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NATURAL KILLER CELL ENRICHED DONOR LYMPHOCYTE INFUSIONS FROM A 3-6/6 HLA MATCHED FAMILY MEMBER FOLLOWING NON-MYELOABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION

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Abstract

Purpose—Infusing Natural Killer (NK) cells following transplantation may allow less infections and relapse with little risk of acute graft versus host disease (aGVHD). We delivered 51 total NK cell enriched donor lymphocyte infusions (DLIs) to 30 patients following a 3-6/6 HLA matched T cell depleted nonmyeloablative allogeneic transplant. The primary endpoint of this study was feasibility and safety.

Methods—Eight weeks following transplantation, donor NK cell enriched DLIs were processed using a CD56+ selecting column (@Miltenyi) with up to 3 fresh infusions allowed. Toxicity, relapse and survival were monitored. T cell phenotype, NK cell functional recovery, and KIR typing were assessed for association with outcomes.

Results—Fourteen matched and sixteen mismatched transplanted patients received a total of 51 NK cell enriched DLIs. Selection resulted in 96% (standard deviation (SD) 8%) purity and 83% (SD 21%) yield in the matched setting and 97% (SD 3%) purity and 77% (SD 24%) yield in the mismatched setting. The median number of CD3− CD56+ NK cells infused was 10.6 (SD 7.91) × 10e6 cells/kg and 9.21 (SD 5.6) × 10e6 cells/kg respectively. The median number of contaminating CD3+CD56− T cells infused was .53 (1.1) × 10e6 and .27 (.78) × 10e6 in the matched and mismatched setting respectively. Only 1 patient each in the matched (n=14) or mismatched (n=16) setting experienced severe aGVHD with little other toxicity attributable to the infusions. Long term responders with multiple NK cell enriched infusions and improved T cell phenotypic recovery had improved duration of responses (p=.0045) and overall survival (p=.0058).

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Conclusions—A one step, high yield process is feasible and results in high doses of NK cells infused with little toxicity. NK cell enriched DLIs result in improved immune recovery and outcomes for some. Future studies must assess whether the improved outcomes are the direct result of the high doses and improved NK cell function or other aspects of immune recovery.

INTRODUCTION

Non-myeloablative stem cell transplantation allows allogeneic immunotherapy to be offered to older, more infirm patients with various types of neoplastic diseases or marrow failure syndromes with high rates of engraftment, low treatment related mortality, and high complete response rates, however long term remission remain elusive.[1,2,3,4,5] Donor lymphocyte infusions (DLIs) have been utilized to improve durability of response, however their use is limited by the risk of acute graft versus host disease (aGVHD) and poor durability of response.[6,7,8] Work by Vago et. al.[9] indicates one reason for the poor response to DLIs may be that residual leukemia cells following transplantation are altered and more resistant to standard donor T-cell anti-tumor effects, suggesting the importance of alternative mechanisms for tumor cell killing. Natural killer (NK) cells may provide such benefit as they may mediate a graft versus tumor (GVT) effect independently of aGVHD.[10,11] However, the low frequency of NK cells in adults (<10% of the DLI sample) has been posited as one reason for relapse.[9] This study investigated the safety and feasibility of infusing a DLI enriched for NK cells to patients following a T-cell depleted, non-myeloablative allogeneic transplant from a 3-6/6 human leukocyte antigen (HLA) matched family member. Corollary studies assessing the effect on T cell phenotype and NK functional immune recovery and the impact of KIR matching were also investigated.

METHODS

Patients and donors

Eligible adult patients were those who engrafted following a fludarabine based T cell depleted non-myeloablative allogeneic transplant regimen with alemtuzumab. The details of this procedure have been previously published.[3] Subsequent to transplantation, an infusion of NK cell enriched DLIs was planned at 6–8 weeks. Donors were the same HLA 3-6/6 matched family member used for the allogeneic transplantation. Two more infusions could be provided at 8 week intervals for up to 3 total infusions for those with high risk diseases (i.e. high risk cytogenetics or those in 2nd or greater remission). Adjustments in scheduling +/−4 weeks were allowed for concerns over patient health, disease status, or logistics of donor/lab availability for graft manipulation. Patients on mycophenolate at the initiation of the NK cell infusions for planned prophylaxis continued until at least 2 weeks following the first NK infusion, and then it was discontinued. Patients with aGVHD had to be successfully treated and on <30mg/d of prednisone (or equivalent) and ≤ Grade 2 aGVHD[12] at time of infusion of NK cells. Patients were evaluated weekly for toxicity following the NCI expanded common toxicity criteria (version 3) [13] until a minimum of 8 weeks following the last infusion, then at least monthly for 3 additional months. Determination of hematopoietic chimerism (by short tandem repeat analysis) and immune reconstitution studies were performed just prior to each NK cell infusion, and every 3 months following the last NK cell infusion. All patients signed informed consent for this IRB approved protocol. (Clinicaltrials.gov # NCT 00586690)

NK cell enriched DLI collection and processing

Donor cells were collected with one apheresis procedure without growth factors, selected for NK cells, and then infused over 30 minutes fresh. Cell processing was performed according to FACT procedures for collection, labeling and handling.[14] The lymphocytes were enriched

for NK cells using a CD56 antibody (CliniMACS CD56 Reagent), CliniMACS^{plus} instrument using established company protocols (Miltenyi Biotec Inc, Auburn, California). Pre- and post processing cell counts, cultures, and viability were assessed. To infuse into patients, the product must have had a viability ≥ 70% and gram stain negative for signs of infection. Patients received acetaminophen and diphenhydramine premedication prior to each NK cell enriched DLI.

The goal for each NK cell enriched DLI was to infuse the most NK cells while maintaining a low risk of aGVHD. We have previously shown the early infusion of DLIs would have <20% chance of GVHD if the infusate contained a maximum of 0.5×10^6 CD3+ 56− cells/kg patient weight in the 3–5/6 matched setting and 1×10^6 CD3+56− cells/kg patient weight in the 6/6 matched sibling setting.[15] Thus, the total dose infused in this study was limited by ensuring the dose of $CD3^+56^-$ cells did not exceed these limits. If mild or moderate aGVHD occurred subsequent to the first NK infusion, the patient was allowed to receive further NK infusions only if the GVHD had resolved to meet initial eligibility criteria.

T cell recovery

Using standard analyses for immunophenotypes, these studies monitored the recovery of $CD3^+$ T cells and $CD56^+$ NK cells. Using multi-parameter 3- and 4-color FACS analysis, the $CD3^+$ T cells were further described for their expression of CD4, CD8, CD45RA and CD45RO. Samples were tested 6 weeks following transplantation and at 3 month intervals from transplant through the first year and 6 month intervals for the second.

NK cell functional assays

As part of this report, we present a more ‘user-friendly’ flow based assay for the assessment of NK cell activity performed pre infusion, 6 weeks, and at subsequent 3 month intervals. K562 (ATCC; # CCL-243) and Raji (ATCC; # CCL-86) cells were exposed to varying ratios of effector peripheral blood mononuclear cells (PBMC) prepared from 50 ml blood collected from the patients. In each assay for NK cell function, the total number of target cells was held constant and three-fold serial dilutions of the effector cells, performed in triplicate, were established. The percent lysis was measured on the target cells directly as a percentage of $7AAD^+$ cells. Background (minimum) 7AAD uptake was calculated from targets incubated without effectors. The percent lysis was simply calculated as: (% sample 7AAD uptake - %minimum 7AAD uptake). The complete details of this simplified procedure and its validation are detailed in the Supplemental Data section on-line. Positive and negative controls were run in tandem with all clinical samples. Samples not felt to be adequate or assays not reliable when run in tandem were discarded and not reported in this data set. Data shown as ‘No’ or ‘0’ lysis were from samples with a large blood specimen tested with either too few lymphocytes to be active or those present were not able to induce measurable lysis with this assay.

Statistical Analyses

This study evaluated the safety of NK cell enriched DLIs using a CD56+ selection technique and the impact of infusions of high doses of such cells on recovery of T cell and NK cell function. The primary clinical endpoints included mortality, occurrence of severe aGVHD or other unacceptable toxicity within 8 weeks of the NK cell enriched DLI, and duration of response. Recognizing that aGVHD in the nonablative setting does not follow the traditional day 100 limit of onset, we utilized the more conservative recommended approach to account for GVHD that clinically presents similar to early aGVHD, even beyond the day 100 historical cutoff, as aGVHD related to the procedure.[16] Unacceptable toxicity was defined as grade ≥ III aGVHD of the gut or liver or Grade IV aGVHD of the skin lasting > 7 days; other Grade 4 toxicity from the procedure in the major organs that lasted > 5 days; or

treatment-related mortality (TRM). The rate of unacceptable toxicity was monitored with stopping rules to permit the study to be closed early if there was evidence that the true unacceptable toxicity rate was ≥ 0.40. Though these infusions are provided early following transplantation and severe toxicity could still have occurred due to the primary transplant procedure, for this study any aGVHD or other toxicities occurring after the first day of infusion of the NK cell enriched DLIs is here as study related.

Though this is a feasibility study, association with laboratory measures of phenotypic T cell subset recovery and NK cell function were undertaken to investigate trends. For this investigation, CD3+ lymphocyte recovery was arbitrarily assessed as 'poor' if there was enough sample for testing but too few lymphocytes to have a meaningful count on flow analysis up to 25% the normal number, 'moderate' with >25% and <75% normal CD3 cell counts and 'good' if ≥75%. NK cell functional ability was categorized as 'poor' for those with adequate sample for testing but with <25% lysis, 'mid level' for 25–75% lysis and 'fully recovered' if ≥75% lysis was encountered in the in vitro assay. Correlation with clinical outcomes was performed using a Cox proportional hazards model while the effect on occurrence of ≥ grade 2 aGVHD was measured based on logistic regression analyses. Low resolution HLA- A, B, C, and high resolution DRB1, DRB3/4/5 and DQB1 were performed on each recipient and related donor allowing assessment of the impact of KIR compatibility with clinical outcomes.

RESULTS

Fifty one total NK cell enriched DLIs were processed and delivered to 30 consecutive patients. Fourteen patients underwent a total of 24 infusions from a 6/6 HLA matched sibling and 16 patients received a total of 27 infusions from a mismatched family member (2- 5/6 matched siblings, 14- 3–4/6 matched family members) from May of 2005 to the present. The first infusion was a median of 2 months from transplant (range 1.5–3), beyond the point at which the low concentration of persistent alemtuzumab is considered able to induce lysis.[17] Six were on low doses of immune suppression during the first infusion. All but 2 subjects had donor engraftment accounting for >80% of their hematopoiesis at the time of first infusion.

Cell Doses

In the matched setting, following the 1 step enhancement with the CD56+ selecting column (Miltenyi@), there was a median CD56+ cell purity of 96% (+/−8%) (range 87 –100%) and yield of 83% (standard deviation (SD) 21%) (range 36–88%) compared to the pre-processing number (Table 1). The median dose of CD3+56+ cells/kg infused was 1.94 × 10e6 (SD 2.22), and 10% of these CD3+/56+ cells also expressed CD4 or 8 (data not shown). The NK cells of interest, CD3−56+, were enriched with a median infused dose of 10.60 (7.91) × 10e6 cells/kg. The median CD3+CD56− cells/kg dose, most often CD4 or CD8+ cells concerning for their potential to cause aGVHD, was assessed to ensure the risk of aGVHD was minimized with the intention to 'cap' this dose at a maximum of 1 × 10e6 CD3+CD56− cells/kg infused. None of the infusions in fact reached this 'cap' as a median of .53 (1.1) × 10e6 CD3+CD56− cells/kg was infused.

In the mismatched setting, following enhancement with the CD56+ selecting column (Miltenyi@), there was a median CD56+ cell purity of 97% (3%) (range 86 –100%) and yield of 77% (24%) (range 36–100%) (Table 1). The median dose of CD3+56+ cells/kg infused was 3.67 (2.41) × 10e6. The median dose of NK CD3−56+ cells/kg infused was 9.21 (5.6) × 10e6. The median number of concerning CD3+CD56− cells/kg infused was .27 (.78) × 10e6 cells/kg. Remembering the total cell dose of these contaminating CD3+56− cells

infused was capped to ensure ≤.5 × 10e6, 6 patients had a total of 10 infusion products 'capped' for this reason.

Of the 14 matched sibling recipients receiving a total of 24 NK cell enriched DLIs; 8 received 1, 2 received 2, and 4 patients received 3 infusions, the maximum allowed on the study. Of those who received <3 infusions, 4 were standard risk patients, 3 due to mild aGVHD, 3 due to relapse of disease. The 16 recipients of mismatched grafts received a total of 27 NK cell enriched DLIs; 9 received 1, 3 received 2, and 4 patients received 3 infusions. Of those who received <3 infusions, 5 were not high risk patients or are still awaiting further infusions, 5 due to mild aGVHD, and 1 due to relapse of disease.

Toxicity

Mild skin aGVHD was experienced by 2 of the 14 matched patients prior to treatment on this study (Table 2A). Six of these patients experienced ≥ grade 1 aGVHD, though only 1 had ≥3–4 (severe) overall aGVHD. The median onset following the first NK cell infusion was 2 months (range 1–7 months). Mild skin aGVHD was experienced by 4 of the 16 mismatched patients prior to enrollment in this study. At the doses provided, ≥ grade 1 aGVHD was experienced by 8 of the 16 patients with skin GVHD being common, though only 1 of the 16 had ≥3–4 overall aGVHD. The median onset of aGVHD following the first NK cell infusion was 1.5 months (range 1–5 months). Only 1 case of severe chronic (c) GVHD was encountered.

Severe non- aGVHD toxicity was uncommon (Table 2B). Only 1 subject in each group (matched and mismatched) had bacterial sepsis, though viral exanthemas remained a significant concern despite the lymphocyte infusions with Polyoma, CMV, VZV, HSV, and Parainfluenza all encountered. Three cases of cardiac dysrythmias (atrial) needing medication for rate control were documented and 1 case of transient renal insufficiency. There was 1 case of significant decrease in donor engraftment, possibly leading to secondary graft failure, not due to evident disease progression.

Durability of Response—The fourteen patients with a matched sibling donor have a median follow up of survivors of 12 months (range 3–33 months) and estimates of a 43% 1 year overall survival with 8 in continuous remission. The sixteen patients with a mismatched related family member donor have a median follow up of survivors of 27 months (range 3–45 months) and preliminary estimates reveal a 42% 1 year overall survival with 8 remaining alive and in remission.

Evaluating outcomes by disease type rather than degree of graft match shows the 19 with a myeloid disease have a 50% 1 year survival and the 11 with a lymphoid disease had a 29% 1 year survival.

Patient NK cell function and the impact of NK cell enriched DLIs—Figure 1 presents the results of the flow based assay measuring NK cell function from a representative patient 2 to 6 months following transplant *without* the NK cell enriched DLI infusions provided in this series. There is minimal ability for recovering NK cells to induce lysis for many months and even 6 months later their function is only modest. This is the typical pattern seen in our patients following this transplant regimen. Functional Analysis of NK activity pre and post the NK cell enriched DLIs provided in this study is represented in Figure 2. While a few patients had measurable function just 6 weeks following transplant and before the NK cell selected boost, this represents a small fraction of the total with most subjects having little measurable activity. In focusing on the poor responders, subsequent NK cell enriched DLIs were associated with improved NK function in 4 of 7 tested patients (Figures 2C and D).

Association of lymphocyte phenotypic recovery and NK functional ability— The majority of patients had poor lymphocyte phenotypic recovery and NK cell functional ability early in transplant recovery (6 weeks) prior to NK cell enriched DLIs. Those with good early phenotypic T cell recovery were most likely to be the same as those with good NK cell functional ability (p=.005 using Fisher's mid – ptest).

In 10 patients with more than 1 infusion evaluated over time for both phenotypic T cell recovery and NK cell functional analysis performed, 70% (95%CI= 35–93%) had continued improvement in T cell phenotypic recovery with at least 50% increase in CD3+ cell counts and 80% (95%CI=44–98%) improved their NK cell function.

Association of lymphocyte phenotypic recovery and NK function with clinical outcomes—Long term responders who had multiple NK cell enriched DLIs and improved T cell phenotypic recovery also had improved duration of remission as measured from day 1 post transplant or day of first NK cell enriched DLI (p=.0045 and .0133 respectively) and better overall survival (p=.0058). It remains undetermined if the improved outcome is specific to the specific infusions, NK cell function specifically, or the expected normal kinetics of immune recovery in long term responding patients. There also was no statistical difference in toxicities or survival endpoints using the ligand to ligand model (Ligand incompatibility), the receptor-ligand model (Missing ligand model), or donor activation of KIR in this small study. (data not shown)

DISCUSSION

Our original report of non-myeloablative therapy with mismatched donors noted with the T cell depleted nonablative transplant the 2 most common causes of death were infections and relapse with a 31% 1 year survival in the mismatched setting.[3] Further, the value of unselected DLIs remains questionable.[15] As the majority of spontaneous tumors are typically non-immunogenic, the elicitation of NK cell mediated major histocompatability complex unrestricted cytotoxicity may be helpful in inducing significant antitumor responses while this cell type does not appear to induce aGVHD.[18,19,20]

Recent evidence has confirmed the phenotype of the early recovering NK cells is altered from the circulating NK cells seen in a normal host. [3,21] Further, Vago et al. have shown that it takes many months to have meaningful NK cell activity following CD34+ selected haploidentical transplantation and suggest the infusion of alloreactive NK cells following transplant, similar to the approach presented herein, might be an important step forward.[22] Recognizing these issues and the promise of NK cell mediated tumor lysis, infusions of autologous or donor NK cells alone or in conjunction with cytokine stimulation have been reported, though clinical benefit in these settings remains limited. [21,23,24,25] These and other prior efforts to exploit NK cells are often costly, time consuming, and the benefit of attaining a highly purified sample remains unclear. Our study utilized a one step positive selection process, with a high yield and CD56+ purity similar to prior reports using more stringent multistep step NK cell manipulation processes.[26,27] In addition, limiting infusions by the CD3+CD56− cells/kg dose still resulted in approximately a 2.5 log increase in NK cells infused, compared with our prior work with unmanipulated grafts, with no significant increase in aGVHD.

Using the flow based assay, our data reveals that few patients recover meaningful NK cell activity early following transplantation, as recently reported in the ablative setting as well.[22] Subsequent to infusion of the NK enriched DLIs there was a notable increase in T cell phenotypic recovery as well as NK cell function in many patients, a reaction further enhanced with continued infusions for many and associated with a significant improvement

in duration of response and survival, suggesting a possible benefit from this approach. We are cognizant that this is simply an association and not necessarily causal. It may simply be that those who were healthier were more able to receive further infusions due to not having toxicities and remaining in remission.

As noted above, this study did not infuse solely purified NK cells as approximately 10% of the infusate was CD3+CD56+ cells. Some of these cells are reported to act as cytokine induced killer cells or gamma-delta T cells and may also have a potent ability to lyse tumor cells. [28,29,30] In addition, it has recently been reported that NK cell function may directly trigger antigen-specific T cell mediated and humoral responses, suggesting significant improvements in immune activity may result from the interactions of NK cells and other T cell subsets.[31] The NK cell functional assay presented in this manuscript will be an important aspect of future studies that assess the causal nature of the improved outcomes relating to general T cell as well as specific NK cell function.

Dunbar et al. have recently reported in the HLA matched setting that those with low NK cell levels 60 days following reduced intensity transplantation had higher relapse or death rates.[32] Further, Pende et al have shown the importance of NK cells as clinical outcomes in haploidentical transplantation was associated with NK cell activity regulated by a balance of activating and inhibiting KIRs[33] and Ruggeri et al. have reported that, in myeloid patients undergoing allogeneic transplant, using a donor with NK alloreactive clones improved survival.[34] These studies reveal that NK cells clearly impact survival, though the mechanisms of action remain unclear. [35,36,37, 38] This feasibility study was not able to determine the relevance of KIR typing in this approach. Each of the 3 different models bear closer examination in larger, comparative, studies evaluating NK cell activity in relation to KIR activity before donor selection can be based on this information.

This is the first such report to show that NK cells can be significantly enriched using a 1 step processing of DLIs and be safely infused in either the HLA matched and mismatched setting early following nonmyeloablative allogeneic transplantation. Infusions had a low risk of inducing severe aGVHD or other severe toxicities. This supports future studies designed to further improve outcomes via manipulation of NK cell function. Importantly, this study does not provide information as to whether the improved recovery or outcome noted here is due specifically to the higher NK cell dose delivered, an issue that needs to be addressed in future randomized trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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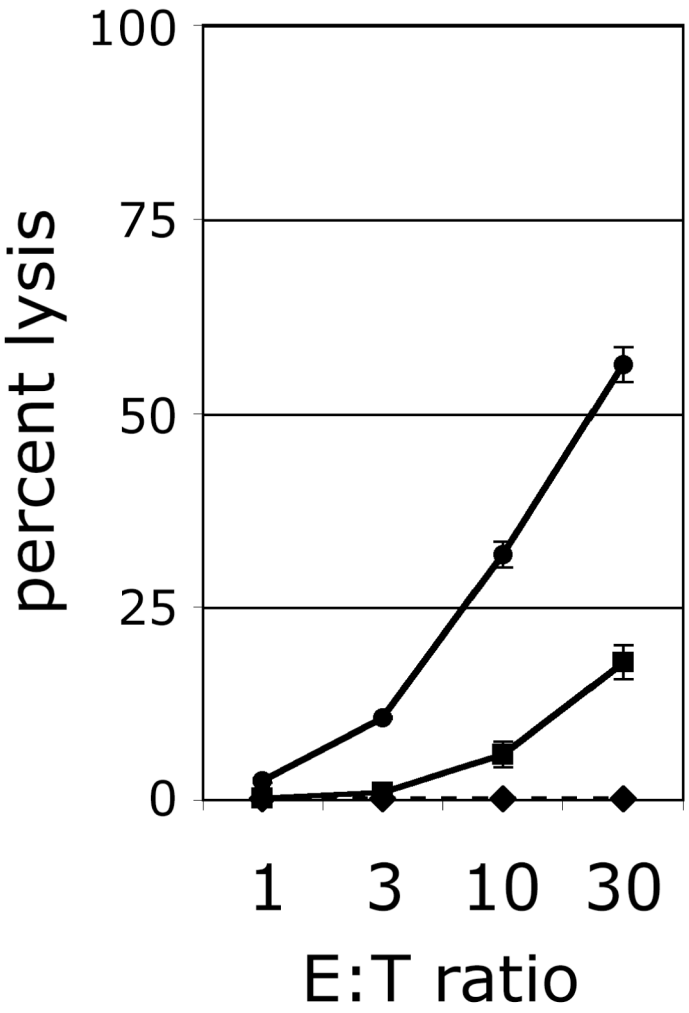

Figure 1.
NK cell function was measured by their capacity to lyse K562 target cells, which is dependent upon the content of $CD56^+$ effector cells within a sample. Improved NK cell ability to lyse the target cells is denoted by a steeper line as effector-to-target cell ratios increase. Typical results following T cell depleted nonmyeloablative therapy are noted here. At 2 months following transplant in this patient there were still not enough lymphocytes to quantify a meaningful response (indicated with a stippled line). There was marginal recovery at approximately 4 months (black squares) and still only modest recovery at 6 months (shown with black triangles). Error bars represent the standard deviation derived from triplicate samples.

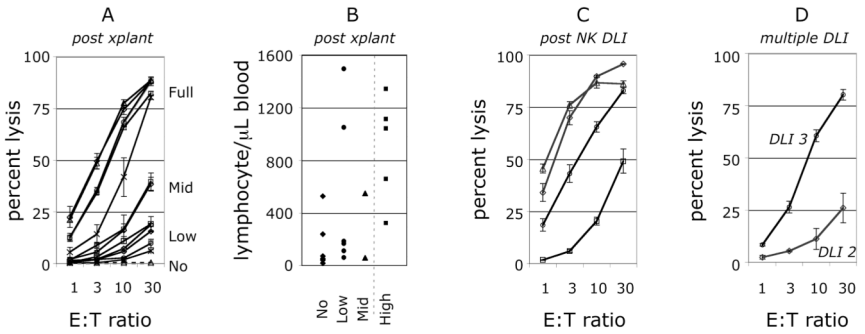

Figure 2. Impact of NK Cell Enriched DLI

Panel A. NK cell function was measured at 6 to 8 weeks post-transplant, immediately prior to receiving NK infusions. At that time, NK cell function had returned fully in only a few patients (Full), while 2 additional patients demonstrated at least some NK cell function (Mid). However, the majority of patients demonstrated low NK cell function (Low) or did not recover sufficient lymphocytes to assay NK function (No, indicated with a stippled line).

Panel B. The total recovery of lymphocytes to the peripheral blood was examined within each patient group. At 6 to 8 weeks post-transplant, lymphocyte recovery was only consistently strong among patients that recovered full NK cell function (Full).

Panel C. The impact of NK cell donor lymphocyte infusion (DLI) was monitored in patients that had not previously responded ('Low' or 'No' NK cell function patients in panel A). Of those patients, 4 responded within 6 to 8 weeks after a single NK cell-DLI.

Panel D. In one patient, NK cell function returned gradually following a 2^{nd} and 3^{rd} DLI. In all panels, the error bars represent the standard deviation derived from triplicate samples.

* the '0' measure for percent lysis refers to patients with functional ability below the level of detection in this assay due to either poor function and/or too few cells recovered to have measurable with this assay.

Table 1

Cell doses infused post processing

Donor	Cell Dose (Standard Deviation)	% PURITY	% YIELD	CD3+CD56−/KG x 10e5	CD3+CD56+/KG x 10e6	CD3− CD56+/KG x 10e6
Matched	Median (SD)	96 (8)	83 (21)	.53 (1.1)	1.94 (2.22)	**10.60 (7.91)**
Mis Matched	Median (SD)	97 (3)	77 (24)	.27 (.78)	3.67 (2.41)	**9.21 (5.56)**

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Table 2

Toxicities for the matched sibling donors, N=14 (24 total NK cell enhanced infusions); Or mismatched family member donors, N=16 (27 total NK cell enhanced infusions)

(A) aGVHD								
Site↓/Grade→	HLA Matched N=14 patients; 24 total infusions				HLA Mismatched N=16; 27 total infusions			
	1	2	3	4	1	2	3	4
Skin	1	1	2	-	3	3	1	-
Gut	2	-	-	-	2	-	-	1
Liver		-	1	-		-	1	-
Overall	2	3	1	-	4	3	-	1

(B) Non- GVHD toxicity						
Organ System↓/CTC Grade→	Matched			Mismatched		
	2	3	4	2	3	4/5
Cardiac-arrythmia	1	-	-	2	-	-
Renal Insufficiency	-	-	-	1	-	-
Infectious	-	-	-		-	
Polyoma cystitis	1	1	-		2	-
CMV Reactivation only	3	-	-	6	-	-
CMV Disease	-	-	-	-	1	-
HSV	-	-	1	1	-	-
Parainfluenza	-	2	-	-	3	-
VZV	1	-	-	-	1	-
Bacterial	-	2	1	2	2	1
Parasitic (acanthamoeba)	-	-	1	-	-	-
Gastritis (non-GVHD)	2	-	-	-	-	-
Hemolytic Anemia		-	-	-	1	-
Pulmonary (non-infectious)	1	-	-	-		-
Post transplant lymphoproliferative disorder (PTLD)	-	-	-	-	-	2

1 case of severe cGVHD noted in the mismatched group