Successful Transfer of Umbilical Cord Blood CD34+ Hematopoietic Stem and Progenitor-derived NK Cells in Older Acute Myeloid Leukemia Patients

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Abstract

**Purpose:** Older acute myeloid leukemia (AML) patients have a poor prognosis; therefore, novel therapies are needed. Allogeneic natural killer (NK) cells have been adoptively transferred with promising clinical results. Here, we report the first-in-human study exploiting a unique scalable NK-cell product generated ex vivo from CD34+ hematopoietic stem and progenitor cells (HSPC) from partially HLA-matched umbilical cord blood units.

**Experimental Design:** Ten older AML patients in morphologic complete remission received an escalating HSPC-NK cell dose (between 3 and 30 × 10^6/kg body weight) after lymphodepleting chemotherapy without cytokine boosting.

**Results:** HSPC-NK cell products contained a median of 75% highly activated NK cells, with <1 × 10^4 T cells/kg and <3 × 10^5 B cells/kg body weight. HSPC-NK cells were well tolerated, and neither graft-versus-host disease nor toxicity was observed. Despite no cytokine boosting being given, transient HSPC-NK cell persistence was clearly found in peripheral blood up to 21% until day 8, which was accompanied by augmented IL15 plasma levels. Moreover, donor chimerism up to 3.5% was found in bone marrow. Interestingly, in vivo HSPC-NK cell maturation was observed, indicated by the rapid acquisition of CD16 and KIR expression, while expression of most activating receptors was sustained. Notably, 2 of 4 patients with minimal residual disease (MRD) in bone marrow before infusion became MRD negative (<0.1%), which lasted for 6 months.

**Conclusions:** These findings indicate that HSPC-NK cell adoptive transfer is a promising, potential "off-the-shelf" translational immunotherapy approach in AML. Clin Cancer Res; 23(15): 4107–18. ©2017 AACR.

Introduction

Acute myeloid leukemia (AML) is prevalent in older adults with a mean age of 69 years (1). Current treatment consists of intensive chemotherapy, hypomethylating agents, low-dose cytarabine, or supportive care (2, 3). Although morphologic complete remission (CR) can be achieved in 40%–60% of older patients (4–6), most patients relapse resulting in a 5-year overall survival (OS) of about 10%. This poor outcome can be attributed to unfavorable cytogenetic and molecular features, and increased incidence of multidrug resistance (7). Intensive treatment modalities, such as allogeneic stem cell transplantation (allo-SCT) are often limited due to comorbidities (8). However, nonmyeloablative conditioning regimens have made allo-SCT feasible for older and medically less fit patients (9, 10). Unfortunately, net results in terms of OS has not improved, because reduction of nonrelapse mortality (NRM) is counterbalanced by higher incidence of relapse-related mortality (11–15). Hence, relapse is still the leading cause of treatment failure in AML patients, who are consolidated with allo-SCT, after having achieved morphologic CR (10, 13, 14).
Persistent minimal residual disease (MRD) has been associated with increased relapse rates and decreased OS in both myeloablative and nonmyeloablative allo-SCT (16–18). Therefore, improved and tolerable conditioning therapies are needed to reduce or eradicate MRD in older AML patients.

Adoptive cell therapy (ACT) exploiting allogeneic natural killer (NK) cells can exert anti-AML activity without inducing graft-versus-host disease (GVHD) and is clinically well tolerated (19–22). Allogeneic NK-cell ACT is performed following cyclophosphamide and fludarabine-based lymphodepleting chemotherapy, thereby preventing immediate rejection of transferred allogeneic NK cells. Moreover, it eliminates tolerogenic immune cells and promotes cytokine availability for in vivo NK-cell persistence and expansion (20, 23). Most previous studies combined NK-cell infusion with administration of IL2 to boost in vivo expansion (19–24). However, high doses of IL2 resulted in significant toxicity. Furthermore, an increase in regulatory T cells (Tregs) was found after IL2 administration even at lower dosage, which could inhibit NK-cell functionality (23–25). Interestingly, studies at the University of Minnesota (Minneapolis, MN) showed that poor prognosis AML patients with relapsed/refractory disease achieved hematologic CR after NK-cell therapy in 26% (5/19) and 53% (8/15) of cases without or with prior IL2-diphtheria toxin fusion protein (IL2DT) treatment to deplete Tregs, respectively (20, 24). This increased CR rate allowed several patients to become eligible for potentially curative allo-SCT (24). Although NK-cell ACT is a promising therapy in AML patients, the desired clinical effect needs further enhancement of the NK-cell–mediated antitumor effect.

Effective ACT requires NK cells to be appropriately activated, available in sufficient numbers, have appreciable persistence in vivo, home to the tumor site, and effectively kill tumor cells. Generally, allogeneic NK-cell products have been enriched from the peripheral blood (PB) of haploidentical donors followed by stimulation with IL2 or IL15. However, this cellular product is a heterogeneous mixture of cells. CD3+ T-cell and CD19+ B-cell depletion results in a cell product with 23%–70% of infused cells being NK cells (20, 23, 24). CD3 depletion followed by CD56-positive selection can result in higher percentages of NK cells (66%–97%), but relatively low NK-cell dosages of 3–5 × 106 cells/kg can be generated using these methods (21, 22, 24). Moreover, these procedures reported T-cell dosages of 5 × 109/kg up to 2 × 1011/kg in adult patients, resulting in an increased risk of GVHD. Therefore, development of more homogeneous and well-defined allogeneic NK-cell products is preferable for adoptive immunotherapy. Previously, we reported a good manufacturing practice (GMP)-compliant culture method for the ex vivo generation of highly active NK cells (26). In this system, CD34+ human stem and progenitor cells (HSPC) isolated from allogeneic umbilical cord blood (UCB) are expanded and differentiated into NK cells in the presence of IL15 and IL2, resulting in a clinically relevant dose of NK cells with a purity >85% (26, 27). These allogeneic HSPC-NK cells show cytolytic activity against AML cells in vitro (26) as well as bone marrow homing capacity and antileukemic effects in vivo (28).

Here, we describe the first-in-human phase I study investigating ACT with escalating dosages of UCB-derived HSPC-NK cells in older AML patients. HSPC-NK cell infusion was performed without IL2 administration to investigate IL15-driven homeostatic NK-cell persistence and tolerability after infusion, and to avoid IL2-mediated Treg expansion potentially affecting donor NK-cell functionality. We demonstrate that ACT using this scalable and potentially “off-the-shelf” HSPC-NK cell product results in transient donor NK-cell persistence, homing to the bone marrow and further in vivo maturation in older AML patients following lymphodepleting chemotherapy. HSPC-NK cells were well tolerated and neither GVHD nor toxicity was induced. Two of 4 patients with detectable MRD before infusion became MRD negative after HSPC-NK cell ACT. Although we cannot separate the effect of the NK-cell infusion from the effect of immunosuppressive chemotherapy on MRD, our results indicate that HSPC-NK cell ACT represents an innovative and promising strategy for patients with AML.

Materials and Methods

Study design

Older AML patients who achieved morphologic CR after remission induction chemotherapy or hypomethylating agents were included. Inclusion criteria were: AML age ≥ 55 years and contraindication for allo-SCT, no HLA-antibodies, CR after first- or second-line chemotherapy, World Health Organization (WHO) performance score < 2 and life expectancy > 6 months. The study protocol has been approved by the National Medical Ethical Committee (CCMO registration NL31699.00020; EudraCT number 2010-018988-41) and the study was registered at the Dutch clinical trial registry (NTR 2818). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

UCB selection

HLA typing of eligible AML patients was performed by the Luminex method using the LAbType SSO Kit (OneLambda). Subsequently, a suitable allogeneic UCB unit displaying the highest HLA match for HLA-A, -B, and -C was selected for HSPC-NK cell product manufacturing (for details, see Supplementary Information). KIR typing of UCB units was performed with the KIR SSO genotyping test (OneLambda). A total of 350 UCB units within...
the Cord Blood Bank Nijmegen containing a mean of $874 \pm 439 \times 10^6$ (range 230–3,070 \times 10^6) viable nucleated cells and $4.66 \pm 3.64 \times 10^6$ (range 2.00–33.95 \times 10^6) viable CD34+ HSPC were available. The UCB requirements as drug substance for HSPC-NK product manufacturing were negative for viral, bacterial, and fungal contamination, and presence of $\geq 2.0 \times 10^6$ viable CD34+ HSPCs.

HSPC-NK cell infusion

To favor HSPC-NK cell persistence, patients received non-myeloabative chemotherapy with cyclophosphamide (Cy) 900 mg/m²/day and fludarabine (Flu) 30 mg/m²/day from day -6 to -3. Following premedication with acetaminophen (500 mg) and clemastine (2 mg), HSPC-NK cells were administered intravenously 3 days after conditioning (day 0). Except for patient 1, all patients received a single dose of pegfilgrastim (6 mg) on day 8 to prevent long-term neutropenia. According to the local guidelines for severe immunocompromised patients, all patients were treated ciprofloxacin 500 mg twice daily and fluconazole 400 mg once daily during neutropenia. In addition, patients were treated with cotrimoxazol 480 mg once daily and valaciclovir 500 mg twice daily until one year after cyclophosphamide and fludarabine conditioning.

Results

Patient characteristics

In this study, we investigated the feasibility, safety, and biological properties of allogeneic HSPC-NK cell infusion following lymphodepleting chemotherapy in older (≥55 years) AML patients, who were not eligible for allo-SCT because of age or comorbidity. A total of 17 patients were enrolled (Fig. 1A). All included patients had a WHO performance status of 0 to 1. Four patients were excluded after consent signing: two patients had anti-HLA class I antibodies, one patient did not reach morphologic CR, and one patient withdrew informed consent. For 13 patients, HSPC-NK cell product manufacturing was initiated. One production was interrupted because of relapse during culturing. Another patient developed a relapse just before the start of HSPC-NK cell production was completed). Eleven remaining patients received cyclophosphamide and fludarabine conditioning, and 10 of 11 HSPC-NK cell products generated met the release criteria. Eventually, 10 patients were treated with escalating dosages of HSPC-NK cells (Fig. 1B). Dosages were based on previous studies with enriched NK-cell products from peripheral blood ranging from $1–5 \times 10^6$/kg using the CD3-depletion/CD56-enrichment method (21) and $2–15 \times 10^6$/kg using the CD3/CD19 depleting method (20). The intended HSPC-NK dosages were 3, 10 and $30 \times 10^6$/kg in 3 cohorts of 3 patients. Patient characteristics and their UCB-donor are displayed in Table 1. Median age of patients was 72 years (range 68–76 years). Patients were all in morphologic CR after one ($n = 1$), two ($n = 4$), or three ($n = 2$) courses of standard intensive chemotherapy (Table 1). Three patients were in morphologic CR after intensive chemotherapy followed by treatment with hypomethylating agents (IUPN7, 8, and 9).

HSPC-NK cell products

For the generation of allogeneic NK-cell products, partially HLA-matched UCB units were selected on the basis of the HLA type of the patient. Matching for HLA-A, -B, and -C for the 10 treated patients was 6/6 for 1 patient, 3/6 for 5 patients, 2/6 for 3 patients and 1/6 for 1 patient. Although KIR mismatch was not prioritized in this study, 7 of 10 patients had a KIR receptor-ligand mismatch, where the UCB donor possessed inhibitory KIR genes for which the HLA ligand was lacking in the patient (Table 1). KIR genotype of the used UCB units is reported in Table 1. Cell numbers at each step of the manufacturing process are shown in Supplementary Table S1. After thawing and CD34 selection, a median of $2.63 \times 10^6$ CD34+ cells (range, 1.15–8.13) were placed in culture. The mean purity of the CD34+ cell product was 74% ± 13% (range 46%–86%). After 42 days of culture, a median of $2.341 \times 10^6$ total nucleated cells (TNC; range 563–5,842) were recovered. Purity of CD56-CD3+ NK cells in the generated cell products was 75% ± 12% (range 40–85) with a median viability of 94% (range 88–99). The percentage of CD3+ T cells and CD19+
Donor HSPC-NK cell persistence

As anticipated, cyclophosphamide and fludarabine conditioning and infusion of HSPC-NK cells was well tolerated and no NK-cell infusion-related toxicities were seen. Furthermore, we did not observe any signs of GVHD. Importantly, no clinical signs of cytokine release syndrome or other unexpected toxicities were observed. Furthermore, no significant increase in IL6 nor ALT levels was observed (data not shown). Cyclophosphamide and fludarabine conditioning did result in expected hematologic toxicity. The median time to neutrophil count of $>0.5 \times 10^9/L$ and to platelet count $>25 \times 10^9/L$ was 21 and 29 days, respectively. Two patients had a neutropenic period $>30$ days, and 4 patients had a platelet count $<25 \times 10^9/L$ for $>30$ days. Six patients developed febrile neutropenia, but all recovered well with antibiotic treatment. An overview of all adverse events $\geq$grade 2 is shown in Supplementary Table S3.

Safety of HSPC-NK cell infusion

Patients were monitored for toxicity and GVHD at specified time points. Administration of cyclophosphamide and fludarabine conditioning and infusion of HSPC-NK cells was well tolerated and no NK-cell infusion-related toxicities were seen. Furthermore, we did not observe any signs of GVHD. Importantly, no clinical signs of cytokine release syndrome or other unexpected toxicities were observed. Furthermore, no significant increase in IL6 nor ALT levels was observed (data not shown). Cyclophosphamide and fludarabine conditioning did result in expected hematologic toxicity. The median time to neutrophil count of $>0.5 \times 10^9/L$ and to platelet count $>25 \times 10^9/L$ was 21 and 29 days, respectively. Two patients had a neutropenic period $>30$ days, and 4 patients had a platelet count $<25 \times 10^9/L$ for $>30$ days. Six patients developed febrile neutropenia, but all recovered well with antibiotic treatment. An overview of all adverse events $\geq$grade 2 is shown in Supplementary Table S3.

Donor HSPC-NK cell persistence

As anticipated, cyclophosphamide and fludarabine conditioning induced lymphocytopenia in all patients (Supplementary Fig. S2A). Temporary lymphodepletion is essential to facilitate NK-cell persistence and proliferation in vivo. Absolute numbers of CD4+ T cells, CD8+ T cells, and CD56+CD3- NK cells were depleted to $<0.1 \times 10^9/L$ at the time of infusion (Supplementary Fig. S2B–S2D). Relatively, depletion of CD8+ T cells was more profound as compared with CD4+ T cells (Supplementary Fig. S2E and S2F). From day 8 onward, repopulation of patient’s NK cells and T cells was observed. Endogenous IL15 plasma level,
which is essential for in vivo survival and expansion of infused NK cells, increased after cyclophosphamide and fludarabine conditioning. We observed a 6-fold increase in IL15 concentration from baseline (18–5 ng/mL) with a peak at day 6 after cyclophosphamide and fludarabine and NK-cell infusion (mean 49–16 ng/mL; Fig. 3A). Although patients did not receive IL2 injections, a relative increase in the percentage of Tregs within the CD4+ T-cell population was observed (Fig. 3B). However, the mean absolute number of Tregs was not augmented.

Successful donor chimerism was detectable in all patients after infusion. At day 6–8, we found chimerism up to 21% in peripheral blood and up to 3.5% in bone marrow (BM) (Fig. 3C and D). Donor chimerism tend to increase when more HSPC-NK cells were infused, although the patient that reached the highest chimerism at day 6 did not receive most NK cells (i.e., UPN5 who received 10 x 10^6/kg). Notably, this patient did reach the highest amount of endogenous IL15. These data demonstrate that HSPC-NK cells transiently persist in peripheral blood and bone marrow following cyclophosphamide and fludarabine conditioning in the absence of additional cytokine boosting.

Transferred HSPC-NK cells mature in vivo by acquisition of CD16 and KIR expression

To investigate HSPC-NK cell phenotype after infusion, multicolor flow cytometry (FCM) analysis was performed at specified time points. Ex vivo–generated HSPC-NK cells express higher levels of CD56, as compared with endogenous CD56dim and CD56bright NK cells, so these cells could be readily detected by FCM in vivo. Interestingly, the phenotype of infused NK cells of three patients (UPN5, 7, and 8) could be analyzed (Fig. 4A). In these patients, a clear population of CD3+CD56bright HSPC-NK cells was observed. This population increased in time, and this increase reflected the measured donor chimerism. Interestingly, CD16 expression on donor HSPC-NK cells increased after infusion.
resulting in frequencies up to 30%–60% at day 6–8 (Fig. 4B; Supplementary Fig. S3A). As reported previously (29), IL15/IL2 expanded HSPC-NK cell products express low levels of KIRs (<5%; Fig. 2B). However, FCM analysis revealed that HSPC-NK cells rapidly acquire KIRs after infusion (Supplementary Fig. S3B). In addition to NK-cell maturation, preservation of an activation phenotype is essential for NK-cell reactivity in vivo. Importantly, we did not observe a significant decrease in the expression of NKp30, NKp46, or NKG2D; however, expression of NKp44 did decrease after infusion (Fig. 4C). These results demonstrate that in vivo persisting HSPC-NK cells further mature into KIR+CD16+ NK cells while preserving an activated phenotype in vivo after adoptive transfer.

Clinical outcome and effect on MRD

At the time of HSPC-NK cell infusion, all patients were in morphologic CR (Table 1). Currently, 4 of the 10 treated patients are disease free for 60, 52, 22, and 16 months, respectively. The other 6 patients relapsed after a mean duration of 364 days after cyclophosphamide and fludarabine and HSPC-NK cell infusion. In most patients, investigations to detect MRD were performed by FCM using leukemia-associated phenotypes (LAP) and/or molecular analysis (Table 2). Before HSPC-NK cell infusion, UPN3 and UPN5 showed a positive qPCR MRD signal in bone marrow and peripheral blood for the TP53 and IDH2 mutation, respectively (Table 2). After infusion, the signals remained detectable and patients relapsed after 4 (UPN3) and 6 (UPN5) months. Furthermore, FCM could detect MRD at day -14 in two patients (UPN7 and 8). This could be confirmed in UPN8 by a positive qPCR detection in bone marrow after adoptive transfer of a RUNX1 mutation. Notably, we observed a clear decrease in the FCM-based MRD signal in UPN7 and 8 (Fig. 5A and B; Table 2). Both patients had been...
treated with azacitidine before start of the study. UPN7 had been treated with one course of chemotherapy, and subsequently received two courses of azacitidine (50 mg/m², 5 days, last dose day -26). At day -14, MRD of 6.7% was measured in bone marrow by FCM (Fig. 5B, top). This patient was treated with cyclophosphamide and fludarabine followed by infusion of 30 × 10⁶/kg HSPC-NK cells. At day 90 after infusion, MRD in bone marrow was <0.01% (Fig. 5A and B). Thereafter, MRD remained low in bone marrow until day 180. However, UPN7 relapsed shortly thereafter, at day 194. Azacitidine therapy was started, but could not be continued due to infectious complications, and the patient died at day +306. UPN8 had received one course of chemotherapy followed by 3 courses of decitabine (20 mg/m², 5 days). Because of persisting disease during decitabine therapy, he was considered ineligible for allo-SCT. Subsequently, therapy was switched to azacitidine (75 mg/m², 7 days for 4 cycles, followed by 50 mg/m², 5 days, last dose day -17). Despite this therapy, MRD of 6.1% was measured at day -20 in bone marrow by FCM (Fig. 5B, bottom), and 6% RUNX1-mutated cells in bone marrow and peripheral blood.

Figure 4. HSPC-NK cells expand and further mature in vivo. The phenotype of infused NK cells was determined by FCM at different time points in UPN 5, 7, and 8. A, The percentage of CD3⁺CD56bright NK cells, a phenotype which corresponds to our HSPC-NK cell product, increased in time in peripheral blood (PB) and bone marrow (BM). Gated on CD45⁺ lymphocytes. B, The percentage of CD16⁺ cells within the CD3⁺CD56bright NK-cell population at different time points in UPN 5, 7, and 8. C, Expression of activating receptors on HSPC-NK cell products before infusion within the CD3⁺CD56bright NK cells in peripheral blood at day 6, and bone marrow at day 8 after infusion in three patients (mean ± SD) are shown. * P < 0.05, one-way ANOVA with Bonferroni post hoc test.
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<th>MRD status Day +7</th>
<th>MRD status Month +3</th>
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NOTE: MRD<sup>a</sup> or relapse is depicted in bold text.
Abbreviations: BM<sup>POS</sup>, negative in bone marrow; CRI, first complete remission; CR3, third complete remission; ND, not determined; NE, not evaluable; PB<sup>POS</sup>, negative in peripheral blood.

<sup>a</sup>Molecular MRD: POS is >0.1% mutated cells, and NEG is ≤0.1% mutated cells; (detection limit qPCR assays is 0.1%).

<sup>b</sup>FCM MRD: POS is >0.01% LAP<sup>+</sup> cells, and NEG is ≤0.01% LAP<sup>+</sup> cells; (detection limit FCM MRD assays is 0.01%).
were detected by qPCR (Table 2). This patient was treated with cyclophosphamide and fludarabine and $17 \times 10^6$/kg HSPC-NK cells. At day 90, MRD in bone marrow was reduced to 0.04% (Fig. 5B, bottom), and RUNX1-mutated cells were <0.1% in peripheral blood (Table 2). UPN8 relapsed thereafter at day 181, and treatment with selinexor was started. However, blast counts rapidly increased despite therapy and the patient passed away at day +301. Collectively, these findings suggest that immunosuppressive chemotherapy followed by a high dose of HSPC-NK cells exerted a transient antileukemic effect.

Figure 5.
Decreased AML blasts in bone marrow (BM) after HSPC-NK cell therapy. In 2 patients, the percentage of AML blasts in bone marrow was measured at different time points by FCM using consensus leukemia-associated phenotypes (LAP). A, Graphic representation of subsequent treatments and their effect on the percentage of AML blasts in the bone marrow in UPN7 and UPN8. B, Decrease in the percentage of LAP$^+$ AML cells of UPN7 (CD45$^+$CD34$^-$/CD133$^+$CD117$^-$ cells) and UPN8 (CD45$^+$CD34$^-$CD133$^+$CD7$^-$ cells) in bone marrow at day 90 after HSPC-NK cell therapy, as compared with day -14 (UPN 7) or day -20 (UPN8).
Discussion

Here, we investigated the safety, toxicity, and biologic properties of ACT with escalating dosages of *ex vivo*-generated, UCB-derived HSPC-NK cells without additional cytokine administration in older AML patients (EudraCT number 2010-018988-41). This is the first time that UCB-derived HSPC-NK cell ACT has been explored in humans. A major advantage of using widely available UCB units for generating NK cells compared with haploidentical peripheral blood donors is the selection of the most optimal NK donor based on KIR haploype B and KIR-L (HLA) genetics. Using a limited number of 350 UCB units, we were able to select a partially HLA-matched UCB unit for most AML patients with either a KIR receptor–ligand mismatch and/or KIR B haploype (Table 1). Ten older AML patients in morphologic CR were successfully infused withalloreactive HSPC-NK cells (between 3 and 30 × 10^6/kg body weight) after cyclophosphamide and fludarabine chemotherapy. We found that this was safe with limited toxicity. In line with earlier studies investigating ACT with allogeneic NK-cell products, we did not observe GVHD or severe infections (19–24). As expected, conditioning with cyclophosphamide and fludarabine induced hematologic toxicity. The median time to absolute neutrophil and platelet count recovery was comparable with the cytopenic period reported earlier by Curti and colleagues (21, 22). In our study, 2 patients had a neutropenic period >30 days, and 4 patients had a platelet count below 25 × 10^9/L for >30 days. So while the majority of patients did recover quickly, heavily pretreated older AML patients are at risk to develop prolonged cytopenia. These results suggest the need for a careful evaluation for the eligibility for cyclophosphamide and fludarabine plus NK-cell therapy, which should be proposed only to patients with sufficient bone marrow reserve.

Patients in the current study were not eligible for allo-SCT at the time of study inclusion. At the start of the study, allo-SCT was not performed in patients >65 years of age at our center. Currently, 4 of 10 treated patients (UPN1, 2, 9, and 10) are still alive and disease free at 60, 52, 22, and 16 months after NK-cell infusion. Moreover, one patient (UPN4) was disease free for 35 months, and 2 of 4 (very) poor risk patients with detectable MRD, became negative (<0.1%) lasting for 6 months. Some of these patients would currently be eligible for allo-SCT, as during our study, NMA allo-SCT was introduced in medical fit AML patients >65 years of age (8, 30). We observed that HSPC-NK cell infusion following cyclophosphamide and fludarabine conditioning can at least temporarily decrease MRD. Although in this study the therapeutic effect of HSPC-NK cells cannot be separated from a cyclophosphamide and fludarabine effect, we believe this therapy could be an attractive strategy to reduce MRD before NMA allo-SCT in patients with morphologic CR. Especially as prolonged cytopenia will not be a limiting factor using this strategy due to the donor stem cell rescue. In addition, HSPC-NK cell therapy could also be applied for consolidating medical less fit younger patients with (very) poor risk AML that undergo NMA allo-SCT.

In this phase 1 study, we performed HSPC-NK cell infusion without IL2 administration. This study design allowed us to separate toxicity induced by cyclophosphamide and fludarabine conditioning and HSPC-NK cell infusion from IL2-mediated toxicity, which can be considerable (31). Besides, it has been reported that Tregs, capable of suppressing NK-cell function, are relative resistant to cytotoxic therapy and expand rapidly after IL2 administration (23, 25, 32, 33). Although we did observe a relative increase in Tregs at day 14 after HSPC-NK cell infusion, the absolute Treg number before and after treatment was unchanged. Importantly, HSPC-NK cells were detectable up to 21% in peripheral blood and 3.5% in bone marrow until day 8 in patients infused with >10 × 10^6/kg. Regarding the HSPC-NK cell dose, 3 patients received 1 × 10^6/kg, 1 received 6 × 10^6/kg, 3 received 10 × 10^6/kg, 1 received 17 × 10^6/kg, and 2 received 30 × 10^6/kg. Furthermore, for two of the included patients (UPN11 and 12), who were not infused due to T-cell impurity in the product (UPN11) or relapse before the start of cyclophosphamide and fludarabine conditioning (UPN12), the achievable HSPC-NK cell dose was 23 and 30 × 10^6/kg, respectively. The highest HSPC-NK cell dose that was given in this study was 30 × 10^6/kg. This is 7.5-fold higher than the medium infused dose of 4 × 10^6/kg CD3-depleted/CD56-enriched PB-NK cells (22) and in the same range of the highest PB-NK cell doses (mean 26 ± 15 × 10^6/kg) using the CD3/CD19 depletion method (24). However, HSPC-NK cell products hardly contain allogeneic T-cell impurity, avoiding the chance of inducing GVHD. The future perspective of exploiting HSPC-NK cell therapy is at least the infusion of 30 × 10^6 HSPC-NK cells/kg, which could be increased by further optimizing the culture protocol.

Notably, donor NK-cell chimerism could be detected in all patients, but NK-cell numbers did not exceed an absolute level of 100 cells/μL in peripheral blood. Miller and colleagues (20) showed that an *in vivo* NK-cell persistence and expansion of >100 cells/μL at day 7 to 14 after infusion and IL2 boosting is correlated with achievement of CR in relapsed/refractory AML patients. This indicates that this surrogate endpoint could be used to evaluate clinical efficacy of NK-cell therapy products. As we did not reach this surrogate endpoint in the current study, further improvement of our therapy is needed, such as boosting *in vivo* expansion using cytokines. As mentioned earlier, IL2 has been used in most reported studies to stimulate NK-cell expansion *in vivo* (19–22, 24). However, as IL15 is crucial for NK-cell survival, proliferation, and effector function (34, 35), and in contrast to IL2 does not promote Treg expansion (36, 37), it is a very attractive cytokine to boost NK-cell expansion. Clinical grade IL15 is available, though its efficacy might be limited by its short half-life and poor bioavailability in lymphoid organs (38). Hence, IL15 superagonist complexes with enhanced biological activity have been developed (39). A promising IL15 superagonist is ALT-803, which includes an activating mutation in IL15, a IL15Rα sushi domain to increase transpresentation, and an IgG1 Fc domain to increase *in vivo* half-life and stability (38). A phase I trial, investigating ALT-803 in patients with relapsed hematologic malignancy after allo-SCT revealed no dose-limiting toxicity (40). In addition, a dose-dependent increase in NK-cell proliferation was observed. For these reasons, the combination with ALT-803 is a promising strategy to boost HSPC-NK cell expansion in future clinical studies.

Next to boosting *in vivo* expansion, the killing potential of HSPC-NK cell products could be further improved to maximize clinical efficacy. Recently, we published an improved HSPC-NK cell generation protocol (29). In this protocol, IL2 is replaced by IL12 during *ex vivo* NK-cell generation. Addition of IL12 to the culture protocol resulted in superior IFNγ production and cytolytic activity of HSPC-NK cells towards AML cells *in vitro*. Importantly, these NK cells demonstrated improved antileukemic responses in mice bearing human AML cells (29). Furthermore, we showed both in mice (28, 29), and now also in patients that HSPC-NK cells rapidly gain CD16 expression *in vivo*. This facilitates the combination with mAbs directed against antigens.
expressed on leukemic blasts such as CD33, CD123, and CLEC12A to exploit antibody-dependent cellular cytotoxicity as well as with TriKEs composed of single-chain scFv against CD16 and CD33 in combination with a modified IL15 cross-linker (41). Another way to make HSPC-NK cells more antigen specific is the redirection toward target antigens expressed by leukemic blasts by chimeric antigen receptors (CAR; ref. 42). This is particularly interesting for HSPC-NK cells as transduction efficacy of expanding CD34+ cells is much more effective compared with viral transduction of peripheral blood–derived NK cells. Consequently, HSPC-NK cell products with improved antitumor potential have great promise for immunotherapy in AML and other cancers. Altogether, we found that ACT using HSPC-NK cell products in older AML patients following lymphodepleting chemotherapy is tolerable with limited toxicity. In addition, we demonstrate that HSPC-NK cells transiently persist, home to the bone marrow, and undergo in vivo maturation into antileukemic effector cells. Interestingly, we found a reduction in MRD in some poor risk AML patients with morphologic CR. This HSPC-NK cell-based immunotherapy could be an adjuvant therapy to decrease or eradicate MRD in AML patients following intensive chemotherapy or treatment with hypomethylating agents, and serve as a consoli-
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Disclosure of Potential Conflicts of Interest
N.M. Schaap is a consultant/advisory board member for Novartis. No potential conflicts of interest were disclosed by the other authors.

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Dolstra et al.


Successful Transfer of Umbilical Cord Blood CD34+ Hematopoietic Stem and Progenitor-derived NK Cells in Older Acute Myeloid Leukemia Patients

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