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

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Clinical Trial informations：this study was approved by the Ethics Committee of Tianjin First Central Hospital, and was registered for clinical trials in the China Clinical Trial Registration Center (Trial registration: ChiCTR2000041024. Registered 16 December 2020, http://www.chictr.org.cn/showproj.aspx?proj=65785).

Disclosures

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Author contribution statement

All authors report substantial contributions to the conception and design of the studies, 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.

Data-sharing statement

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

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 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, we successfully generated NK cells with CD22 ligands, referred to as "MsNK" cells. These glyco-engineered 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.

To obtain an adequate quantity of NK cells, umbilical cord blood (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 (Fig. S1A). The CD22 ligand was introduced into the 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, prolonging the incubation time to 48 and 72 hours resulted in higher levels of CD22 binding to the NK cells (Fig. 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 (Fig. S1E, F). Therefore, for further study, the NK cells were incubated with 2 mM MPB-sia1 for 48 hours. Confocal microscopy confirmed that over 99% of the NK cells expressed CD22 ligands after incubation (Fig. 1A). Phenotypic analysis showed no differential markers between MsNK cells and unmodified NK cells (Fig. 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 (Fig. 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 IL-15), we observed the persistence of CD22 ligand expression on MsNK cells. However, we found that IL-15 did not prolong their persistence (Fig. S2D).

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 (Fig. 1B). Furthermore, the co-incubated supernatants were collected to analyze IFN-γ levels, which were significantly increased after interaction with Raji and BALL-1 cells compared to unmodified NK cells (Fig. 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 (Fig. 1D-F). These results were confirmed using NK cells from at least 2 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 (Fig. S2D). These results 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-cell acute lymphoblastic leukemia (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. Lymphodepleting chemotherapy with fludarabine and cyclophosphamide was administered to both patients for three days (Table 1), followed by three infusions of MsNK cells at a dose of 3×10^7/kg with a one-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 interventio. No symptoms of neurotoxicity or hemophagocytic lymphohistiocytosis were observed. Additionally, there were no cases of graft-versus-host disease (GVHD) despite HLA mismatches between the MsNK cells and the patients. Transient and reversible hematological toxicity events, primarily related to lymphodepleting chemotherapy, were observe. 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 Table S1. None of the patients required admission to the intensive care unit for management of MsNK cell-related adverse events.

After two weeks of treatment, Patient 1 achieved partial remission (PR), 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 (Fig. 2A). However, the patient experienced disease progression after one month of treatment. Subsequently, the patient received anti-CD19 chimeric antigen receptor (CAR) T cell therapy and achieved a complete response. 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 (Fig. 2A). The patient died due to disease progression after two 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 (Fig. 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 (Fig. 2B). STR data indicated the rapid disappearance of allogeneic NK cells, suggesting potential autologous immune rejection of the allogeneic NK cells by the patients (Fig. 2C). Inflammatory cytokine levels, including interleukin-6 and IFN-γ, showed only a slight increase, which corresponded to the mild CRS observed after MsNK cell infusion (Fig. 2D, E). There were no significant abnormalities in total bilirubin or other indicators related to

GVHD-related liver injury during treatment (Fig. 2F, 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 umbilical cord blood (UCB)-derived NK cells. In both in vitro and in vivo experiments, MsNK cells demonstrated superior CD22 target-dependent killing efficacy compared to unmodified NK cells. Two patients with B-cell acute lymphoblastic leukemia (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 comparable cytotoxicity to peripheral blood (PB)-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 KIR 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 cell acute lymphoblastic leukemia.

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

Patient ID	1	2
Age/Sex	46/F	53/F
Disease type	B-ALL	B-ALL
Gene mutation	N/A	WT1
Karyotype	46，XX, t（9；22）（q34；q11) [20]	Normal
Prior lines of treatment	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 (days)	Died of post-transplant organ failure (431)	Died of disease progression (43)

ID: identification number; F: female; B-ALL: B-cell acute lymphoblastic leukemia; N/A., not available; VDCP: Vincristine, daunorubicin, cyclophosphamide, prednisone; VDLP: Vincristine, daunorubicin, L-asparaginase, prednisone; CRS: cytokine release syndrome; CRES: CART-cell-related encephalopathy syndrome. GVHD: graft-versus-host disease; PR: partial response; SD: stable disease.

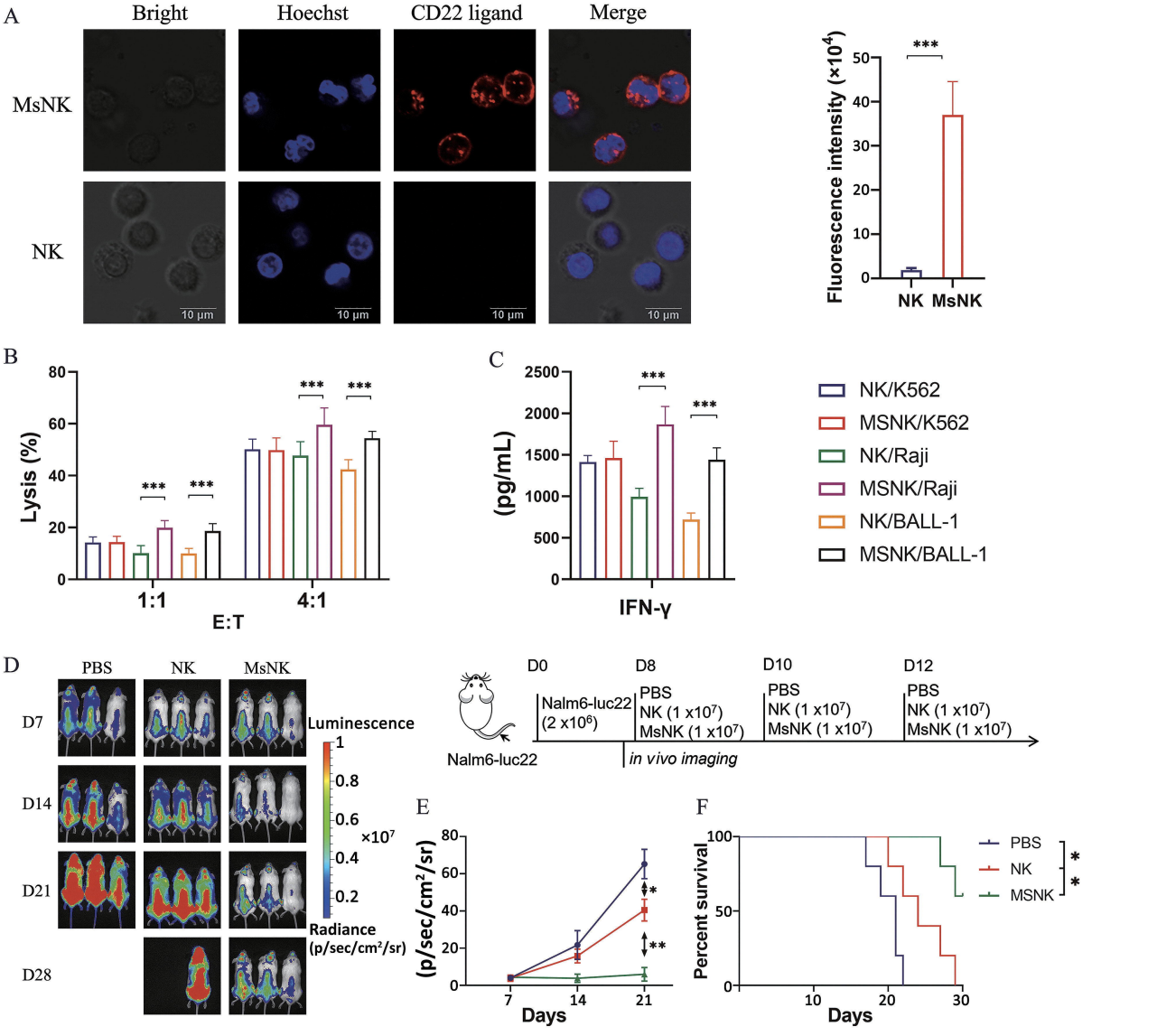
CRS was graded per a modified grading system proposed by Lee et al(15).

Individual symptoms of CRS were graded per CTCAE version 5.0.

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 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 IFN-γ content of the supernatants was analyzed (Experiments were conducted using cells from three different donors; mean ± 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, 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 three mice in each group are given). E, shows the quantitative plot of leukemia burden of each group of mice at different time points (n = 5, mean ± SD, *P < 0.05, **P < 0.01). H, survival time of mice treated with PBS, NK cells, and MsNK cells (n = 5, *P < 0.05).

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 NK cells was monitored in peripheral blood. C, the proportion of infused cord blood-derived

allogeneic NK cells in PB nucleated cells was determined by STR. F, D, data on interleukin-6 (IL-6) and interferon-gamma (IFN-γ) during treatment in patients. F, data on liver function-related parameters (total bilirubin) during treatment.


A
Bright
Hoechst
CD22 ligand
Merge
MsNK
NK
10 μm
Fluorescence intensity (×10^4)
***
NK
MsNK
B
Lysis (%)
E:T
1:1
4:1
C
(pg/mL)
IFN-γ
NK/K562
MSNK/K562
NK/Raji
MSNK/Raji
NK/BALL-1
MSNK/BALL-1
D
PBS
NK
MsNK
D7
D14
D21
D28
Luminescence
×10^7
Radiance (p/sec/cm²/sr)
D0
D8
D10
D12
Nalm6-luc22 (2 x10^6)
PBS
NK (1 x10^7)
MsNK (1 x10^7)
Nalm6-luc22
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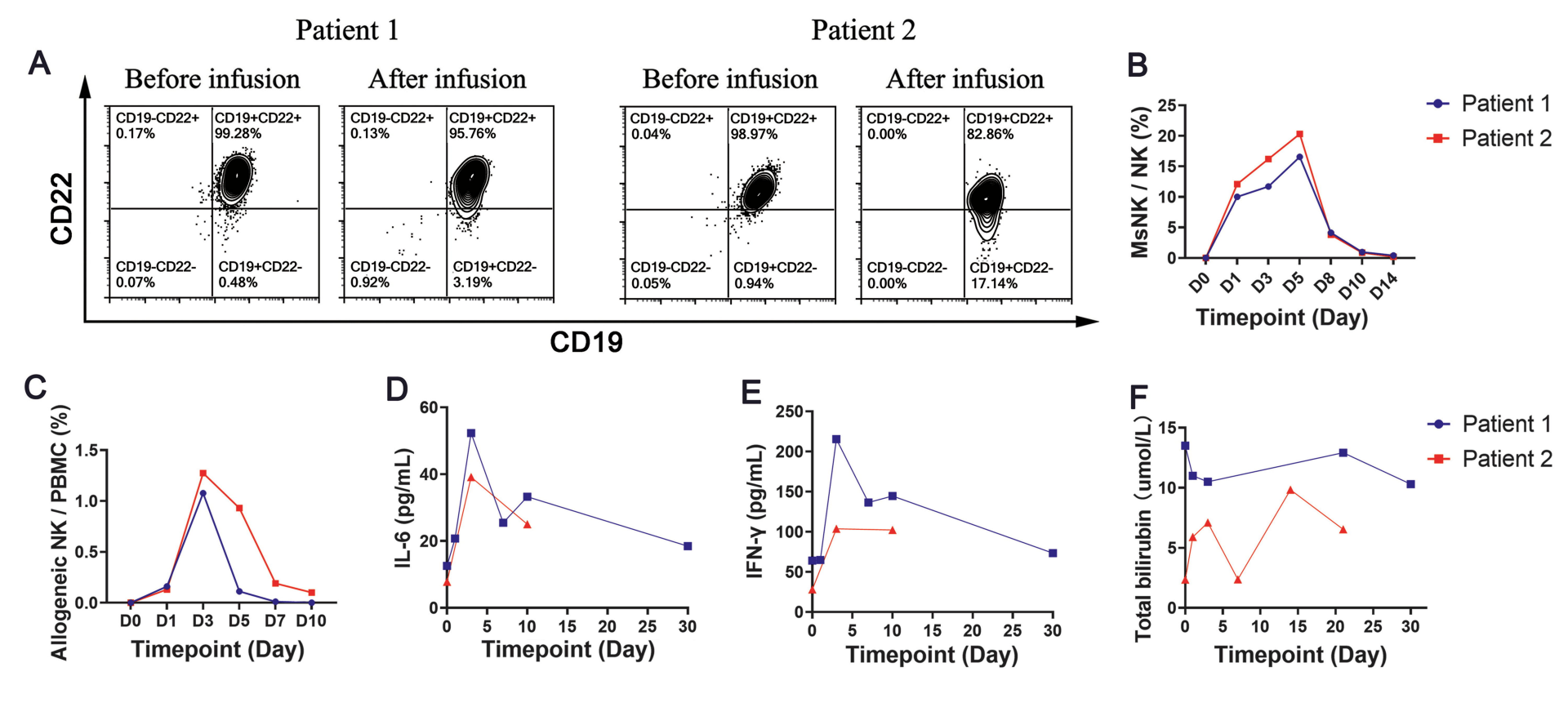
E
(p/sec/cm²/sr)
Days
F
Percent survival
Days
PBS
NK
MSNK


Patient 1
Patient 2
A
Before infusion
After infusion
Before infusion
After infusion
CD19-CD22+ 0.17%
CD19+CD22+ 99.28%
CD19-CD22- 0.07%
CD19+CD22- 0.48%
CD19-CD22+ 0.13%
CD19+CD22+ 95.76%
CD19-CD22- 0.92%
CD19+CD22- 3.19%
CD19-CD22+ 0.04%
CD19+CD22+ 98.97%
CD19-CD22- 0.05%
CD19+CD22- 0.94%
CD19-CD22+ 0.00%
CD19+CD22+ 82.86%
CD19-CD22- 0.00%
CD19+CD22- 17.14%
CD22
CD19
B
MsNK / NK (%)
Timepoint (Day)
D0 D1 D3 D5 D8 D10 D14
Patient 1
Patient 2
C
Allogeneic NK / PBMC (%)
Timepoint (Day)
D0 D1 D3 D5 D7 D10
D
IL-6 (pg/mL)
Timepoint (Day)
E
IFN-γ (pg/mL)
Timepoint (Day)
F
Total bilirubin（umol/L）
Timepoint (Day)
Patient 1
Patient 2

Supplementary Materials

Table S1. Treatment-emergent adverse events

Event \ Patient	Patient 1	Patient 2
Fever	Grade 2	Grade 2
Encephalopathy	No	No
Neutropenia	Grade 4	Grade 4
Anemia	Grade 3	Grade 3
Thrombocytopenia	Grade 3	Grade 4
Hypoxia	No	No
Somnolence	No	No
Acute kidney injury	No	No
Agitation	No	No
Ascites	No	No
Aspartate aminotransferase increased	No	No
Cardiac failure	No	No
Delirium	No	No
Fatigue	No	No
Hemorrhage intracranial	No	No
Hypocalcemia	No	No
Hyponatremia	No	No
Hypophosphatemia	No	No
Hypotension	No	No
Metabolic acidosis	No	No
Oral herpes	No	No
Pseudomonal sepsis	No	No
Restlessness	No	No
Tremor	No	No
Urinary tract infection	No	No
dedma	No	No

Treatment-emergent adverse events were graded per CTCAE version 5.0.

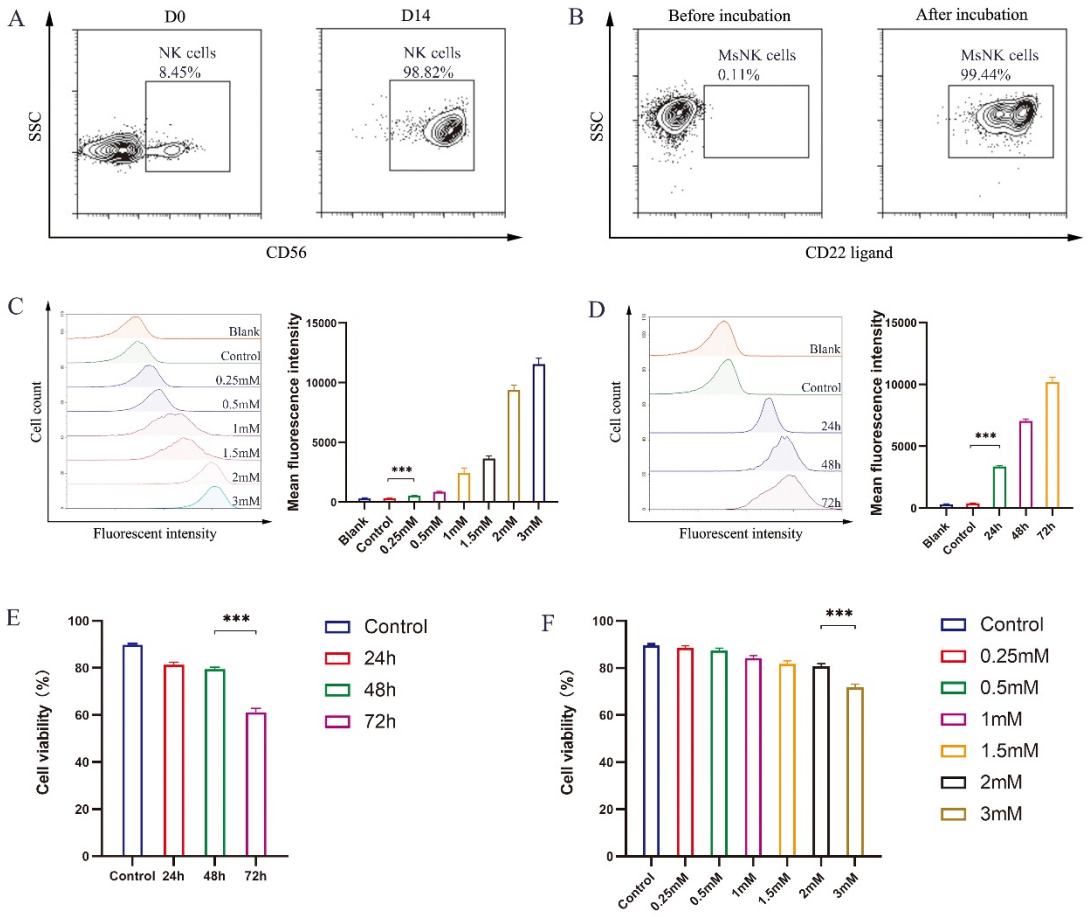


Figure S1. Successful production of glyco-engineered “MsNK” cells. A, representative flow cytometric diagram of NK cells before and 14 days after amplification. B, representative flow cytometry diagram of CD22 ligand expression in NK cells incubated with 2 mM MPB-sia1 for 48 h. C, flow cytometric diagram and statistical results of CD22 ligand expression after incubation with NK cells at different doses of MPB-sia1 for 24 h. D, flow cytometric diagram of CD22 ligand expression after 2 mM MPB-sia1 incubation with NK cells for different time and statistical results. E, NK cell activity after incubation of NK cells with 2mM MPB-sia1 for different times. F, NK cell viability after incubation of NK cells with different concentrations of MPB-sia1 for 24 hours. (Experiments were conducted using cells from

three different donors; Mean ± SD; ***P< 0.001).

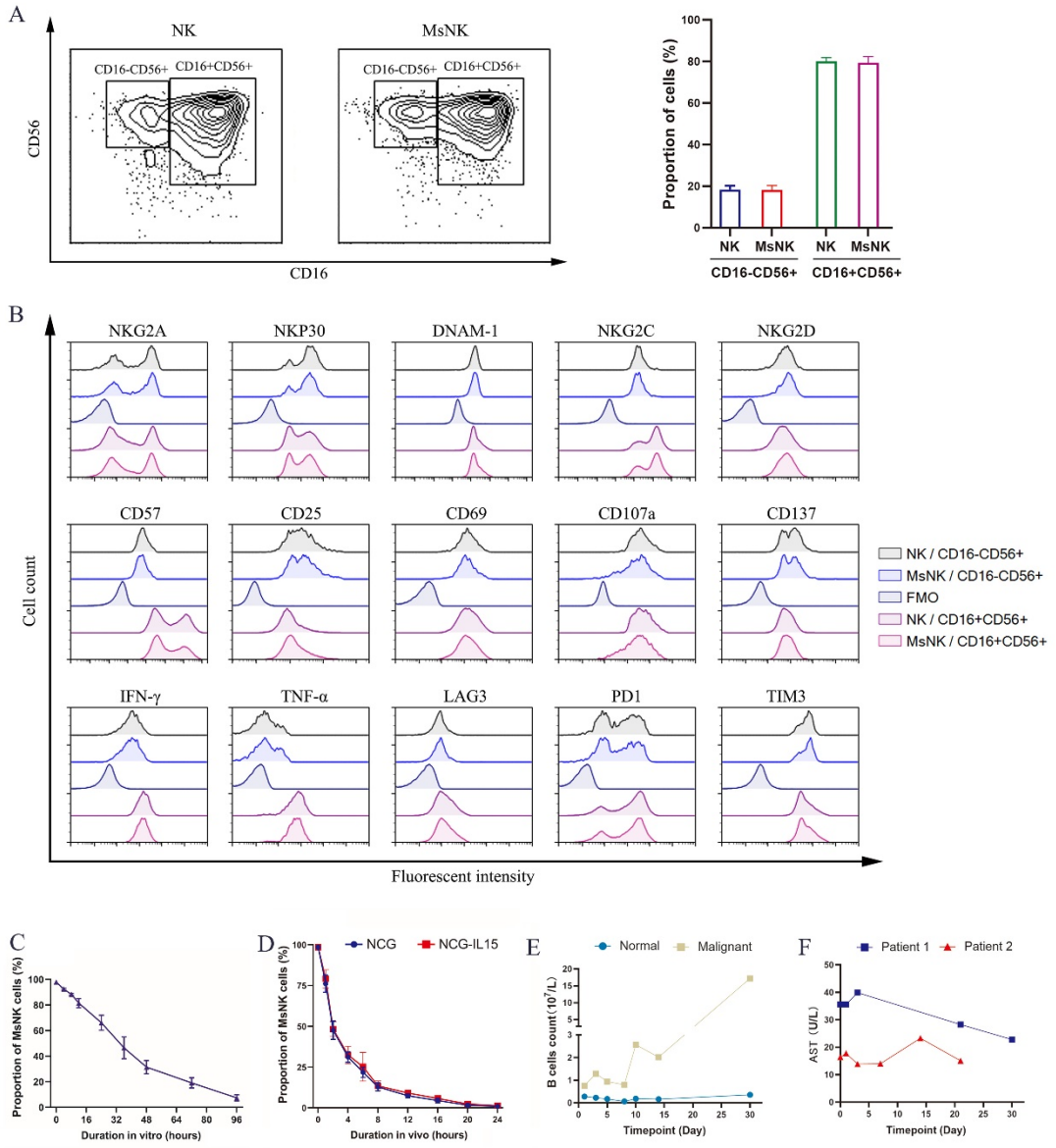


Figure S2. The phenotype of MsNK cells tended to be constant before and after production. A, Representative flow cytometry diagram of unmodified NK cells and MsNK cells labeled with CD16 and CD56 antibodies and proportion of subsets (Experiments were conducted using cells from three different donors; mean ± SD). B, representative flow

cytometry diagram of the expression of markers in each subgroup of unmodified NK cells and MsNK cells labeled with CD16 and CD56 antibodies. C, Detection of CD22 ligand expression on MsNK cells by flow cytometry at different time points after removal of MPB-sia1 from cell culture medium. (Experiments were conducted using cells from three different donors; Mean ± SD). D, 1×10^7MsNK cells were injected into NCG mice and NCG-IL15 mice respectively, and the proportion of MSNK-positive cells in mice was continuously detected at different time points by flow cytometry (n=3). E, numbers of malignant and normal B cells in PB of patient 1 during treatment. F, data on liver function-related parameters (aspartate aminotransferase (AST)) during treatment.