Results of a pilot study on immunotherapy with *ex vivo* expanded haploidentical natural killer cells for children with leukemia

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SUMMARY

The results of a pilot study on immunotherapy with *ex vivo* expanded haploidentical natural killer (NK) cells for children with leukemia are described in the article. Ten patients from 1 to 17 years with refractory or relapsed AML and ALL were enrolled in the study. NK cells were infused after the end a block of chemotherapy with median dose of 70.2 (12-109)*10^6/ kg. NK related adverse effects included fever (20%), hepatic failure (20%), cardiovascular reaction (10%), peripheral neuropathy (10%), and CRS (10%). In all cases, adverse reactions did not exceed grade 2. The peak of donor NK cells expansion *in vivo* was observed at day +6. The overall response rate was 50%. Six patients were alive without evidence of disease with a median follow-up of 1890 days. Registered at clinicaltrials.gov as NCT04327037.

Keywords: natural killer (NK) cells, immunotherapy, leukemia.

INTRODUCTION

Natural killer (NK) cells were identified more than 40 years ago and usually characterized as the cells of the innate immunity with the ability to kill tumor and virally infected cells without previous sensitization [1]. The first clinical attempt to use NK cells to combat tumors was the immunotherapy with lymphokine-activated killer (LAK) cells [2]. This method included leukapheresis, *ex vivo* activation of harvested cells for 3-5 days with high doses of IL-2 and re-infusion the cells into the patients with IL-2 administration. Current evidence suggested that the cytotoxic effect of LAK was mainly mediated by NK cells [3]. However, LAK cells predominantly contain polyclonal T-lymphocytes that outgrow NK cells with respect to numbers in the presence of the high doses of IL-2. In addition, activity of autologous NK cells are typically dysfunctional due to interactions of inhibitory KIR receptors with self MHC I molecules [4] or/and NK suppression by tumor microenvironment and exhaustion in cancer [5]. Taken together, these observations could explain the modest results of LAK therapy. In general, this approach resulted about 20 % partial and complete responses [6].

However, after a number of observations of anti-tumor effect of NK cells particularly in bone marrow transplantation settings [7] and appearing the technologies allowing to get a pure population of cells have resumed an interest to NK cell immunotherapy. Immunomagnetic isolation from peripheral blood [8], differentiation with cytokines from umbilical cord blood [9] or induced pluripotent stem cells (iPSC) [10] are available today for clinical purposes.

Another way to get NK cells for immunotherapy is the expansion with stimulatory/feeder cells such as irradiated autologous peripheral blood mononuclear cells, EBV-transformed lymphoblastoid cell lines or genetically engineered feeder cells [11].

There are numerous clinical trials being conducted at different stages and statuses to investigate NK cell immunotherapy for oncological diseases. The design and results of recent

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clinical trials have been reviewed elsewhere [12-14]. Generally, NK immunotherapy is safe and has a low incidence of toxicity [15]. The majority of patients who receive NK cell therapy experience grade 1 or 2 infusion reactions [16].

It was suggested earlier that NK cell dosing could be a significant factor in determining therapeutic response [17]. Currently, the greatest rate of NK cell expansion from peripheral blood mononuclear cells (PBMCs) has been achieved with feeder cell lines expressing mbIL-21 and the 4-1BB ligand [18-20]. We previously reported the creation of our own K562-41BBL-mbIL21 feeder line [21]. NK cells obtained with this line have a phenotype of activated and relatively undifferentiated cells with enhanced cytolytic activity against cancer cell lines [22]. Here we describe the result of our pilot study of immunotherapy with *ex vivo* expanded haploidentical NK cells for children with leukemia (NCT04327037).

MATERIALS AND METHODS

Patients

Ten patients from 1 to 17 years old (median 9.4 years) with acute myeloid leukaemia (AML; n = 6), acute lymphoblastic leukaemia (ALL; n = 4) were enrolled in the study. The study protocol has been approved by the local Ethics committee (approval number 30/12/19) and the study was registered at clinicaltrials.gov (NCT04327037). All subjects signed written informed consent in accordance with the Declaration of Helsinki.

Donors

Peripheral blood samples of 10 haploidentical donors (5 females and 5 males) with a median age of 39 years (range 27-50) were the source of peripheral blood mononuclear cells (PBMCs) for NK cell expansion. The inclusion criteria for donors were: HLA-haploidentical relative donor; age > 18 years; negativity for HIV, hepatitis B and C; and absence of pregnancy or breast feeding.

Donor selection

In the cases of several available donors the procedure of donor selection was performed based on NK alloreactivity. Donor was classified as alloreactive against recipient when donor NK cells possess an educated KIR receptor for recipient KIR missing ligand.

The presence of KIR ligand was determined based on the HLA class I typing of the recipient cells and classification of HLA I alleles into KIR ligand with database (http://www.ebi.ac.uk/ipd/kir/ligand.html) and published recommendations [23].

Genotyping of donor KIR receptors was assessed with Olerup SSP KIR Genotyping Kit (Olerup, Stockholm, Sweden). KIR surface expression was also confirmed with monoclonal antibodies by flow cytometry.

NK expansion and quality control

For expansion, a total of 100-250 ml of whole peripheral blood of the selected donors was withdrawn by venipuncture. PBMCs were isolated by gradient centrifugation. Donor NK cells were expanded by co-culturing the PBMCs with the sub-lethally irradiated (100 Gy) feeder cell line K-562 modified for expression of mbIL-21 and 4-1BBL (K562-mbIL21-41BBL), which was obtained in our laboratory [21].

Monoclonal antibodies (all from Beckman Coulter) to CD3 (FITC, clone UCHT1), CD56 (PE-Cy5, clone N901), CD69 (PE-Cy7, clone TP1.55.3) and NKp44 (CD336, PE, clone Z231) surface antigens were used to monitor cell growth, purity and activation. T cells, NK cells and NK-like T (NKT) cells were identified by their CD3+CD56-, CD3-CD56+ and CD3+CD56+ phenotypes, respectively.

Quality control testing for obtained NK cells included: cell viability, immunophenotyping, bacteriological control, feeder cell contamination and oncogenic transformation controls [22].

Release criteria for cell products were: the absence of microbial contamination, the viability of cells (>70 %) and NK cell content (>80 %).

NK immunotherapy

NK cells were infused after the end a block of chemotherapy during the period of cytopenia not earlier than 48 h after the last dose of chemotherapy. 8 out of 10 patients received individual blocks of therapy (Table 1), 2 patients received non-myeloablative conditioning regimen with cyclophosphamide 60 mg/kg intravenously (IV) once on day -7 and fludarabine 25 mg/m^2 IV from -6 to -2 days.

Following premedication with clemastine (at age dose) NK cells were administered IV on day 0. According to the protocol the patients received six doses of interleukin-2 (1×10^6 U/m^2) by subcutaneous injections after premedication with paracetamol (at age dose) every second day starting on day -1.

Hematological and immune recovery

Complete blood counts were monitored every day during the period of leukopenia. For hematological recovery we analyzed time (days) to leukocytes ($>1\times10^9$/L) and platelets ($>50\times10^9$/L) counts without transfusion support.

Immune monitoring performed before immunotherapy course and at the days 0, 2, 6, 10, 14 and 21 after cells infusion. Subpopulations of lymphocytes were characterized by multicolor flow cytometry with antibodies directed against CD45, CD3, CD56, CD4, CD8, CD19, HLA-DR, CD69, CD25, CD127. Percentages and absolute counts of NK-, B-, T-cells as well as T-helper, T-cytotoxic and T-regulatory cells, activated T- and NK cells were assessed. All antibodies were from Beckman Coulter or EXBIO. Analysis was performed with Navios or DxFlex flow cytometer (Beckman Coulter).

Analysis of donor NK cell persistence

Detection of donor NK chimerism was performed after PBMCs isolation at days 2, 6, 10, 14 and 21 after cell therapy by InDel-real time PCR assay with the specific primers and probes published earlier [25, 26]. In described cases donor chimerism was also assessed in bone marrow samples after PBMCs isolation. Before quantification, the donor and the recipient were genotyped for all 23 genetic markers. An allele was considered as informative when it was positive in the donor and negative

in the recipient. The positivity for alleles was defined by a Ct value ranging between 20 to 26, whereas the negativity for a specific allele was assessed by a Ct value exceeding 36.

The following formula was used to calculate chimerism [25],

$$\text{Chimerism, \%} = 100\% \times (1 + E)\text{-}(\Delta CtU\text{-}\Delta CtC)$$

where ΔCtU is ΔCt in the unknown sample; ΔCtC is ΔCt in the calibrator sample; E is PCR efficiency of the target DNA sequence.

Absolute number of persisting donor NK cells was calculated with the following formula:

(absolute lymphocyte count/mL) × (% donor chimerism using InDel-real time PCR testing)/100.

Statistical analysis

Incidence of Treatment-Emergent Adverse Events was classified according to the CTCAE version 4.0. Outcome follow up was measured from the day of NK infusion to progression of disease, death, or the last follow-up.

RESULTS

Patients and disease characteristics

A total of 10 children from 1 to 17 years old with acute leukemia (4 ALL and 6 AML) were enrolled in this study. Eight patients were with disease relapse or had primary refractory disease, whereas two patients (P3, P10) were with primary AML and were treated with NK cells according to individual scheme due to comorbidities. Two patients were in relapse after allogeneic hematopoietic stem cell transplantation (HSCT) (P2, P6). Clinical characteristics of patients are summarized in Table 1.

The group of patients included in this trial was heterogeneous in terms of previous treatment protocols, number of chemotherapy cycles (median 3), and conditioning chemotherapy blocks before NK infusion. Only two patients received non-myeloablative conditioning regimen with cyclophosphamide and fludarabine which usually used before NK infusion.

Characteristics of donors and NK cell products

Haploidentical parent donors were used in all cases. Five donors had NK alloreactivity against recipient cells. Characteristics of donors are shown in Table 2.

Eleven NK cell products were obtained for 10 patients. The purity of NK cells in the final cell products was 97 % (range, 89.0-99.1) in spite of low initial content of NK cells (11.5 %, range 4.2-29.4) and NK expansion without procedure of CD3 depletion. The median duration of expansion was 19 days (range, 12-25). At the end of culturing, the median fold expansion of NK cells was 251.3 (range, 102.9-647). Median viability of infused cells was 86 % (range, 70-93).

Seven patients received one dose of freshly obtained NK cells when 3 patients received two doses of cells (first dose – freshly obtained cells, second – cryopreserved). Median dose of infused NK cells was 70.2 (12-109)*10^6/kg. Median dose of total CD3+ (CD56+ and CD56-) cells was 0.9 (0.4-4.3)*10^6/kg. More detailed characteristics of infused cells are shown in Table 3.

Table 1: Clinical characteristic of patients

Patients	Age	Sex	Diagnosis	Molecular aberrations	Disease status	Cycles of prior therapy	Chemotherapy before NK
P1	8	m	ALL, BIV	c-myc-IgH	Molecular relapse	7	ICE +Rituximab
P2	2	f	ALL, BI	MLL-AF4	Relapse 2	3	FLAG-Ida
P3	4	m	AML, M2	AML1/ETO	MRD+	3	Cy+Flu
P4	10	m	T-ALL, TII	nd	Relapse, progression	4	ARA-C, Cy, PEG-ASP, Nelarabin, medrol
P5	3	f	B-ALL, BII	nd	Relapse, progression	3	VPR
P6	13	m	AML-MR, M1	FLT3-ITD, WTmut	Relapse	4	Cy+Flu
P7	14	m	AML, M2	CBFb/MYH11	Refractory	3	FLAG-Ida
P8	17	f	AML-MR, M2	FLT3-ITD	Refractory	2	FLAI
P9	11	m	AML, M4	FLT3-ITD	Refractory	3	FLAG+Decitabine+ Vorinostat, Sorafenib
P10	1	m	AML, M5	46,XY,t(1;8;11) (q21;q22;q23)[20] MLL/ND	MRD+	2	HD-ARA-C+Ida

Note: ALL – acute lymphoblastic leukemia, AML – acute myeloid leukemia, AML-MR – acute myeloid leukemia myelodysplasia related; MRD – minimal residual disease; ARA-C – cytarabine, Cy – cyclophosphamide, PEG-ASP – pegylated asparaginase. ICE – ifosphomide, carboplatine, etoposide; FLAG – fludarabine, cytarabine, G-CSF; FLAG-Ida – FLAG, idarubicine; Cy+Flu – cyclophosphamide, fludarabine; VPR – vincristine, prednisolone; FLAI - fludarabine, cytarabine, idarubicine; HD-ARA-C+Ida – high doses of cytarabine, idarubicine.

Table 2: Donor characteristics

Patients	Donor	Donor age, years	Missing KIR ligand	Alloreactivity	Donor KIR haplotype	Donor KIR haplotype score
P1	Mother	35.8	Yes / C2	Yes	telAA/cen AA	Neutral
P2	Father	29.5	Yes / C2	Yes	telAA/cenAB1	Better
P3	Father	33.7	No	No	telAA/cenAB2	Better
P4	Father	46.4	Yes / C2	Yes	telAA/cenAA	Neutral
P5	Father	29.3	No	No	telAA/cenAB2	Better
P6	Mother	39.1	Yes / C1	No	telAA/cenAA	Neutral
P7	Mother	40.6	Yes / C2	No	telAB/cenAA	Neutral
P8	Mother	50.4	Yes / C1	ND	telAA/cenAA	Neutral
P9	Father	44.3	Yes / Bw4	Yes	telAA/cenBB	Best
P10	Mother	27.4	Yes / Bw4	Yes	telAA/cenAB2	Better

Table 3: NK cell product characteristics

Patients	NK doses	Doses of infused cells					
		NK %	NK *10⁶/ kg	CD3+56- %	CD3+CD56- *10⁶/ kg	CD3+CD56+ %	CD3+CD56+ *10⁶/ kg
P1	1	96.8	68	1.8	1.24	1.0	0.69
P2	1	96.0	45.8	1.6	0.73	1.6	0.73
P3	1	98.5	73.9	0.5	0.375	0.6	0.45
P4	1	89.0	35.8	0.7	0.28	10	4.0
P5	1	97.0	100	1.1	1.13	0.3	0.31
P6	1 2^{cryo}	98.5 98.4	70.2 27	0.5 0.8	0.37 0.22	0.7 0.6	0.49 0.15
P7	1	98.3	74	1.3	0.38	0.4	0.3
P8	1 2^{cryo}	94.0 92.9	55.4 11.9	0.7 1.8	0.41 0.23	3.8 5.1	2.2 0.66
P9	1 2^{cryo}	98.8 99.1	73.8 88.2	0.4 0.5	0.3 0.4	0.8 0.4	0.59 0.36
P10	1	96.3	109	1.1	1.2	1.3	1.5
Median		**97**	**70.2**	**0.7**	**0.69**	**0.8**	**0.6**

Table 4: Il-2 related reactions

Patients	Number of IL-2 doses	General disorders and administration site conditions		Transition to intravenous administration
		Injection site reactions	Fever	
P1	4	Yes	No	Yes
P2	0	Not used	Not used	Not used
P3	6	No	No	No
P4	6	Yes	No	Yes
P5	3	Yes	No	Yes
P6	4	Yes	No	Yes
P7	6	No	No	No
P8	6	No	No	No
P9	1	Yes	Yes	No
P10	4	No	Yes	No

The majority of obtained NK cells had the phenotype of immature activated cells (NKG2A+, double bright CD56++CD16++, CD57-) expressing NKp30, NKp44, NKp46, NKG2D, CD69, HLA-DR and CD96 [22]. Expanded NK cells also exhibited high cytolytic activity against leukaemia cell lines, high degranulation activity and production of cytokines. Median cytotoxicity against K562 cells at the effector:target ratio 5:1 was 89.0 % (range, 52.1-97.5). In addition, 20.2 % (range, 5.6-36.1) NK cells produced IFNγ after stimulation tests with K562 cells.

Safety of NK cell infusion

The primary objective of our pilot study was the safety of expanded haploidentical NK cells. We analyzed IL-2 related and NK cells infusion related reactions separately. Adverse effects related with IL-2 injections are shown in Table 4.

lL-2 was initially administered subcutaneously for 9 patients. Local reactions (erythema, edema, pain) at the injection sites were the most frequent adverse reaction (5 patients out of 9, grade 1-2). For this reason, for 4 patients subsequent doses of IL-2 were administered intravenously. Only two patients had fiver in relation to IL-2 injection (20 %). Pain in the injection site, absence of donor chimerism were the reason for IL-2 withdrawal. Thus, the median number of IL-2 injections during the course of immunotherapy was 4 (0-6).

An overview of adverse events attributed to NK cell infusions are shown in Table 5.

The most observed symptoms were fever (20%) and increased level of AST/ALT (20%). All described events were grade 1 or 2.

One patient (P1) in 30 minutes after NK infusion showed a reaction which was classified as cytokine release syndrome (CRS, grade 2) with chills, fiver, shortness of breath and cyanosis. This reaction was resolved with one injection of glucocorticoid (methylprednisolone 1 mg/kg).

One patient (P9) after 6 hours of cells infusion had the episode of hypotension (grade 2).

Accordingly, infusions of expanded NK cells were well tolerated, adverse reactions did not exceed grade 2, cases of graft-versus-host disease (GvHD) were not reported. Acute episodes of adverse reactions were reported during the first 24 hours after NK infusion.

Immune recovery

The second prospective end point of the study was the time of hematological and immune recovery.

Median rest days between last dose of chemotherapy and NK infusion was 11 (range, 2-17). Hematopoietic cell recovery

Table 5: NK cell infusion-related reactions

System organ class (SOC)/Adverse events	Grade 1	Grade 2	Grade 3, 4
General disorders and administration site conditions Fever	–	2 (P1, P10)	–
Vascular disorders Hypotension	–	1 (P9)	–
Nervous system disorders Peripheral neuropathy	1 (P9)	–	–
Hepatobiliary disorders Hepatic failure	2 (P4, P10)	–	–
Immune system disorders CRS	–	1 (P1)	–

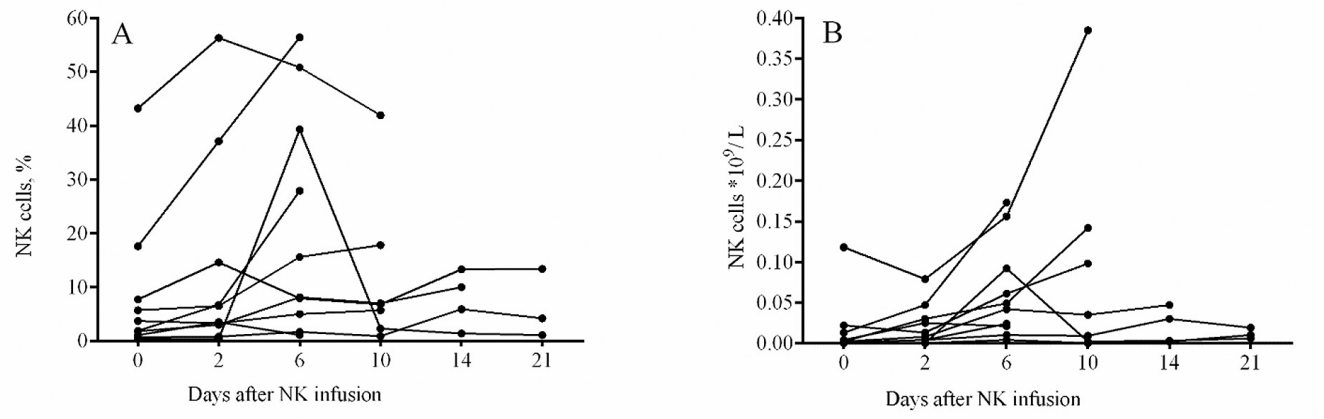

Figure 1: Dynamic of NK cell number in peripheral blood of patients after NK infusion

A – percent of NK cells within lymphocyte population; B – absolute number of NK cells.

after cells administration was rapid. The median time to absolute leukocyte count >1.0×10^9/L and platelet count >50×10^9/L was 4 (range, 0-52) and 5 (range, 0-9) days, respectively. Two patients had a leukopenia period >30 days after NK infusion (P2 was underwent HSCT at day +12; P8 had leukopenia period 52 days). Similar, two patients did not recovered platelets by the day 30 because of HSCT (P2) and early death (day +18, P5).

Also, number of NK, T cell subpopulations, activated T and NK cells were monitored during 21 days after immunotherapy. NK cell number kinetics in peripheral blood is presented in Figure 1.

Generally, we observed the increasing of percent and absolute number of NK cells after infusion with peaked level at day 6. On the day 0, 2, 6, 10, 14, 21 after cells infusion the median content of NK cells was 2.8% (range, 0.3-43), 5% (range, 0.4-56.3), 11.9% (range, 1.1-56.4), 6.8% (range, 0.9-41.9), 7.9% (range, 1.4-13.3), 4.2% (range, 1.1-13.4), correspondingly (Figure 1A). The median absolute blood NK cell counts on the day 0, 2, 6, 10, 14, 21 was 2 cells/μL (range, 0.1-118), 11 cells /μL (range, 0.1-79), 46 cells /μL (range, 4-173), 35 cells/μL (range, 0.5-385), 17 cells/μL (range, 2-47), 10 cells/μL (range, 6-19), correspondingly (Figure 1B).

Dynamic of T cell showed gradual decreasing percent of CD4+ and increasing of CD8+ T cells after NK infusion. At day 0 percent of CD4+ and CD8+ T cells was 66.9 % (range, 4.2-96.2) and 16.1 % (range, 4.3-44.5), while at 21 day – 48.2% (range, 30.8-75.2) and 38.1 % (range, 15.3-67.7), correspondently. Also, we noted increasing of percent and absolute number of Treg cells with peaked count at 6-10 days with subsequent decreasing. Moreover, it was found the increasing of absolute number of activated NK (CD69+) and T cells (CD69+, HLA-DR+). Maximal number of activated cells was detected at day 10.

Persistence of donor NK cells

Another prospective end point of the study was the analysis of *in vivo* donor NK cell persistence during 3 weeks after NK cell infusion. Analysis of donor chimerism was performed for 9 patients (for patient P2 the target was not found). Absolute number of persisting donor NK cells is presented in the Figure 2.

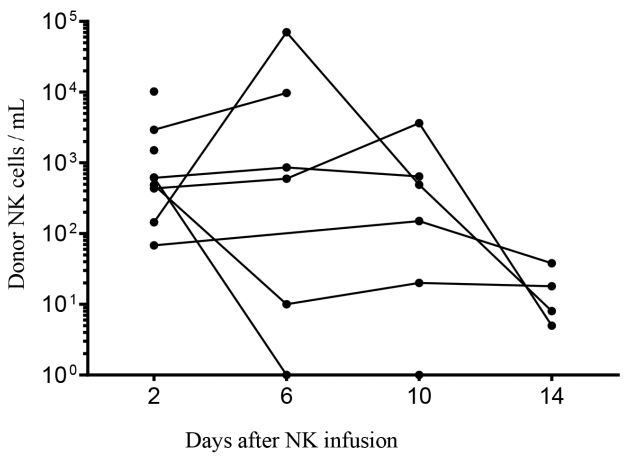

Figure 2: Persistence of donor NK cells after NK cell infusion

Peak of persisting donor NK cells was observed on day 6. The median levels of donor NK cells at days 2, 6, 10, 14 were 616, 730, 320 and 13 cells/mL, correspondently. At day 21 in 2 out of 3 investigated cases we did not find donor cells.

Also, it was possible to evaluate the donor chimerism in the bone marrow samples of some patients. Donor cells were detected in bone marrow at day 4 (P5, 7.9%), day 10 (P4, 0.03%), day 21 – in two (P8, 0.4%; P7, 0.008%) out 4 assayed samples after NK infusions. In one case (P8) donor NK cells were detected (0.01%) in bone marrow sample at day +43.

Clinical response

Clinical responses after one course of NK therapy are represented in Table 6.

8 out of 10 patients showed decreasing blast cells content in bone marrow according to results of morphological assays and/or MRD measurements. Complete remission (CR) after one course of immunotherapy was achieved in 5 out of 10 patients (CR – 3 patients, CRm – 2 patients). Within the group of responders after a course of NK therapy one patient finished his treatment (P3) without HSCT; one patient (P1) received autologous HSCT; another one (P9) received matched related HSCT; two patients (P7, P10) were administered the second course of NK therapy followed by matched unrelated HSCT. Patients P2, which decreased blast cells content after NK therapy was transplanted from haploidentical donor without reaching a remission. In April of 2025 6 patients were alive without evidence of disease with median follow up 1890 days.

The disease progression was the cause of death of 3 patients. One of them (P8) in spite of a decreasing blast cells after a course of NK immunotherapy did not get the second course because of infection status. One patient (P6) after NK immunotherapy received one block of chemotherapy followed by haploidentical HSCT and dyed from chronic GvHD (lung fibrosis) complicated with COVID-19.

DISCUSSION

The use of natural killer cells as a cellular immunotherapy has increased over the past decade, especially for patients with hematologic malignancies. Within the different cell sources and approaches expansion of peripheral blood NK cells stimulated with engineered feeder cells allows to get higher NK cell doses with less cost. K-562-based feeder cell lines expressing costimulatory ligand 4-1BBL and membrane bound (mb) cytokines interleukin (IL)-15 or IL-21 are commonly used in clinical trials [27]. When comparing feeder lines based on mbIL-15 and mbIL-21, mbIL21 supports greater proliferation of NK cells than mbIL15 [18, 21, 28], creating the possibility to generate substantially more NK cells from a single withdrawal of peripheral blood. We have previously reported the creation of K-562-based cell line transduced to express 4-1BBL and mbIL-21 [21]. Here we described the results of pilot trial of immunotherapy with *ex vivo* expanded natural killer cells in the presence of locally generated feeder cell line. In this trial paediatric patients with acute leukemia were treated with one or two doses of haploidentical NK cells. Median infused NK cells dose was 70.2*10^6 cells/kg, which was higher that was described earlier for paediatric patients. Spanish investigators

used expanded with K-562-based feeder line with mbIL-15 reported median used dose 6.7*10^6 cells/kg for patients with relapsed or refractory leukemia [29] and 36.4*10^6 cells/kg [30] for patients with primary AML. Other groups used immunomagnetic approach to get NK cells and infused median 18.6*10^6 cells/kg for children with relapsed leukemia [31] and 12.5*10^6 cells/kg for consolidation remission in AML [32]. In the cited publications and the others describing NK immunotherapy it was not reported about major side effects attributed to the NK cell infusion. Usually, toxicity associated with NK immunotherapy includes infusion reactions, chills, headache, fatigue, cardiovascular reaction [15, 16]. Fever (20%) and hepatic failure (20%) were the most reported reactions in our group of patients. One patient developed reaction classified as CRS, which stopped with glucocorticoid. We explain this reaction as combining effect of rituximab included in conditioning chemotherapy and high dose of NK cells (68*10^6 cells/kg). Earlier, Bachanova V. et al. [33] described well tolerated single dose infusion of haploidentical NK cells in combination with IL-2 and rituximab for patients with non-Hodgkin lymphoma. However, in this study NK cells were immunomagnetically isolated, 24h activated and infused in dose 5-32.7*10^6 NK cells/kg, moreover the patients were administered methylprednisolone from -2 to +9 days.

The main concern of allogeneic NK cells products is T-cells contamination and graft-versus-host disease (GvHD) as a consequence. In our study median dose of total CD3+ (T-cells and NKT cells) cells was 0.9 (0.4-4.3)*10^6/kg. In another study where allogeneic NK cells were expanded without CD3 depletion, the doses of T-cell and NKT-cells were 0.49 (0-11) *10^6/kg and 0.3 (0-14.6) *10^6/kg, respectively [29]. However, GvHD has not been reported in our group of patients and in the vast majority of other studies utilising allogenic feeder-based expanded NK cells. So far, the onset of severe GvHD has been described in only one trial. Specifically, IL-15/4-1BBL-expanded NK cells were used as NK-DLI after T cell-depleted peripheral blood stem cell transplantation; in this case, the T-cell (1-2 *10^4/kg) and NK cell (1*10^5/kg) doses were very low [34]. Possible factors that may explain the unexpected GvHD rates include the timing of NK-cell infusion, the lack of posttransplant immunosuppression, unrelated donors [35]. In recent publication it was described the safety and efficacy of high doses (1×10^5–1×10^8 cells/kg/dose) of donor mb-IL21 expanded NK cells which were administered on days -2, +7, and +28 in haploidentical transplant setting [36].

In majority of protocols infusion of NK cells followed by IL-2 administration to sustain NK cells proliferation and persistence [29, 31, 32, 33]. In our protocol it was scheduled six IL-2 subcutaneous injections. However, half of the patients had injection site reactions, that was the reason for changing form of administering of IL-2 from subcutaneous to intravenous. Therefore, IL-2 related reactions and low levels of donor chimerism were the indications for IL-2 withdrawal. Only 4 patients received all six doses of IL-2.

Table 6: Clinical response after a course of NK immunotherapy

Patients	Blast cells (%) and MRD level in BM		Remission	Treatment after NK	Outcome, follow up, days	Cause of death
	before	after				
P1	0.75 MRDpos	0.5 MRDneg	Yes, CRm	auto-HSCT	Alive, +1838	
P2	70.5	35	No	HSCT	Alive, +1793	Relapse, +444 day
P3	3.0^10^{-5}	Negative	Yes, CRm	None	Alive, +1673	
P4	47	22.8	No	HR-2 + Daratumumab	Death, +113	Progression
P5	88	86	No	Salvage therapy (Vincristine, prednisolone)	Death, +18	Progression
P6	3 WTmut positive	6 WTmut positive	No	FLAG, HSCT	Death, +616	TRM (COVID-19 infection, cGvHD)
P7	43 4.6^10^{-2}	1.75 4.5^10^{-3}	Yes, CR	FLAG-Ida+ NK, HSCT	Alive, +1367	
P8	47.7	9.5	No	Salvage therapy (5-azacitidine)	Death, +260	Progression
P9	64.3 FLT3-ITD positive	1.75 FLT3-ITD positive	Yes, CR	HSCT	Alive, +1164	
P10	5 4.3[fl]	1.25 0.4[fl]	Yes, CR	AME-H+ NK, HSCT	Alive, +1044	

Note: MRD – minimal residual disease, HSCT – hematopoietic stem cell transplantation, HR-2 – mitoxantrone, vinorelbine, doxorubicine, prednisolone; AME-H – high doses of cytarabine, mitoxantrone, etoposide; FLAG-Ida – fludarabine, cytarabine, G-CSF, idarubicine. fl – MRD level was measured by flow cytometry. CR – morphologic complete remission, CRm – molecular complete remission. cGvHD – chronic graft-versus-host disease, TRM – transplant-related mortality.

Besides, previously it was shown that IL-2 also stimulates rapid expansion of host regulatory T-cells (Tregs) suppressing allogeneic NK cell proliferation [37, 38]. Moreover, the depletion of host Tregs cells with IL-2-diphtheria fusion protein (IL2DT) extend persistence of allogeneic NK cells and improve the efficacy of cell therapy [38]. We also detected increasing of percentage and absolute number of Tregs with peaked count at 6-10 days after NK infusion. Taking into account noted IL-2 related local reactions and Tregs expansion our subsequent trials (NCT05334693, NCT05272293) do not include IL-2 administration.

We observed *in vivo* expansion donor NK cells in peripheral blood of patients during the first week after infusion with peak level at day +6. However, after +10 day the level of donor cells decreased. Donor NK cells were also detected in bone marrow of patients at different time points. In the other studies the peaks of donor chimerism in peripheral blood were detected between 7-14 days [39, 31, 32]. Short period of NK cell persistence is the weakness of this type of immunotherapy. Thereby, improving of donor cells persistence is possible through the multiple cell infusions. Encouraging clinical results were demonstrated for patients with high-risk R/R AML treated with six NK cells infusions [40].

Clinical response rates in clinical studies were variable: the therapeutic response depended on the diagnosis, stage of disease, timing of immunotherapy, NK alloreactivity, cell dose and frequency of infusions [29, 31, 32, 36, 40]. In our small and heterogeneous group of patients, the overall response rate was 50%. Six patients were alive without evidence of disease with a median follow-up of 1890 days.

Donor NK cell alloreactivity is one of the factors affecting the clinical response to this type of cell therapy. We did not find any obvious influence of NK alloreactivity or KIR haplotype score on clinical response, which could be due to the small number of patients. However, it worth to note, that in the group of responder 100% of them had AML, 60 % had alloreactive educated donor NK cells and in 60% cases donor cells had better/best KIR score.

In conclusion, immunotherapy with *ex vivo* expanded haploidentical NK cells was well tolerated, adverse reactions did not exceed grade 2, without cases of GvHD. Episodes of adverse reactions were reported during the first 24 hours after NK infusions, late reactions were not observed. Peak of persisting donor NK cells was observed on day 6. Administration of several NK infusions could improve *in vivo* expansion of cells and would allow not to use IL-2. The NK cell therapy showed an overall response rate of 50%. Patients with relapsed or refractory AML will benefit from this therapy.

CONFLICTS OF INTEREST

None declared.

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Результаты пилотного исследования иммунотерапии *ex vivo* экспансированными гаплоидентичными естественными киллерными клетками при лейкозах у детей

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РЕЗЮМЕ

В статье представлены результаты пилотного исследования по применению *ex vivo* экспансированных естественных киллерных (ЕК) клеток при лейкозах у детей. Десять пациентов в возрасте от 1 года до 17 лет с рефрактерными или рецидивными формами ОМЛ и ОЛЛ были включены в исследование. ЕК клетки вводили после окончания блока химиотерапии в медианной дозе 70.2 (12-109)*10^6/кг. Побочными эффектами после введения ЕК клеток были лихорадка (20%), печеночная недостаточность (20%), осложнения со стороны сердечно-сосудистой системы (10%), периферическая нейропатия (10%) и цитокиновый шторм (10%). Во всех случаях побочные реакции не превышали 2 степени. Пик *in vivo* экспансии донорских ЕК клеток наблюдали на +6 день. Общий клинический ответ составил 50%. Шесть пациентов живы без признаков болезни с медианой наблюдения 1890 дней. Исследование зарегистрировано на clinicaltrials.gov: NCT04327037.

Ключевые слова: естественные киллерные клетки, иммунотерапия, лейкоз.