FULL-LENGTH ARTICLE

Clinical Research

Cellular immunotherapy with multiple infusions of in vitro-expanded haploidentical natural killer cells after autologous transplantation for patients with plasma cell myeloma

Astrid Tschan-Plessl^{1,*,*}, Christian P. Kalberer^{1,*}, Ronja Wieboldt¹, Martin Stern¹, Uwe Siegler¹, Aleksandra Wodnar-Filipowicz², Sabine Gerull¹, Jörg Halter¹, Dominik Heim¹, André Tichelli¹, Dimitrios A. Tsakiris¹, Karl-Johan Malmberg^{3,4}, Jakob R. Passweg^{1,*}, Alessia Bottos^{1,*}

¹ Clinical and Diagnostic Hematology, University Hospital Basel, Basel, Switzerland

² Stem Cell Center of Competence, University Basel, Basel, Switzerland

³ Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway

⁴ Department of Medicine, Huddinge, Center for Infectious Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

ARTICLE INFO

Article History:

Received 27 July 2020

Accepted 22 September 2020

ABSTRACT

Background aims: To investigate the feasibility and safety of haploidentical natural killer (NK) cell infusions as consolidation immunotherapy after autologous stem cell transplant (ASCT) in patients with plasma cell myeloma.

Methods: Ten patients (median age, 59 years) received induction treatment followed by high-dose melphalan (200 mg/m²) at day −1, ASCT at day 0 and increasing NK cell doses (1.5 × 10⁶, 1.5 × 10⁷ and multiple doses of 1.0 × 10⁸ cells/kg body weight) from day +1 to day +30 after ASCT. NK cells were harvested and purified from peripheral blood of haploidentical donors and expanded for 19 days with interleukin (IL)-2 and IL-15 under Good Manufacturing Practice conditions.

Results: NK cell numbers increased 56.0-fold (37.4- to 75.5-fold). Patients received a median of 3.8 × 10⁸ (0.9–5.7 × 10⁸) NK cells/kg body weight in six (three to eight) infusions. Multiparametric mass cytometry analysis demonstrated an altered surface receptor repertoire of expanded NK cells with increased degranulation and cytokine production activities but diminished expression of perforin. Donor NK cells were detectable in the peripheral blood, peaking 1 h after each dose (up to 90% donor NK cells). The treatment was safe and well tolerated, without evidence of graft-versus-host disease. Comparison with a control patient population receiving ASCT without NK cell infusions showed no significant difference in relapse, progression-free survival and overall survival.

Conclusions: This study demonstrates reliable manufacturing of high numbers of activated NK cells for multiple-dose infusions and safe administration of these cellular products. The trial was registered at ClinicalTrials.gov (identifier no. NCT01040026).

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Introduction

Plasma cell myeloma (PCM)¹ is a malignant disease characterized by the neoplastic proliferation of plasma cells. It occurs with an annual

incidence of four to five per 100 000 in the European population [1,2]. Standard treatment for newly diagnosed, symptomatic PCM in younger patients is induction therapy with a combination of proteasome inhibitors, immunomodulatory drugs and dexamethasone, followed by high-

** Correspondence: Astrid Tschan-Plessl, M.D., University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland.

* These authors contributed equally to this work.

E-mail address: astrid.tschan-plessl@usb.ch (A. Tschan-Plessl).

¹ ASCT Autologous stem cell transplantation BW Body weight CD Cluster of differentiation CR Complete remission CyTOF Cytometry by time of flight DMSO Dimethyl sulfoxide GMP Good manufacturing practice GvHD Graft versus Host Disease HLA Human leukocyte antigen HSA Human serum albumin HSCT Hematopoietic stem cell transplantation IFN Interferon Ig Immunoglobulin IL Interleukin ISS International Staging System IMWG International Myeloma Working Group KIR Killer cell immunoglobulin-like receptor mAb Monoclonal antibody MM Multiple myeloma NK cell Natural killer cell PBS phosphate buffered saline PCM Plasma cell myeloma PR Partial remission RD Revlimid (lenalidomide), dexamethasone TNF Tumor necrosis factor VEGF Vascular endothelial growth factor VGPR Very good partial remission VCD Velcade (Bortezomib), cyclophosphamide, dexamethasone VTD Velcade (Bortezomib), thalidomide, dexamethasone

dose chemotherapy and autologous stem cell transplantation (ASCT) [3–6]. Although this approach has been successful in increasing response rates and prolonging survival, PCM remains an incurable disease, and patients eventually relapse. Therefore, new treatment strategies are needed.

Natural killer (NK) cells form part of the innate immune system and have been widely implicated in the elimination of neoplastic and virally infected cells. NK cells were initially identified based on their capacity to eliminate tumor cells *in vitro* and *in vivo* without previous antigen stimulation [7–9]. Their constitutive cytotoxic potential has been shown *in vitro* and *in vivo* against various tumor targets, including PCM [10–12]. Indirect and direct points of evidence supporting NK cell cancer immunosurveillance *in vivo* stem from a large epidemiological study associating reduced NK cell cytotoxicity with increased tumor incidence [13] and from a pioneering study on anti-leukemic NK cell alloreactivity after haploidentical hematopoietic stem cell transplantation [14]. Importantly, NK cell dysfunction has been reported in patients with PCM, arguing for immune escape elicited by the neoplastic disease [15–21]. Furthermore, the clinical efficacy of standard anti-myeloma drugs such as immunomodulatory drugs and proteasome inhibitors relies in part on restoring NK cell effector functions [22]. There are clinical data correlating immune reconstitution of NK cells after ASCT with prolonged remission [23].

Based on these findings, immunotherapeutic strategies using NK cells to target PCM and other types of cancer have gained significant interest in the past years, and several clinical trials on hematopoietic malignancies as well as solid tumors in different therapeutic settings have been carried out. NK cell manufacturing has been performed using various methods, with [24–27] or without [28–30] short-term activation or after *in vitro* expansion with cytokines and/or feeder cells [31–38].

The authors have previously demonstrated that haploidentical NK cells can be administered without relevant toxicity in the context of haploidentical hematopoietic stem cell transplantation [39,40], and other groups have shown *in vivo* persistence and/or remission induction in selected patients in both the transplant and non-transplant settings [24,25,27,28,36,41].

The authors have described a protocol for the expansion of NK cells compliant with Good Manufacturing Practice (GMP) regulations [42]. The developed protocol of a 19-day expansion in culture bags

with IL-2, IL-15, irradiated autologous feeder cells and the anti-CD3 antibody OKT3 produced high numbers of activated NK cells. Here the authors describe the results of a phase 1/2 study of large-scale infusions of *in vitro*-expanded and activated haploidentical NK cells in 10 patients with PCM.

Methods

Study design, patient inclusion criteria and study endpoints

This phase 1/2 single-center, open-label clinical trial was approved by the local ethics committee Ethikkommission Nordwest- und Zentralschweiz and registered at ClinicalTrials.gov (identifier no. NCT01040026). Patients and related family NK cell donors were included in the study after obtaining written informed consent for experimentation with human subjects. Patient inclusion criteria were age between 18 and 70 years, PCM with indication for ASCT and availability of a related HLA haploidentical donor (sibling or child). Donor inclusion criteria were age above 18 years and being fit for cell donation and apheresis.

The study design is outlined in Figure 1. Patients diagnosed with PCM received standard induction therapy (Table 1). After autologous hematopoietic stem cell mobilization and cryopreservation, NK cells were purified from leukapheresis of haploidentical NK cell donors. During *in vitro* expansion of NK cells, patients received an additional one to two cycles of induction therapy. Patients were consolidated with high-dose melphalan (200 mg/m²), ASCT and a maximum of 10 NK cell infusions, given at 2- to 3-day intervals three times per week, starting at day +1 after ASCT. Cryopreserved NK cell units were thawed at the bedside and infused rapidly. Patients did not receive post-ASCT standard consolidation or maintenance treatment for PCM.

Primary endpoints of the study were (i) tolerability of NK cell infusions, defined as dose-limiting toxicity (according to the World Health Organization toxicity scale), early or late clinical adverse reactions; (ii) feasibility of NK cell infusion (cell collection, NK cell enrichment, expansion and cryopreservation under GMP conditions, administration of up to 10 NK cell infusions without exceeding the cumulative T-cell limit of 1.0×10^5 cells/kg body weight); and (iii) safety of NK cell infusion in terms of engraftment of autologous stem cells.

Figure 1. Study outline. Description of patient and donor study procedures. Cryo, cryopreservation; HSC, hematopoietic stem cell. (Color version of figure is available online).

Table 1
Patient characteristics.

Patient	Sex	Age (years)	Body weight (kg)	Diagnosis	Stage DS	ISS	Cytogenetics	Induction therapy	Remission at ASCT	NK cell donor, age (years)	KIR-ligand-mismatch
1	M	62	77	Lambda light chain disease	III A	I	del17p, del13q, t(11;14)	4x VTD	CR	Son, 32	C1
2	M	42	94	IgA kappa	III A	I	+11q13	4x VTD	PR	Son, 22	–
3	M	59	74	IgG kappa	III A	III	complex: +1, ...	5x VTD	VGPR	Son, 27	–
4	F	58	53	IgD lambda	III A	III	complex: +1, t(4;14), ...	4x VTD	VGPR	Brother, 62	–
5	M	48	89	IgA kappa	III B	III	–13	4x VCD	PR	Son, 22	–
6	M	46	82	IgG lambda	III B	III	complex: t(4;14), +1, –13, ...	4x VTD	VGPR	Daughter, 21	Bw4/3DL1 not expressed
7	F	63	57	IgG lambda	III A	II	complex: t(4;14), ...	3x VTD, 1x RD	PR	Son, 35	–
8	F	59	91	IgG lambda	III A	III	complex: –13, partial deletion of chromosomes 1, 2, 4, 6, 8, 14, 16, 19, 20, 22	4x VTD	VGPR	Daughter, 37	Bw4
9	M	60	94	IgG lambda	III A	II	complex: +8, –14, partial deletion/insertion of chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 14, 17, 19	4x VTD	PR	Son, 37	–
10	M	57	78	IgA lambda	III A	I	ND at study enrollment. At diagnosis: t(4;14), + part.17p	4x VTD	CR	Brother, 53	–

ASCT, autologous stem cell transplantation; CR, complete response; del, deletion; DS, Durie-Salmon; F, female; Ig, immunoglobulin; ISS, International Staging System; KIR, killer cell immunoglobulin-like receptor; M, male; ND, not done; PR, partial response; RD, lenalidomide, dexamethasone; VCD, bortezomib, cyclophosphamide, dexamethasone; VGPR, very good partial response; VTD, bortezomib, thalidomide, dexamethasone.

NK cell expansion and cryopreservation

The NK cell expansion protocol has been described previously [42]. In brief, NK cells were purified from 12 to 15 L unstimulated leukapheresis by an immunomagnetic selection procedure using the CliniMACS device (Miltenyi, Bergisch Gladbach, Germany), with a CD3⁺ cell depletion step, followed by CD56⁺ cell enrichment. NK cells were cultured in air-permeable 500-mL tissue culture bags (Perma-Life cell culture bags; OriGen, Austin, TX, USA) in GMP medium (stem cell growth medium; CellGenix, Freiburg, Germany) containing 5% human AB⁺ serum (Blutspendezentrum, Bern, Switzerland), 200 U/mL IL-2 (Proleukin; Novartis, Basel, Switzerland) and 10 ng/mL IL-15 (CellGenix). Anti-CD3 monoclonal antibody (clone OKT3; Miltenyi) was added once on day 1. Autologous feeder cells (i.e., pooled leukapheresis cells from the negative fractions of the NK isolation procedure) were irradiated with 30 Gy and added to the NK cells at a ratio of 7–10:1. Fresh culture medium was added on day 8, day 12 and day 15, and the content of one bag was equally split into two bags on day 8 and day 15. At the end of culture (day 19 or day 20), NK cells were aliquoted and cryopreserved in liquid nitrogen in escalating doses (1.5×10^6 , 1.5×10^7 and maximal eight doses of 1.0×10^8 cells/kg body weight). The cryopreservation solution contained phosphate-buffered saline, 2% human serum albumin and 7.5% dimethyl sulfoxide.

NK cell expansion: quality control and release criteria

Quality controls were regularly performed with samples taken on day 0, day 8, day 12, day 15 and day 19 of culture. Sterility was tested with the Bact/Alert FN/FA microbial detection system for the presence of aerobic and facultative anaerobic micro-organisms (Biomérieux, Inc, Durham, NC, USA). Cell number and cellular

composition of cultures were assessed by flow cytometric single platform analysis with Trucount tubes (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were stained with anti-CD3 antibody (clone UCHT1; BD Biosciences) and BD Multitest six-color TBNK reagent (BD Biosciences) containing anti-CD3 (clone SK7), CD8, CD19, CD4, CD16/56 and CD45 antibodies. Samples taken from five of all expansion cell bags were used to estimate the total number and expansion fold of NK cells in the entire expansion culture.

Viability was determined at several time points during expansion culture before freezing and directly after thawing each NK cell dose using the Live/Dead fixable aqua dead cell stain kit (Invitrogen Molecular Probes, Eugene, OR, USA). Data acquisition and analysis were performed using FACSCanto (BD Biosciences) and FACSDiva software (BD Biosciences).

The release criteria of the NK cell product were defined as, minimally, three NK cell portions, sterile, undetectable *Mycoplasma* (Biolytix, Witterswil, Switzerland) and endotoxin level of <2 IE/mL (Spitalpharmazie, Basel, Switzerland) and a viability of >65%. The cumulative T-cell dose limit was set to 1×10^5 T cells/kg body weight.

Phenotype and function of expanded NK cells

Before (day 0) and after (day 19/20) expansion cultures, donor NK cells were analyzed for phenotypic and functional characteristics using mass cytometry. For functional assay, donor NK cells were thawed and rested for 2 h in complete cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum; Sigma-Aldrich, St Louis, MO, USA). NK cells were co-cultured with different tumor target cell lines—K562, U266, 721.221, 721.221 loaded with rituximab (Roche, Basel, Switzerland) – for a total of 5 h at an E:T ratio of 4:1 in

the presence of anti-CD107a-151Eu (clone H4A3; Fluidigm, San Francisco, CA, USA), anti-LFA-1-162Dy (clone m24; BioLegend, San Diego, CA, USA) and protein transport inhibitor (GolgiStop and GolgiPlug; BD Biosciences).

For mass cytometry analysis, cells were stained with Cell-ID Intercalator-Rh103 (Fluidigm) for viability testing, followed by Fc-blocking reagent and a surface antibody cocktail (see supplementary Table 1). Subsequently, cells were fixed in phosphate-buffered saline (free of calcium and magnesium ions) with 2% paraformaldehyde, permeabilized, barcoded using the Cell-ID 20-Plex Pd barcoding kit (Fluidigm) and pooled for bulk analysis. Samples were then transferred to methanol and stored at -20°C . On the acquisition day, cells were stained with an intracellular antibody cocktail (see supplementary Table 1) and labeled with Cell-ID Intercalator-Ir (Fluidigm).

Samples were supplemented with EQ four-element calibration beads (Fluidigm) and acquired on a CyTOF 2 (Fluidigm) at an event rate of <400 per second. FCS files were normalized using Helios software (Fluidigm), debarcoded either manually or using Helios software and analyzed with the Cytobank platform (Cytobank Inc, Santa Clara, CA, USA), FlowJo v10 (Tree Star, Ashland, OR, USA) and Prism 6 (GraphPad Software Inc, San Diego, CA, USA).

HLA and KIR genotyping

HLA genotyping of patients and family members was performed using either LinkSeq HLA typing kits (Linkage Biosciences, San Francisco, CA, USA) or HLA-FluoGene kits (inno-train Diagnostik, Kronberg, Germany). KIR genotyping of NK cell donors and patients was performed using the LinkSeq KIR typing kit (Linkage Biosciences).

NK cell monitoring

NK cells and other lymphocyte subsets in patients' blood after NK infusions were quantified using TruCount tubes (BD Biosciences), BD Multitest six-color TBNK reagent (BD Biosciences) and FACSDiva software (BD Biosciences) according to the manufacturer's instructions. Donors' and patients' NK cells were distinguished and quantified by short tandem repeat chimerism analysis (AmpFISTR Profiler Plus polymerase chain reaction amplification kit; Thermo Fisher Scientific, Waltham, MA, USA) with DNA isolated from NK cells purified using RoboSep (Stem Cell Technologies, Vancouver, Canada). The sensitivity of the chimerism assay was 1–2%.

Cytokine profile in vivo

Patient serum was collected after high-speed centrifugation of blood samples and stored at -70°C or in liquid nitrogen ($<-180^{\circ}\text{C}$) for long-term storage. Cytokine analysis in serum was performed by Thermo Fisher Scientific after design of a ProcartaPlex 19-plex with the following analytes: caspase-3, granulocyte colony-stimulating factor, granzyme A, indoleamine-2,3-dioxygenase, interferon (IFN) α , IFN- γ , IL-2, IL-6, IL-10, IL-12/IL-23p40, IL-15, IL-18, IL-21, macrophage inflammatory protein 1 α (MIP-1 α), oncostatin M, stromal cell-derived factor 1 α (SDF-1 α), tumor necrosis factor α (TNF- α), vascular endothelial growth factor (VEGF) A.

Clinical assessment

Graft-versus-host disease (GVHD) was assessed clinically and classified using modified Glucksberg criteria. If necessary, GVHD was confirmed by biopsy of the involved organ and assessment of T-cell chimerism in peripheral blood. Response assessment was performed using bone marrow aspiration, cytology, histology, immunophenotyping and protein electrophoresis/immunofixation and a free light chain assay at regular intervals post-treatment.

Statistical analysis, control cohort

Study patients were compared with matched control PCM patients ($n = 28$) treated at the University Hospital Basel between 2010 and 2016. The control group was matched for sequential number of ASCTs, remission before ASCT, Durie-Salmon stage and International Staging System stage. Matching by cytogenetic risk group was not possible because of missing data in the control group. Each patient was matched to two to three controls. To optimize the number of controls per patient, matching was for four criteria in 14 patient control pairs and three criteria in 14 patient control pairs (International Staging System stage being the variable most commonly mismatched).

Results

Patient, disease and treatment characteristics

As per protocol, 10 patients (seven males, median age, 58.5 years) with newly diagnosed ($n = 9$) or relapsed ($n = 1$) PCM were enrolled in the study (Table 1) between 2012 and 2016. All patients were scheduled for high-dose chemotherapy with melphalan (200 mg/m²), followed by ASCT. Patients received a median of four cycles of induction chemotherapy with bortezomib, thalidomide and dexamethasone; bortezomib, cyclophosphamide and dexamethasone; or lenalidomide and dexamethasone. Remission stage at transplant was 2x complete response, 4x very good partial response and 4x partial response. All patients underwent ASCT and received up to eight NK cell infusions and up to 5.7×10^8 NK cells/kg body weight (Table 2). Response assessment was undertaken according to International Myeloma Working Group criteria [43,44].

Donor selection and NK cell expansion

All enrolled patients had a haploidentical family donor. A KIR-ligand-mismatch in the graft-versus-host direction along with cell surface expression of the respective KIR on donor cells was present in two of 10 patients (Table 1). Results of KIR and HLA genotyping of donors and patients are summarized in supplementary Table 2.

NK cell infusions were prepared as specified earlier. Highly enriched NK cells (mean, 90.3% NK cells and 0.004% T cells) were obtained starting from an average of 206.8×10^8 total white blood cells. Mean NK cell purification efficiency was 52%, and mean T-cell depletion efficiency was 6.4 log (Table 3). Expansion cultures were initiated with $1.2\text{--}6.7 \times 10^8$ NK cells and a feeder:NK ratio of 8–10:1. NK expansion was 37- to 76-fold (mean, 56-fold), yielding 64.6×10^8 to 460×10^8 NK cells (mean, 270×10^8 cells) (Table 4, Figure 2; also see supplementary Table 3).

In three NK cell products, for patients #8, #9 and #10, the T-cell content was above the limit of 1×10^5 T cells/kg body weight (mean, 8.2×10^5 cells/kg body weight). An additional T-cell depletion step successfully reduced the T-cell content below the target limit (mean, 0.14×10^5 T cells/kg body weight, mean, 149-fold reduction); however, at the expense of a significant NK cell loss (#8, pre: 433, post: 344×10^8 cells, #9, pre: 508, post: 460×10^8 cells, #10, pre: 453, post: 352×10^8 cells).

For cryopreservation, the NK product was split into aliquots of increasing cell numbers: 1.5×10^6 cells/kg body weight, 1.5×10^7 cells/kg body weight and 1×10^8 cells/kg body weight. In total, 57 NK cell doses were cryopreserved (i.e., three to eight doses per patient were produced) (Table 4; also see supplementary Table 3). NK cell viability at collection was 84% on average (range, 64–94%) (see supplementary Table 3). NK products were cryopreserved for a maximum of 41 days, and viability at thawing was 77% on average (range, 53–89%), demonstrating maintenance of NK cell viability after cryopreservation.

Table 2
Treatment details.

Patient	NKI applied (no.)	Total NK cells (x10e8/kg)	Time after ASCT (days)		Stem cell dose (CD34+ cells x10e6/kg)	Neutrophil engraftment (days)
			First NKI	Last NKI		
1	8	5.7	4	18	4.5	15
2	3	0.9	2	21	5.6	21
3	3	0.9	2	7	3.1	14
4	6	3.7	2	14	6.8	15
5	6	4	3	17	2.1	17
6	6	3.4	2	14	2.5	22
7	5	2.5	2	11	2.1	13
8	5	3.2	1	14	3.9	24
9	7	4.9	1	18	2.6	19
10	7	4.5	1	15	3.8	15
Mean	5.6	3.4	2	15	3.7	17.5

NKI, NK cell infusion.

Table 3
NK cell selection.

Patient	Apheresis			Purified viable NK cells		NK cell isolation efficiency (%)	T-cell content (%)	T-cell depletion (log reduction)
	Processed volume (L)	Viable WBCs (x10e8)	Viable NK cells (x10e8)	x10e8	Purity (%)			
1	15	221	28.2	16.7	97.4	59	0.002	5.55
2	15	207	10.8	1.2	96.3	11	0.028	5.56
3	15	291	4.4	1.6	62.8	36	0.002	6.54
4	15	122	8.7	3.6	88.0	41	0.001	6.58
5	15	208	15.2	6.4	92.6	42	0.000	6.97
6	15	144	10.5	8.1	95.8	77	0.000	8.10
7	15	189	5.9	4.1	89.1	70	0.002	6.27
8	12	216	12.3	7.6	97.2	62	0.002	5.80
9	12	223	11.4	7.7	89.6	68	0.000	5.64
10	12	248	15.7	7.6	93.9	48	0.001	7.16
Mean	14	207	12.3	6.5	90.3	52	0.004	6.42

WBCs, white blood cells.

Table 4
In vitro NK cell expansion.

Patient	Start day 0		Harvest day 19+20							
			CD56+ NK cells			Bags (no.)	Volume (L)	Expansion fold	NKI (no.)	T cells(x10 ⁵ cells/kg BW)
	x10 ⁸	Purity (%)	x10 ⁸	x10 ⁸ cells/kg BW	Purity (%)					
1	6.7	97.4	437	5.7	85.3	48	13.4	65.0	8	0.6
2	1.2	96.5	84.7	0.9	99.9	16	4.5	75.7	3	0.4
3	1.6	62.8	64.6	0.9	99.7	20	5.6	39.6	3	0.3
4	3.3	88.0	184	3.7	99.9	24	6.7	56.1	6	0.6
5	6.0	92.6	348	4.0	97.6	42	11.8	57.9	6	0.18
6	6.2	95.8	282	3.4	97.8	44	12.3	45.8	6	0.16
7	3.8	89.1	141	2.5	99.1	28	7.8	37.4	5	0.13
8	6.7	97.2	344	3.8	99.8	48	13.4	51.1	6	0.39 ^a
9	6.7	89.6	460	4.9	99.9	48	13.0	68.5	7	0.03 ^a
10	5.6	93.9	352	4.9	99.9	40	11.2	62.8	7	<0.01 ^a
Mean	4.8	90.3	270	3.5	97.9	36	10.0	56.0	5.7	0.28

BW, body weight; NKI, NK cell infusion.

^a Secondary T-cell depletion.

Phenotype and function of expanded NK cells

Mass cytometry and multidimensional analysis of NK cells before and after *in vitro* expansion revealed a profound skewing of the cellular phenotype post-expansion, with increased expression of CD56 and upregulation of activating receptors NKG2D and NKP44 as well as inhibitory receptors NKG2A and CD96, whereas activating receptor DNAM-1 was decreased. Strikingly, expression of perforin and CD16 was reduced. Expanded NK cells displayed a less differentiated phenotype, with only a very low fraction of cells expressing the differentiation marker CD57.

Expression of the bone marrow homing receptor CXCR4 was markedly reduced, as was expression of the IL-2/IL-15 receptor β -chain CD122. The substrate receptors CD71 and CD98 as well as the cell adhesion molecule CD2 and the ectoenzyme CD38 increased during expansion. Expression of PD-1, TNF-related apoptosis-inducing ligand and CD137 tended to increase at a low level. Expression of a number of receptors, including NKG2C, KIR2DL1, KIR2DL2L3, KIR3DL1, KIR2DS1, KIR2DS4, TIGIT, TIM-3 and CD25, as well as granzyme B, was stable (Figure 3A,B).

In a missing-self reaction against K562 cells, bulk expanded NK cells displayed increased degranulation, TNF- α and MIP-1 β

Figure 2. *In vitro* NK cell expansion. Values are estimates from samples taken from five of all culture bags and extrapolated to the entire expansion culture (Color version of figure is available online).

production, whereas IFN- γ production remained stable. Degranulation and cytokine production of expanded NK cells in response to myeloma cell line U266 were roughly unchanged compared with *ex vivo* NK cells (Figure 3C).

NK cell infusion

NK cell infusions were given between day 1 and day 21 after ASCT (Table 2). For patient #2, the last NK infusion was given with a 16-day interval because of an acute infection. Of the 57 cryopreserved NK cell doses, one (dose six of six of patient #8) was withheld because of the appearance of donor-specific anti-HLA antibodies after the fifth infusion.

Follow-up analysis: NK persistence in vivo

Peripheral blood samples were collected 1 h and 20 h after NK cell infusions. Molecular chimerism analysis was performed on 71/110 blood samples (see supplementary Table 4). Eight of 110 samples were excluded from the analysis because of low NK cell purity of <30% after isolation, and 31/110 samples were not collected or could not be analyzed because of insufficient DNA extracted from isolated NK cells. The purity of the evaluable NK cell samples was $68.4 \pm 19.7\%$ (mean $\pm$ standard deviation, $n = 71$). After infusion of the lowest NK cell dose of 1.5×10^6 cells/kg body weight, donor NK cells were not detectable in peripheral blood of patients. By contrast, after the first maximal dose of 1.0×10^8 NK cells/kg body weight, donor-derived NK cells were readily detectable in eight of 10 patients at 1 h after infusion (up to 90% of total NK cells) (Figure 4; also see supplementary Table 4). Twenty hours after infusion, donor NK cells decreased significantly and were still detectable in seven of nine samples (up to 24% of total NK cells), suggesting that donor NK cells do not persist or expand in peripheral blood. Total NK cell numbers increased over time due to successful immune reconstitution after ASCT (see supplementary Figure 1), explaining the decreased relative donor NK chimerism after NK infusions five to seven (Figure 4).

Cytokines in vivo after ASCT and during the course of NK therapy

Serum samples were analyzed for the concentration of specific cytokines prior to ASCT and at 1 h and 20 h after NK infusions. Thirty-five samples were available. A multiplex assay was performed to quantify the concentration of caspase-3, granulocyte colony-stimulating factor, granzyme A, indoleamine-2,3-dioxygenase, IFN- α , IFN- γ , IL-2, IL-6, IL-

10, IL-12/IL-23p40, IL-15, IL-18, IL-21, MIP-1 α , oncostatin M, SDF-1 α , TNF- α and VEGF-A. Only a few cytokines were detectable. Among the markers analyzed, the authors observed a stable concentration of SDF-1 in the peripheral blood, whereas VEGF, IL-10 and IL-18 tended to increase over the treatment duration (see supplementary Figure 2).

Engraftment and safety

In line with the good tolerability of NK cell infusions reported in past clinical trials [25–27,29,36,37,40,41], the authors did not observe any immediate or late side effects associated with the NK cell infusions. Importantly, no signs of acute or chronic GVHD were observed. Neutrophil engraftment occurred at a median of day 15 in study patients and day 17 in controls ($P = 0.47$). Altogether, the authors' clinical experience indicates that the dose-limiting toxicity of NK cell infusions has not been reached with 1×10^8 cells/kg body weight even when given multiple times. Supplementary Figure 1 shows NK, T- and B-cell reconstitution after ASCT in treated patients.

Treatment response and survival

Outcome of study patients was compared with controls as described. Median follow-up of surviving study patients was 1946 days (1129–2455 days, 5.3 years, 3.1–6.7 years), and median follow-up of controls was 1477 days (180–2978 days, 4.0 years, 0.5–8.2 years) ($P = 0.7$). Figure 5A–C shows overall survival, progression-free survival and relapse probability for patients and controls. At 5 years after treatment, overall survival was $50 \pm 32\%$ (95% confidence interval) in study patients and $57 \pm 24\%$ ($P = 0.24$) in controls. Respective progression-free survival estimates were $17 \pm 29\%$ in study patients and $40 \pm 20\%$ in controls ($P = 0.66$). Disease progression was $81 \pm 33\%$ in study patients and $58 \pm 20\%$ in controls ($P = 0.82$). Differences are not statistically significant; however, study patients tended to do rather worse than controls. In contrast to study patients, control patients had received standard consolidation and maintenance treatment after ASCT. Study patients did not receive drug consolidation or maintenance treatment. The small number of treated patients precludes any correlation of response with number of NK cells infused or associations with KIR mismatches and other characteristics.

Figure 3. Multiparametric CyTOF analysis of NK cells pre- (day 0) and post- (day 19/20) *in vitro* expansion (n = 8/10). (A) Marker intensity plots for t-SNE clustering displaying bulk NK cells. (B) Graphs displaying percentage of positive NK cells and median mass intensity of selected parameters pre- and post-expansion. (C) Functional response of NK cells co-cultured with K562 and U266 cell lines. CyTOF, cytometry by time-of-flight. (Color version of figure is available online).

Discussion

This study demonstrates that high doses of haploidentical *in vitro*-expanded and activated NK cells can be produced and safely given in the context of ASCT in patients with PCM. NK cells were expanded 56-fold over 19–20 days. Cells were given in multiple increasing doses in the early phase of ASCT. No untoward side effects were observed, and autologous hematopoietic stem cells engrafted as expected. Donor-derived NK cells were found in the circulation. Expanded NK cells displayed markedly altered phenotypical and functional properties compared with *ex vivo* NK cells.

In comparison to a historical patient cohort, the replacement of standard consolidation and maintenance treatment post-ASCT with

expanded and activated NK cells did not result in a clinically evident benefit. NK cells were expanded using IL-2 and IL-15, anti-CD3 monoclonal antibody and irradiated autologous feeder cells and were harvested after a relatively long culture time of 19–20 days, resulting in a comparably high number of up to 6×10^8 NK cells/kg body weight.

Cytokine-expanded NK cells displayed increased expression of CD56, certain inhibitory and activating receptors, substrate receptors and effector molecules (NKG2A, NKG2D, NKp44, CD2, CD96, CD38, CD71, CD98, TNF-related apoptosis-inducing ligand), whereas other activating, cytokine and chemokine receptors decreased (CD16, DNAM-1, CXCR4, CD122) and others appeared to be unchanged (KIRs, NKG2C, TIGIT, TIM-3, granzyme B), part of which has been observed previously by the authors' group and others [42,45].

Figure 4. NK cell persistence. NK cell chimerism 1 h and 20 h after NKI. NKI, NK cell infusion. (Color version of figure is available online).

Importantly, the authors observed a decreased expression of perforin in both surface density and percentage of positive cells, the functional consequence of which remains to be determined. Cellular function as analyzed by a degranulation and cytokine production assay increased or remained at least stable. The increased expression of crucial inhibitory receptors such as NKG2A and the reduced expression of the bone marrow homing receptor CXCR4 as well as the effector molecule perforin might implicate several problem areas *in vivo*: once reinfused into patients, the expanded NK cells might be inhibited in an HLA-E-dependent manner and might not reach the organ of interest, in this setting the bone marrow. Also, the reduced expression of perforin might lead to decreased apoptosis induction of target cells. The

authors' current efforts to optimize the expansion protocol aim to resolve these problems.

NK cells can be isolated from different sources [46], and infusion of cord blood-expanded NK cells in combination with autologous stem cell transplant obtained promising results in a phase 1/2 study in multiple myeloma [47]. Attempts to expand NK cells *in vitro* include other cytokines besides IL-2 and IL-15, such as the addition of IL-21 to boost NK cell cytotoxicity or a combination of IL-12, IL-15 and IL-18 to induce memory-like NK cells [45,48,49].

Other protocols apply co-cultures with different types of tumor cell lines, such as Epstein-Barr virus-transformed lymphoblastoid cells [50,51], the leukemia cell line K562 genetically modified to

Figure 5. (A) Overall survival at 5 years: $50 \pm 32\%$ in study patients, $57 \pm 24\%$ in controls ($P = 0.24$). (B) Progression-free survival at 5 years: $17 \pm 29\%$ in study patients, $40 \pm 20\%$ in controls ($P = 0.66$). (C) Relapse probability at 5 years: $81 \pm 33\%$ in study patients, $58 \pm 20\%$ in controls ($P = 0.82$). Red line: patients. Blue line: controls. (Color version of figure is available online).

express 4-1BB ligand [32,52] plus membrane-bound IL-15 [11,53] or HLA-E-expressing feeder cells, to generate adaptive NK cells [54]. Even though a direct comparison of the clinical utility of these protocols is lacking, some of these studies were able to demonstrate promising *in vivo* activity. Moreover, the protocol applying IL-12, IL-15 and IL-18 demonstrated favorable *in vivo* expansion and persistence of infused NK cells over 7–14 days [45]. As the authors' expansion protocol is long and labor-intensive, current efforts are addressing the question of shortening expansion durations while retaining expansion yields through the application of different cytokine combinations.

A further crucial issue for the effectiveness of NK cell therapies is the *in vivo* persistence of expanded cells. Transfused NK cells could readily be detected in the peripheral blood of patients but ultimately disappeared from the circulation. Currently, it is not known how long transfused cells need to persist in the circulation for beneficial effects to occur and whether disappearance reflects desired relocation to the marrow space or disappearance due to short half-life or by trapping (e.g., in the lungs). Circulating expanded NK cells were recovered repeatedly, arguing against immunological elimination as in this case non-recovery after the first few doses would have been expected.

Donors chosen for this study were haploidentical relatives of the patients. This choice is justified by the fact that in other settings, in particular acute myeloid leukemia, potent anti-leukemic effects have been demonstrated using haploidentical donors [14], and by the general availability of haploidentical family members to support the treatment of their relatives. Ultimately, only two of 10 patients had a donor with KIR-ligand-mismatch and surface expression of the available respective KIR, potentially explaining the lack of an evident clinical benefit. Alternative donor types include autologous or fully mismatched random donors. Arguments favoring the application of autologous NK cells include lack of immune-mediated rejection, availability and motivation for donation. By contrast, autologous cells have been described to be deficient in the expression of activating receptors necessary for tumor cell recognition [17], are inhibited by an inhibitory KIR repertoire tolerized against autologous HLA class I molecules and can often only be collected after therapy has already been initiated. The functional consequence of reduced activating receptor expression could potentially be overcome by cytokine stimulation, as autologous NK cells of PCM patients were recently described to significantly enhance their cytotoxicity after IL-2/IL-15 stimulation [55].

Although random donor NK cells carry the disadvantage of immune-mediated rejection and the necessity for more intensive immunosuppression, obvious advantages are availability and the possibility to select a donor with appropriate KIR repertoire. At present, NK cell donor choice remains a matter of investigation, and no specific setting is known to be clearly superior to others.

The concept of administration of allogeneic haploidentical expanded and activated NK cells as immunotherapy in the setting of ASCT requires justification. The authors reasoned that conditioning for ASCT was potentially favorable to the *in vivo* survival of expanded NK cells and that the cytokine milieu in aplasia, with reduced cellular competition for, for example, IL-15, would favor persistence and further expansion of the transfused cells. The potential incurred risks were non-engraftment of autologous hematopoietic stem cells in the presence of haploidentical NK cells and detrimental effects of NK cells in causing GVHD or other immunologic complications. None of these events were observed, although the population was limited in size.

Concluding on therapeutic aspects, no clinical benefit of expanded and activated NK cells could be demonstrated in this setting when comparing the study patients to matched controls from the institutions' database. Although differences were not significant, there was rather inferior disease control in the study patients, and this is most likely explained by the absence of post-ASCT standard consolidation and maintenance treatment, which was not intended in the study

patients. Obviously, at this stage it is impossible to conclude which factors best explain why this study was negative from a clinical point of view, whether it was the lack of maintenance treatment, the expansion conditions, donor choice, lack of KIR-ligand-mismatch in a majority of patient-donor pairs, the setting of administering NK cells early after ASCT or other unknown factors. Finally, and importantly, this was a phase 1/2 study of feasibility not powered to detect clinical efficacy.

In summary, the authors' data demonstrate feasibility of high-dose NK cell production for clinical use. No NK cell-mediated toxicities were recorded. Biological monitoring revealed *in vivo* persistence of expanded NK cells. Analysis of expanded NK cells showed evidence of potentially increased killing capacity. Compared with an historical cohort of PCM patients at the authors' institution, outcome was not improved.

The authors' future projects to improve the clinical outcome of PCM patients treated with *in vitro*-expanded NK cells include combining these with standard consolidation and maintenance treatment, optimisation of the expansion conditions to improve *in vivo* persistence of infused cells and genetical modification of NK cells to facilitate bone marrow homing.

Funding

This work was supported by the Swiss National Fund for Research (JRP; grant no. 32003B_149647) and the Swiss Cancer League (AT-P; grant no. BIL KFS-3745-08-2015). This work was further supported by a network grant from the European Commission (JRP; grant no. H2020-MSC-ITN-765104-MATURE-NK). RW is a fellow on the project.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: JRP, MS, AW-F, AT, DT. Acquisition of data: AT-P, CPK, RW, MS, US, SG, JH, DH, DT, JRP, AB. Analysis and interpretation of data: AT-P, CPK, RW, K-JM, JRP, AB. Drafting or revising the manuscript: AT-P, CPK, RW, JRP, AB. All authors have approved the final article.

Acknowledgments

The authors thank Gideon Hönger for KIR genotyping.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.09.009.

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