Supplementary Information

Supplementary materials and methods

GMP-compliant manufacturing of the HSPC-NK cell products

In all UCB units, the red blood cell count had been reduced before storage using standard EloHAES® separation, and the mononuclear cells had been washed, cryopreserved and stored in liquid nitrogen.*(1)* On day -42 before infusion, the selected UCB unit was thawed at 37°C and resuspended in thawing buffer consisting of CliniMACS PBS/EDTA buffer (Miltenyi Biotech, Bergisch Gladbach, Germany), 5% human serum (HS; Sanquin blood bank, Amsterdam, the Netherlands), 3.5 mM $MgCl_2$ (Pharmacy Department, RUNMC, Nijmegen, The Netherlands) and 100 U/ml Pulmozyme (Roche, Almere, The Netherlands) as described previously.*(2)* After thawing, $CD34^+$ HSPCs were enriched using the CliniMACS Cell Selection Device (Miltenyi) and directly used for NK cell generation according to the culture method described previously.*(2)* Briefly, the cells were cultured in the Radboudumc clean room facility under GMP conditions in VueLife™ culture bags (Cellgenix, Freiburg, Germany) in Glycostem Basal Growth Medium (GBGM®; Glycostem Therapeutics, 's-Hertogenbosch, The Netherlands) supplemented with 10% HS (Sanquin Bloodbank, Nijmegen, the Netherlands), a low-dose cytokine cocktail consisting of GM-CSF (Neupogen; Amgen, Breda, the Netherlands), G-SCF and IL-6 (both Cellgenix). At day 0-9, the medium was additionally supplemented with a high-dose cytokine cocktail containing SCF, Flt3L, TPO and IL-7 (all CellGenix), and between day 10 and 14 TPO was replaced by IL-15. After 14 days cells were differentiated by replacing Flt3L by IL-2 (Proleukin®; Chiron, München, Germany). During the first 14 days of culture, low molecular weight heparin (LMWH; Clivarin®; Abott, Wiesbaden, Germany) was added to the media. Around day 14 cells were transferred to the WAVE Bioreactor™ System 2/10 (GE Health, Uppsala, Sweden). The

conditions of the bioreactor were as follows: temperature 37°C, CO_2 5%, airflow 0.1-0.2 L/min, rocking rate 10/min, rocking angle of 6°. After 42 days, NK cell products were harvested and washed. A sample of the final product was used for lot release testing. Before lot release, the product was required to have > 70% $CD56^+CD3^-$ cells, > 70% viability ($7AAD^-$) of $CD56^+CD3^-$ cells, > 30% positivity for NKG2A, NKp30, NKp44, NKp46 and $NKG2D^+$ on $CD56^+CD3^-$ cells, < $1x10^4$ $CD3^+$ T cells/kg, < $3x10^5$ $CD19^+$ B cells/kg and negative in-process bacterial and fungal cultures. Sterility testing was performed on the final products and reported 14 days after conditional release by the QP. All manufactured HSPC-NK products were negative for bacterial and fungal contamination.

Chimerism assay

PB and BM chimerism was determined by real-time quantitative PCR using single nucleotide polymorphisms (SNPs) and/or the *SMCY* gene as described previously.*(3)* Briefly, UCB units and AML patients were screened for discriminating SNPs. Quantification was based on real-time PCR with allele-specific primers for DNA sequences containing the discriminating SNP and target DNA-specific probes. Quantitative analysis was performed by generating calibration functions from cycle thresholds (C_t) obtained by real-time PCR of DNA serially diluted in water. The detection limit of the used assays were 0.1%. Donor chimerism was expressed as the percentage of donor cells within total PB or BM cells.

Immunophenotyping

The cell count, purity, and viability of the HSPC-NK cell product was determined using a single-platform FCM assay with the following antibodies CD45-ECD (J33), CD3-FITC (SK7), CD19-FITC (J3-119), CD34-PE (581) (all Beckman Coulter, Woerden, the Netherlands), CD56-PE (NCAM16.2), CD3 (SK7) (both BD Biosciences, San Jose, CA,

USA), 7AAD (Sigma, St Louis, MO, USA) and counting beads (Beckman Coulter). Ten-color analysis was used to determine the phenotype of HSPC-NK cell products as well as PB/BM samples from patients before and after treatment. The following monoclonal antibodies were used in the appropriate concentration: CD16-FITC (NP15, BD-bioscience, Franklin Lakes, NJ, USA), CD336-PE (Z231), CD3-ECD (UCHT-1), CD337-PEcy5 (Z25), CD355-PEcy7 (BAB281), CD314-APC (ON72), CD244-APC700 (C1.7), CD56-APC750 (N901), CD159a-PB (Z199), CD45-KO (J.33), CD85j-PEcy5.5 (HP-F1), CD158b-PEcy7 (GL183), CD158e-APC (Z27.3.7), CD158a-APC700 (EB6B), CD45RA-FITC (ALB11), HLA-DR-PE (Immu-357), CD45RO-ECD (UCHL1), CD5-PEcy5.5 (BL1a), CD27-PEcy7 (1A4CD27), CD56-APC (N901), CD8-APC700 (B9.11), CD3-APC750 (UCHT-1), CD4-PB (13B8.2) (all Beckman Coulter) and CD159c-PE (134591, R&D systems, Minneapolis, MN, USA). Treg numbers were determined using anti-human FoxP3 Staining Kit (BD Biosciences) according to the manufacturer's instructions. The acquisition was perfomed on the Navios^{TM} flow cytometer and the data were further analyzed using Kaluza^{TM} software (both from Beckman Coulter).

Serum cytokine concentrations

IL-15 concentrations were determined on frozen plasma by ELISA (R&D systems, Abingdon, Oxon, UK) as described previously.*(2)*

Flow cytometry based cytotoxicity and degranulation assay

Functional analysis of HSPC-NK cell products was assessed retrospectively using cryopreserved cells which were thawed and cultured for six days before use in basal medium supplemented with 10% HS, 1000 U/ml IL-2, and 20 ng/ml IL-15. Flow cytometry based cytotoxicity assays were performed as previously described.*(4)* Briefly, CFSE-labeled target

cells were resuspended in IMDM/10% FCS to a final concentration of 1-3 x 10^5/ml. HSPC-NK cells were washed with PBS and resuspended in IMDM/2% HS to a final concentration of 1-10 x 10^5/ml. Target cells were co-cultured at different E:T ratios in 96-well flat-bottomed plates. NK and target cells alone were plated as controls. After 24 or 48 hr incubation at 37^{o}C, cells were harvested and the number of viable target cells was quantified by flow cytometry. Target cell survival was calculated as follows: % survival = (absolute number of viable $CFSE^+$ target cells co-cultured with NK cells / absolute number of viable $CFSE^+$ target cells cultured in medium)*100. The percentage of specific killing was 100 minus % survival. Co-cultures of NK with primary AML cells were supplemented with IL-3 (50 ng/mL; Cellgenix), SCF (20 ng/mL; Immunotools), Flt3L (20 ng/mL; Cellgenix), G-CSF (20 ng/mL; Amgen), and IL-15 (5 ng/mL). Killing of K562 cells was measured following overnight incubation with HSPC-NK cells using increasing E:T ratios. In addition, killing of primary AML blasts was analyzed for three HSPC-NK product/patient pairs for which BM cells collected and cryopreserved at diagnosis were available. Degranulation of HSPC-NK cells during co-culture was measured by cell surface expression of CD107a. After 18 hrs of incubation at 37^{o}C, the percentage of $CD107a^+$ cells was determined by FCM.

MRD analysis

FCM-based detection of MRD was performed using consensus leukemia-associated (immune)phenotypes (LAPs) as described previously.*(5)* LAPs were established in newly diagnosed AML in a two-step approach. In the first step, FCM panels were used to define the immunophenotype of the blasts in whole BM or PB at diagnosis. In this so-called first run, aberrant expression patterns of individual antigens were defined, and from these runs relevant antigens for the putative LAPs for MRD assessment (one LAP per tube) were defined and tested in a second run. Sensitive LAPs were used for MRD detection.

AML patients were standard analyzed for NPM1, FLT3-ITD and CEPBA mutations. In addition, AML patients without these molecular aberrations were screened with the Ion AmpliSeq™ AML panel and subsequent sequencing on the Ion Torrent PGM (ThermoFisher Scientific, Waltham, MA, USA), according to manufacturer's instruction. This panel utilizes 237 amplicons to analyze 19 genes implicated in AML (entire coding regions: *CEPBA*, *DNMT3A*, *GATA2*, *TET2*, *TP53*; hotspot regions: *ASXL1*, *BRAF*, *CBL*, *FLT3*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *WT1*). Molecular MRD quantification was performed by real-time PCR with allele-specific primers for DNA sequences containing the patient-specific mutation and target DNA-specific probes. Quantitative analysis is performed by generating calibration functions from cycle thresholds (C_t) obtained by real-time PCR of DNA from the AML patient at diagnosis serially diluted in DNA from healthy donor BM DNA. The detection limit of the assays was 0.1%. Molecular MRD was expressed as the percentage of mutated cells within total PB or BM cells.

Statistical analysis

Results are presented as median values, ranges and/or mean ± SD. To compare expression of activation receptors before and after infusion the one-way analysis of variance with Bonferroni post-hoc test was used. A *P*-value of <0.05 was considered statistically significant.

References

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Supplementary Table 1. Characteristics of HSPC-NK cell products.

UPN	Cryopreserved UCB		After thawing			After CD34 selection		HSPC-NK products after expansion and preparation						
	Total NC ($x10^6$)	Total $CD34^+$ ($x10^6$)	Total NC ($x10^6$)	Total $CD34^+$ ($x10^6$)	$CD34^+$ Recovery (%)	Total $CD34^+$ ($x10^6$)	Purity $CD34^+$ (%)	Total NC ($x10^6$)	Purity $CD56^+CD3^-$ (%)	Viability $CD56^+CD3^-$ (%)	Infused $CD56^+CD3^-$ ($x10^6$)	$CD56^+CD3^-$ per kg ($x10^6$/kg)	$CD3^+$ per kg ($x10^5$/kg)	$CD19^+$ per kg ($x10^5$/kg)
1	2122	4.14	1137	2.78	67	2.68	81	2012	75	93	220	3	0.01	0.00
2	1415	2.90	690	2.07	71	1.76	85	563	81	99	324	3	0.00	0.00
3	545	2.02	313	1.47	73	1.21	71	3343	71	99	189	3	0.00	0.00
4	1133	5.55	633	3.04	55	3.47	82	3173	58	99	650	10	0.00	0.05
5	1221	5.55	561	3.14	57	2.58	86	4984	74	94	530	10	0.00	0.48
6	1106	3.37	511	1.64	49	1.57	52	2069	74	97	770	10	0.03	0.62
7	545	6.22	566	6.33	102	4.30	83	2156	79	93	1693	30	0.00	0.80
8	460	4.19	350	3.11	74	1.95	65	1820	65	96	1191	17	0.00	0.47
9	2147	15.90	1305	9.27	58	8.13	77	1198	40	88	510	6	0.05	1.10
10	1020	10.04	659	8.56	85	7.41	82	5842	79	91	2190	30	0.07	0.10
11	680	3.40	429	1.93	57	1.15	46	2525	85	94	Not infused due to exceeding the $CD3^+$ T cell impurity criteria; achieved manufacturing dose $23x10^6$/kg		0.43	0.02
12	3070	11.05	1984	6.35	57	6.58	82	3832	77	94	Not infused due to relapse prior Cy/Flu conditioning; achieved manufacturing dose $30x10^6$/kg		0.00	0.20
Median	1120	4.87	600	3.08	63	2.63	82	2341	75	94	NA	NA	0.00	0.15
Mean ± SD	1289 ±793	6.19 ±4.11	762 ± 485	4.14 ± 2.75	67 ± 15	3.57 ±2.49	74 ± 13	2793 ± 1528	75 ±12	95 ±3	NA	NA	0.05 ±0.12	0.32 ±0.37

Abbreviations: UCB, umbilical cord blood; NC, nucleated cells; SD, standard deviation; NA, not applicable.

Supplementary Table 2. Functional activity of HSPC-NK cell products.

UPN	% Killing E:T ratio 3:1	% $CD107a^+$ NK cells	% $IFN\gamma^+$ NK cells
1	72%	23	0.3
2	69%	28	1.0
3	82%	30	2.5
4	84%	24	0.6
5	84%	41	6.4
6	94%	29	1.5
7	81%	25	2.3
8	N/A	N/A	N/A
9	N/A	N/A	N/A
10	87%	24	2.9
11	84%	27	4.0
12	N/A	29	7.0
Median	84 %	28 %	2.4 %
Mean ± SD	82 ± 7	28± 5	2.9 ± 2.3

Functional activity of HSPC-NK cell products, which had been cryopreserved at the day of infusion, was analyzed after thawing and re-culture. For this, cells were thawed and cultured for six days in basal medium supplemented with 10% HS, 1000 U/ml IL-2 and 20 ng/ml IL-15. At day 6, NK cells were washed and specific killing of K562 cells by HSPC-NK cells was analysed after overnight co-culture in a FCM-based killing assay. The percentages of degranulating ($CD107a^+$) and IFNγ-producing HSPC-NK cells were determined following 4 hr stimulation with K562 cells at the single cell level by FCM. N/A, not analyzed.

Supplementary Table 3. Adverse events associated with Cy/Flu regimen.

Event	***Number of treated patients (N=10)***	
	Grade 3	**Grade 4**
Neutropenia		10 (100%)
Trombocytopenia		10 (100%)
Anemia	9 (90%)	
Febrile neutropenia	6 (60%)	
Hypokalemia	3 (30%)	
Urticaria (before infusion of HSPC-NK cells)	1 (10%)	
Flank pain (before infusion of HSPC-NK cells)	1 (10%)	
Syncope	1 (10%)	
ALAT increased (149 days after infusion)	1 (10%)	

Abbreviations: Cy, cyclophosphamide; Flu, fludarabine; HSPC, hematopoietic stem and progenitor cell; NK, natural killer; ALAT, alanine aminotransferase.

Figure S1. Functional activity of HSPC-NK cell products. Functional activity of HSPC-NK cell products, which had been cryopreserved at the day of infusion, was analyzed. Cells were thawed and cultured for six days in basal medium supplemented with 10% HS, 1000 U/ml IL-2 and 20 ng/ml IL-15. Cells were incubated with K562 cells. Specific killing of K562 cells by HSPC-NK cells after overnight co-culture was analysed in a FCM-based killing assay.

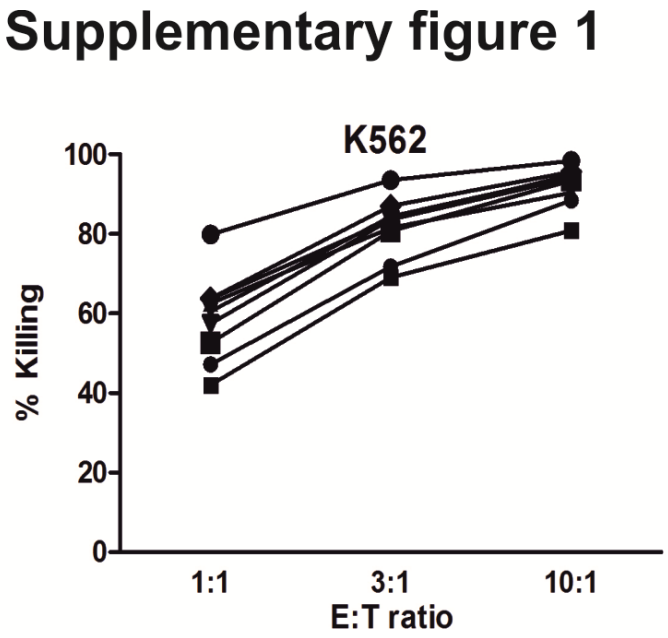

Figure S2. Cy/Flu conditioning induces temporary lymphocytopenia. (**A**) Lymphocyte numbers were measured in PB at different time points between day -7 and day 90. (**B-D**) The absolute NK cell (**B**), $CD4^+$ T cell (**C**) and $CD8^+$ T cell (**D**) count was determined by measuring the percentage within the lymphocyte population by FCM. (**E-F**) The percentage of $CD4^+$ (**E**) and $CD8^+$ (**F**) T cells within the lymphocyte population was determined by FCM. Mean ± SD of 10 patients. < depicts the threshold of detection of lymphocyte count.

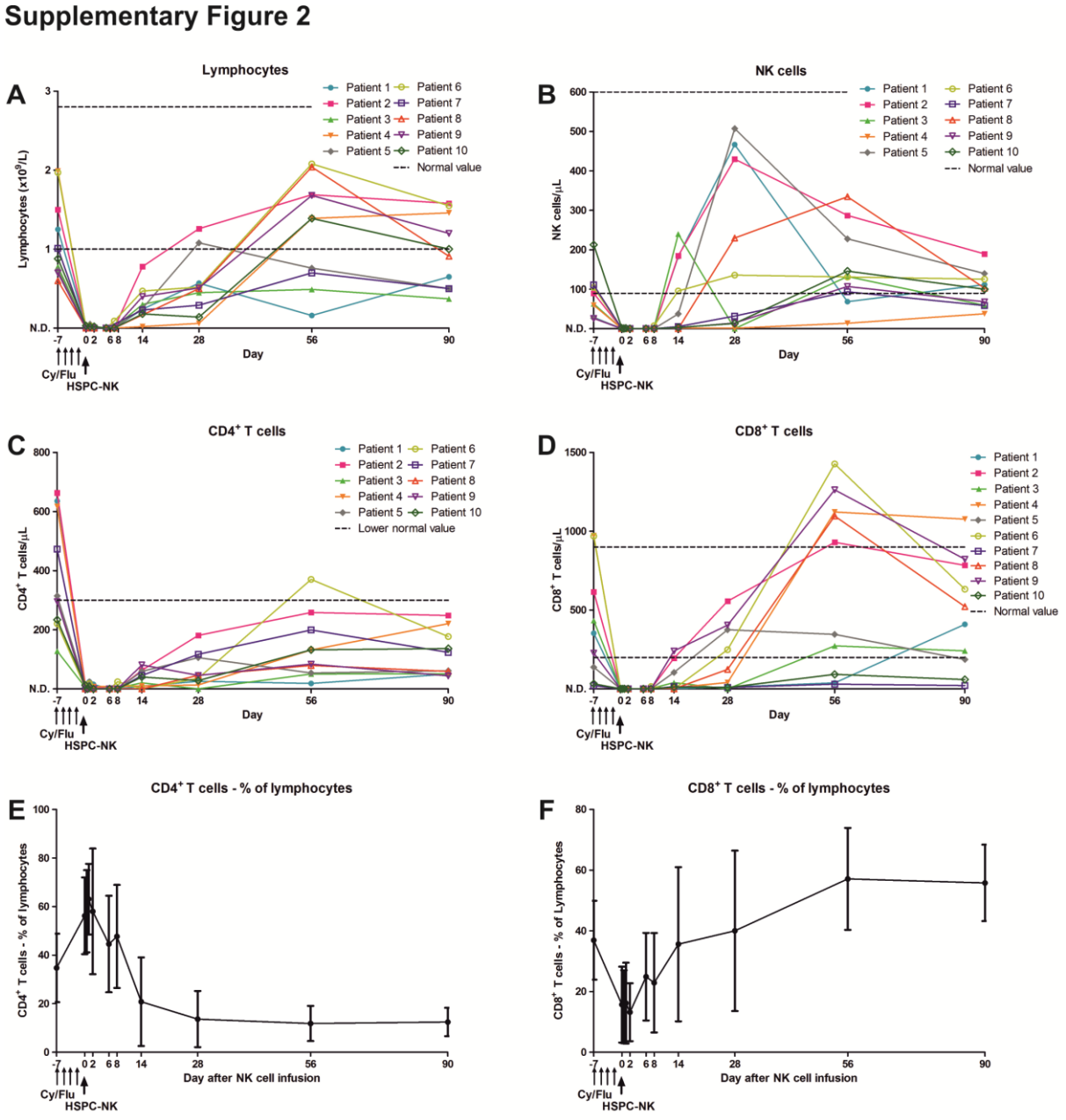

Figure S3. HSPC-NK cells mature *in vivo.* After NK cell infusion, the phenotype of $CD3^-$ $CD56^{verybright}$ NK cells, a phenotype which corresponds to our HSPC-NK cell product, was determined by FCM at different time points in UPN 5, 7 and 8. (**A**) The percentage of $CD16^+$ cells within the $CD56^{verybright}$ population at different time points in patient 5, 7 and 8. (**B**) KIR (CD158a, b and e) expression within the $CD3^-CD56^{verybright}$ cell population at day 2 and 8 after infusion in UPN5 and UPN8.