CD22-targeted glyco-engineered natural killer cells offer a further treatment option for B-cell acute lymphoblastic leukemia

The safety and efficacy of natural killer (NK) cell therapy have been demonstrated in clinical trials, highlighting its potential as an off-the-shelf product with diverse clinical application.[1,2] NK cells differ from T cells in that they can be activated without the need for antigen presentation and can directly eliminate various cancer cells.[3] By binding to ligands on target cells, NK cells can rapidly distinguish between normal and cancer cells, activating and initiating the cytotoxic activity against cancer cells. Importantly, NK cells exhibit minimal toxic side effects on patients during cancer therapy, and allogeneic NK cells do not induce graft-*versus*-host disease (GVHD).[4] CD22 is an inhibitory receptor highly expressed in various B-cell malignancies.[5] Therapeutic approaches targeting CD22, such as CD22-specific chimeric antigen receptor (CAR) T cells and antibody-drug conjugates, have demonstrated clinical efficacy in treating B-cell lymphoma and leukemia.[6,7] In this study, through glycoengineering of primary NK cells derived from umbilical cord blood (UCB), we successfully generated NK cells with CD22 ligands, referred to as "MsNK" cells. These glycoengineered MsNK cells exhibited the ability to selectively kill CD22-positive B-cell lymphoma and leukemia cells both *in vitro* and *in vivo*. In a phase I clinical trial, where two patients with B-cell acute lymphoblastic leukemia (B-ALL) received infusions of MsNK cells, no significant toxicity was observed in either patient, and one patient achieved partial remission.

In order to obtain an adequate quantity of NK cells, UCB mononuclear cells were isolated and cultured using an NK-cell amplification kit. After 14 days of expansion, over 1×10^{10} cells with a purity of more than 98% were obtained (*Online Supplementary Figure S1A*). The CD22 ligand was introduced into NK cells via metabolic engineering using a modified sialic acid derivative called MPB-sia1,[8] in a dose- and time-dependent manner. Increasing the concentration of MPB-sia1 enhanced the expression level of the CD22 ligand on the cell surface and prolonging the incubation time to 48 and 72 hours resulted in higher levels of CD22 binding to the NK cells (*Online Supplementary Figure S1B, D*). However, cell viability was significantly reduced when the concentration of MPB-sia1 exceeded 2 mM or when the incubation time exceeded 48 hours (*Online Supplementary Figure S1E, F*). Therefore, for further study, NK cells were incubated with 2 mM MPB-sia1 for 48 hours. Confocal microscopy confirmed that over 99% of NK cells expressed CD22 ligands after incubation (Figure 1A). Phenotypic analysis showed no differential markers between MsNK cells and unmodified NK cells (*Online Supplementary Figure S2A, B*). The persistence of CD22 ligands on the MsNK cells was analyzed, and approximately 30% of the ligands remained on the cells 48 hours after the removal of MPB-sia1 from the cell culture medium (*Online Supplementary Figure S2C*). Several studies have suggested that IL-15 can enhance NK-cell persistence in preclinical and clinical investigations.[1,2,9,10] In our experiments conducted in NCG-IL15 mice (which endogenously secrete human interleukin [IL]-15), we observed the persistence of CD22 ligand expression on MsNK cells. However, we found that IL-15 did not prolong their persistence (*Online Supplementary Figure S2D*).

In order to assess cytotoxicity, MsNK cells were co-incubated with CD22-expressing Raji and BALL-1 cells, as well as non-CD22-expressing K562 cells. Compared to unmodified NK cells, MsNK cells demonstrated significantly enhanced cytotoxicity against Raji and BALL-1 cells, while no significant cytotoxicity was observed against CD22-negative K562 cells (Figure 1B). Furthermore, the co-incubated supernatants were collected to analyze interferon-γ (IFN-γ) levels, which were significantly increased after interaction with Raji and BALL-1 cells compared to unmodified NK cells (Figure 1C). *In vivo* analysis using a leukemia mouse model established by injecting luciferase-engineered and CD22-overexpressing Nalm6 cells (human B-ALL cell line) demonstrated that mice injected with MsNK cells exhibited significantly slower tumor growth and extended survival compared to mice injected with PBS or unmodified NK cells (Figure 1D-F). These results were confirmed using NK cells from at least two independent donors. The persistence of CD22 ligands on MsNK cells *in vivo* was found to be short, with approximately 50% of the ligands remaining on the cells after 12 hours, indicating the need for multiple infusions for effective treatment (*Online Supplementary Figure S2D*). The results stated above suggest that MsNK cells hold promise as a treatment strategy for CD22-positive B-cell malignancies.

Following approval by the Ethics Committee of Tianjin First Central Hospital, the clinical study was registered for trials at the China Clinical Trial Registration Center (ChiCTR2000041024, http://www.chictr.org.cn/showproj.aspx?proj=65785). Two patients diagnosed with B-ALL, having undergone multiple lines of treatment with evident disease progression, were enrolled in the study (Table 1). Both patients had high-risk characteristics and refractory diseases. Flow cytometry analysis confirmed that over 90% of the tumor cells in both patients expressed CD22.

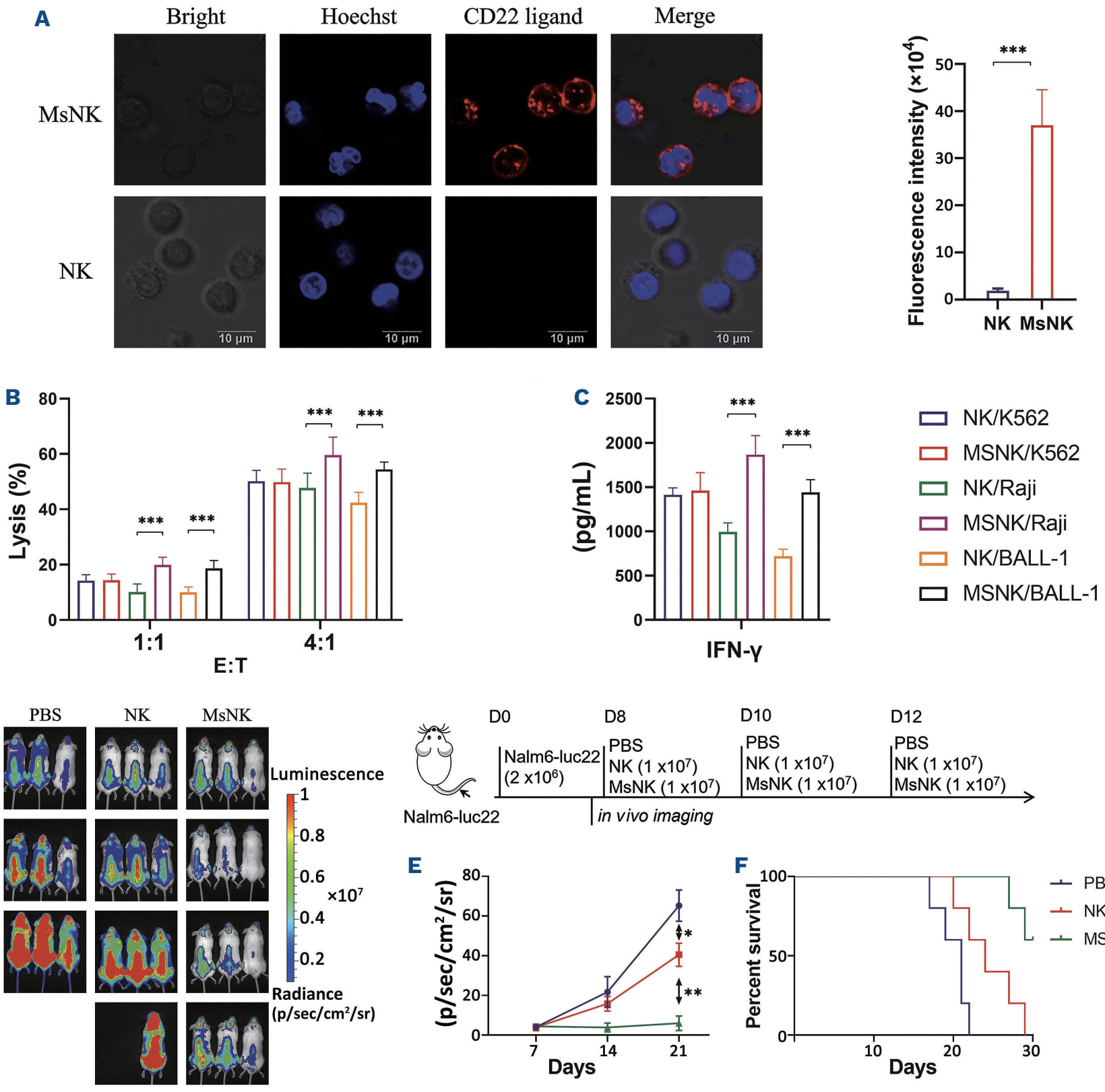

Figure 1. MsNK cells can target CD22-positive tumor cells *in vitro* and *in vivo*. (A) Confocal microscopy imaging showing the expression and statistical results of the CD22 ligand. (B) Unmodified natural killer (NK) cells and MsNK cells were co-incubated with K562, Raji and BALL-1 cells, respectively, at different effector cell target cell ratios to evaluate their specific cytotoxicity. (C) Supernatants from the co-incubation (effector cell:target cell =1:1) were collected and the interferon-γ (INF-γ) content of the supernatants was analyzed (experiments were conducted using cells from 3 different donors; mean ± standard deviation [SD]; ***P<0.001). (D) Luciferase engineering and CD22-overexpressing Nalm6 (Nalm6-Luc22) leukemia cells were injected into NSG mice through the tail vein to establish leukemia mouse models. On days 8, 10, and 12 after tumor inoculation, phosphate-buffered saline (PBS) was injected into the tail vein, and after NK cells and MsNK cells were treated, the tumor load of mice was detected by *in vivo* imaging technique at the specified time points (N=5, representative pictures of 3 mice in each group are given). (E) Quantitative plot of leukemia burden of each group of mice at different time points (N=5, mean ± SD; *P<0.05; **P<0.01). (F) Survival time of mice treated with PBS, NK cells, and MsNK cells (N=5; *P<0.05).

Lymphodepleting chemotherapy with fludarabine and cyclophosphamide was administered to both patients for 3 days (Table 1), followed by three infusions of MsNK cells at a dose of 3×10^7/kg with a 1-day interval. Post-treatment management was determined based on the treating physician's assessment at day 30. Both patients exhibited grade 1 cytokine release syndrome (CRS), characterized by transient fever that resolved without medical intervention. No symptoms of neurotoxicity or hemophagocytic lymphohistiocytosis were observed. Additionally, there were no cases of GVHD despite human leukocyte antigen (HLA) mismatches between the MsNK cells and the patients.

Table 1. Characteristics of patients and outcomes of MsNK cell therapy.

Characteristic	Patient 1	Patient 2
Age in years/sex	46/F	53/F
Disease type	B-ALL	B-ALL
Gene mutation	NA	WT1
Karyotype	46,XX,t(9;22)(q34;q11) [20]	Normal
Prior lines of treatment, N	6	7
Extramedullary invasion	Yes	No
Pretreatment disease burden %	59.77	93.70
CD22 positivity %	99.45	99.01
Initial therapy	VDCP+Imatinib	VDLP
Lymphodepletion	FC	FC
CRS/grade	1	1
CRES/grade	0	0
GVHD	No	No
MsNK in the infusion %	98.85	97.60
MsNK dose/kg	9×10^7	9×10^7
HLA matching loci	2/6	1/6
KIR matching	No	No
Response	PR	SD
Status at last follow-up (time point in days)	Died of post-transplant organ failure (431)	Died of disease progression (43)

Cytokine release syndrome (CRS) was graded per a modified grading system proposed by Lee *et al.*[15] Individual symptoms of CRS were graded per CTCAE version 5.0. F: female; B-ALL: B-cell acute lymphoblastic leukemia; NA: not available; VDCP: vincristine, daunorubicin, cyclophosphamide, prednisone; VDLP: vincristine, daunorubicin, L-asparaginase, prednisone; CRS: cytokine release syndrome; CRES: chimeric antigen receptor T-cell-related encephalopathy syndrome; GVHD: graft-*versus*-host disease; NK: natural killer; PR: partial response; SD: stable disease.

Transient and reversible hematological toxicity events, primarily related to lymphodepleting chemotherapy, were observed. Therefore, it was difficult to determine if the infusion of MsNK cells resulted in hematological toxicity. All adverse events observed in the study are listed in the *Online Supplementary Table S1*. None of the patients required admission to the intensive care unit for management of MsNK cell-related adverse events.

After 2 weeks of treatment, patient 1 achieved partial remission, with a reduction in the proportion of bone marrow tumor cells. There was a slight decrease in tumor cell CD22 expression post-treatment, while CD19 remained strongly expressed (Figure 2A). However, the patient experienced disease progression after 1 month of treatment. Subsequently, the patient received anti-CD19 CAR T-cell therapy and achieved a complete response. In order to ensure long-term survival, the patient underwent unrelated donor hematopoietic stem cell transplantation but ultimately succumbed to post-transplant organ failure. Patient 2 had a high tumor burden at enrollment, and no decrease in tumor burden was observed during treatment evaluation. There was a trend of decreased CD22 expression on tumor cells post-treatment, while CD19 remained strongly expressed (Figure 2A). The patient died due to disease progression after 2 months of treatment. CD22-positive B cells were detected in the peripheral blood only in patient 1, and there was a transient decrease in these cells following MsNK cell infusion (*Online Supplementary Figure S2E*). B-cell counts did not return to normal levels in either patient during follow-up. Flow cytometry and short tandem repeat (STR) assays were used to dynamically measure the metabolism of MsNK and NK cells in the patients. The proportion of MsNK cells among the total NK cells in patients peaked on the sixth day post-infusion, reaching approximately 20%, followed by a rapid decline (Figure 2B). STR data indicated the rapid disappearance of allogeneic NK cells, suggesting potential autologous immune rejection of the allogeneic NK cells by the patients (Figure 2C). Inflammatory cytokine levels, including IL-6 and IFN-γ, showed only a slight increase, which corresponded to the mild cytokine-release syndrome (CRS) observed after MsNK cell infusion (Figure 2D, E). There were no significant abnormalities in total bilirubin or other indicators related to GVHD-related liver injury during treatment (Figure 2F; *Online Supplementary Figure S2F*). No cases of GVHD, rash, or diarrhea were observed.

NK cells, known for their potent anti-cancer activity, face limitations in intrinsic targeting ability against cancer cells. Glycoengineering was employed to enhance this capability through CD22 ligands, resulting in the generation of MsNK cells from UCB-derived NK cells. In both *in vitro* and *in vivo*

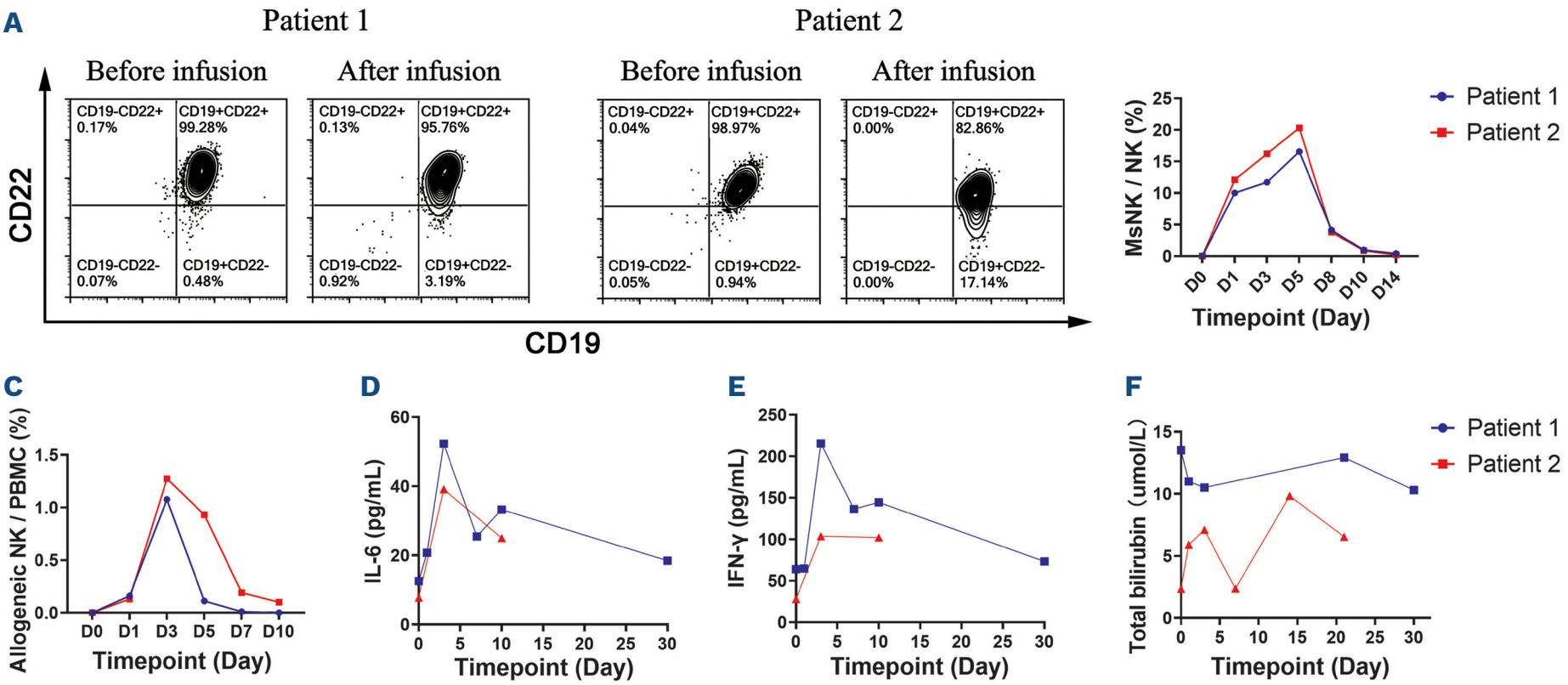

Figure 2. Dynamic index analysis of the patient during MsNK cell infusion. (A) Expression of CD19 and CD22 on tumor cells in the bone marrow of patients before and 14 days after treatment. (B) The proportion of MsNK cells among all natural killer (NK) cells was monitored in peripheral blood (PB). (C) The proportion of infused cord blood-derived allogeneic NK cells in PB nucleated cells was determined by short tandem repeat (STR). (D, E) Data on interleukin-6 (IL-6) and interferon-γ (IFN-γ) during treatment in patients. (F) Data on liver function-related parameters (total bilirubin) during treatment. PDMC: peripheral blood mononuclear cells.

experiments, MsNK cells demonstrated superior CD22 target-dependent killing efficacy compared to unmodified NK cells. Two patients with B-ALL were treated with MsNK cell therapy, confirming safety and efficacy.

UCB was chosen as a source for MsNK cells due to stable availability and cytotoxicity comparable to peripheral blood NK cells.[1,11] A 1-day interval infusion mode was adopted due to the short half-life of MsNK cells. In the clinical study, the dose of infused MsNK cells was guided by the treatment doses established in previous CAR NK-cell therapies.[2] Our research indicates that the administered dose of MsNK cells did not reach the maximum tolerated dose. Given the short duration of MsNK cell persistence and the mild observed toxicity, higher doses may potentially benefit patients. HLA and killer immunoglobulin-like receptor mismatches might contribute to the cytotoxicity of allogeneic NK cells against tumors.[12,13] However, these mismatches may also trigger the recognition and elimination of allogeneic MsNK cells by the patient's autologous immune system,[14] as suggested by our STR data. Therefore, implementing more profound lymphodepleting chemotherapy before infusion could potentially enhance the persistence of allogeneic MsNK cells within the patient's body.

Patient 1 achieved complete remission after subsequent CD19 CAR T-cell therapy, even though the treatment target differed. This indirect observation suggests that the efficacy of MsNK cells might be weaker compared to CAR T cells. Furthermore, our preclinical and clinical investigations both revealed the rapid *in vivo* metabolism and loss of CD22 ligands on MsNK cells, indicating their potential suitability for patients with low tumor burdens. Additionally, the observed mild treatment toxicity in patients suggests that MsNK cells have significant potential when combined with other therapeutic modalities. In conclusion, glycoengineering emerges as a promising approach to enhance NK-cell targeting in cancer therapy. With its lower toxicity, further exploration of higher doses or combination therapies could provide a complementary strategy for the treatment of B-ALL.

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https://doi.org/10.3324/haematol.2023.284241

Received: September 4, 2023.
Accepted: April 22, 2024.
Early view: May 2, 2024.

Disclosures

The compound MPB-sia1 was provided by Xiamen NuokangDe Biotechnology Co. Ltd. Additionally, a patent related to this project has been filed (patent application number: 201810139361.2). The authors have no conflicts of interest to disclose.

Contributions

All authors report substantial contributions to the conception and design of the study, acquisition of data, or the analysis or interpretation of data; have critically reviewed the manuscript; approved the final version; agree with all content; and accept accountability for the overall work.

Funding

This work was supported by grants from the Science and Technology Project of Tianjin Municipal Health Committee (TJWJ2022QN030 and TJWJ2022XK018 to MZ), and the General Project of the National Natural Science Foundation of China (81970180 to MZ), and Key Projects of Tianjin Applied Basic Research and Multi-Investment Fund (21JCZDJC01240), and Key Science and Technology Support Project of Tianjin Science and Technology Bureau (20YFZCSY00800 to MZ), and Tianjin Key Medical Discipline (Specialty) Construction Project (TJYXZDXK-056B), and Fujian Natural Science Foundation of China (2021J011354 to ZF), and Medical Innovation Project of Fujian Provincial Health and Family Planning Commission (2021CXB020 to ZF), and Tianjin Municipal Natural Science Foundation (22JCQNJC00820 to WL), Youth Science Fund Project of National Natural Science Foundation of China (82300171 to XJ), as well as China Postdoctoral Science 13 Foundation (2022M722748 to XJ).

Data-sharing statement

The data that support the findings of this study are available on request from the corresponding author.

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