

# Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation

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**This pilot study tested feasibility of natural killer cell purification and infusion (NK-DLI) in patients after haploidentical hematopoietic stem cell transplantation (HSCT). The aim was to obtain  $\geq 1.0 \times 10^7/\text{kg}$  CD56+/CD3- NK cells and  $< 1.0 \times 10^5/\text{kg}$  CD3+ T cells. Mononuclear cells were collected by 101 leukapheresis. A two-step *ex vivo* procedure was used to purify NK cells, using an immunomagnetic T-cell depletion, followed by NK-cell enrichment. Five patients with high-risk myeloid malignancies were included, presenting 3–12 months after a haploidentical HSCT with mixed chimerism (3), impending graft failure (1) or early relapse (1). The purified product contained a median of  $1.61 \times 10^7/\text{kg}$  (range 0.21–2.2) NK cells and  $0.29 \times 10^5/\text{kg}$  (0.11–1.1) T cells. A purity of NK cells of 97% (78–99), a recovery of 35.5% (13–75), and a T-cell depletion of 3.55 log (2.9–4.5) was achieved. Infusions were well tolerated and none of the patients developed graft-versus-host disease. We observed an increase in donor chimerism in 2/5, stable mixed chimerism, decreasing chimerism and relapse of AML in one patient each. Selection of NK-DLI is technically feasible. NK cells are well tolerated when used as adoptive immunotherapy in recipients of haploidentical HSCT.**

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## Introduction

Haploidentical hematopoietic stem cell transplantation (HSCT) is used in patients without matched donors.<sup>1–4</sup> Nearly all patients have a haploidentical donor within the family and, in contrast, to an unrelated donor search, the haploidentical family donor is rapidly available. The role of natural killer (NK)-cell alloreactivity in haploidentical HSCT<sup>5</sup> to promote engraftment and graft-versus-leukemia (GvL) effects is increasingly recognized.

In haploidentical HSCT, where T-cell-mediated effects are eliminated by T-cell depletion, alloreactivity is provided by NK-cell mismatches. In patients with acute myeloid leukemia, donor vs host NK-cell alloreactivity is associated with a remarkable GvL effect, apparently without increased risks of graft-versus-host disease (GvHD). This and conversely, the absence of host-versus-graft NK-cell alloreactivity may be explained by NK-cell sensitivity to class I polymorphism being restricted to hematopoietic cells. Other tissues may lack the ligands to activate NK cells. Thus, NK cells may, mediated by killer cell immunoglobulin-like receptors (KIR), prevent rejection and promote GvL effects.<sup>6–9</sup>

We investigated in a pilot protocol the feasibility of preparing and infusing purified, T-cell-depleted, donor NK-lymphocytes

(NK-DLI) to consolidate incomplete engraftment in patients after haploidentical HSCT. We hypothesized that purified NK-lymphocytes would preferentially recognize hematopoietic host cells, and promote engraftment without GvHD.

## Patients and methods

Objectives were to collect, purify and infuse NK-DLI in patients with incomplete engraftment, or relapse in the absence of GvHD after haploidentical HSCT. Targeted cell doses were  $\geq 1.0 \times 10^7/\text{kg}$  CD56+/CD3- NK cells, with  $< 1.0 \times 10^5/\text{kg}$  contaminating CD3+ T cells. The study is approved by the Local Ethics Committee.

## Transplantation protocol

The haplo-HSCT protocol has been described previously<sup>10</sup> and includes: G-CSF-mobilized peripheral stem cells, a T-cell-depleted ( $< 1.0 \times 10^5/\text{kg}$  CD3+) graft with a high dose of CD34+ cells ( $\geq 10 \times 10^6/\text{kg}$ ), a pretransplant conditioning regimen with etoposide, cyclophosphamide, ATG and 12 Gy of fractionated total-body irradiation, post-transplant immunosuppression with OKT3 and cyclosporine for 10 and 14 days. Donor cell engraftment after HSCT was monitored in whole blood 30, 60 and 90 days post-transplant, and three monthly thereafter, using a PCR-assay analyzing polymorphic short tandem repeats (STRs).

## Patients

Five of 16 consecutive recipients of haploidentical HSCT between January 2000 and July 2003 were included. Characteristics of these patients are listed in Table 1. All were children or young adults with high-risk hematological malignancies. Donors were parents. NK alloreactivity predicted based on HLA typing was present in 3/5 patients. All received a high stem cell dose, with low T-cell numbers; and all had rapid neutrophil, platelet and red cell engraftment, without developing GvHD. One patient had secondary graft failure at 5 months (UPN960). Indication for NK-DLI was incomplete engraftment in three, early relapse in one (UPN953) and graft failure in one patient (UPN960).

## NK cell collection, processing and infusion

Mononuclear cells were collected from the original donor by 101 leukapheresis and stored at 4°C overnight. The two-step *ex vivo* purification procedure (CliniMACS<sup>®</sup> cell selection system) included first a T-cell depletion, and second, an NK-cell selection. At each step, cells were analyzed for CD3, CD56 and CD19 by flow cytometry. In case of a CD56+/CD3-

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cell dose  $\geq 1.8 \times 10^7/\text{kg}$  or a contamination of T cells  $\geq 1.0 \times 10^5/\text{kg}$ , the processed NK product was split into two units. The first unit was infused immediately and the second was cryopreserved. NK-DLIs were performed as an outpatient procedure. Cryopreserved NK units were thawed at the bedside and infused rapidly. Patients were monitored for immediate adverse reactions, GvHD and infections. Donor chimerism for engraftment after HSCT and monitoring of NK-DLI was assessed by a PCR-based assay analyzing polymorphic STR markers. Amplified alleles were separated by capillary electrophoresis and peak surface area was used to quantitate chimerism. Whole-blood chimerism was measured prior to and monthly post-transplant. The sensitivity of the technique allows detecting a minor patient fraction of 1% of the entire leukocyte population.

### Technical aspects

For removal of CD3+ T cells, mononuclear cells were incubated for 30 min at room temperature with the anti-CD3 antibody, directly conjugated to magnetic microbeads. (CliniMACS T-Cell CD3 MicroBead<sup>®</sup>). The program DEPLETION 2.1 was used for automated cell separation. CD3- cells were collected and enriched for CD56+ NK cells by adding CliniMACS CD56 MicroBeads, incubating for 30 min and running the program ENRICHMENT 1.1.

### Results

Six products were collected from five donors. For patient UPN 931, a second harvest was performed 6 months after NK-DLI. The overall processing time lasted 8–10 h. After processing, purity of CD56+/CD3- cells was 97.3% (median; range 77.9–98.9) with a recovery of NK cells of 35.5% (13.1–75.0). The main loss of CD56+ cells occurred during the first step, with a reduction from  $164 \times 10^7$  (17–301) CD56+/CD3- NK cells before to  $53 \times 10^7$  (13–68) after the second step (Table 1). Overall, a T-cell depletion of 3.55 (2.9–4.5) log was achieved. The total T-cell count was reduced from  $481 \times 10^7$  (284–1539) to  $0.134 \times 10^7$  (0.05–0.518).

Of the six products four were split – three because of high NK cells, one because of high T cells, one because of both – to obtain 10 infusable products. Prior to splitting, CD56+/CD3- cells were  $1.61 \times 10^7/\text{kg}$  (median; range 0.21–2.20) and CD3+ T-cell count was  $0.29 \times 10^5/\text{kg}$  (0.11–1.10). The infused products contained  $0.93 \times 10^7/\text{kg}$  (0.21–1.41) CD56+/CD3- cells, with a T-cell contamination of  $0.22 \times 10^5/\text{kg}$  (0.11–0.55). One product (UPN960) had a very low number of NK cells, despite identical leukapheresis. This donor had a low preapheresis lymphocyte ( $1.13 \times 10^9/\text{l}$ ) and NK-cell counts (CD56+/CD3-, 68/ $\mu\text{l}$ ).

There were three NK products available for patient UPN931, one for UPN960 and two for all others. Nine of the 10 NK donor lymphocyte products were used for infusion. During infusion of the fresh or thawed NK products, no immediate adverse reactions were observed. None of the five patients developed clinical signs of acute or chronic GvHD after NK-DLI. There were no infectious complications and so far no other late effects attributable to NK-DLI have been observed. As of January 1, 2004, four of the five patients are alive, well and in continuous remission 8–18 months after the first NK-DLI (median follow-up, 12 months). One patient had rapidly progressive AML (UPN953) after NK-DLI and did not receive a second dose. After two NK-DLI, UPN924 has possibly stabilized at a donor chimerism

of 35% and UPN853 continued to drop to 70% (Figure 1). UPN931 had an increase in donor chimerism to 100% and because of a subsequent drop has received a third dose of NK-DLI 6 months after the first dose. She remains a complete chimera. UPN960 received NK-DLI followed by a second stem cell dose because of impending graft failure. She responded to this treatment by increasing donor chimerism from 26 to 66%, and neutrophil counts from  $0.04$  to  $0.65 \times 10^9/\text{l}$  but remains transfusion dependent.

### Discussion

This study shows that *ex vivo* purification of donor NK cells from a leukapheresis product is technically feasible, and an adequate number of CD56+, highly CD3-depleted cells can be obtained and infused without immediate adverse events and without inducing GvHD. The processing of donor lymphocyte cells is time consuming, and requires specific knowledge and skills in graft engineering. Finally, our preliminary data suggest that NK-DLI may revert impending rejection in some patients.

This pilot study allowed to set up technical conditions to generate a product with high purity of NK cells and a maximal depletion of T cells, which can be used in a clinical setting. This clinical scale method for isolation of T-cell-depleted CD56+ donor NK-lymphocytes has been described previously, but purified NK donor lymphocyte have only been used rarely as adoptive immunotherapy after HSCT.<sup>11,12</sup> Whereas NK-cell purity was 97% and T-cell depletion was by 3.5 logs, we observed a considerable loss of NK cells during cell engineering. Overnight storing of collected cells may have contributed to this. Overnight storing was chosen in order to guarantee completion of *in vitro* processing and donor infusion on the same day, including the flow cytometry analysis and, if necessary, the cryopreservation of the split product. The target cell number was fixed somewhat arbitrarily to  $1.0 \times 10^7/\text{kg}$  NK cells. This was based on the experience acquired with standard DLI.<sup>13,14</sup> In 15 unmanipulated DLI obtained by 4–6 l leukapheresis, we collected  $48 \times 10^7$  (12–115) NK cells. Increasing the apheresis volume could permit the collection of sufficient numbers of NK cells in view of their subsequent preparation. Prerequisites to achieve an adequate number of NK cells for NK-DLI are a high number of CD56+ cells in the leukapheresis product and efficiency of *in vitro* processing.<sup>15,16</sup> The only product with low NK-cell numbers was derived from a donor with lymphopenia and low NK count.

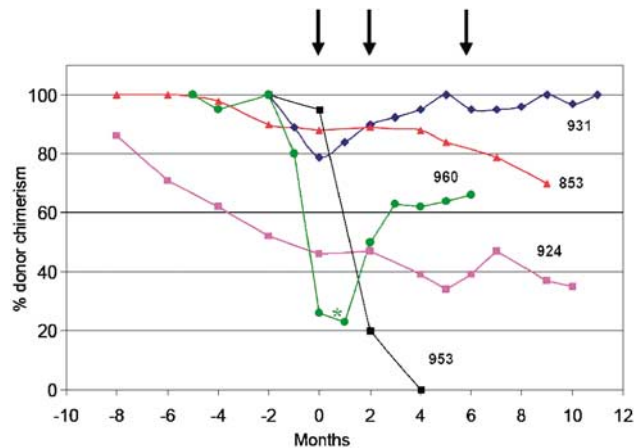
Monitoring of chimerism before and after NK-DLI was performed on whole blood. Analysis of subpopulations is not available. Early after haploidentical HSCT, the majority of peripheral blood cells are granulocytes and lymphocyte reconstitution is usually slow with NK cells recovering faster than T-cell subsets. In this pilot study, the timing of NK-DLI after HSCT was quite variable.

We cannot exclude that effects observed were due to residual T cells infused rather than NK cells. The upper limit of acceptable T-cell contamination had been set at  $1.0 \times 10^5/\text{kg}$  BW in the protocol. The upper limit is the same for the haploidentical HSCT as for the NK-DLI product. In fact, patients received a median of  $0.22 \times 10^5/\text{kg}$  contaminating CD3+ cells, and none of the products contained more than  $0.55 \times 10^5/\text{kg}$  CD3+ cells, which is a T-cell dose considered acceptable in some protocols for haploidentical HSCT. Further reductions of contaminating T cells could be obtained by additional processing, at the expense of an increase in processing time, however.

**Table 1** Patients and donor lymphocyte products characteristics showing results of each processing step

Patients	UPN 853	UPN 924	UPN 931	UPN 953	UPN 960	Median	
Gender	F	M	F	M	F		
Age (years)	5	3	25	16	24	16	
Disease	AML M5a	AML M7	AML M4	AML M5	CML		
Disease state	CR1	CR1	2nd Relapse	CR2	2nd CP		
Weight (kg)	17	18	48	35	60	35	
Donor	Father	Mother	Father	Mother	Mother		
<i>KIR alloreactivity</i>							
Present	Yes	No	Yes	Yes	No		
Donor KIR ligand missing in the recipient	HLA-Bw4	—	Group 2 HLA-C	Group 2 HLA-C+HLA-Bw4	—		
<i>Graft</i>							
CD34+ (× 10 <sup>6</sup> /kg)	80.8	21.6	34.9	14.8	11.2	34.9	
CD3+ (× 10 <sup>5</sup> /kg)	0.42	0.001	0.55	0.04	0.1	0.1	
Engraftment (day post-transplant)	15	14	9	12	11	12	
Interval HSCt to NK-DLI (months)	26	12	3	4	8	8	
Reasons for NK-DLI	Mixed chimerism	Mixed chimerism	Mixed chimerism	Early relapse	Graft failure		
Chimerism at the time of NK-DLI (% donor)	88	46	79	95	26	79	
<i>Products</i>	853	924	931a	931b	953	960	Median
<i>Cell number after leukapheresis</i>							
MNC (× 10 <sup>7</sup> )	1191	2724	899	1239	1788	1012	1215
CD3+ T cells (× 10 <sup>7</sup> )	542	1539	284	384	928	420	481
CD56+ NK cells (× 10 <sup>7</sup> )	94	301	177	151	221	17	164
<i>Results after 1st step (T-cell depletion)</i>							
MNC (× 10 <sup>7</sup> )	319	234	363	495	285	422	341
CD3+ T cells							
Absolute number (× 10 <sup>7</sup> )	0.79	0.45	0.06	1.6	0.1	0.78	0.59
Log depletion	2.8	3.5	3.7	2.4	3.9	2.7	3.15
CD56+ NK cells							
Absolute number (× 10 <sup>7</sup> )	34	94	81	103	89	NA	89
Recovery	36%	31%	46%	68%	40%	NA	40%
Purity	10.70%	40.20%	22.30%	20.80%	31.20%	NA	22.30%
<i>Results after 2nd step (NK enrichment)</i>							
MNC (× 10 <sup>7</sup> )	31	41	69	67	70	16	54
CD3+ T cells							
Absolute number (× 10 <sup>7</sup> )	0.198	0.05	0.053	0.518	0.087	0.181	0.134
Total log depletion	3.4	4.5	3.7	2.9	4	3.4	3.55
CD56+ NK cells							
Absolute number (× 10 <sup>7</sup> )	31	40	68	66	67	13	53
Recovery	33%	13%	38%	43%	30%	75%	35.50%
Purity	98.90%	96.80%	98.80%	97.90%	95.40%	77.90%	97.35%
<i>NK-DLI</i>							
Cells after processing							
CD56+/CD3− cells × 10 <sup>7</sup> /kg	1.81	2.2	1.41	1.37	1.9	0.21	1.61
CD3+ cells × 10 <sup>5</sup> /kg	1.1	0.28	0.11	1.08	0.25	0.3	0.29
Product split	Yes	Yes	No	Yes	Yes	No	
Cells infused							
CD56+/CD3− cells × 10 <sup>7</sup> /kg	0.9	1.1	1.41	0.69	0.95	0.21	0.93
CD3+ cells × 10 <sup>5</sup> /kg	0.55	0.14	0.11	0.54	0.12	0.3	0.22

CR: complete remission; CP: chronic phase; NK-DLI: natural killer donor cell infusion; MNC: mononuclear cells; NA: not applicable.



**Figure 1** Percent donor chimerism in the five patients before and after treatment with NK-DLI. Arrows (on top) represent time of NK-DLI, UPN953 had only one infusion (represented by the first arrow); UPN853, 924, 960 had two infusions (represented by the first and second arrow) and UPN931 had three infusions (represented by all three arrows). UPN960 received an additional stem cell boost (asterisks). The X-axis represents time prior to and after the first NK-DLI in months.

Infusion of NK-DLI was well tolerated and none of the patient had GvHD. NK-DLI resulted in increasing donor chimerism in two patients, but one of them had received an additional stem cell dose after NK-DLI. Stabilization of donor chimerism was found in one patient and decreasing donor chimerism in two; one of them had early relapse. This study was not designed to test the efficacy of NK-DLI; nevertheless, early results appear encouraging. This pilot study has several limitations. The patient number was small and heterogeneous. We do not have data on long-term follow-up. We did viability testing on the NK cells but we do not have data on their functionality. Finally, KIR alloreactivity was estimated based on HLA typing and not measured directly in these donor–recipient pairs.

In conclusion, purification of NK cells for infusion is technically feasible and possibly safe. To evaluate the role of NK-DLI in consolidation of engraftment and prevention of relapse, we have started a larger phase II study, evaluating pre-emptive donor NK-cell infusions after haploidentical HSCT.

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