



OPEN Phase I study of safety and efficacy of allogeneic natural killer cell therapy in relapsed/refractory neuroblastomas post autologous hematopoietic stem cell transplantation

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Despite low incidence, neuroblastoma, an immunologically cold tumor, is the most common extracranial solid neoplasm in pediatrics. In relapsed/refractory cases, the benefits of autologous hematopoietic stem cell transplantation (auto-HSCT) and other therapies are limited. Natural killer (NK) cells apply cytotoxicity against tumor cells independently of antigen-presenting cells and the adaptive immune system. The primary endpoint of this trial was to assess the safety of the injection of allogeneic, ex vivo-expanded and primed NK cells in relapsed/refractory neuroblastoma patients after auto-HSCT. The secondary endpoint included the efficacy of this intervention in controlling tumors. NK cells were isolated and primed ex vivo (by adding interleukin [IL]-2, IL-15, and IL-21) in a GMP-compliant CliniMACS system and administered to four patients with relapsed/refractory MYCN-positive neuroblastoma. NK cell injections (1 and 5×10^7 cells/kg in the first and second injections, respectively) were safe, and no acute or sub-acute adverse events were observed. During the follow-up period, one complete response (CR) and one partial response (PR) were observed, while two cases exhibited progressive disease (PD). In follow-up evaluations, two died due to disease progression, including the case with a PR. The patient with CR had regular growth at the 31-month follow-up, and another patient with PD is still alive and receiving chemotherapies 20 months after therapy. This therapy is an appealing and feasible approach for managing refractory neuroblastomas post-HSCT. Further studies are needed to explore its efficacy with higher doses and more frequent administrations for high-risk neuroblastomas and other immunologically cold tumors.

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Abbreviations

Auto-HSCT	Autologous stem cell transplantation
MIBG	Metaiodobenzylguanidine
NK	Natural killer
IL	Interleukin
CR	Complete response
PR	Partial response

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PD	Progressive disease
OS	Overall survival
TME	Tumor microenvironment
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
TMB	Tumor mutational burden
APC	Antigen-presenting cell
KIR	Killer immunoglobulin-like receptor
ADCC	Antibody-dependent cellular cytotoxicity
F/U	Follow-up
CT	Computed tomography
CONSORT	Consolidated Standards of Reporting Trials
GMP	Good manufacturing practice
PBMC	Peripheral blood mononuclear cells
TNF- α	Tumor necrosis factor- α
IFN- γ	Interferon- γ
ELISA	Enzyme-linked immunosorbent assay
IV	Intravenous
LAL	Limulus amoebocyte lysate
SD	Stable disease
INRC	International Neuroblastoma Response Criteria
RECIST	Response Evaluation Criteria in Solid Tumors
mL	Milliliters
ng	Nanogram
CEM	Carboplatin, etoposide, and melphalan
Dx	Diagnosis
LN	Lymph node
No	Number
Pt	Patient
BM	Bone marrow
BMA/BMB	Bone marrow aspiration and biopsy
N/A	Not assessed
GVHD	Graft-versus-host disease
GM-CSF	Granulocyte/monocyte colony-stimulating factor
CAR	Chimeric antigen receptor
LAK	Lymphokine-activated killer
mAb	Monoclonal antibody

As the most encountered extracranial solid malignancies in childhood¹, neuroblastomas comprise a heterogeneous group of tumors with variable clinicopathological and prognostic features². Despite their rarity, with an incidence of 10.2 cases per million children younger than 15 years of age³, these neural crest-originated tumors are among the main cause of cancer-related mortalities during childhood³. The prognosis of patients with neuroblastoma varies considerably and depends on age at the time of diagnosis, the presence of high-risk genetic aberrations, tumor size, lymph node involvement, and distant metastases. Accordingly, observation and close monitoring may be sufficient in certain scenarios². However, approximately 50% of the diagnoses were categorized as high-risk at the time of diagnosis. While the 5-year overall survival (OS) of low- and intermediate-risk disease has reached over 90%, the tide turns for high-risk and relapsed/refractory disease^{4,5}. As a result of the establishment of myeloablative therapy and autologous hematopoietic stem cell transplantation (auto-HSCT) followed by isotretinoin treatment for residual disease, the five-year OS of such patients has risen to more than 50%^{4,6}. However, it is estimated that half of the cases that undergo auto-HSCT will suffer a relapse of malignancy, and robust therapeutic approaches against this scenario are not well-established⁷.

Neuroblastomas are considered immune-cold tumors; T-cell and natural killer (NK) cell infiltrations within neuroblastomas are considerably lower than those of most other tumors, and the infiltrated cells express higher levels of inhibitory receptors, causing their exhaustion^{8–10}. Moreover, the tumor microenvironment (TME) of neuroblastomas is enriched with immunosuppressive myeloid-derived suppressor cells (MDSCs) and cancer-associated fibroblasts^{8,11}. Above these, neuroblastomas have diminished expression of major histocompatibility complex (MHC, also known as human leukocyte antigen [HLA]) class I and NK-activating ligands and generally harbor a low tumor mutational burden (TMB)^{11,12}.

The cardinal impacts of the immune system in defeating neoplastic growth are undeniable; particularly for neuroblastomas, as higher NK cell and T-cell infiltrations and higher MHC-I expression status correlate with a more favorable prognosis^{11,13}. A large body of evidence suggests that NK cells, as innate immune cells, do not rely on signals from antigen-presenting cells (APCs) and instead recognize their target cells based on reduced and/or allogeneic or haploidentical HLA-I expression without the need for prior sensitization. NK cell activation during their maturation is regulated by inhibitory killer immunoglobulin-like receptors (KIRs, a process known as licensing), among other mechanisms¹⁴, and their interactions with self-MHC-I prevent further NK cell activation¹⁴. Therefore, in recent years, NK cells from different sources and with various expansion/priming methods have been harvested to combat a wide variety of hematological malignancies and solid tumors^{15–26}.

In fact, the superiority of chimeric antigen receptor (CAR) NK cells over CAR T-cells in exerting comparable anti-tumoral effects with lesser complications has recently been shown¹⁸. Moreover, nonmodified NK cells can be harvested for extended periods without losing anti-tumor and memory-like features, making them intriguing tools for feasible and cost-effective cancer immunotherapies¹⁷.

Based on these observations, it can be postulated that NK cells can exhibit considerable antitumor activity against neuroblastoma cells. Observations from preclinical^{27,28} and clinical studies are consistent with the efficacy of ex vivo-primed NK cells in treating neuroblastomas^{29–31}. In addition, NK cells are integral mediators of antibody-dependent cellular cytotoxicity (ADCC) and, as a result, putatively enhance the efficacy of anti-disialoganglioside (i.e., anti-GD2) therapies^{32,33}. The impact of NK cell therapy alone on the fate of post-auto-HSCT relapsed neuroblastomas has not yet been addressed. Therefore, this study was designed to delineate the safety and efficacy of allogeneic NK cell transfer in the management of relapsed neuroblastomas.

Results

NK cells were effectively separated from the peripheral blood of allogeneic donors (Fig. 1). Likewise, ex vivo NK cell expansion and priming procedures were functional (Tables 1 and 2).

Of the five identified patients, four met the eligibility criteria, consented to participate in the trial, and received two doses of allogeneic ex vivo expanded and activated NK cells. The fifth patient was excluded due to the presence of bone marrow involvement at both biopsy sites. The baseline characteristics of the enrolled patients are shown in Table 3.

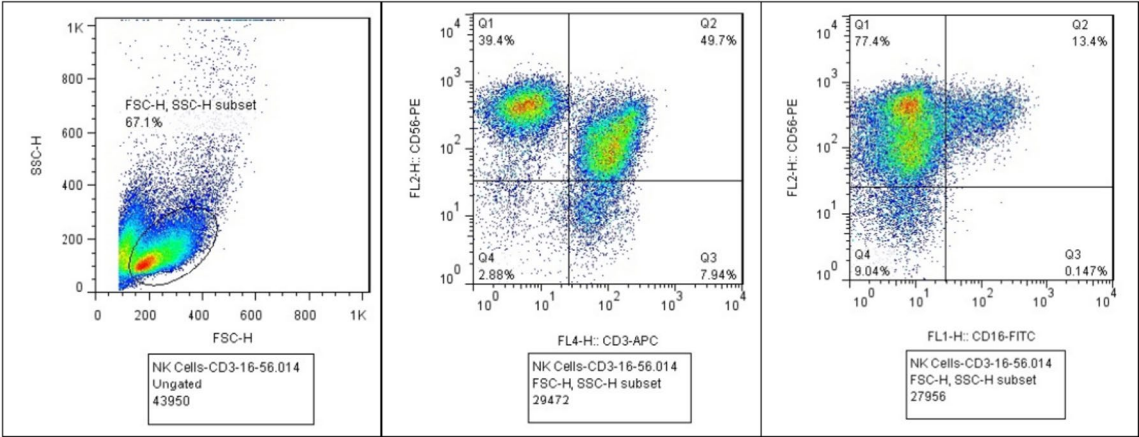


Fig. 1. Flow cytometry analysis of NK cell separation efficacy.

Effector/target ratio	Cytotoxicity (%) ^a	
	CHLA255 cell line	SK-N-SH cell line
1:1	21	29
2:1	39	44
5:1	56	62
10:1	81	93

Table 1. Cytotoxicity of IL-2-and IL-15-activated peripheral blood NK cells on human NB cell lines. *IL* interleukin; *NB* neuroblastoma; *NK* natural killer. ^aPercent of neuroblastoma cells’ death.

Composition	Cytokine release (ng/mL)	
	IFN-γ	TNF-α
PBMCs	0.7	0.1
Naïve NK cells	1	0.2
IL-2-activated NK	3.2	0.8
IL-2- and IL-15-activated NK cells	6	1.4

Table 2. Cytokine release assays for PBMCs and primed and non-primed NK cells. *IL* interleukin; *IFN-γ* interferon-gamma; *mL* milliliters; *ng* nanograms; *NK* natural killer; *PBMC* peripheral blood mononuclear cell; *TNF-α* tumor necrosis factor-alpha.

Baseline and auto-HSCT features					Details of response to NK cell therapy									
Pt. no	Age at Dx. of NB (years)	Involvement Hx before HSCT	MYCN amplification	Dx. to HSCT interval (m)	Response duration (m)	Preconditioning regimen	Best response	HSCT to NK interval (m)	Response duration (m)	Recurrence site(s)	Complication(s)	Best overall response	F/U duration (m)	Final outcome
1	2	Diffuse skeletal and soft tissue	+	12	11	MIBG ^b and CEM	CR	12	9	Bone	None	PR	15	Dead (PD)
2	3	Diffuse skeletal and BM ^a	+	24	–	CEM	PD	5	18	None	Anemia and thrombocytopenia (resolved)	CR	31	Alive
3	5	Diffuse skeletal, soft tissue (LNs), and BM ^a	+	9	12	MIBG ^b and CEM	CR	20	–	Soft tissue	None	None	20	Alive
4	5	Adrenal gland and soft tissue (LNs)	+	19	6	MIBG ^b and CEM	PR	10	–	Soft tissue	Neutropenic fever	None	7	Dead (PD)

Table 3. Baseline characteristics and clinical responses to auto-HSCT and NK cell therapy. *CEM* carboplatin, etoposide, and melphalan; *CR* complete response; *Dx* diagnosis; *F/U* follow-up; *HSCT* hematopoietic stem cell transplantation; *LN* lymph node; *m* months; *MIBG* metaiodobenzylguanidine; *NK* natural killer cell therapy; *No* number; *PD* progressive disease; *PR* partial response; *Pt* patient; *SD* stable disease. ^aResolved at the time of transplantation. ^bHigh dose (0.67 gigabecquerel [18 millicurie]/kg).

Overall, the treatment was safe, and no acute (e.g., anaphylaxis or cytokine storm) or subacute (e.g., fever, hemolysis, urticaria, etc.) toxicities were observed. All enrolled patients received two doses of allogeneic NK cells (total dose of 6×10^7 cells/kg). The efficacy of this treatment was modest; one patient exhibited CR, and another patient had PR during the F/U period (Tables 3 and 4).

The first patient was a four-year-old boy who presented with new bone metastases 11 months after a successful auto-HSCT. The patient received NK cell therapy one month after the diagnosis of new metastases. In the two-month F/U evaluations, he exhibited signs of progressive disease (PD), but at the three-month F/U, a considerable reduction in the burden of ^{131}I -MIBG-avid lesions was noted. The MIBG scans of this patient are depicted in Fig. 2. This PR lasted nine months; however, one year after therapy, the patient presented with new bone lesions. His parents refused to receive any further treatment, and 15 months after NK cell therapy, the patient died due to PD.

The second patient, a 5-year-old girl, underwent auto-HSCT two years after the diagnosis of neuroblastoma and, after observing no response to this therapy, received allogeneic NK cells five months later. After NK cell therapy, despite an initial progression in bone metastases, she showed some degree of regression in 4-month scans, and her disease remained stable in 7-month scans. Nevertheless, despite receiving no anti-neuroblastoma therapies except isotretinoin, her ^{131}I -MIBG scans demonstrated resolution of bone and soft tissue metastases 13 months after treatment with NK cells, suggestive of CR. Accordingly, three months after receiving NK cells, she gained weight and reached developmental landmarks in a regular manner in subsequent F/Us.

In the third patient, despite the observation of some degree of response in bone metastases, new ^{131}I -MIBG-avid soft-tissue lesions appeared in the F/U evaluations, suggesting PD. However, his three- and five-month Curie scores showed a reduction of more than 50%. The patient subsequently started receiving conventional chemotherapies five months after NK cell injection. He was alive at the latest F/U (20 months after NK cell injections) and received conventional chemotherapies against neuroblastoma.

The last case exhibited prominent signs of progressive disease in subsequent evaluations after NK cell injection. Accordingly, he was admitted to the oncology ward several times to receive conventional chemotherapy

Pt. no	F/U after NK therapy (months)	^{131}I -MIBG scan	Curie score	CT-scans	BMA/BMB
1	-1	An avid lesion in right paravertebral area	1	Unremarkable	NL
	2	Possible diffuse skeletal involvement and suspicious left paracaval LN	10	Enhancing right paravertebral soft tissue lesion (9*7 mm) with marginal calcification	NL
	3	Small right paravertebral lesion	3	Similar as above	NL
	6	Reduced avidity of right paravertebral lesion	1	Similar as above	NL
	9	Stable MIBG-avid right paravertebral lesion	1	One right paravertebral soft tissue region (9*7 mm)	NL
	14	Intense diffuse skeletal metastases	25	Multiple paraaortic and retrocrural reactive LNs	NL
2	-2	Scattered skeletal lesions	21	Unremarkable	NL
	+2	Same as above	20	Unremarkable	NL
	+3	Same as above	23	BM heterogeneity in pelvic bone and vertebral bodies	NL
	+6	Scattered skeletal lesions and one LN adjacent to the pancreatic head (suggestive of SD)	20	Heterogeneous BM of lower lumbar and iliac bones	NL
	+13	NL	0	N/A	NL
	+18	NL	0	No progression since the last scan	NL
	+22	NL	0	N/A	NL
3	-3	Scattered skeletal and soft tissue lesions	12	Multiple left paraaortic and mesenteric LNs; solid masses in the retrocaval area and the right sacrum	N/A
	3	Focal skeletal lesions, mild activities in left paraaortic and left common iliac LNs	4	N/A	NL
	5	Prominent soft tissue mass with intense avidity in the precaval region; several LNs in the aortocaval region	4	N/A	N/A
4	-1	LNs in the preaortic, aortocaval, and precaval regions	6	N/A	NL
	+1	Diffuse skeletal and paraaortic lesions	18	Ill-defined soft tissue mass in the left paraaortic region with multiple paraaortic metastatic LNs	NL
	+3	Diffuse skeletal and paraaortic lesions, with intensification of paraaortic LN activities	20	Multiple paraaortic metastatic LNs along with left paraaortic soft tissue mass with progressive extension to the adrenal gland	NL
	+6	Similar to above	20	Paraaortic metastatic LNs and bilateral paraaortic soft tissue lesions	NL

Table 4. Imaging and bone marrow evaluation findings of enrolled patients before and after NK cell therapy. BM bone marrow; BMA/BMB bone marrow aspiration and biopsy; CT computed tomography; F/U follow-up; LN lymph node; MIBG metaiodobenzylguanidine; NK natural killer cell therapy; N/A not assessed; PD progressive disease; PR partial response; Pt patient; RECIST Response Evaluation Criteria in Solid Tumors; SD stable disease.

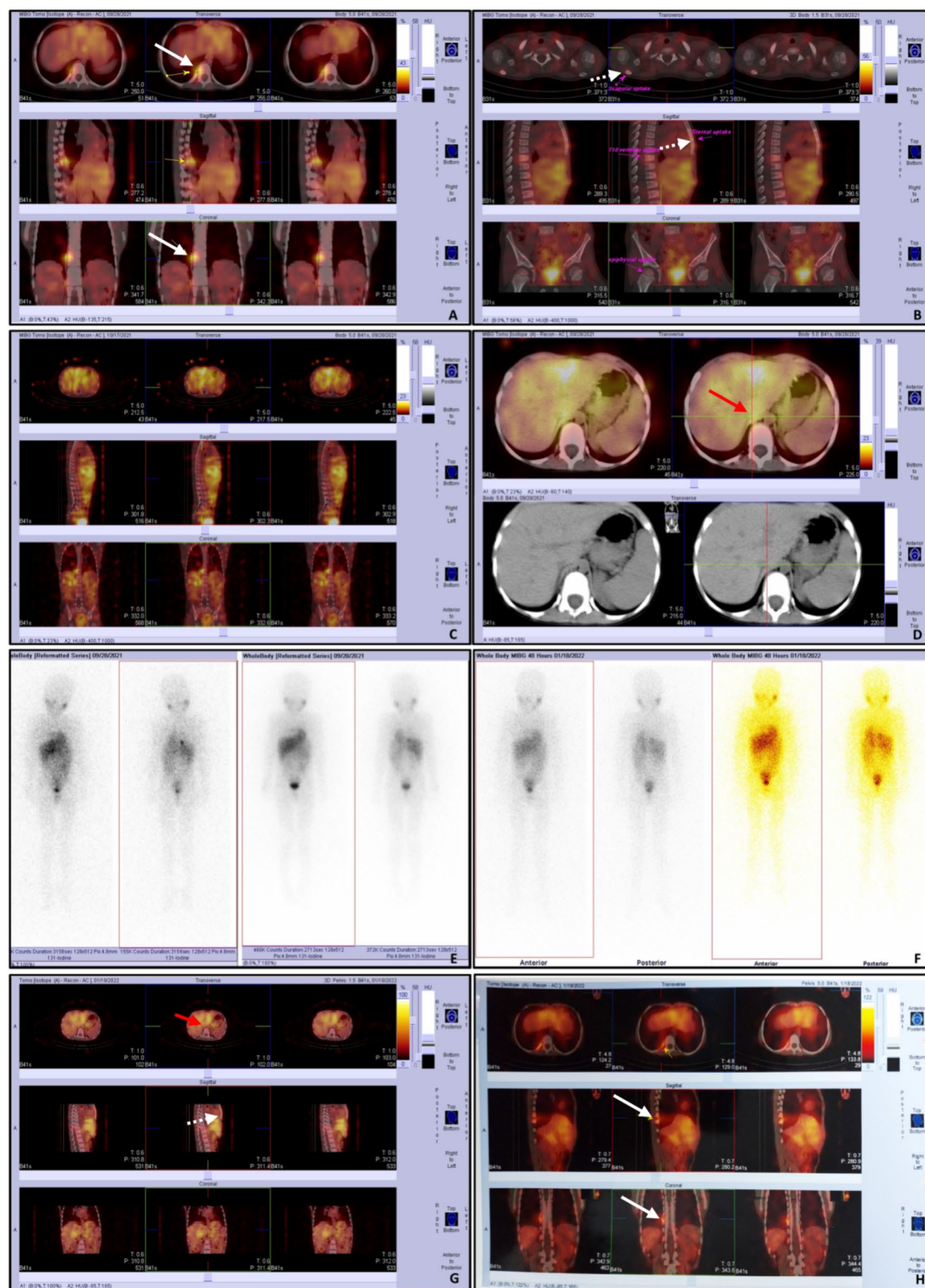


Fig. 2. Partial response of the first case to NK cell therapy. (A, H) A decrease in the uptake of the right paravertebral lesion (at the level of T9–T10) is evident about four months after NK cell therapy (solid white arrows). (B, G) The diffuse uptake by bone is decreased four months after receiving NK cells (dotted white arrows). (B–D, G) MIBG uptake by a suspicious lymph node in the left paracaval region is dampened (red arrows). (E, F) A decrease in the general MIBG uptake within the torso can be seen.

and to manage the associated complications. Approximately seven months after therapy, he presented with an episode of neutropenic fever and died of this complication.

Notably, none of the patients presented with bone marrow metastases after NK cell therapy. The safety profile of our NK cell therapy was acceptable; none of the enrolled cases were afflicted with acute or chronic graft-versus-host disease (GVHD), and excluding the last case, the other three did not present with infectious complications or reactivation of viral infections. The second patient had mild, manageable anemia and thrombocytopenia, which were triggered by the conditioning regimen before the transfusion of NK cells.

Discussion

As briefly mentioned, NK cells are the main effector components of the innate immune system that exert anti-tumor and antiviral responses directly by inducing apoptosis through Fas signaling and secreting lytic granules containing perforin and granzymes and indirectly by secreting a vast number of proinflammatory cytokines (such as TNF- α , IFN- γ , and granulocyte/monocyte colony-stimulating factor [GM-CSF]) and chemokines^{14,34,35}. Of note, NK cells are not dependent on the stimulating signals from APCs¹⁴, which, at least in theory, can be of great importance in defeating neuroblastoma cells, owing to their low TMB, neoantigen, and MHC-I expression. In addition, NK cell-derived exosomes and their secreted microRNAs have been demonstrated to alleviate neuroblastoma-induced immunosuppression³⁶.

Although the application of adoptive NK cell transfer in pediatric tumors has not been widely explored, several immune-based therapies (particularly CAR T-cells and immune checkpoint inhibitors) have emerged as promising approaches for treating hematologic malignancies^{37–39}. However, CAR T-cell therapies are quite expensive, require meticulous preparation processes, have considerable side effects (namely, cytokine release syndrome), and demonstrate limited efficacy against solid tumors³⁷. Therefore, there is an unmet need for safe, feasible, and effective cancer immune cell therapies, and adoptive NK cell transfer merits further investigation.

In 1985, Rosenberg et al. demonstrated the relative efficacy of the combined administration of interleukin (IL)-2 and autologous lymphokine-activated killer (LAK) cells in controlling the progression of metastatic cancers^{40,41}. Afterward, in 2002, Ruggeri et al. reported that KIR-mismatched NK cells from HSCT donors exhibited graft-versus-leukemia effects and, unlike T-cells, did not induce GVHD⁴². This report establishes the rationale for allogeneic KIR-mismatched NK cell therapy in the clinical setting.

However, despite the promising outcomes of adoptive NK cell transfer strategies in managing hematologic malignancies, their activity against solid tumors faces several hurdles. The neoplastic cells and TME of most solid tumors harbor considerable degrees of heterogeneity^{43,44}, which can negatively affect immune cell functions. In addition, their infiltration into tumoral tissues is suboptimal, a challenge that has yet to be encountered⁴⁴. The immunosuppressive characteristics of the TME (e.g., accumulation of regulatory T-cells, immunosuppressive cytokines, and hypoxia) are other factors that can critically compromise the antitumor abilities of immune cells⁴⁴. Additionally, traditional approaches to transferring NK cells do not yield long-lasting NK cell activity in vivo.

Hence, unprecedented progress has ensued in their ex vivo expansion and activation and characterization of their cardinal regulators (including KIRs). For instance, an in vitro investigation exhibited that KIR-incompatible allogeneic NK cells are superior in exerting anti-tumor functions against renal cell carcinoma and melanoma cells than autologous NK cells⁴⁵. In fact, KIR genes exhibit profound variations in terms of copy numbers and allelic pleomorphisms⁴⁶. Accordingly, the KIR genotype can simply be categorized as A or B, in which the A haplotype mainly contains inhibitory KIRs, while the B haplotype contains several activating KIRs⁴⁶. Intriguingly, several investigations have demonstrated that KIR haplotypes predict the prognosis and the conversion of MDS to AML⁴⁷; the incidence of leukemia⁴⁸ and solid tumors⁴⁹; the prognosis of classical Hodgkin's lymphoma⁵⁰; the prognosis and response to targeted therapies in colorectal cancer^{51,52}; and the response of high-risk NB to auto-HSCT⁵³. Moreover, higher frequencies of inhibitory KIRs have been associated with an increased risk of the development of NBs⁵⁴. Subsequent trials after the Ruggeri et al. study⁴² demonstrated the efficacy of haplo-identical and donor-recipient KIR-mismatched NK-cells treatment along with IL-2 in the resolution of AML^{55,56}. Interestingly, KIR genotyping of bone marrow donors is proposed to be effective in promoting the prognosis of patients with AML who received reduced-intensity conditioning before transplantation, regardless of HLA typing⁵⁷. It should also be noted that although most of these reports have implicated better outcomes with the presence of activating KIRs, the tide turns in the context of cancer immunotherapies, i.e., inhibitory KIRs have been associated with better responses to such therapies. For instance, a recent investigation on KIR/KIR-ligand genotyping of NB patients who had received anti-GD2 therapies determined that the presence of inhibitory KIR2DL2 and KIR3DL1 and their ligands (HLA-C1 and HLA-Bw4, respectively) significantly improves the event-free survival (EFS) and OS⁵⁸.

One of the more studied and promising approaches to increase the efficacy and stemness of adoptive NK cell therapies is priming them with cytokines and growth factors. Pre-clinical and clinical experiments have shown that the addition of IL-2, IL-15, IL-18, IL-21, or IL-27 to the culture media of NK cells can promote their cytotoxic and cytokine-producing capacities and induce a memory-like phenotype, and infusion of IL-2 following NK cell transfer can enhance their proliferation and engraftment in vivo¹⁹. Accordingly, in 2004 Ishikawa et al.⁵⁹ attempted to co-culture autologous, patient-derived NK cells with irradiated human feeder cell line (HFWT) in RHAM- α medium and IL-2. They subsequently administered these cells along with low-dose IFN- β by intravenous with or without focal injections to patients with recurrent malignant gliomas, and an acceptable efficacy and safety profile was observed⁵⁹. However, transfer of autologous, ex vivo activated NK cells did not show an acceptable efficacy in progressive stage IV melanoma or renal cell cancer⁶⁰ and unresectable, locally advanced, and/or metastatic gastrointestinal cancers⁶¹, which was attributed to a lack of activation in the peripheral blood⁶⁰.

Given the importance of KIR-HLA interactions in determining the activation or inhibition of NK cells and the GvL phenomenon, subsequent trials have aimed to investigate the safety and efficacy of allogeneic NK cells. For instance, a trial on relapsed/refractory non-Hodgkin lymphomas and multiple myeloma cases used ex vivo expanded allogeneic NK cells with nicotinamide and IL-15 and injected them with low-dose IL-2 and rituximab

or elotuzumab to enhance their *in vivo* proliferation and ADCC. Expectedly, this adoptive NK cell transfer exhibited promising efficacies as well, given the advanced status of the disease in enrolled cases⁶².

Reports on the efficacy of adoptive NK cell therapy for pediatric solid malignancies are scarce²², and in the case of neuroblastoma, most studies have combined NK cell transfer with anti-GD2 therapies. However, adoptive NK cell transfer approaches have yielded promising outcomes in pediatric bone and soft tissue sarcomas, which are immunologically cold, similar to neuroblastomas^{20,37}.

Much effort has been made to enhance the prognosis of patients with high-risk neuroblastomas, and cancer immunotherapies have shown promising outcomes in this venue. Monoclonal antibodies (mAbs) against GD2 (such as dinutuximab), along with cytokine therapies (including IL-2), can improve survival outcomes^{63,64} by priming NK cell- and neutrophil-mediated ADCC and direct and complement-mediated cytotoxicity². For instance, in those with newly diagnosed neuroblastoma, the addition of non-primed haploidentical NK cells ($4.1\text{--}113.98 \times 10^6$ CD56⁺ cells/kg) to auto-HSCT, hu14.18K322A (an anti-GD2 mAb), GM-CSF, and IL-2 combination therapy (as consolidation therapy) appeared tolerable and effective and did not induce significant toxicity²³. However, the phase II trial of this study did not observe any added benefits from NK cell transfers, which was attributed to the lack of *ex vivo* activation and transient engraftment^{21,23}.

Focusing on more advanced stages of the disease, early trials on recurrent/refractory neuroblastoma have shown the safety and relative efficacy of haploidentical NK cell therapy (with doses of $< 1 \times 10^6$ to 59.5×10^6 CD3⁺CD56⁺ cells/kg) combined with anti-GD2 mAbs^{25,26}, IL-2, conventional chemotherapies²⁵, with response rates of 61.5%²⁵ and 29%^{25,26}. However, anti-GD2 therapies are expensive and not widely available⁶⁵. Putting anti-GD2 mAbs aside, Choi et al.²⁴ enrolled seven patients with relapse/progression of neuroblastoma after auto-HSCT to receive haploidentical NK cells from their parents. The patients received a dose of 3×10^7 /kg of *ex vivo* expanded NK cells in three doses, along with IL-2. Acute (7/7) and chronic (5/7) GVHD and cytomegalovirus (7/7), BK virus (7/7), and Epstein-Barr virus reactivation were common complications after NK cell transfer, and of note, CD16⁺CD56⁺CD3⁺ comprised the preponderance of lymphocytes until one-month post-transplantation²⁴. One patient died due to infectious complications, two showed CR, and the other four cases exhibited SD at the three-month F/U, implicating the promising outcomes of haploidentical NK cell and IL-2 therapy. At the end of F/Us, one patient remained alive (after salvage therapy), whereas the other five died due to disease progression²⁴.

NK cell expansion, activation, and injection are indeed feasible and cost-effective methods of cancer immunotherapy. We and others have previously shown the safety, efficacy, and feasibility of allogeneic NK cell transfer against solid tumors in experimental^{66–68} and clinical⁶⁹ settings. As discussed above, there are several approaches for priming NK cells *ex vivo* and *in vitro*. Preclinical and clinical experiments have shown that the addition of IL-2, IL-15, IL-18, and IL-21 to the culture medium of NK cells promotes their cytotoxic and cytokine-producing capacities and induces a memory-like phenotype, and infusion of IL-2 following NK cell transfer can enhance their proliferation and engraftment *in vivo*^{19,59}. In our study, we used a combination of IL-2, IL-15, and IL-21, as they have been shown to promote the *ex vivo* expansion of NK cells more effectively⁷⁰. These NK cells exhibit acceptable cytokine release and cytolytic activity *in vitro*. We also utilized a low-intensity conditioning regimen and applied a lower dose of NK cells than in previous studies. In fact, these might be the reasons for the absence of adverse reactions and complications (namely, GVHD and cytokine release syndrome) in our patients. Only one patient exhibited serious side effects (neutropenic fever), and notably, we could not reliably attribute it to NK cell transfer.

While the sample size of our trial was small, and robust conclusions could not be drawn, NK cell therapy alone was modestly effective against recurrent neuroblastomas after auto-HSCT. One patient achieved CR (which lasted for 18 months), and the other achieved PR which lasted for nine months. Two patients (i.e., those with CR and PD in a soft tissue lesion) were alive in 26-month and 15-month F/Us, respectively. Of note, the former exhibited normal growth and functional status. Therefore, considering its favorable safety profile, allogeneic NK cell transfer is a promising therapy for patients with neuroblastoma, even as neoadjuvant therapy for high-risk patients.

This study has several limitations. We did not assess the efficacy of NK cell engraftment, the composition of peripheral blood lymphocytes and other immune cells, or the infiltration rate of transplanted cells into tumoral tissues following injections. Likewise, the cytotoxicity and cytokine release capacities of NK cells were not investigated in F/U evaluations. Finally, considering the small sample size of this study, robust conclusions could not be drawn reliably.

In conclusion, we report the results of a pilot trial on the administration of allogeneic *ex vivo* expanded NK cells in patients with relapsed neuroblastoma after auto-HSCT. The treatment was safe, feasible, and cost-effective (compared to CAR T-cell and anti-GD2 therapies) and, considering the high-risk nature of the disease, showed a relatively acceptable efficacy in controlling disease progression. Further studies with larger enrolled cases, higher doses of NK cells, and more frequent injections are warranted to evaluate the efficacy of this therapy in the adjuvant (after the failure of or in combination with auto-HSCT) and even neoadjuvant settings for high-risk cases and to characterize the intratumoral infiltration and cytotoxicity of allogeneic NK cells after transfer.

Materials and methods

Study design and patient selection

We conducted an open-label, nonrandomized phase I trial to assess the safety and efficacy of allogeneic NK cell injection in pediatric patients (aged between 2 and 15 years) with relapsed/refractory neuroblastomas after auto-HSCT. All patients without evidence of bone marrow metastases who had received an auto-HSCT in our center between February 2020 and June 2021 and had evidence of relapsed/refractory disease in the subsequent follow-up (F/U) studies were evaluated for enrollment in this trial, according to the inclusion and exclusion criteria presented in Supplementary Table S1 online. As neuroblastoma is a rare pediatric cancer, we did not

consider randomization, blinding, or the introduction of a control arm. Similarly, statistical calculations of sample size were not applied in this study.

Written informed consent was obtained from peripheral blood donors and each patient's parents/legal guardians after a detailed clarification of the experimental nature of the trial and the anonymity of the enrolled individuals. This trial was conducted following the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Tehran University of Medical Sciences (ethics registration code, IR.TUMS.MEDICINE.REC.1399.817). The findings of this trial are described according to the Consolidated Standards of Reporting Trials (CONSORT) recommendations.

NK cell isolation

NK cell separation was conducted using a good manufacturing practice (GMP)-compliant closed system (CliniMACS Prodigy, Miltenyi Biotec, Germany) designed for clinical applications. Apheresis (10–15 L, using the Spectra Optia Apheresis System, Terumo Blood and Cell Technologies) was performed on eligible healthy donors. Approximately 80–120 cm³ of blood was taken, which was then transferred to transfer bags (Baxter AG, Switzerland). After dilution with PBS/EDTA buffer and 0.5% human albumin serum, the solutions were centrifuged (300 × *g* for 15 min at room temperature) to isolate peripheral blood mononuclear cells (PBMCs). The buffy coat containing PBMCs was harvested and resuspended in EDTA/PBS and 0.5% human albumin and incubated with CD3 (1 vial for a total of 40 × 10⁹ nucleated cells) and CD56 (1 vial for a total of 40 × 10⁹ nucleated cells) microbeads for 30 min each at room temperature to deplete T-cells and enrich NK cells, respectively, using CliniMACS Tubing Set LS (CliniMACS Prodigy, Miltenyi Biotec, Germany).

NK cell viability, cytokine release, and cytolytic assays

To assess the viability of the isolated and cultured NK cells, we combined 20 µL of the cell suspension with 20 µL of 0.4% trypan blue. The resulting suspension was read using a Neubauer improved cell counting chamber according to established methods.

The release of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) was assessed by culturing NK cells in a 96-well enzyme-linked immunosorbent assay (ELISA) plate (10,000 per well) and adding NK MACS GMP medium, 20 ng/mL interleukin (IL)-2, and 50 ng/mL IL-15. After 3 days, cell viability was assessed by trypan blue staining, and the solution was centrifuged. The supernatant was then used for ELISA.

To assess the cytotoxicity of the cultured NK cells, we applied the lactate dehydrogenase assay. NK cells were exposed to tumoral cells (CHLA255 and SK-N-SH human neuroblastoma cell lines, Pasteur Institute of Iran, Tehran, Iran) at different ratios (1:1, 1:2, 1:5, and 1:10; target cells:effector cells), and after two hours of coculture, the solutions were centrifuged (250 × *g* for 10 min). The supernatant was then passed through 0.22 µm sterile syringe filters, and 100 µL of the filtered solution was transferred to diagnostic plates (Roche Diagnostics, Germany). The reactions were conducted for 30 min in the dark, and the results were read using an ELISA reader with 492 nm filters. The readings were compared with the corresponding values of the background, low, and high control groups.

NK cell culture

The isolated NK cells were initially transferred to T25 flasks containing NK MACS GMP medium (Miltenyi Biotec, Germany) supplemented with 5% human albumin solution (Valley Biomedical, USA), IL-2 (500 IU/mL, Miltenyi Biotec, Germany), IL-15 (140 IU/mL, Miltenyi Biotec, Germany), and IL-21 (1 IU/mL, Miltenyi Biotec, Germany). NK cells were cultured for 48 h at 37 °C and 5% CO₂. After 48 h, the cultured cells were transferred to culture bags (VueLife). In compliance with the manufacturer's guidelines, fresh expansion NK MACS medium, in addition to IL-2 and IL-15, was added to the medium without removing the older medium. The volume of the newly added culture medium and cytokines was determined according to the cell count of each bag^{70,71}. NK cells were cultured for a total duration of 22 ± 3 days.

NK cell administration

After reviewing the inclusion and exclusion criteria, eligible patients were hospitalized and received cyclophosphamide (60 mg/kg, for two hours of continuous intravenous [IV] infusion) on days –5 and –4. On day zero, the first dose of NK cells (1 × 10⁷ cells/kg) was infused intravenously for one hour with 200 mL of physiological serum containing 2% human albumin solution (Albumedix, United Kingdom). If the first dose was tolerated, the second escalated dose of NK cells (5 × 10⁷ cells/kg) was administered three weeks later using the same protocol. After each injection, the patients were closely monitored for signs and symptoms of possible allergic and acute reactions (fever, angioedema, hives, etc.), cardiopulmonary decompensation, and infectious complications for one week. Subsequently, routine antifungal and antiviral drugs were prescribed as the preventive strategy for opportunistic infections, and isotretinoin treatment was reinstituted.

NK cells were first isolated by centrifugation of the culture medium at 500 × *g* for 10 min. Afterward, we assessed their viability, cytotoxicity, and cytokine release, as described previously. In addition, karyotyping, viral, bacterial, and mycoplasma infection control, and bacterial endotoxin release control (using the limulus amoebocyte lysate [LAL] test) were performed before infusion.

Outcome measure and endpoints

The primary objective of this trial was to assess the safety and tolerability of allogeneic NK cells in children with relapsed/refractory neuroblastomas following auto-HSCT. Secondary objectives included the assessment of primary and/or metastatic tumor responses to allogeneic NK cells (defined as complete response [CR], partial

response [PR], or stable disease [SD]), according to the recommendations of the revised International Neuroblastoma Response Criteria (INRC)⁷².

One week after the injection of NK cells, the patients were discharged, and their parents were instructed on regular outpatient visits to the pediatric stem cell transplantation clinic at least once a week for the first month after the injection of NK cells. Subsequent F/U visits were planned according to the INRC recommendations. Assessments included computed tomography (CT) scans and bone marrow biopsies (bilateral aspirates and bilateral trephine biopsies) at 3 and 6 months and iodine-131 (¹³¹I)-metaiodobenzylguanidine (MIBG) scans at 1, 3, and 6 months after the injection of the last dose of NK cells. More frequent assessments would be performed on the appearance of new signs and/or symptoms of disease or the development of complications.

Expert pediatric radiologists and a nuclear medicine specialist interpreted the CT and ¹³¹I-MIBG scans. Two expert pathologists evaluated the bone marrow aspirates and biopsies. A semiquantitative scoring system was adopted to assess ¹³¹I-MIBG scans based on the Curies scale⁷³. The response of the primary tumor and bone, bone marrow, and soft tissue metastases to treatment was evaluated by adopting changes in the Curies scores of ¹³¹I-MIBG scans, Response Evaluation Criteria in Solid Tumors (RECIST) of CT scans⁷², and pathological assessment of bone marrow samples⁷⁴.

Ethics approval and consent to participate

All procedures performed in this study were approved by the appropriate ethics committee and performed in compliance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from the patients' parents or legal guardians before participation in the study. This study (Phase I study of safety and efficacy of allogeneic natural killer cell therapy in relapsed/refractory neuroblastoma post autologous hematopoietic stem cell transplantation) was approved by the ethics committee of Tehran University of Medical Sciences (Reference Number: IR.TUMS.MEDICINE.REC.1399.817, approved November 21, 2020).

Data availability

Detailed information on patients' MIBG and CT scan findings is available from the corresponding author upon reasonable request. All other data generated or analyzed during this study are included in this published article and its supplementary information.

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Author contributions

R.M. conceptualized the trial, designed the study protocol, and performed the functional assays of NK cells. P.M.S. gathered patient data, interpreted the data, drafted the manuscript, and designed the figures. M.B. conceptualized the trial and performed the follow-up visits as a transplantation specialist. S.S. obtained and cultured NK cells. A.S. obtained and cultured NK cells. L.J. partially contributed to functional assays of NK cells. A.K. partially contributed to NK cell isolation. Z.N. administered NK cells to the enrolled patients and was responsible for administering conditioning regimens. A.A.H. conceptualized the study, designed the study protocol, performed F/U visits (as the head of transplantation specialists), critically appraised the draft, and supervised the project. All the authors have read and approved the final manuscript. The parents or legal guardians of the included cases consented to the participation of their children in this trial and the anonymous publication of their data.

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Competing interests

The authors declare no competing interests.

Additional information

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