor-versus-recipient alloreactive NK clones were 9.5 ± 3.1% for HLA-C group 2 mismatched pairs and 7.5± 3% for HLA-C group 1 mismatched pairs, respectively. In contrast, the donor who was mismatched for Bw4 did not possess NK alloreactive clones against HLA-Bw4-negative targets.

NK cell purification
A median of 17.10 x 10⁶ CD56⁺CD3⁻ cells/kg (range 3.80-42.50) was collected from PB in one leukapheresis. Immunomagnetic selection provided a cell population with a median purity of 93.5% (range 66.4-99.2) and a median recovery of 53.05% (range 30.9-72.8) for infusion (Table 1). Positive NK cell selection resulted medianly in 3.03 log T-cell depletion (range 2.1-4.5). Each positive selection required one column and one CD56⁺ tubing set. Target NK cell dose recovery (5 x 10⁶/kg) was obtained in 10/13 patients (77%) and the minimum NK cell dose (10⁶/kg) in 13/13 patients. To prevent the GVH effect in weakly immunosuppressed patients, we established a maximum of 10⁵/kg T cells in the final cell product. Since CD56⁺ cell purification significantly enriched CD56⁺CD3⁺ double positive T cells (Fig. 1A), in most patients, we were not able to infuse the total number of collected CD56⁺CD3⁻ NK cells. Thus, a median of 2.74 x 10⁶ CD3⁻CD56⁺ cells/kg (range 1.11-5)
were infused. 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 (Fig. 1B).

**Safety of NK cell therapy**

After administration of Flu/Cy chemotherapy and infusion of highly purified NK cells, hematopoietic cell recovery was rapid and similar to what is observed after a standard chemotherapy cycle. The median times to 0.5 absolute neutrophil count $\times 10^9$/L and to 20 x $10^9$ Platelets/L were 18 and 20 days, respectively. Few infections were documented and hospital stays were short (data not shown). No clinical and/or laboratory signs of GVHD were observed. All patients received all IL-2 injections as scheduled. In some cases, local side effects, such as mild erythema were observed at the injection site.

**Analysis of post-infusion NK cell repertoire**

The Flu/Cy preparative regimen caused lymphopenia$^{19}$ and homeostatic augmentation of endogenous IL-15 which is essential for in vivo expansion and survival of NK cells. In our patients, IL-15 concentration peaked on day 3 after NK cell infusion (Fig. 2A). Post-infusion NK cell number kinetics in PB is reported in Fig. 2B and shows that NK cell count increased when IL-15 concentration decreased. The donor chimerism assay in PB and BM demonstrated that donor-derived cells were present in PB and BM after NK cell infusion, peaking on days 10 and 5, respectively (Fig. 2C and D). Although a statistically significant correlation between serum IL-15 levels and PB donor chimerism was not found ($r=0.4$, $p=0.1$), in some cases, the rise in IL-15 serum level was followed by the increase in donor chimerism. Moreover, since several mechanisms may regulate NK cell expansion/clearance, including alloimmunization of recipient’s T cells against donor antigens,$^{13}$ especially after repeated infusions, we specifically analyzed the results of the only patient who was treated with two subsequent NK cell infusions and we observed that IL-15 concentration and the percentage of donor chimerism were different after the first and the second infusion. In particular, NK cells of donor origin were demonstrated up to day 17 after the first infusion, while donor NK cells were detected for only 5 days after the second infusion (data not shown). These data paralleled with higher and durable concentrations of serum IL-15 after the first than after the second infusion (Fig.1S).
Alloreactivity of post-infusion NK cells

The kinetics of alloreactive NK cell subsets was documented by flow cytometry and by limiting dilution cloning of circulating NK cells during the first month after the NK cell infusion. Fig. 3A illustrates the kinetics of KIR2DL2/3/S2+/NKG2A− cells from 7 HLA-C1 positive donors infused into HLA-C1 negative recipients. Such cells are potentially alloreactive as they contain only KIR2DL2/3+ NK cells, that are alloreactive against targets from C1-negative recipients. Fig 3B shows the kinetics of KIR2DL1/S1+/NKG2A− cells from 5 HLA-C2 positive donors infused into HLA-C2 negative recipients. Such cells are potentially alloreactive as they contain only KIR2DL1+ NK cells that are alloreactive against targets from C2-negative recipients. Moreover, to directly document donor NK cell function in vivo, NK cell clones were obtained from NK cell-infused patients and their alloreactivity against recipient PHA blasts was determined in a standard 51Cr-release assay. In Fig. 3C-D, each bar represents the degree of alloreactivity (% specific lysis against recipient PHA blasts) exerted by one individual clone. All alloreactive clones obtained from all patients are shown. In particular, Fig. 3C shows alloreactive NK clones detected in 5 HLA-C1 negative recipients after NK cell infusion from HLA-C1 positive donors. In agreement with the functional results, immunofluorescence analyses of KIR and NKG2A receptors in two randomly selected alloreactive NK cell clones demonstrated such clones express, as their only inhibitory receptor for self, the KIR for which there is no class I ligand in the recipient (Fig. 4). They are representative of 20 tested that exhibited the same phenotype.

The frequencies of alloreactive NK clones detected in patients shortly after the NK cell infusion tended to reproduce the frequency originally detected in the donors (Fig. 5). As expected from the relatively modest immune suppressive treatment delivered to the patients and the relatively small number of NK cells infused, the frequencies of alloreactive NK cells detected in patients decreased over time. Chimerism analyses performed in five randomly selected alloreactive NK clones demonstrated they were of donor origin. No alloreactive NK clones were detected after infusing NK cells from the HLA-Bw4-mismatched donor who did not possess detectable frequencies of donor-versus-recipient alloreactive NK clones. Altogether, these data indicate there was no preferential outgrowth of recipient versus donor clones, nor that IL-2 treatment stimulated recipient rather than donor NK cells and boosted non-ligand-specific NK cell cytotoxicity. Finally, alloreactive NK clones isolated from patient 8 (see table 1) were tested for lysis of the patient’s
Criopreserved primary leukemic cells. All alloreactive clones killed the patient’s leukemic cells.

**Clinical outcome**

One of the 5 patients with active/progressive disease obtained a CR, which lasted for 6 months. Disease persisted in the other 4 patients who ultimately died. Both patients in molecular relapse, as evaluated by increasing levels of leukemia-associated (Wilms’ tumor 1 gene, *WT1*) and leukemia-specific (*CBF-MYH11*) transcripts, achieved CR, which lasted 9 and 4 months, respectively. Since donor NK cells were available, one patient received an additional cycle of Flu/Cy therapy followed by NK infusion at the time of second molecular relapse, which again resulted in molecular CR (Fig. 6). Three of the 6 patients who were treated in CR, are disease-free after 34, 32 and 18 months respectively. The other 3 patients relapsed soon after NK cell infusion.
DISCUSSION

The present study, involving elderly patients with high risk AML, reports the biological and clinical results of KIR-ligand mismatched NK cell adoptive immunotherapy after immunosuppressive chemotherapy. Major end-points were evaluation of feasibility and safety as well as the procedure’s anti-leukemia efficacy and potential. Miller and collaborators were the first to infuse NK cells in adult AML patients with active/progressive disease.\textsuperscript{12} Although clinical responses were observed in some cases, the allogeneic grafts included enriched NK cells and a mixed population of different cell subsets, including T cells, B cells and monocytes, which could have played an additional role in the therapeutic effect of cellular infusion. Thus, the contribution of NK cells was not fully elucidated. Furthermore, NK-cell donors were not selected according to KIR-ligand mismatches, although retrospectively best responses were observed in KIR-ligand mismatched patients.

Our results demonstrate that the whole procedure is feasible and safe in elderly AML patients. Since approximately 40% of screened patients had KIR-ligand mismatched donors, KIR-ligand mismatched NK therapy can be applied to a significant fraction of AML patients. NK cells engrafted and hematological toxicity was tolerable, certainly not worse than it might be expected after an immunosuppressive/myelotoxic chemotherapy regimen that included Flu and Cy. Indeed, the NK cell infusion was not associated with any additional toxicity as neutropenia was acceptable, not complicated by significant infections and importantly, no signs of GvHD were documented in any patient. After infusion of haploidentical KIR-ligand mismatched NK cells, donor NK cells were detected in PB and BM and functional alloreactive NK clones were found in patients’ blood during the first month post-NK infusion.

Although this pilot study was designed to assess the feasibility and toxicity of cellular adoptive immunotherapy, our clinical results suggest NK-cell based therapy has a potential clinical benefit for patients with poor prognosis AML. As expected, patients who seemed to benefit from the procedure were treated in CR and/or very early in molecular relapse. Indeed, after 34, 32 and 18 months follow-up, 3/6 of patients who were infused in CR are leukemia-free. The disappearance of the leukemia-associated transcript in patients in molecular relapse suggests that immunosuppressive chemotherapy followed by NK cells exerted an anti-leukemic effect.
In a pediatric cohort of AML patients, who underwent NK therapy after an immunosuppressive regimen, the 2-year event-free survival was 100%. Notably, unlike our elderly patients with a median age of 62 years, who were at high risk of relapse, all the children were considered at low-risk of relapse, with a significant fraction harboring good-prognosis cytogenetics. Furthermore, as children weigh less than adults, the median number of infused NK cells was significantly higher than in the present trial, although the separation procedure was the same. These differences may partially explain the discrepancy in clinical results and suggest that in adult patients the clinical effect of NK therapy may be implemented by increasing the number of infused NK cells.

In conclusion, further studies are highly warranted to specifically assess the role of NK therapy in the post-remission management of adult AML patients as present results demonstrate that adoptive immunotherapy with haploidentical NK cells, obtained from KIR-ligand mismatched donors is feasible and safe in elderly patients with high risk AML. Furthermore, this is the first study to document the post-NK cell infusion emergence and persistence over time of functional donor-versus-recipient NK cell alloreactivity. As toxicity appears similar to a standard chemotherapy-based consolidation cycle, NK therapy may be a promising strategy for consolidating CR in high-risk elderly who are not candidates for stem cell transplantation. Indeed, a phase II study with a greater NK cell dose and the option of multiple NK infusions is currently being conducted at our Institutions.
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Contributions: Antonio Curti contributed to study design, was responsible for inpatient and outpatient care and wrote the paper; Loredana Ruggeri was responsible for NK immunological tests and contributed to write the paper; Elena Urbani and Sara Trabanelli helped with immunological tests; Alessandra D’Addio, Stefania Paolini, Alessandro Isidori and Sarah Parisi contributed to inpatient and outpatient care and helped with data collection; Andrea Bontadini and Fiorenza Fruet contributed to genetic analyses; Elisa Dan and Maria Rosa Motta contributed to NK cell purification; Valeria Giudice contributed to cell collection; Giuseppe Bandini, Giovanni Martinelli and Michele Baccarani helped with inpatient care and manuscript revision; Andrea Velardi contributed to study design and to write the paper; Roberto M. Lemoli was the principal investigator of the clinical trial, contributed to study design, helped with patient care and with manuscript revision.

Conflict-of-interest disclosure: All the Authors of this research have no conflict of interest to disclose.

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REFERENCES


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

Figure 1. Donor NK cell purification. Panel A. Flow-cytometry analysis of NK cells after T-cell depletion and after CD56⁺ cell positive selection. The results are representative of the whole patient population. Panel B. NK cells obtained after purification were tested against K562 cells in a flow-cytometry-based cytotoxicity test. The results represent the mean ± SD of 6 independent experiments.

Figure 2. Increase in IL-15 concentration and donor chimerism in lympho-ablated recipients after NK cell infusion. Panel A. IL-15 concentration was evaluated in the serum of all patients at different time-points after NK cell infusion. Each curve corresponds to one different patient; Panel B. CD56⁺CD3⁻ cells were enumerated in the PB of all patients by flow-cytometry at different time-points after NK cell infusion. Baseline corresponds to the day before chemotherapy was started. The results are the mean ± SD of all patients; Panel C-D. The percentage of donor chimerism, as tested on whole blood, was evaluated in the PB (C) and BM (D) by VNTR analysis in all treated patients (n=13). Ten/13 patients were fully evaluable all over the different time-points, whereas in the remaining patients, particularly during the neutropenic phase after chemotherapy, cell concentration did not allow a reliable VNTR analysis. Here are reported the results of all positive patients (n=7 for PB and n=4 for BM). In the remaining evaluable patients, no donor chimerism was observed.

Figure 3. Post NK cell infusion detection of donor-versus-recipient alloreactive NK cell repertoires. Panel A. Time kinetics of KIR2DL2/3/S2+/NKG2A⁻ NK cells from 7 HLA-C1 positive donors infused into HLA-C1 negative recipients. Such cells are potentially alloreactive as they contain KIR2DL2/3⁺ only NK cells (which are alloreactive against C1-negative recipients). Panel B. Time kinetics of KIR2DL1/S1⁺/NKG2A⁻ NK cells from 5 HLA-C2 positive donors infused into HLA-C2 negative recipients. Such cells are potentially alloreactive as they contain KIR2DL1⁺ only NK cells (which are alloreactive against C2-negative recipients). Panels C-D. 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 ⁵¹Cr-release assay. Each bar represents the degree of alloreactivity ( % specific lysis against recipient PHA blasts) exerted by one individual clone. All alloreactive clones, exhibiting ≥ 30% specific lysis, obtained from all
patients are shown. Panel C. NK clones detected in 5 HLA-C1 negative recipients after NK cell infusion from HLA-C1 positive donors. Panel D. NK clones detected in 3 HLA-C2 negative recipients after NK cell infusion from HLA-C2 positive donors.

**Figure 4. KIR and NKG2A receptor expression by alloreactive NK cell clones.** Immunofluorescence analyses of two representative alloreactive NK clones are shown. Such clones express, as their only inhibitory receptor for self, the KIR for which there is no class I ligand in the recipient. Panel A. An NK clone from HLA-C1 positive donor detected in HLA-C1 negative recipient is KIR2DL2/3/S2* and KIR2DL1/3DL1/NKG2A−. Panel B. An NK clone from HLA-C2 positive donor detected in HLA-C2 negative recipient is KIR2DL1+ and KIR2DL2/3/S2/3DL1/NKG2A−. These two clones are representative of 20 tested that exhibited the same phenotype.

**Figure 5. Post NK cell infusion kinetics of donor-versus-recipient alloreactive NK cell clones.** Each bar represents mean ± SD of frequencies of NK clones that killed recipient targets detected in donors and in recipients at days +3, +9, +12, +18 and +20, after NK cell infusion.

**Figure 6. Clinical outcome after NK cell infusion of one patient with AML harbouring inv(16) in early molecular relapse.** Since adequate numbers of donor NK cells were available, a second NK infusion, following immunosuppressive chemotherapy, was performed at the time of the second relapse. The figure shows the value of both WT1 and CBF molecular transcript at different time-points.
Figure 1

A

After T-cell depletion

CD56

CD3

After NK positive selection

B

% IgG

K562/NK ratio

10/1 6/1 2/1 1/2
Figure 2

A

B

C

D
Figure 3

A

B

cells/μL

days post NK infusion

days post NK infusion

C

D

% lysis

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Figure 4

A

B
Figure 5

![Bar graph showing the percentage of allogeneic NK clones over time following NK infusion.](image-url)
Figure 6
Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high-risk acute myeloid leukemia patients

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