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Phase II Study of Haploidentical Natural Killer Cell Infusion for Treatment of Relapsed or Persistent Myeloid Malignancies Following Allogeneic Hematopoietic Cell Transplantation

Brian C. Shaffer, MD[1], **Jean-Benoit Le Ludec, PhD**[3], **Christopher Forlenza, MD**[2], **Ann A. Jakubowski, MD, PhD**[1], **Miguel-Angel Perales, MD**[1], **James W. Young, MD**[1,3], and **Katharine C. Hsu, MD, PhD**[1,3]

[1]Memorial Sloan Kettering Cancer Center, Adult Blood & Marrow Transplant Service, Weill Cornell Medical College, New York, NY

[2]Memorial Sloan Kettering Cancer Center, Department of Pediatric Medicine, Division of Hematology/Oncology, New York, NY

[3]Sloan Kettering Institute, New York, NY

Abstract

We conducted a phase 2 study to determine the efficacy of HLA-haploidentical related donor NK cells following cyclophosphamide based lymphodepletion in patients with relapsed or progressive acute myelogenous leukemia (AML) or myelodysplastic syndrome (MDS) following allogeneic HCT. Eight patients (2 with MDS, 6 with AML) were treated with cyclophosphamide 50 mg/kg on day −3 and day −2 prior to infusion of NK cells isolated from a haploidentical related donor. One person additionally received fludarabine 25 mg/m^2/d × 4. Six doses of 1 million units of interleukin-2 (IL-2) were administered on alternating days beginning on day −1. The median number of infused NK cells was 10.6×10^6/kg (range 4.3–22.4) and the median number of CD3 cells was 2.1×10^3/kg (range 1.9–40). NK infusions were well tolerated with a median time to neutrophil recovery of 19 days (range 7-not reached) and no incidence of graft *versus* host disease after NK infusion. One patient with AML and one patient with MDS achieved a complete response but relapsed at 1.7 and 1.8 months, respectively. One patient with MDS had resolution of dysplastic features but persistence of clonal karyotype abnormalities. This patient remains stable at 65 months post NK cell therapy. The median survival was 12.9 months (range 0.8–65.3 months). Chimerism analysis of CD3−/CD56+ peripheral blood cells did not detect circulating haploidentical NK cells after infusion. NK phenotyping was performed on seven patients during and after IL-2 infusion. We found a slight trend towards greater expression of KIR2DL2/2DL3/2DS2 (5% versus 28%, P = 0.03) at 14 days in patients who survived longer than 6 months from NK cell infusion (N = 4) when compared to those who died within 6 months of NK

Corresponding author: Dr. Katharine C. Hsu, MD, PhD; Tel: (646) 888-2667, Fax: (646) 422-0298, hsuk@mskcc.org.

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cell therapy (N=3). In summary, these data support the safety of haploidentical NK cell infusion after allogeneic HCT.

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) can result in durable remission of malignancies that arise from myeloid progenitor cells such as myelodysplastic syndrome (MDS), acute myelogenous leukemia (AML), or chronic myelogenous leukemia (CML). This is due to an increasingly recognized graft *versus* leukemia (GVL) effect mediated by alloreactive donor lymphocytes, including T-cells and natural killer (NK) cells.(1, 2) Unfortunately, relapse occurs in 40% of patients undergoing HCT for myeloid malignancies and there are limited options for these individuals.(3, 4) Chemotherapy may result in a subsequent remission but is poorly tolerated in transplant survivors who typically have a tenuous immune function and poor performance status. A second strategy to address relapse is the infusion of unmanipulated donor lymphocytes (DLI) to increase the potential for GVL. DLI results in remission of CML in approximately 70–90%; however, responses are much less frequent in MDS and occur only rarely in AML.(4–6) DLI may also induce serious graft *versus* host disease (GVHD), and thus should be used with caution. Available therapies to treat relapsed myeloid malignancy, particularly MDS and AML, therefore have low efficacy and can incur serious adverse effects. Consequently, there are few long-terms survivors of relapse after HCT.

Natural killer (NK) cells play a key role in mediating the GVL effect against myeloid malignancies, particularly AML.(7–11) An array of activating and inhibitory cell surface receptors control NK effector function against target cells, including NKG2A, NKG2D, natural cytotoxicity receptors, and the killer Ig-like receptors (KIR).(12, 13) Among these, the latter have gained considerable attention following reports associating donor KIR genotypes with NK alloreactivity and leukemia control in murine and human HLA mismatched allo HCT.(10, 14–16) NK cells stochastically express inhibitory KIR that interact with specific epitopes in class 1 HLA on target cells. KIR2DL2/3 recognize HLA-C characterized by Lys80 (HLA-C1 allotypes); KIR2DL1 recognizes HLA-C characterized by Asn80 (HLA-C2 allotypes); and KIR3DL1 recognizes HLA-B and HLA-A allotypes with the Bw4 motif.(17, 18) Additionally, the activating KIR2DS1 recognizes HLA-C2.(19) Malignant cells lacking HLA capable of binding inhibitory KIR will induce a net activating signal in NK cells leading to greater effector function in the latter. This effect is pronounced when the inhibitory KIR ligand is expressed in the transplant donor, a phenomenon referred to as activation of licensed NK cells recognizing "missing self" HLA determinants in the host.(14) NK effector function may therefore be enhanced by the intentional use of HLA-mismatched donors.

In the current study we tested whether HLA-haploidentical purified NK cells can exert a GVL effect without inducing GVHD in patients with relapsed myeloid malignancy after HLA-matched HCT. Adoptive transfer of purified NK cells offers the advantage of leukemotoxicity without the risk of GVHD, a frequent complication of unmodified DLI. Moreover, administration of a purified NK product enables the use of highly HLA-

mismatched NK cell donors, promoting the likelihood of greater anti-tumor effect. The primary endpoint of the study is to determine the feasibility and safety of adoptive transfer of haploidentical NK cells for patients with myeloid malignancy that has relapsed following HCT from an HLA-matched sibling or unrelated donor. Such a strategy may capitalize on the inherent strengths of adoptive cell transfer after HCT in that it is well tolerated and has the potential to maximize the relatively lymphodepleted post transplant recipient in order to induce GVL without serious toxicity.

METHODS

Subject eligibility, response assessment, and treatment plan

Patients of any age who had relapsed or persistent AML, MDS, or blastic CML following allogeneic HCT and who were determined to be ineligible for second HCT were eligible for this study. The study was approved by the Institution Review Board and regulatory authorities. All patients gave informed consent. The study was registered at ClinicalTrial.gov with the identifier NCT00526292. Patients were required to have 5% bone marrow involvement, determined by morphology, karyotype, or fluorescent *in-situ* hybridization (FISH). Patients with measurable extramedullary disease were excluded. Patients who received other treatments for relapsed disease were not excluded, as long as patients met bone marrow criteria prior to NK infusion. Patients with GVHD were not excluded provided they did not receive systemic immunosuppression for two weeks prior to enrollment. Toxicities were graded according to the National Cancer Institutes Common Terminology Criteria for Adverse Events, v3.0. Clinical responses were assessed by established criteria.(20, 21) Response was evaluated by bone marrow aspiration/biopsy at days +30, +100, +200, and +365. Neutrophil engraftment is defined as the first day the blood neutrophil count was determined to be >500 for 3 or more consecutive days.

Cytoreductive therapy, IL-2, and supportive care

Cytoreductive chemotherapy included cyclophosphamide 50 mg/kg/day intravenously on days −3 and −2. One patient received cyclophosphamide on days −6 and −5 in addition to fludarabine 25 mg/m^2/day on days −6 through −2. In order to promote *in vivo* expansion of donor NK cells, patients received IL-2 at a dose of 1 million units per m^2 every 48 hours for six doses beginning on day −1. NK cells were infused on day 0. Patients were treated with prophylactic antimicrobials for Pneumocystis *jiroveci*, Herpesviridae, and candida species during therapy according to institutional guidelines.

Donor selection, leukapheresis, and immunomagnetic isolation of donor-derived NK cells for adoptive transfer

Eligible donors were HLA-haploidentical family members who met standard criteria for cell donation based on FACT/NMDP guidelines and who provided informed consent. All donors underwent KIR typing as described below. The selected donor underwent a standard 10-liter apheresis the day prior to the anticipated cell infusion. Isolated peripheral blood mononuclear cells (PBMC) were used to enrich NK cells using a 2-step procedure on the CliniMACS clinical cell selection device (Miltenyi Biotech, Gladbach, Germany) as follows: PBMC were first depleted of CD3+ cells (CD3 Reagent, Miltenyi Biotec, Auburn,

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CA). The CD3-negative cell product was collected, washed once and underwent a positive selection for CD56+ cells (CD56 Reagent, Miltenyi Biotec, Auburn, CA). Viability was assessed and phenotyping was performed to evaluate the absolute numbers of CD45+, CD3+, and CD56+ cells. Cytokines were not used during *in vitro* NK cell processing. NK cells were considered acceptable for administration if the total number of CD3+ cells did not exceed $2{\times}10^5$/kg, the CD56 enrichment resulted in 90% CD3−/CD56+ pure cell product, the viability was 70%, endotoxin was 5 EU/mL, and mycoplasma, gram stain and bacterial/fungal cultures were negative. Sterility samples from the final infused product were obtained and monitored in culture for 14 days.

Immunophenotyping and cell culture conditions

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Effector cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% sodium pyruvate, and 1% 2-mercaptoethanol, supplemented with human IL-2 (Proleukin, Prometheus Laboratories Inc.; San Diego, CA) at 200 U/ml and incubated at 37°C with 5% CO_2 for 12 to 16 hours prior to assaying function. CD107 mobilization and intracellular IFN-γ production were used as indicators of effector cell activation.(22–24) PBMCs (5×10^5/well) or NK cells (1×10^5/well) were incubated with the human erythroleukemia cell line K562 (ATCC; Manassas, VA) serving as target cells at ratios of 1:5 and 1:1, respectively, for 4 hours in 96-well U-bottom plates with 200 μL medium, described above, per well. APCH7-conjugated anti-CD107a (clone H4A3, BD Biosciences; Franklin Lakes, NJ) was added to each well prior to incubation. Cells were stained with anti-CD3-BV650 (clone SK7, BD Biosciences), anti-CD56-ECD (clone N901, Beckman Coulter; Brea, CA); anti-KIR2DL1/2DS1-PEcy5.5 (clone EB6B, Beckman Coulter), anti-KIR2DL2/2DL3/2DS2-FITC (clone CH-L, BD Biosciences), anti-KIR3DL1/S1-APC (clone Z27, Beckman Coulter), anti-KIR3DL1–Alexa Fluor 700 (clone DX9, Biolegend; San Diego, CA); anti-NKG2A-PEcy7 (clone Z199, Beckman Coulter); anti-LIR-1-PE (clone HP-F1, Beckman Coulter). For IFN-γ evaluation, brefeldin-A (2 μg/ml, Sigma-Aldrich; St. Louis, MO) and GolgiStop (1 μg/ml, BD Biosciences) were added to the mixture 1 hour following incubation, and FIX & PERM (Invitrogen; Carlsbad, CA) was used for staining. Cells were analyzed using multicolor flow cytometry on a FACS LSRFortessa instrument with FACS Diva software (BD Biosciences). Results were interpreted using FlowJo software (FlowJo; Ashland, OR) and GraphPad Prism (GraphPad Software; La Jolla, CA).

RESULTS

Study population

The study population is described in Table 1. The median age at treatment was 19.0 years (range 1.9–55.9). Seven patients underwent transplantation from a HLA-matched sibling and one from a HLA-matched unrelated donor. The median time to relapse after HCT was 3.5 months (range 1–94 months). The median time from HCT to NK infusion was 6.8 months (range 3.9–152 months). Five of eight patients had cytoreductive chemotherapy prior to NK infusion. One patient with extramedullary AML had surgical cytoreduction but had residual disease at the time study enrollment. One patient was treated with azacytidine followed by

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infusion of donor lymphocytes without response prior study enrollment. Two patients had GVHD of the skin at the time of enrollment but did not require systemic immunosuppressive therapy. The remaining patients had been tapered from systemic immunosuppression prior to enrollment without evidence of GVHD.

NK Cell Enrichment

Leukocytes were successfully collected from all donors. The median number of total nucleated cells (TNC) collected was 130×10^8 (106–147). The CD3−CD56+ cell content ranged from 4.7–10% of the TNC. After immunomagnetic enrichment the number of contaminating CD3+ cells composed <0.1% of the cell product in all instances. The median (range) dose of CD3+ cells/recipient kg was 2.0×10^3 ($0.9–40 \times 10^3$) and CD3−CD56+ cells/recipient kg was 11×10^6 ($4.3–22.4 \times 10^6$) Table 2. The CD3−CD56+ viability ranged from 82–100%.

Response, neutrophil recovery, and toxicity

Survival of patients treated is shown in Figure 1A. Three patients achieved responses. Patients 5 and 8 developed CR after treatment, with relapse at 1.7 and 1.8 months post treatment, respectively. Both patients survived 20.2 months after haploidentical NK cell transfer. Patient 1 had donor-derived MDS associated with deletion of chromosome 20q (del20q) after HCT for lymphoma. After haploidentical NK cell transfer the patient had resolution of dysplastic features but persistent del20q. Among the 5 patients without response the median survival was 5.4 months (range 0.8–30.2 months). Patient 2 underwent second allo HCT 18 months after NK infusion. He relapsed 9.0 months from second transplant and died after failed re-induction 30.2 and 12.0 months from NK infusion and second HCT, respectively.

The median time to neutrophil recovery was 19 days, (Figure 1B). One patient died without neutrophil recovery due to progressive leukemia. No patient developed infusion reaction to the NK cell product. Two patients experienced non-hematologic adverse events: patient 1 developed grade 4 streptococcal lower respiratory tract infection during neutropenia that required intubation and mechanical ventilation, but later resolved. Patient 4 developed grade 2 colitis secondary to Clostridium *difficile* that resolved with directed therapy. All patients were dependent on blood and platelet transfusions at the time of study enrollment. Among the two patients with cutaneous GVHD, one person developed resolution of symptoms after treatment. There were no cases of GVHD after NK infusion.

NK Cell Recovery, Licensing, Chimerism and Immunophenotype

We performed donor/recipient/3rd party chimerism studies on purified CD3−/CD56+ blood cells using polymerase chain reaction amplification of short tandem repeats (STR) at 3, 7, 14, 30, and 60 days post NK cell infusion. We did not detect haploidentical NK cells in any patient post infusion. Four patients had detectable allo HCT donor derived CD3−/CD56+ cells after NK cell infusion. The remaining patient had recipient derived NK cells. In order to examine the effects of IL-2 infusion on NK cell function we examined the phenotype of blood NK cells after infusion in 7 patients (Figure 2). One patient did not undergo NK phenotyping due to early relapse. We found no association between expression of specific

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NK phenotypes and overall response. Expression of inhibitory KIR was not increased in patients missing respective ligands. We found a slight trend towards greater expression of KIR2DL2/2DL3/2DS2 (5% versus 28%, P = 0.03) and LIR1 (8% versus 29%, P = 0.09) at 14 days in patients who survived longer than 6 months from NK cell infusion (N = 4) when compared to those who died within 6 months of NK cell infusion (N=3). NK effector function at early time points was not associated with response nor was the relative size of the CD56+ peripheral blood population.

DISCUSSION

In the current study we report outcomes in eight patients treated with cyclophosphamide followed by haploidentical NK cells for relapsed AML or MDS after HCT. Here we demonstrate transient responses in 2 individuals and morphologic resolution of dysplasia in a third individual. These results are consistent with other reports of individuals treated with adoptive NK cell infusion after transplantation.(25, 26) One important difference in our study is the use of haploidentical NK cells after HLA-matched HCT. Despite undergoing lymphocyte depleting chemotherapy, we noted no episodes of GVHD after HLA-mismatched NK cell infusion. These results are supportive of the safety of this cell product in a high-risk population of individuals.

The two-step immunomagnetic enrichment of NK cells resulted in a highly pure NK cell product with good viability as described above. We were able to achieve a higher dose of NK cells in the pediatric patients compared to the adult patients. Whether greater numbers of infused NK cells would improve efficacy is unknown. Early reports of adoptive NK cell therapies in children suggest higher overall response rate (27), which may be due to the higher number of infused NK cells per recipient weight in children; however, this was not seen in our smaller series. Several groups are investigating *ex vivo* NK expansion using platforms both with and without feeder cells.(28–30) Ex vivo expansion may result in a lower activation threshold, but could also decrease mediators of homing necessary to direct NK cells to tumor sites.(31) More importantly, recent evidence suggests that certain *ex vivo* expansion methods may increase the likelihood of acute GVHD after NK cell infusion.(32) Further work and perhaps comparative studies are warranted in the future to evaluate freshly collected *versus* cultured NK cell products.

A major limitation to NK cell infusion is the difficulty detecting donor NK cells after transfer, presumed to reflect poor *in vivo* persistence. (33) Haploidentical donor NK cells were not detected in the blood of any patient in this series. Lack of persistence of transferred NK cells may be attributed to either an inadequate number of infused cells, inhibition from cell populations such as T-regulatory or myeloid suppressor cells, or competition for cytokines from other lymphocyte populations. We did not examine blood cytokines or T-regulatory cell populations in the patients in this series, however, IL-2 infusion here may have increased their number and function in study subjects, paradoxically inhibiting NK cell function. Bachanova and colleagues recently reported that T-regulatory-directed therapy with IL-2/diphtheria toxin recombinant protein increased the persistence of donor NK cells after transfer.(34) While feasible, such an approach requires caution in the post-HCT patient due to the potential to induce GVHD. The use of activating cytokines may induce GVHD in

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the context of allogeneic NK cell products.(32) Therefore, caution is warranted, particularly with the use of activated, HLA-mismatched cell products. Here we were able to demonstrate safety but not efficacy in this setting. To address this problem, NK cells may be more effective as prophylaxis against overt relapse, an approach that has recently yielded promising results in 16 individuals who received donor NK cells after T-cell depleted haploidentical transplantation.(26)

We examined the phenotype and function of blood NK cells after haploidentical NK cell infusion. Chimerism studies demonstrate that the NK cells studied here were recipient or original alloHCT donor, therefore, these results are relevant to the activation state during IL-2 therapy but do not reflect function of the infused cell product. We did not note any trends in NK phenotyping, including for KIR, in patients with or without a response. Effector function was relatively stable over time and was not associated with clinical response. Interestingly, lack of a KIR ligand (HLA-C1, C2, or Bw4) in the recipient was not associated with detectable expansion of cognate KIR+ NK cells. Our sample size is too small to draw conclusions with respect to the role of missing ligand in haploidentical NK cell transfer. Curti and colleagues treated 13 AML patients with haploidentical NK cells following cyclophosphamide and fludarabine, using recipient HLA and donor KIR gene expression to select recipients lacking KIR-ligands for donor KIR. One individual with active disease experienced a transient response and 3/6 individuals treated in CR remained so in long-term follow up. These findings require larger scale studies to confirm whether donor KIR profile may be used to increase efficacy of adoptive NK cell products.

In summary these data are supportive of the safety of haploidentical NK cells for myeloid malignancy after HCT. Further efforts to improve the efficacy of this product will incorporate donor selection to maximize NK alloreactivity, improved timing of NK cell infusion, and *ex vivo* manipulations to improve NK numbers and activation state.

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Highlights

- Haploidentical NK cells were administered to allogeneic transplant recipients
- Responses were seen in 3 of 8 treated patients with relapsed malignancy
- No patient developed graft *versus* host disease
- Haploidentical NK cells did not persistent despite immunocompromised recipients

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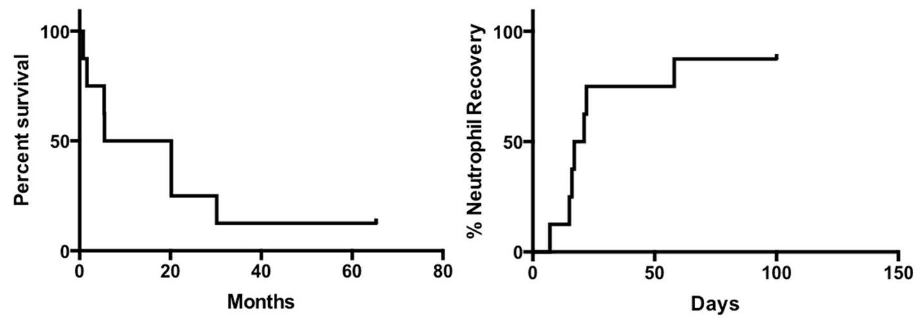

Figure 1.
Overall survival (A) and neutrophil recovery (B) in treated patients.

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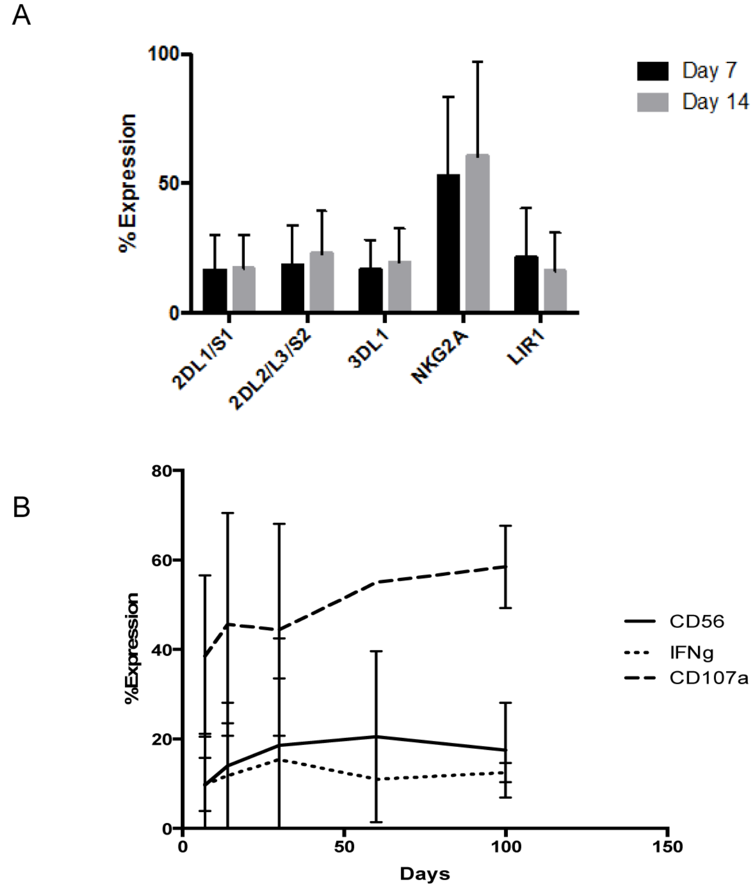

Figure 2.

Peripheral blood NK cells were obtained from study subjects after haploidentical cell infusion. Chimerism analysis demonstrated either autologous or original allo HCT donor recovery in all subjects. (A) KIR expression on these cells was consistent during and after IL-2 administration. (B) The fraction of CD3−/CD56+ NK cells as well as their activation state consistent after haploidentical NK cell infusion. NK activation state was assessed via CD107a expression and IFN-γ release after a 24 co-incubation with K562 cells.

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TABLE 1

Characteristics of Study Participants

Patient	Disease	HCT Donor	NK Donor	Age (y)	Karyotype	Pre-treatment blasts	Time to relapse (m)	Time relapse to NK infusion (m)	Acute GVHD (grade)	Therapy for post HCT relapse
1	MDS	Matched sib	Child	53.8	del 20q	0%	93.6	58.0	None	DLI, azacitidine
2	AML	Matched sib	Sibling	55.9	del 20q	0%	33.2	10.0	Skin (2)	Surgical resection
3	MPAL*	Matched sib	Parent	29.7	Complex	1%	5.2	1.3	None	Chemotherapy
4	AML	Matched Sib	Parent	8.4	t(9;11), del 20q	75%	2.4	1.5	Skin (2)	Chemotherapy
5	AML	Matched sib	Parent	2.0	t(1;5), +20q	21%	3.1	5.6	None	Chemotherapy
6	AML	Matched sib	Parent	1.9	t(9p;12p)	23%	3.0	4.2	None	Chemotherapy
7	AML	Matched sib	Parent	2.5	t(3q;11p)	71%	3.8	2.7	None	Chemotherapy
8	MDS	URD	Sibling	32.3	Complex	8%	1.0	4.3	None	None

*Mixed phenotype acute leukemia

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TABLE 2

Donor Cell Dose and KIR Gene Profile

Patient	CD3−/CD56+ per kg ×10^{6}	CD3+/kg × 10^{3}	Viability %	CD3−CD56+ %	CD3−CD56+ Yield %	Donor KIR Haplotype	Recipient Missing Ligand
1	9.2	1.9	93	98.6	64.4	B,B	None
2	4.3	0.9	82	97.8	59.0	A,B	Bw4
3	5.77	0.58	84	99.9	40.2	B,B	C2
4	12	7.2	92	99.7	86.8	A,B	Bw4, C2*
5	20	3.3	89	99.8	73.0	A,B	None
6	19.9	40	88	99.9	41.3	A,A	Bw4, C2*
7	22.4	2.0	100	96.7	50.9	A,B	None
8	5.36	2.2	88	98.5	41.7	A,B	Bw4

* Ligand(s) expressed in donor