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Haploidentical Natural Killer Cells Infused before Allogeneic Stem Cell Transplantation for Myeloid Malignancies: A Phase I Trial

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A B S T R A C T

Allogeneic stem cell transplantation is an effective treatment for high-risk myeloid malignancies, but relapse remains the major post-transplantation cause of treatment failure. Alloreactive natural killer (NK) cells mediate a potent antileukemic effect and may also enhance engraftment and reduce graft-versus-host disease (GVHD). Haploidentical transplantations provide a setting in which NK cell alloreactivity can be manipulated, but they are associated with high rates of GVHD. We performed a phase I study infusing escalating doses of NK cells from an HLA haploidentical–related donor—selected for alloreactivity when possible—as a component of the preparative regimen for allotransplantation from a separate HLA-identical donor. The goal of infusing third-party alloreactive NK cells was to augment the antileukemic effect of the transplantation without worsening GVHD and, thus, improve the overall outcome of hematopoietic transplantation. Twenty-one patients with high-risk acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), or chronic myelogenous leukemia refractory or beyond first remission received a preparative regimen with busulfan and fludarabine followed by infusion of apheresis-derived, antibody-selected, and IL-2–activated NK cells. Doses were initially based on total nucleated cell (TNC) content and later based on $CD56^+$ cells to reduce variability. $CD56^+$ content ranged from .02 to 8.32 $\times$ 10^6/kg. IL-2, .5 $\times$ 10^6 units/m^2 subcutaneously was administered daily for 5 days in the final cohort (n = 10). $CD3^+$ cells in the NK cell product were required to be $< 10^5$/kg. Median relapse-free, overall, and GVHD-free/relapse-free survival for all patients enrolled was 102, 233, and 89 days, respectively. Five patients are alive, 5 patients died of transplantation-related causes, and 11 patients died of relapse. Despite the small sample size, survival was highly associated with $CD56^+$ cells delivered (P = .022) and development of $\geq$ grade 3 GVHD (P = .006). There were nonsignificant trends toward higher survival rates in those receiving NK cells from KIR ligand–mismatched donors and KIR-B haplotype donors. There was no association with disease type, remission at time of transplantation, or KIR content. GVHD was not associated with TNC, $CD56^+$, or $CD3^+$ cells infused in the NK cell product or the stem cell product. This trial demonstrates a lack of major toxicity attributable to third-party NK cell infusions delivered in combination with an HLA-compatible allogeneic transplantation. The infusion of haploidentical alloreactive NK cells was well tolerated and did not interfere with engraftment or increase the rate of GVHD after allogeneic hematopoietic transplantation. Durable complete remissions occurred in 5 patients at high risk for disease recurrence. This approach is being further developed in a phase I/II trial with ex vivo–expanded NK cells to increase the NK cell dose with the objective of reducing relapse and improving the outcome of allogeneic hematopoietic transplantation for AML/MDS.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is effective for myeloid malignancies supporting administration of high-dose chemotherapy and inducing an immunologic

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graft-versus-leukemia (GVL) effect. However, relapse remains the major post-transplantation cause of treatment failure [1].

Natural killer (NK) cells have been appreciated as contributing to the GVL effect without directly causing graft-versus-host disease (GVHD) [2]. NK cell number, as measured by the dose in the stem cell graft or recovery after transplantation, has been associated with a decreased relapse rate [3,4]. NK cells are governed by activating and inhibitory receptors. NK cells can be selected for increased alloreactivity by mismatch of licensed inhibitory receptors in a setting of missing HLA ligands (KIR receptor:ligand mismatch); these cells may have more potent GVL activity and may also enhance engraftment and reduce GVHD [5] by eliminating host T cells and antigen-presenting cells required for priming a GVHD response [6]. When GVHD is established, however, NK cells may cooperate with the adaptive immune response and exacerbate GVHD [7]. In addition to the release of inhibition caused by missing self, NK cells respond to activating signals to trigger lysis of tumor targets. Activating ligands of natural killer group D 2 (NKG2D) (MHC class I polypeptide-related sequence A (MIC) and UL16 binding protein (ULBP) family members) are upregulated by virus-infected and malignant cells as a consequence of stress [8] and may be further upregulated through genotoxic stress caused by radiation or chemotherapy, sensitizing tumors to NK cell lysis [9].

Haploidentical donors may be selected for the presence of KIR-ligand mismatch, thereby establishing a setting in which the donor NK cells are reactive against recipient tumor cells because of a missing KIR ligand. Haploidentical stem cell transplantation has historically been complicated by excessive GVHD, infection, and treatment-related mortality [10]. We hypothesized that haploidentical third-party NK cells could be added to an HLA-identical hematopoietic transplantation to increase GVL effects without exacerbating GVHD. We designed a phase I clinical trial to determine whether haploidentical NK cells could be safely administered after high-dose chemotherapy and before an HLA-matched allogeneic HSCT, a time of maximum stress sensitization and minimum disease burden.

MATERIALS AND METHODS

Patient Population

Twenty-one patients with high-risk myeloid malignancies were enrolled on protocol 2005-0508 (NCT00402558, phase I dose escalation) or 2010-0099 (NCT01390402, phase II expansion) to receive a 10/10 HLA–matched allograft between October 2006 and June 2013. *High-risk disease* was defined as acute myeloid leukemia (AML) past first remission or primary induction failure, myelodysplastic syndrome (MDS) with intermediate or high-risk international prognostic scoring system score, or chronic myelogenous leukemia (CML) that had failed control with tyrosine kinase inhibitor or in accelerated or blast phase. Patients were required to meet standard institutional transplantation criteria for cardiac, liver, renal, and pulmonary organ function, and they were initially required to be $\leq$ 60 years of age. The age requirement was later extended to age $\leq$ 70 years.

Hematopoietic progenitor cells were obtained from granulocyte colony–stimulating factor–mobilized peripheral blood collected by apheresis. The protocol required collecting peripheral blood stem cells of at least 6×10^6 $CD34^+$ cells/kg recipient weight (goal 8×10^6 $CD34^+$ cells/kg). On day 0, 4×10^6 $CD34^+$ cells/kg were infused, retaining at least 2×10^6 $CD34^+$ cells/kg as a backup in case of graft failure. Donors were related (n = 13) or unrelated (n = 8). Hematopoietic stem cells procured from unrelated donors were obtained through the National Marrow Donor Program.

For the purpose of selecting haploidentical NK cell donors from available family members, NK cell alloreactivity was initially determined using the KIR ligand:ligand mismatch model, defined as the presence of a KIR-ligand (HLA group C1, C2, or Bw4) in the donor that was missing in the recipient. KIR genotyping for the selected donor was then obtained to confirm KIR mismatch by establishing the presence of the KIR-receptor gene relevant to the mismatched KIR ligand. Donors were required to have mismatch for the phase I dose-escalation study. Because many studies report that KIR:KIR ligand mismatch is not required for a response to treatment with NK cells, the phase II expansion study preferred—but did not require—a mismatched donor when possible.

The protocols were approved by the institutional review board of MD Anderson Cancer Center and conducted under Investigational New Drug applications from the Food and Drug Administration. All patients and NK cell donors provided written informed consent to the protocol.

Transplantation Regimen

All patients received fludarabine 40 mg/m^2/day and busulfan 130 mg/m^2/day (adjusted for ideal body weight) for 4 days on days −13 through −10. NK cells were infused on day −8. Thymoglobulin (Sanofi, Bridgewater, NJ) 1.5 mg/kg/day was given on days −3 to −1 to all patients for GVHD prophylaxis and to prevent the NK cells from hindering engraftment. Tacrolimus was started on day −2 and discontinued after 3 months if there was no evidence of GVHD. Methotrexate 5 mg/m^2 was given on days 1, 2, 6, and 11. All patients received granulocyte colony–stimulating factor 5 mcg/kg/day starting on day 7 and until absolute neutrophil counts were $> 500 \times 10^9$/L for 3 consecutive days. Standard antimicrobial prophylaxis was provided with voriconazole, pentamidine or trimethoprim-sulfamethoxazole, and acyclovir or valacyclovir for fungal, pneumocystis jiroveci, and herpes simplex, respectively.

NK cell product

The NK cell product was produced from a steady state apheresis product of up to 2×10^{10} peripheral blood mononuclear cells by first depleting T cells using the CliniMACS (Miltenyi Biotec, Auburn, CA) device and magnetic-activated cell sorting colloidal super-paramagnetic anti-CD3 monoclonal antibody (Miltenyi Biotec, Auburn, CA). A second-step CD56-positive selection was performed for the first 3 patients but discontinued thereafter to improve cell yield. The NK cell product was then cultured overnight ($\sim$16 hours) in complete media containing 1000 IU/mL recombinant human IL-2 (Proleukin; Chiron, Emerysville, CA) and washed twice with normal saline using a COBE 2991 cell processor (COBE BCT, Lakewood, CO) before intravenous infusion.

Patients were treated in 4 dose levels of the NK cell–enriched product based on total nucleated cell (TNC) content, as follows: (1) 10^6 cells/kg, (2) 5×10^6/kg, (3) 3×10^7/kg, and (4) 3×10^7/kg (or all cells collected) followed by systemic IL-2 .5 million units/m^2 subcutaneously daily for 5 days. A subsequent phase II study in CML patients used a fixed dose of 5×10^6 $CD56^+$/kg, which was the maximum dose that could be routinely obtained from a steady-state apheresis from normal donors. The median number of NK cells infused at dose levels 3 and 4 was 5×10^6/kg (range, .97 to 8.32). $CD3^+$ cells in the NK cell product were required to be $< 10^5$/kg (median infused, 0×10^5/kg; range, 0 to 1.7).

Trial Design

The primary objective of 2008-0508 was to assess the safety of infusing alloreactive NK cells from a haploidentical relative and to determine the maximum tolerated dose (MTD) of these cells given in combination with busulfan, fludarabine, Thymoglobulin, and allogeneic transplantation from a separate HLA-identical related donor for treatment of AML/MDS. The secondary objectives were to determine if infusion of alloreactive haploidentical NK cells with busulfan and fludarabine/antithymocyte globulin will improve progression-free survival after allogeneic stem cell transplantation from an HLA-compatible donor compared with historical controls, and to determine the rate of engraftment, GVHD, immune reconstitution, and survival after infusion of alloreactive haploidentical NK cells.

The trial was designed as a phase I dose-escalation study followed by a cohort expansion at the identified MTD, or maximum feasible dose if no MTD were reached. The continual reassessment method was used for dose finding, with a target probability for transplantation-related mortality of .30, the baseline rate seen historically in this patient population for this regimen without NK cells.

HLA and KIR Typing and Determination of KIR Content/Matching

Patients and stem cell and NK cell donors were HLA typed at the intermediate-resolution level for alleles at HLA-A, -B, -C, -DRB1, and -DQB1 loci by PCR amplification and oligonucleotide hybridization using commercial kits from Invitrogen (Carlsbad, CA), ELPHA, and/or One Lambda (Canoga Park, CA). The patients and selected donors were typed for the same loci by high-resolution methods using PCR amplification and nucleotide sequencing (Abbott, Abbott Park, IL, or Protrans, Hockenheim, Germany).

KIR genotyping was performed for the selected NK cell donors with reverse sequence-specific oligonucleotide methodology using fluorescently labeled beads conjugated to oligonucleotide probes (One Lambda). KIR typing was performed in 17 of the 21 donors. The revised typing kit allowing

discrimination of functional (Func) versus deletion (Del) variants of KIR2DL4 was used for 15 donors. KIR typing was not performed for stem cell donors.

KIR-ligand:ligand mismatch was predicted using the KIR Ligand Calculator maintained by the European Bioinformatics Institute of the European Molecular Biology Labs (http://www.ebi.ac.uk/ipd/kir/ligand.html). KIR receptor-ligand mismatch was refined by the ligand-ligand mismatch prediction on the basis of the KIR-receptor gene being present in the donor as determined by sequence-specific oligonucleotide genotyping.

KIR-B content was determined using the B Content Calculator maintained by European Molecular Biology Labs (http://www.ebi.ac.uk/ipd/kir/donor_b_content.html). B content was calculated using absence/presence of KIR2DL4 regardless of the Del/Func variant, or by considering the DEL variant as equivalent to being absent. Activating KIR content was determined by scoring the total number of activating KIR genes as determined by the KIR genotyping of the NK cell donor. All short (DS)-designated KIR and KIR2DL4 were considered activating. KIR2DL4 was not counted as activating if the KIR2DL4-Del variant was confirmed. Donor-recipient pairs that did not have KIR typing performed were not included in the B content or activating KIR analysis.

NK cell Function/Phenotype

Aliquots of NK cells produced for the 6 patients on protocol 2010-0099 were cryopreserved for additional testing of function and phenotype. Murine antihuman NKp30, NKp44, NKp46, CD3, CD11b, CD16, CD27, CD56, CD160, NKG2D, DNAM-1, and 2B4 and isotype control mAb were obtained from BD Biosciences (Bedford, MA). Murine anti-human KIR2DL2/3 was obtained from Miltenyi (Auburn, CA). Murine antihuman KIR2DL1 and KIR3DL1 were obtained from R&D Systems (Minneapolis, MN). For direct surface staining, cells were thawed and rested overnight in media containing 100 IU/mL IL-2, then incubated with indicated antibodies for 30 minutes at 4°C, washed, and resuspended in staining buffer. Data were acquired using a FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

As previously described, 721.221 cell lines were obtained [11]. The parental K562 cell line was obtained from the American Type Culture Collection. NK cell cytotoxicity was determined using the calcein release assay as previously described [12]. Briefly, target cells were labeled with .5 μg/mL to 5 μg/mL (titrated for each target cell line) of calcein-AM (Sigma-Aldrich) for 1 hour at 37°C with occasional shaking. Cells were cocultured at the indicated effector-to-target ratios and incubated at 37°C for 4 hours. After incubation, 100 μL of the supernatant was harvested and transferred to a new plate. Absorbance at 570 nm was determined using a SpectraMax Plus^{384} spectrophotometer. The percent lysis was calculated according to the formula ([experimental release−spontaneous release]/[maximum release−spontaneous release]) × 100.

Statistics

Descriptive statistics are presented for patients, NK cell donors, and stem cell donors. The Kaplan-Meier method was used to estimate engraftment kinetics, overall survival, relapse-free survival (RFS), and GVHD-free and RFS (GRFS) [13]. The log-rank test was used to assess differences between groups for RFS. All statistical analyses were performed using Prism 6 for Mac (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Demographics

Patient characteristics are summarized in Tables 1 and 2. Twenty-one patients with high-risk AML, MDS, or CML were enrolled. The median age was 51 years (range, 2 to 63). Fourteen had active disease and 7 were in a first or second complete remission/complete cytogenetic response (CCR) at the time of transplantation. Patients with AML, MDS, and CML were enrolled, although all CML patients were enrolled on dose level 4 and accounted for 7 of the 10 patients on that dose level.

All stem cell donors were matched for 10/10 HLA A, B, C, DRB1, and DQB1 alleles. Eight stem cell donors were unrelated and 13 were siblings.

NK cell donors were all haploidentical related donors. Six were parents, 9 were siblings, and 6 were children. Because of protocol requirements during the dose-escalation phase, 16 NK donor-recipient pairs had KIR mismatch and 5 did not. Three were mismatched for group C1, 8 for group C2, 4 for Bw4, and 1 for both group C1 and Bw4.

Table 1
Patient Demographics and Graft Characteristics

Characteristic	Value
Age, median (range), yr	51 (2-63)
Gender, n (%)	
M	15 (71)
F	6 (29)
Disease type, n (%)	
AML	8 (38)
MDS	6 (29)
RAEB-1	3 (14)
CMML	1 (5)
Unclassified/other	2 (10)
Treatment-related	3 (14)
CML	7 (33)
Accelerated phase	2 (10)
Chronic phase	4 (19)
Complex cytogenetics	1 (5)
Monosomy 7	1 (5)
Cytogenetics (AML/MDS), n (%)	
Poor	6 (29)
Intermediate	3 (14)
Good	5 (24)
Remission status at transplantation, n (%)	
NR	14 (67)
CR/CCR	7 (33)
Prior therapies, median (range)	2 (0-6, 1 prior transplantation)
HSCT donor, n (%)	
Matched sibling	13 (62)
Matched unrelated	8 (38)
NK cell donor, n (%)	
Parent	6 (29)
Sibling	9 (43)
Child	6 (29)
NK product, median (range)	
TNC, × 10^6/kg	29.88 (.98-42.5)
CD3, × 10^6/kg	0 (0-.17)
CD3, %	.02 (0-.62)
CD14, × 10^6/kg	11.41 (.24-20.75)*
CD14, %	41.77 (9.43-53.15)*
CD19, × 10^6/kg	6.2 (.1-21.99)*
CD19, %	21.84 (9.62-73.04)*
$CD56^+CD3^-$, × 10^6/kg	2.96 (.02-8.32)
$CD56^+CD3^-$ %	14.1 (1.5-27.5)*
Stem cell product	
TNC, × 10^8/kg	8.07 (1.05-20.21)
CD3, × 10^6/kg	201 (11.02-886.7)
CD34, × 10^6/kg	4.8 (3.63-12.47)
NK product cell dose level/kg, n (survivors)	
10^6	6 (1)
5×10^6	2 (0)
3×10^7	3 (1)
3×10^7 + IL-2	10 (3)

M indicates male; F, female; RAEB, refractory anemia with excess blasts; CMML, chronic myelomonocytic leukemia.

* Excludes CD56-selected products. The 3 CD56-selected product were > 90% $CD56^+$.

Cell Products

The stem cell product infused contained a relatively high TNC dose and $CD34^+$ cell dose, with a median of 8.07×10^8/kg (range, 1.05 to 20.21) and 4.8×10^6/kg (range, 3.63 to 12.47), respectively. The T cell dose was similarly high, with a mean 201×10^6/kg (range, 11.02 to 886.7) (Tables 1, 2).

The NK cell products were delivered at the doses prescribed by the protocol according to TNC in the phase I trial and according to $CD56^+$ in the phase 2 trial. The median $CD56^+CD3^-$ content of the NK cell products was 15.2%, with a wide variation in NK cell content, as might be expected from the wide variability in peripheral blood NK cells in the normal population. The T cell depletion of the apheresis product was 99.8% efficient, reducing the median $CD3^+$ cell

Table 2
Patient Disease Characteristics, Survival, NK Cell Product Characteristics, and Donor Classification

Protocol	Sex	Age at Transplantation	Diagnosis	Cytogenetic/Molecular	Prior Lines of Therapy	KIR-L MM	KIR-B (Func/Del)	KIR Content	Selection Method	Dose Level	IL-2	TNC/kg, $\times 10^6$	$CD3^-CD56^+$/kg, $\times 10^6$	$CD3^+$/kg, $\times 10^6$	Alive	Cause of Death	EFS
2005-0508	M	2	AML-M7	47,XY,−4,+7(q36),−9,−15,−17,+(18)(q23),ad(19)(p13.3),+21c,+3mar; 42-47,idem,+r,+0-4mar	5	C2	Best	7	CD3 depletion, CD56 selection	10^6 TNC/kg	No	.99	.96	.006	N	Persistence of disease	8
2005-0508	F	25	AML-M4	46,X,−X,−10,add(10)(q22),add(12)(p12),+14,+mar[4],i(21)(q10)add(21)(p11.2)+mar; FLT-3(+)	3	matched	Neutral	2	CD3 depletion, CD56 selection	10^6 TNC/kg	No	1.02	.92	.000	N	Relapse	89
2005-0508	M	36	MDS/AML	46,XY,del(3)(p12p23),del(4)(q21q23),−7,add(17)(p13),+mar	0	C2	Neutral	4	CD3 depletion, CD56 selection	10^6 TNC/kg	No	1.03	.98	.000	N	Relapse	84
2005-0508	M	2	AML-M5	46,XY,der(5)t(5;8)(q35;q13),del(6)(q23q25)[14]; MLL(+)	2	C1	Better (better)	6	CD3 depletion	10^6 TNC/kg	No	1.02	.02	.000	N	Relapse	131
2005-0508	M	21	AML-M2	46,XY	2	C2	ND	ND	CD3 depletion	10^6 TNC/kg	No	.98	.23	.000	Y		2251
2005-0508	F	31	AML-M5	46,XX; RAS(+)	5	C2	Better	5	CD3 depletion	10^6 TNC/kg	No	.99	.09	.000	N	Relapse	38
2005-0508	M	53	tMDS-RAEB-1	46,XY	2	Bw4	ND	ND	CD3 depletion	5×10^6 TNC/kg	No	5.02	.44	.011	N	Relapse	60
2005-0508	M	53	tMDS	45,XY,del(1)(q21),der(3)t(1;3)(q21;p21)ins(3;5)(p13;?),der(5)t(3;5)(p21;q13),del(7)(q22q34),−10,−12	1	Bw4	Neutral	2	CD3 depletion	5×10^6 TNC/kg	No	5.04	.97	.004	N	Persistence of disease	51
2005-0508	M	57	CMML	46,XY[20]; RAS(+); FLT-3(+)	1	C2	Neutral (neutral)	2	CD3 depletion	3×10^7 TNC/kg	No	30.2	5.15	.000	Y		1812
2005-0508	M	53	tMDS	45,Y,−X,add(3)(p13),add(5)(q31),del(6)(q13),del(7)(q22q34),−12,−16,+mar1,+mar2	0	Bw4	Neutral	4	CD3 depletion	3×10^7 TNC/kg	No	30.1	4.77	.000	N	Persistence of disease	33
2005-0508	F	51	AML-M4	46,XX, inv(16)(p13.1q22)	2	C2	Neutral	5	CD3 depletion	3×10^7 TNC/kg	No	30.0	.97	.000	N	Sepsis	84
2005-0508	M	57	MDS-RAEB-1	46,XY	2	C1	ND	ND	CD3 depletion	3×10^7 TNC/kg	Yes	30.3	8.32	.173	N	Relapse	997
2005-0508	M	60	MDS-RAEB-1	46,XY	1	C2, Bw4	Neutral (better)	1	CD3 depletion	3×10^7 TNC/kg	Yes	30.0	4.04	.000	Y		1473
2005-0508	F	55	CML	46,XX,t(9;22)(q34;q11.2); 46,sl,inv(3)(q21q26.2); 46,sl,del(11)(q23q25); 47,sl,+8	3	C2	ND	ND	CD3 depletion	3×10^7 TNC/kg	Yes	29.8	1.87	.000	N	Relapse	42
2005-0508	M	63	MDS/AML-M0	46,XY	2	C1	Neutral (better)	4	CD3 depletion	3×10^7 TNC/kg	Yes	30.0	6.95	.024	Y		343
2010-0099	F	50	CML	46,XX,t(9;22)(q34;q11.2)	5	matched	Neutral (neutral)	2	CD3 depletion	5×10^6 NK/kg	Yes	35.6	5.03	.068	N	Chronic GVHD, cirrhosis, liver failure	144
2010-0099	F	42	CML	46,XX,t(9;22)(q34;q11.2); BCR-ABL(+)	2	Bw4	Neutral (Better)	4	CD3 depletion	5×10^6 NK/kg	Yes	27.2	4.97	.027	Y		767
2010-0099	M	55	CML	BCR-ABL(+)	2	matched	Better (better)	6	CD3 depletion	5×10^6 NK/kg	Yes	34.5	5.01	.014	N	Relapsed, developed aGVHD after DLI off study	102

(*continued on next page*)

Table 2
(*continued*)

Protocol	Sex	Age at Transplantation	Diagnosis	Cytogenetic/Molecular	Prior Lines of Therapy	KIR-L MM	KIR-B (Func/Del)	KIR Content	Selection Method	Dose Level	IL-2	TNC/kg, $\times 10^6$	$CD3^-CD56^+$/kg, $\times 10^6$	$CD3^+$/kg, $\times 10^6$	Alive	Cause of Death	EFS
2010-0099	M	27	CML	Monosomy 7; BCR-ABL(+)	2	matched	Neutral (indeterminate)	1	CD3 depletion	5×10^6 NK/kg	Yes	37.8	5.00	.034	N	Relapse	76
2010-0099	M	25	CML	46,XY,t(9;22)(q34;q11.2)	2	matched	Better (better)	5	CD3 depletion	5×10^6 NK/kg	Yes	42.5	5.03	.000	N	Relapsed, developed aGVHD post DLI off study	233
2010-0099	M	53	CML	46,XY,t(9;22)(q34;q11.2); BCR-ABL(+)	6	C2	Neutral	2	CD3 depletion	5×10^6 NK/kg	Yes	22.0	5.00	.000	N	Relapse	180

KIR-L MM indicates KIR ligand mismatch; EFS, event-free survival; N, no; ND, not done; Y, yes; tMDS, therapy-related myelodysplastic syndrome; aGVHD, acute graft-versus-host disease; DLI, donor lymphocyte infusion.

content from 53.7% to .015%, resulting in a mean T cell dose of the NK cell products that was 1/10,000 that of the stem cell products (.018 versus 233 $\times$ 10^6/kg). The NK cell products also varied widely in distribution of $CD16^+$ versus $CD16^-$ subsets (Figure 1A), cytotoxic function (Figure 1B), and KIR and CD160 expression. There was minimal variability in the expression of activating receptors or the relative proportions of immature versus mature subsets as defined by CD11b and CD27 (Figure 1C). Surprisingly, the percentage of NK cells expressing a given inhibitory receptor was not associated with whether that KIR was licensed (as predicted by the donor HLA/KIR-ligand genotype) (Figure 1D).

NK cell Infusions

Only mild toxicities occurred with the NK cell infusions. Two patients experienced grade 2 fever, 2 patients experienced grade 1 rigor/chills, and 1 patient experienced a grade 2 allergic reaction for which the infusion was held and diphenhydramine given before completing the infusion. The 10 patients at dose level 4 tolerated IL-2 systemic treatment, .5 million units/m^2 subcutaneously daily for 5 days without major toxicity. Four patients experienced grade 1 or 2 fevers from IL-2. Other toxicities documented during the transplantation and post-transplantation follow-up were similar to that reported for this preparative regimen without NK cells.

Transplantation Outcomes

Transplantation outcomes are summarized in Table 3 and Figure 2. Overall survival was low for this cohort (Figure 2A). GVHD was not increased, with only 5 patients developing a maximum acute GVHD of grade 2, and 2 patients with grade 3 GVHD. No patients developed grade 4 GVHD. Six patients developed chronic GVHD, 5 of whom eventually met criteria for chronic extensive. Eleven of the 14 patients with active disease at the time of transplantation achieved a complete remission. Neutrophil engraftment occurred in all patients (median 12 days) (Figure 2B), with the exception of 1 early death before engraftment. None had graft failure. Median platelet engraftment occurred by day 14 (Figure 2B). Two patients failed to achieve platelet engraftment; in both patients, failure was due to relapse before day 100.

There was 1 early death before engraftment at day +8 due to resistant acute leukemia. One patient died from infection 2 months after transplantation. Two patients who relapsed without developing GVHD were taken off study and subsequently died of acute GVHD after receiving a donor lymphocyte infusion. One patient with pre-existing cirrhosis died of chronic liver GVHD. The remaining 10 deaths were due to relapse. The day 100 transplantation-related mortality was 9%, similar to expectations for conventional hematopoietic transplantations in this high-risk patient population.

Predictive Models

In this group of patients with very high-risk myeloid malignancies with refractory disease, there was no difference in survival according to the pretransplantation diagnosis (Figure 3A) or remission status at the time of transplantation (Figure 3B). Despite the fact that patients with CML were over-represented among those treated at higher dose levels, these patients did most poorly in the study (Figure 3A). Diseases of the 5 survivors consisted of 1 each of AML, MDS, MDS transformed to AML, CMML, and CML (chronic myelogenous leukemia) (Table 2). There were no survivors who did not develop grade 3 or greater GVHD or chronic

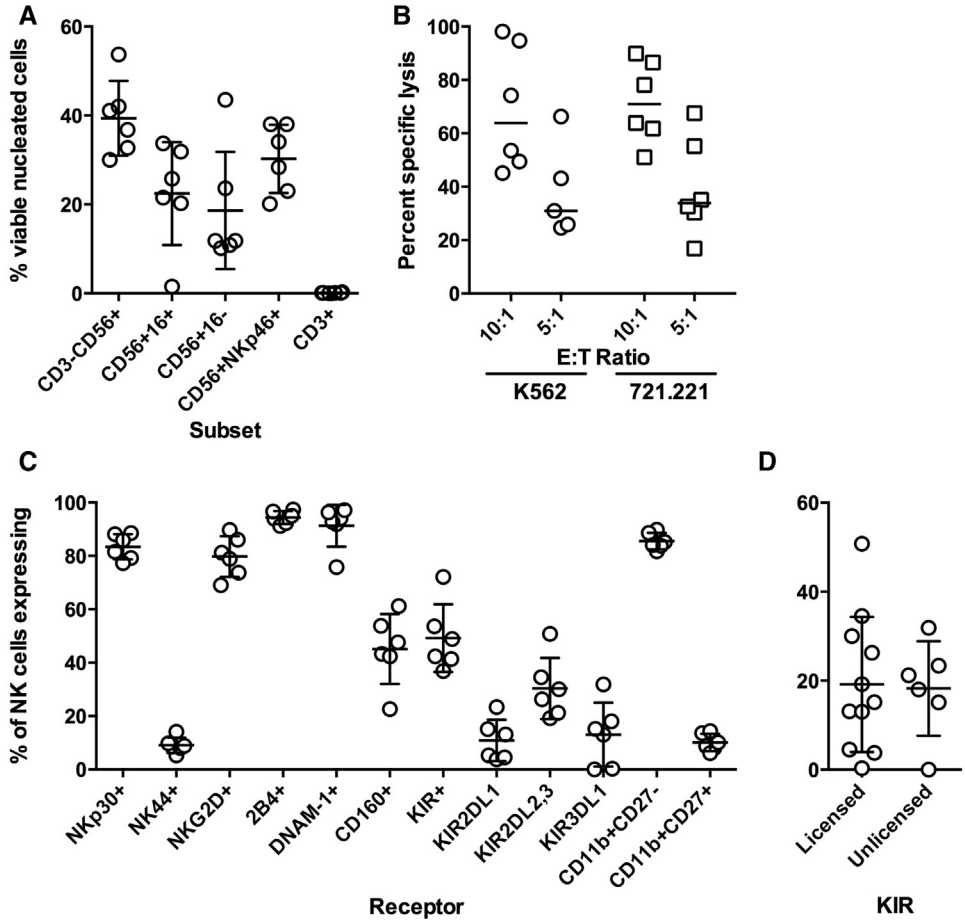

Figure 1. Product characteristics. (A) NK cell and T cell content of the NK cell product as determined by flow cytometry. Total NK cell content was determined by $CD3^{-}CD56^{+}$ or as NK cell subsets ($CD16^{+/-}$) or coexpressing NKp46. (B) Cytotoxicity of the product after overnight activation with IL-2 as measured against HLA class I–negative targets K562 and 721.221. (C) Percent of NK cells expressing NK cell activating and inhibitory receptors and maturation markers. (D) Percent of NK cells expressing KIR, grouped according to predicted licensing by donor HLA.

GVHD (GRFS, Figure 2A), resulting in a strong statistical association ($P = .006$) between RFS and GVHD (Figure 3C), as has been reported in many studies.

Table 3
Patient Outcomes

Outcome	Value
Engraftment, n (%)	20 (100)
Chimerism, n (%)	
Full donor	11 (55)
Mixed	9 (45)
Time, median (range), d	
Neutrophil	12 (5-23)
Platelet	13.5 (7-34)
aGVHD, max grade, n (%)	
1	3 (15)
2	5 (25)
3	2 (10)
cGVHD, n (%)	6 (30)
Final cause of death, n (%)	
Sepsis	1 (5)
Relapse, n (%)	12 (60)
GVHD, n (%)	3 (15)
Survival, median (range), d	
Overall	233 (8-2251)
RFS	102 (8-2251)
GRFS	89 (8-343)

cGVHD indicates chronic graft-versus-host disease.
Data presented are n (%), unless otherwise indicated.
Reported as % of 20 evaluable patients.

Survival was associated with higher $CD3^{-}CD56^{+}$ NK cell dose ($P = .0217$) (Figure 3D), but because NK cell content varied in the NK cell product, survival was not associated with assigned TNC dose level ($P = .34$). Monocyte dose correlated closely with TNC and, similarly, did not correlate with survival ($P = .38$). Despite a positive association of survival with both GVHD and NK cell dose, GVHD was not independently associated with TNC ($P = .24$), NK ($P = .40$), or T cell content ($P = .31$) of the NK cell infusion, nor with T cell content of the stem cell infusion ($P = .28$).

There was a trend towards improved survival in those who received KIR-mismatched NK cells, but the small number of patients with KIR-matched donors in this study (n = 5) prevented a strong association ($P = .26$; 1-sided Fisher exact test for survival, $P = .15$) (Figure 4A). Results were not different when based on the ligand:ligand or KIR:ligand models, as in no cases did the KIR genotyping results change the mismatch predicted by the ligand-ligand model. No donors lacked KIR2DL1 or both KIR2DL2 and KIR2DL3. One donor lacked KIR3DL1 but was not mismatched for Bw4 with the recipient. There was no association of survival with the number of activating KIR genes in the NK cell donor (Figure 3B). The association with KIR B content as determined by the Minnesota model [14] depended on whether KIR2DS4 Func/Del variants were considered. Without this consideration, there was a nonsignificant trend in patients receiving NK cells from better/best donors having lower

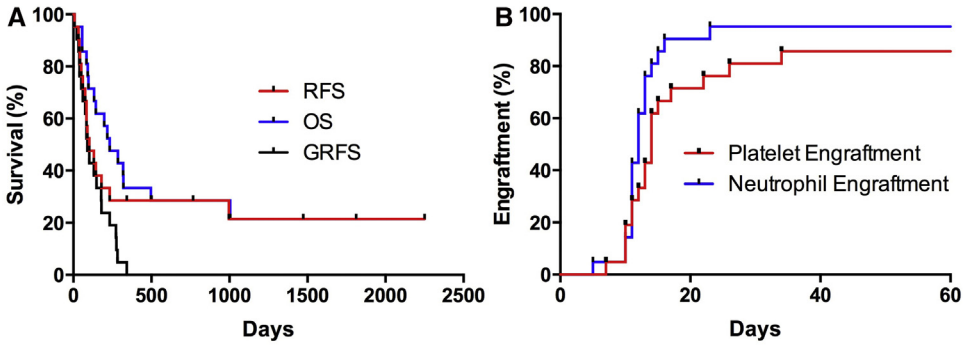

Figure 2. Clinical outcomes. (A) Survival curves showing relapse-free, overall, and GVHD-free/relapse-free (GRFS) survival for all patients enrolled. GFRS was determined described in Holtan et al. (B) Cumulative incidence of neutrophil engraftment and platelet engraftment for all patients enrolled.

survival (Figure 4C), whereas this association was reversed when the Del variants were recategorized as though they were KIR2DS4-negative (Figure 4D), which is more consistent with previously reported clinical associations and functional models.

DISCUSSION

We treated 21 patients with escalating doses of third-party NK cells from a haploidentical related donor after conditioning chemotherapy and before stem cell infusion from an HLA-matched donor with the goal of augmenting GVL effects without exacerbating GVHD. We did not identify a MTD of NK cells up to the maximum feasible dose attainable by apheresis and CD3 depletion of the product. This resulted in approximately 3×10^7 TNC/kg containing approximately 3×10^6 $CD56^+$ $CD3^-$ cells/kg, consistent with other reported NK cell studies utilizing apheresis-derived products [15-18]. This approach was associated with 100% engraftment and a relatively low rate of $\geq$ grade 3 acute GVHD (10%), similar to that observed with conventional hematopoietic transplantations.

The association of high NK cell content in the stem cell graft [3,19] and early NK cell reconstitution [4,20] with improved RFS in HSCT suggest that the early antileukemic effect of NK cells is important, and to our knowledge this is the first report describing NK cell adoptive immunotherapy after myeloablative chemotherapy and before stem cell infusion. This study demonstrates the safety and feasibility of this approach, with no increase in adverse events. Other studies have demonstrated that NK cells derived from the stem cell donor can be delivered after engraftment. NK cells have been infused 2 months after related donor (matched or mismatched) HSCT [21], 2 and 3 weeks after haploidentical HSCT [22], or 3, 40, and 100 days after haploidentical HSCT [23]. Third-party haploidentical NK cells have also been delivered approximately 3 months after autologous HSCT [15] or matched allogeneic HSCT [24].

Allogeneic NK cells in the nontransplantation setting have been associated with prolonged neutropenia presumably from a direct effect against normal hematopoietic progenitors [25,26]. In this study, delivering NK cells before transplantation, we chose to give Thymoglobulin 5 days after

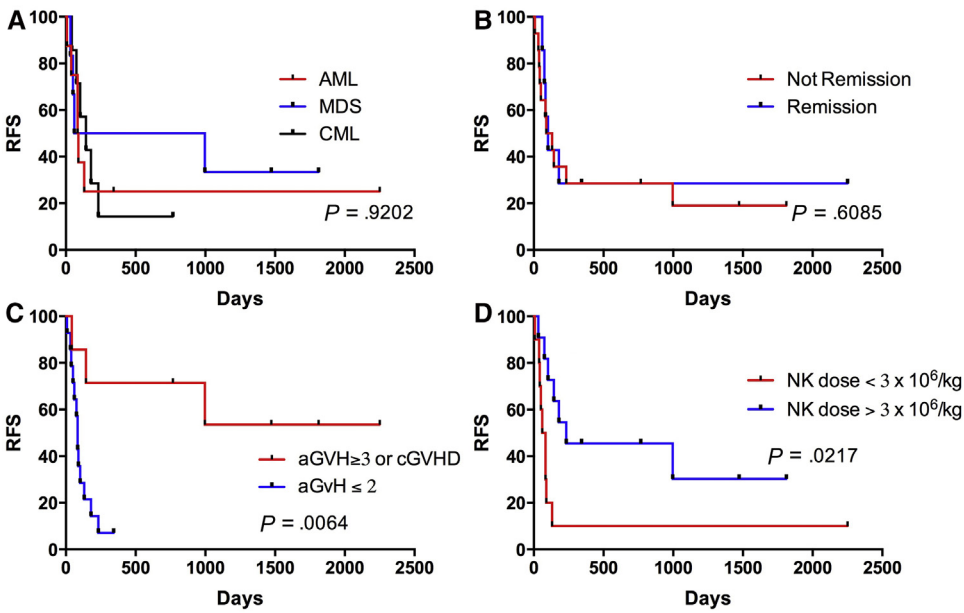

Figure 3. Relapse-free survival as a function of disease type, status, GVHD, and NK cell dose. (A) Survival plotted according to malignant diagnosis at the time of transplantation (eg, patients with MDS-related AML are included in the AML cohort). (B) Survival according to whether patients were in remission at the time of starting transplantation conditioning. (C) Survival according to the development of GVHD. Patients who developed $\geq$ grade 3 acute GVHD, chronic GVHD, or both were considered together as 1 cohort (as in Figure 2). (D) Survival plotted according to dose of NK cells ($CD56^+$) received.

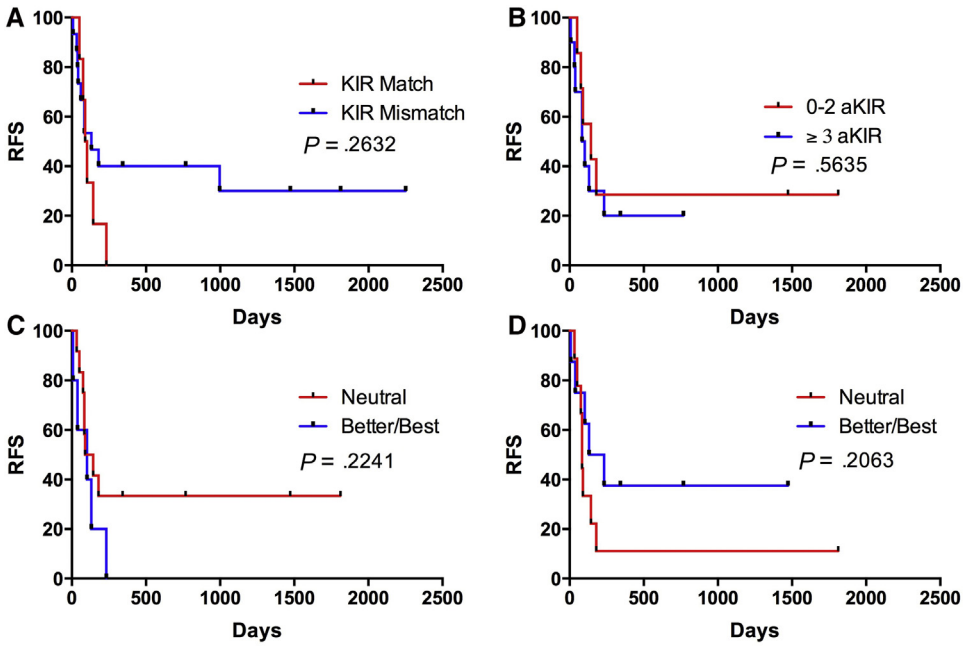

Figure 4. Correlation of relapse-free survival with NK cell–related donor:recipient characteristics. (A) Survival plotted according to the existence of KIR ligand-ligand mismatch between NK cell donor and patient. (B) Survival as stratified according to the number of activating KIR genes in the NK cell donor. (C) Survival stratified according to KIR B content without respect to Func/Del variants of KIR2DL4. (D) Survival stratified according to KIR B content when considering KIR2DL4Del variant as absence of KIR2DL4. n = 17 for B, C, D.

the NK cells and before stem cell infusion to reduce the likelihood that the third-party NK cells would adversely affect engraftment. Graft failure or delayed engraftment did not occur. However, the administration of Thymoglobulin also limited the duration of effect for the NK cells. Given that we saw no graft failure and relapse continued to be the primary cause of treatment failure, elimination of the Thymoglobulin should be studied to favor a more sustained antileukemia response.

Despite the low rates of acute GVHD, there was still a strong overall association with acute GVHD/chronic GVHD and RFS, as has been seen in many studies of hematopoietic transplantation for myeloid malignancies. There was also a nonstatistical trend toward survival association with both NK cell dose and KIR mismatch, raising the prospect for improved outcomes if higher cell doses can be achieved and if KIR-MM can be accomplished for a greater proportion of patients, such as with the use of KIR-blocking antibodies [27]. The beneficial effect of KIR mismatch has been more consistently reported in the context of haploidentical HSCT. There is a proposed mechanism of alloreactive NK cells to reduce GVHD [6,28,29], but there are also reports of KIR mismatch and increased risk of GVHD in some settings [30,31]. The lack of a KIR ligand has also been associated with increased GVHD in matched allotransplantations [32]. Thus, the relationship of NK cell alloreactivity, GVHD, and survival is complex and it is not possible in this study to determine whether the impact of NK cells on survival is mediated through GVHD or independent of it. Given the competing impact of GVHD on relapse and quality of life, a modified criteria for assessing GRFS has been proposed [13] and should be used to assess treatments designed to augment GVL effects without GVHD.

This study prioritized the identification of KIR-mismatched donors on the basis HLA and KIR genotyping. Surprisingly, in the samples available for testing we found no correlation with licensing on the size of the alloreactive NK cell subset. Thus, despite the hypothesized benefit of enhanced alloreactivity with KIR-mismatched donors, the relative frequency of alloreactive NK cells may need to be considered as part of the donor selection criteria in future studies.

In conclusion, we demonstrate the safety and feasibility of early pretransplantation adoptive transfer of NK cells with a goal to improve the antileukemic potency of a well-established transplantation regimen. No toxicity occurred with the maximal cell doses that can be obtained by apheresis from normal donors. Efficacy was potentially limited by the relatively low dose of NK cells that could be obtained. We are undertaking a follow-up clinical trial that aims to dramatically increase the number of NK cells available for infusion through ex vivo propagation [33,34], utilize cord blood and HLA-matched sources for the NK cells for those without mismatched haploidentical donors, and eliminate Thymoglobulin to increase NK cell persistence. Larger controlled studies are required to determine if the addition of NK cells to stem cell transplantation will improve leukemia-free survival for patients with hematologic malignancies.

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