Pembrolizumab plus allogeneic NK cells in advanced non-small cell lung cancer patients

Mao Lin[1, 2#], Haihua Luo[1], Shuzhen Liang[3], Jibing Chen[2], Aihua Liu[4*], Lizhi Niu[2, 5*], Yong Jiang[1*]

[1]Guangdong Provincial Key Laboratory of Proteomics, State Key Laboratory of Organ Failure Research, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China

[2]Biological Treatment Center, Fuda Cancer Hospital, Jinan University, Guangzhou 510665, China

[3]Medical Research Centre, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China

[4]Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, Guangzhou, China

[5]Cancer Center, Fuda Cancer Hospital, Jinan University, Guangzhou 510665, China

[*]*Correspondence to*:

Prof. Aihua Liu, Department of Respiratory and Critical Care Medicine, Nanfang Hospital, Southern Medical University, North Guangzhou Road, Baiyun District, Guangzhou, China. Tel: +86 (20) 61641575; E-mail: lah47158@163.com.

Prof. Lizhi Niu, Cancer Center, Fuda Cancer Hospital, Jinan University, Tangde Xi Road, Tianhe District, Guangzhou 510665, China. Tel: +86 (20) 38993994; E-mail: 18922210657@163.com.

Prof. Yong Jiang, Guangdong Provincial Key Laboratory of Proteomics, State Key Laboratory of Organ Failure Research, School of Basic Medical Sciences, Southern Medical University, Shatai South Road, Baiyun District, Guangzhou 510515, China. Tel: +86 (20) 61648231; E-mail: jiang48231@163.com.

Supplementary data

Supplementary Methods

High-activity NK cell preparation. A human high-activity natural killer (HANK) cell in vitro preparation kit (Hank Bioengineering Co., Ltd, Shenzhen, China) was used to prepare natural killer (NK) cells with high quantity, purity, and activity from peripheral blood mononuclear cells (PBMCs). This kit contains NK cell synergist (#HK-A3), plasma treatment solution (#HK-001), lymphocyte culture fluid additives (#HK-002), serum-free medium additives (#HK-004), and cell infusion additives (#HK-005).

HANK cells were prepared under good manufacturing practice (GMP) conditions using clinical-grade reagents. Briefly, the synergist for human NK cell culture in vitro was used to co-stimulate expansion and activation of NK cells from PBMCs according to the manufacturer's protocol. In detail, the NK cell culture medium was prepared with lymphocyte culture medium and complemented with adequate additives and 2.5% plasma. Peripheral blood (80 mL) from a donor who provided informed consent was collected for centrifugation at 600 ×g for 15 min. The supernatant was collected in a 50-mL conical tube, followed by incubation in a 56°C water bath for 30 min to inactivate the plasma fraction. The temperature was cooled to 37°C, and a 1/20-volume of room temperature plasma treatment solution was added. The sample tube was incubated at room temperature for 1-2 h and centrifuged at 400 ×g for 10 min. The supernatant was then transferred to a new 50-mL conical tube and stored at 4°C for preparation of HANK cell culture solution.

The middle layer was transferred to a 50-mL centrifuge tube for separating human PBMCs. A volume of 40 mL normal saline was added, and after thorough shaking, the mixture was divided into eight centrifuge tubes each containing 5 mL of lymphocyte separation solution (#HK-006). After centrifugation at 400 ×g for 20 min, the intermediate layer was transferred to a 50-mL centrifuge tube and washed twice with saline.

The NK synergist containing membrane chimeric active cellular factors (1) was stored in liquid nitrogen and recovered in a 37°C water bath for 1 min (keep shaking to make it melt as quickly as

possible). Then the synergist was centrifuged at 350 ×g for 5 min, the supernatant was removed, and the precipitate was washed twice with normal saline, followed by resuspension in a tube with 3 mL HANK cell culture medium. NK cell culture was performed following the manufacturer's instructions. On day 1, 4×10^7 PBMCs, interleukin (IL)-2 (10 IU/mL), and resuscitated NK synergist (5 ml) were mixed with 40 mL HANK cell culture medium and cultured in a T175 culture flask at 37°C in a 5% CO_2 atmosphere. On day 3, the cultured cells were refreshed with 60 mL HANK cell culture medium. On day 5, the cells were refreshed with 60 mL HANK cell culture medium at a cell density of 1×10^6 cells/mL. On day 7, the cultured cells were refreshed with HANK cell culture medium and the cells were counted. If the cell number was more than 6×10^7, the synergist was added to the cultured cells and the cells were split into 2 culture flasks. Otherwise, the cell culture was extended until the cell number reached the minimum requirement. On day 9, the cultured cells in each flask were split into 3 flasks and refreshed with HANK cell culture medium to a final volume of 150 mL. Cell quality control inspection was conducted to certify the cell quality (Supplementary Table 2). Then, the cultured cells were added with fresh HANK cell culture medium to a final volume of 200 mL on day 11. The cell density was approximately 7.5×10^6 cells/mL prior to harvest on day 12.

Cytotoxicity assays. Chromium-51 (^{51}Cr) release assay was performed to assess the cytotoxicity of NK cells. ^{51}Cr solution ($Na_2{}^{51}CrO_4$) (PerkinElmer, Boston, MA, USA) has the capability to enter proliferating cells and bind firmly to cytoplasmic proteins. ^{51}Cr is released when the ^{51}Cr-tagged cells are damaged or die. The cytotoxicity of NK cells was calculated by measuring ^{51}Cr emitted into the supernatant from damaged or dead target cells. The K562 cell line (ATCC, Manassas, USA) has attained widespread use as a highly sensitive in vitro target for the NK cell killing assay.

The effector cells (E) and target cells (T) were mixed at ratios of 20:1, 10:1, or 5:1 with a final volume of 0.2 mL in a 96-well culture plate to test the effect of NK cells. We used a spontaneous release well (0.1 mL target cells plus 0.1 mL RPMI-1640 medium) and a maximum release well (0.1 mL target cells plus 0.1 mL 2% SDS) as controls. Cells were incubated at 37°C in 5% CO_2 for

4 h. The supernatant (0.1 mL) from each well was aspirated and transferred to an EP tube for the measurement of counts per minute (CPM) value with a γ-counter. We used the method reported by Girart, *et al.* to calculate the NK cell cytotoxicity (2). The percentage of cytotoxicity = 100 x [(experimental release – spontaneous release)/(maximum release – spontaneous release)].

Detection of NK cell surface receptors. The activity of NK cells is well controlled by the integration of activating and inhibitory signals. NK cells from normal donor blood are usually in an inhibited state, but they become active under the action of $CD4^+$ T cells, dendritic cells, monocytes, or NKT cells. Various cytokines, including IL-1β, IL-2, IL-12, IL-15, IL-18, and TNF-α contribute to NK cell activation in a MHC complex class I-independent manner. The cells were analyzed by FACS with fluorophore-conjugated antibodies, including fluorescein isothiocyanate (FITC)-labeled hCD3 (#561806), hCD16 (#561308), hCD158b (#559784), and hCD158e1 (NKB1, #564103) (BD Biosciences, San Diego, CA) and phycoerythrin (PE)-labeled hCD56 (#561903), hCD158a (#556063), hCD158b1/b2 (KIR-NKAT2, #556071) (BD Biosciences, San Diego, CA, USA), NKG2D (#A08934), NKp30 (#IM3709), NKp46 (#IM3711), and NKp44 (#IM3710) (Coulter, Fullerton, CA, USA).

Donor selection. The donors were selected based on genotyping mismatch between the killer cell immunoglobulin-like receptor (KIR) of the allogenic donor and the human leukocyte antigen (HLA) class I molecules of patients. Once being identified, the donor would provide peripheral blood for subsequent infusions to an individual patient. Genotyping was performed by the polymerase chain reaction with sequence specific primers (PCR-SSP) method with the peripheral blood from allogenic donors and the recipient using a TIANamp Blood DNA Kit (#DP318, Tiangen Biotech Co., Ltd., Beijing, China) and KIR/HLA-Cw Genotyping Low Resolution Kit (#Super009-005-006, Super Biotechnology Developing Co., Ltd., Tianjin, China).

Human genomic DNA was extracted from whole blood. A total of 800 μL of cell lysis (CL) buffer was mixed with 400 μL anticoagulated blood, followed by vortexing for 5-10 s and 3-5 min incubation at room temperature. Then the mixture was centrifuged at 10,000 ×g for 1 min, and the

supernatant was removed. The precipitate was mixed thoroughly with 200 μL saline. Then, 10 μL proteinase K and 200 μL CL buffer were added, and the mixture was vortexed and incubated at 55-60°C for 20 min. After addition of 200 μL anhydrous ethanol, the mixture was vortexed thoroughly for 5-10 s and transferred to a purification column, followed by centrifugation at 8,000 ×g for 1 min to bind the DNA. The purification column was treated with 500 μL washing buffer for deproteinization and centrifuged at 10,000 ×g for 1 min to wash the column. This step was repeated once, and the purification column was centrifuged at 13,000 ×g for 2-3 min. After drying for 5-10 min, 100-150 μL preheated eluent was added to the middle membrane of the purification column, and the column was incubated at room temperature for 3-5 min, followed by centrifugation at 10,000 ×g for 1 min. The eluted DNA had an A260/A280 ratio no less than 1.8 and was stored in a -20°C freezer for subsequent experiments.

KIR and HLA class I molecule typing. The KIR/HLA-Cw Genotyping Low Resolution Kit (Super Biotechnology Developing Co., Ltd.) was used for KIR and HLA class I molecule typing. After dilution with dNTP-Buffer, approximately 1 μg DNA was used as a template for PCR amplification according to the following parameters: pre-denaturation, 95°C for 5 min; 30 cycles' amplification: denaturation at 95°C for 30 s, annealing at 68°C for 50 s, and extension at 72°C for 45 s; and final extension, 72°C for 45 min. PCR reactions were stored at 4°C. The PCR products were resolved in a 2.5% agarose gel and photographed by a gel imaging system (BIO-RAD, Hercules, CA, USA). The results were interpreted using the genotyping table provided by the kit.

Detection of programmed death-1 (PD-1). Peripheral blood (6 mL) was obtained from patients and healthy donors before or 90 days after treatment and analyzed with a FACSCanto™ II (BD Biosciences, San Jose, CA, USA) flow cytometer to detect PD-1 by staining with fluorophore-conjugated antibodies, including hCD3-FITC (#561806), hCD56-PE (#561903), and hPD1-APC (#558694) (BD Biosciences).

HANK cell infusion. On day 12, cells were counted for total cell number, which could reach 8-10 × 10^9 (3) and checked for release criteria (Supplementary Table 3). Approximately 3 × 10^9 HANK

cells were harvested from two T175 culture flasks and transferred into a 200 mL transfusion bag for intravenous infusion at a concentration of 2×10^7 cells/mL, which was adjusted by a mixed solution of 150 mL saline, 2 mL human serum albumin, and 6 mL immune cell infusion additive (#HK-005). The rest cells were cultured with fresh medium and harvested for daily infusion on days 13 and 14. After infusion within 4 h, the patients rested for 30 min. The preparation and infusion process of HANK cells is shown in Supplementary Figure 7.

Supplementary Results

Expansion of HANK cells. On the beginning of the twelfth day, approximately 8-10 billion NK cells were harvested from 80 mL of peripheral blood. NK cells before expansion and on day 12 after expansion were stained with PE- or FITC-coupled antibodies against CD56 or CD3, which are the typical markers for NK and T cells, respectively, followed by analysis by flow cytometry. A representative donor result displayed that on day 12, the proportion of $CD56^+CD3^-$ cells increased from 4.8% (before expansion) to 90.1% (after expansion), whereas that of $CD56^-CD3^+$ cells decreased from 54.7% to 5.7% (Supplementary Figure 6A). Overall data of 55 donors showed that the total cell number was increased from 4.0×10^7 cells before expansion to 10.73×10^9 on day 12 after expansion, and the NK cell number was also significantly increased on day 12 after expansion in comparison with that before expansion (Supplementary Figure 6B,C,D).

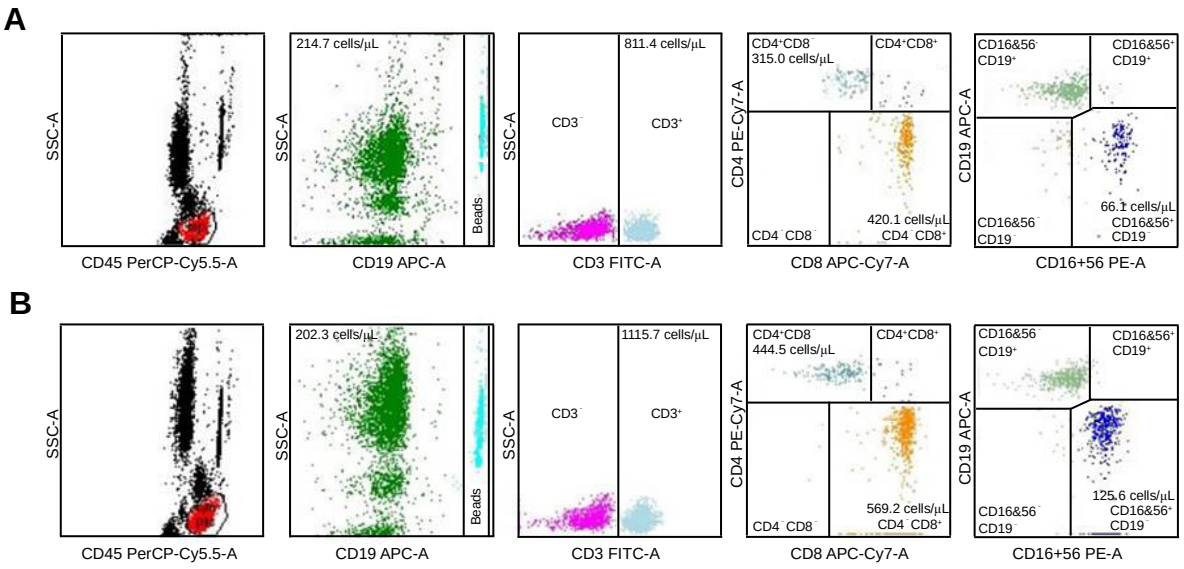
Cytotoxicity of expanded NK cells. In the cytotoxicity assay, NK cells displayed very strong cytotoxicity with an E/T of 20:1 (Supplementary Figure 3), which reached the requirement of cell viability (≥80%).

Activity of expanded NK cells. The expression rates of the inhibitory receptors on the expanded NK cells were lower than those on the PBMCs before expansion (Supplementary Figure 8A). The expression rates of the activating receptors on the expanded NK cells were much higher than those on the PBMCs before expansion, especially the $NKG2D^+/CD56^+$ cells, which increased

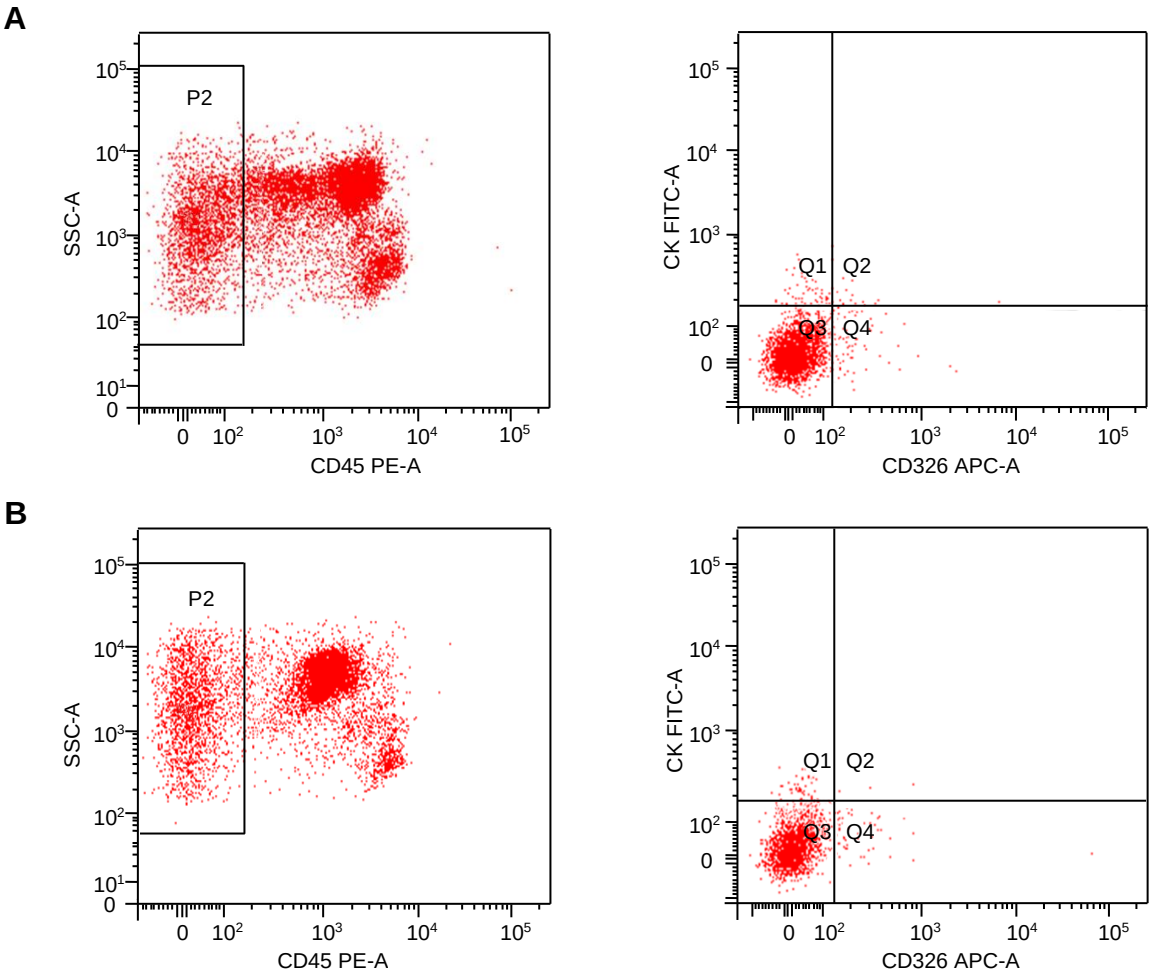
from 23% in the PMBCs to more than 80% in the expanded NK cells (Supplementary Figure 8B).

Genotyping of recipients and donors. KIR/KIR-ligand mismatch is defined as the absence of one or more HLA alleles known to be ligands for the inhibitory KIR typing (4, 5). Two KIR/HLA-Cw patterns, KIR2DL1 and KIR2DL2/KIR2DL3, were obtained in our study (Supplementary Table 4). The specific KIR and KIR-ligand genotypes for all patients administered NK cell therapy are shown in Supplementary Table 5.

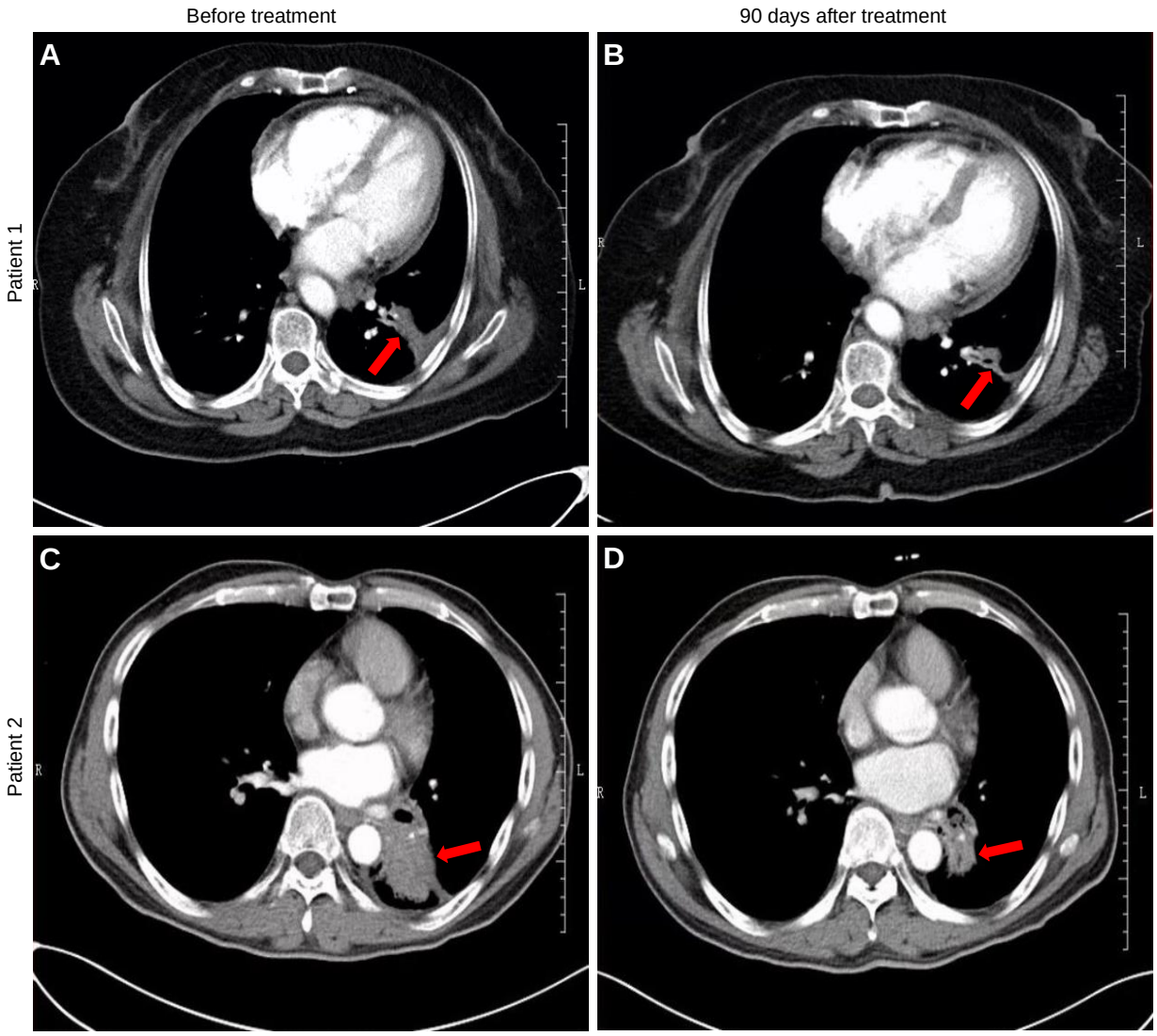
PD-1 detection. PBMCs were obtained from the peripheral blood of 20 healthy donors and 109 non-small cell lung cancer (NSCLC) patients. PD-1 expression on NK cells and T cells obtained from NSCLC patients was significantly higher than that in healthy donors. We further evaluated the PD-1 expression levels before and 90 days after treatment, and we found that the percentage of PD-1-positive cells decreased significantly after treatment (Supplementary Figure 4).



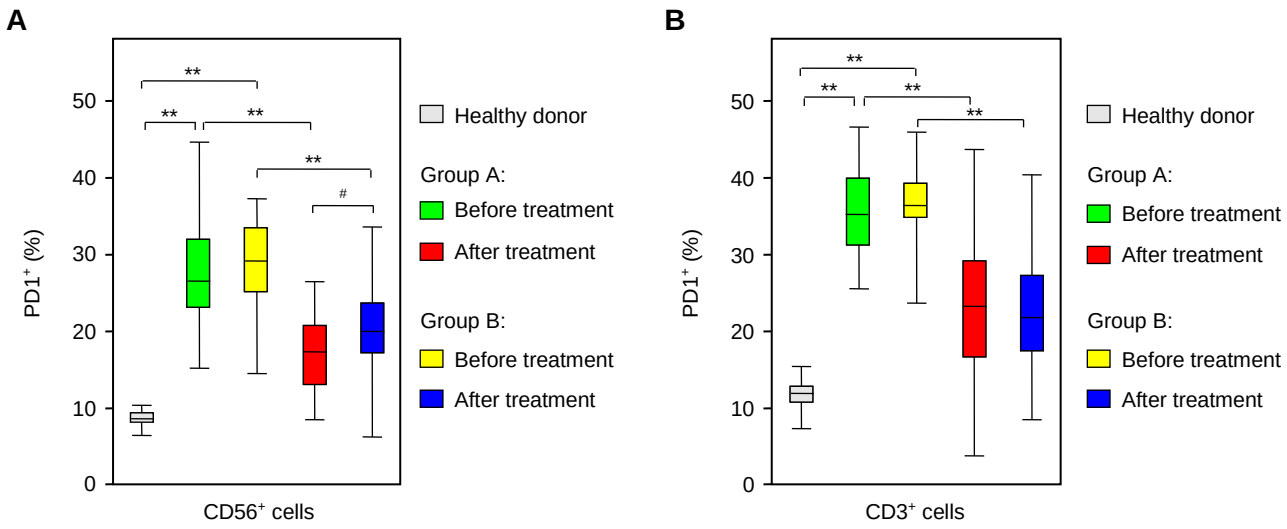
Supplementary Figure 1. Flow cytometry results for lymphocytes from a representative patient who received combination therapy. (A) The absolute numbers of total or subpopulation T cells, NK cells, and B cells before combination therapy. (B) The absolute numbers of total or subpopulation T cells, NK cells, and B cells after combination therapy.



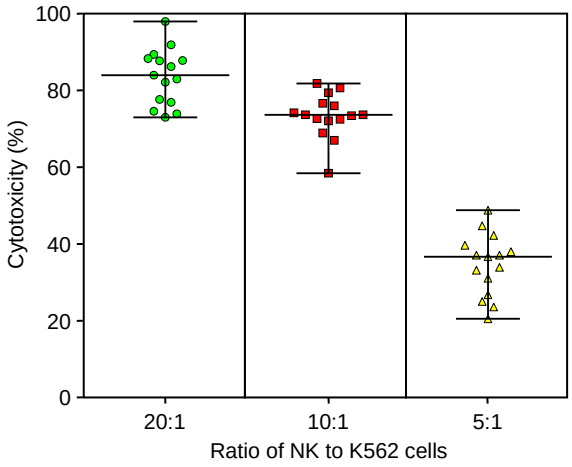
Supplementary Figure 2. CTC analysis in a representative patient who received combination therapy. (A) The number of CTCs before combination therapy. (B) The number of CTCs 90 days after combination therapy.



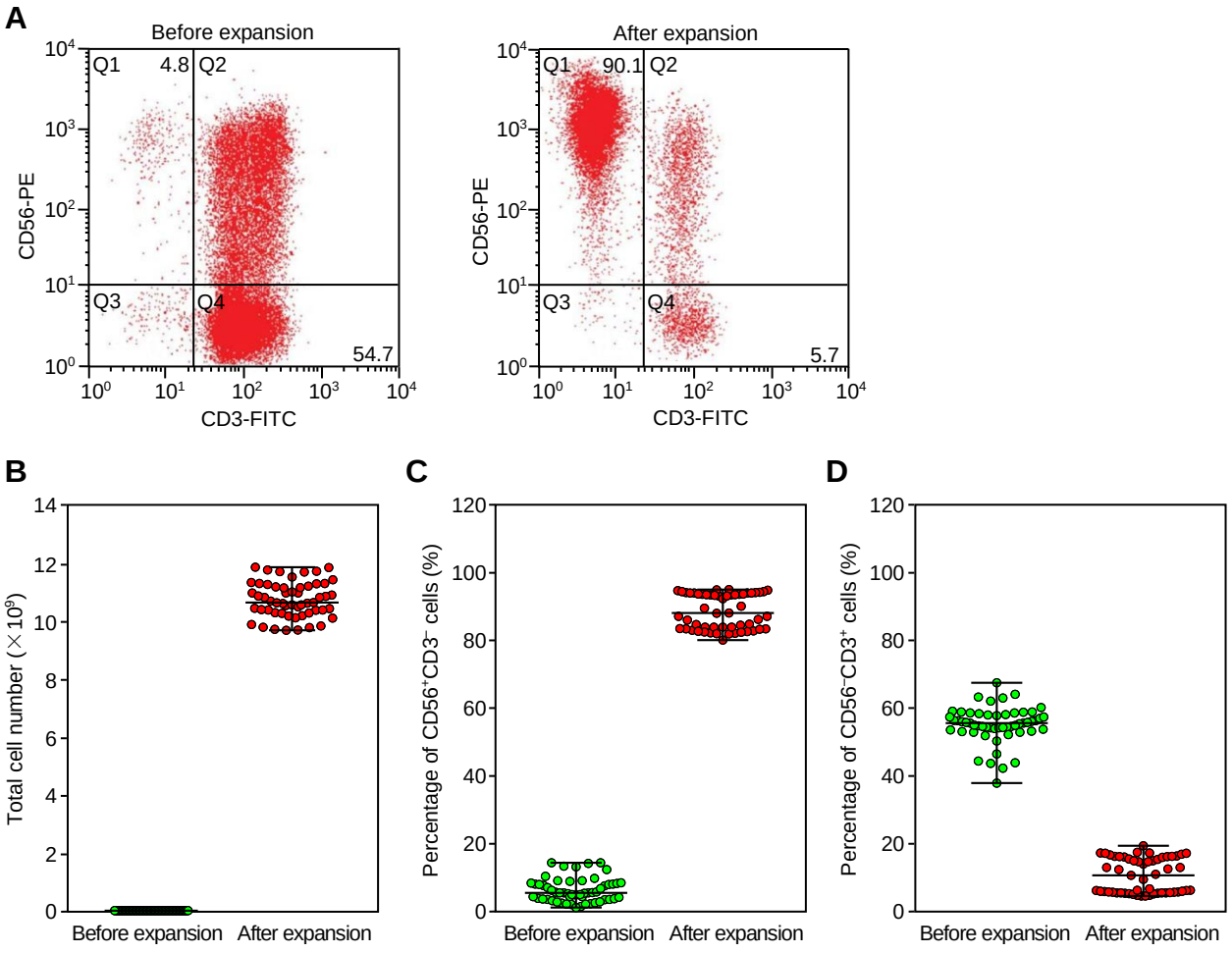
Supplementary Figure 3. Representative CT images before treatment and 90 days after treatment in two Group A patients who achieved PR. (A, B) Patient 1: a 68-year-old woman with stage IVA with a 3.8 × 1.8 × 2.4-cm sheet tumor in the inferior lobe of the left lung before treatment; CT showed that the tumor had decreased to 2.5 × 1.2 × 2.1 cm on day 90 after treatment. (C, D) Patient 2: a 57-year-old man with stage IVA with a 6.6 × 5.5 × 6.2-cm mass tumor in the inferior lobe of the left lung before treatment; the tumor had decreased to 6.1 × 4.1 × 4.8 cm on day 90 after treatment (red arrows indicate the tumor).



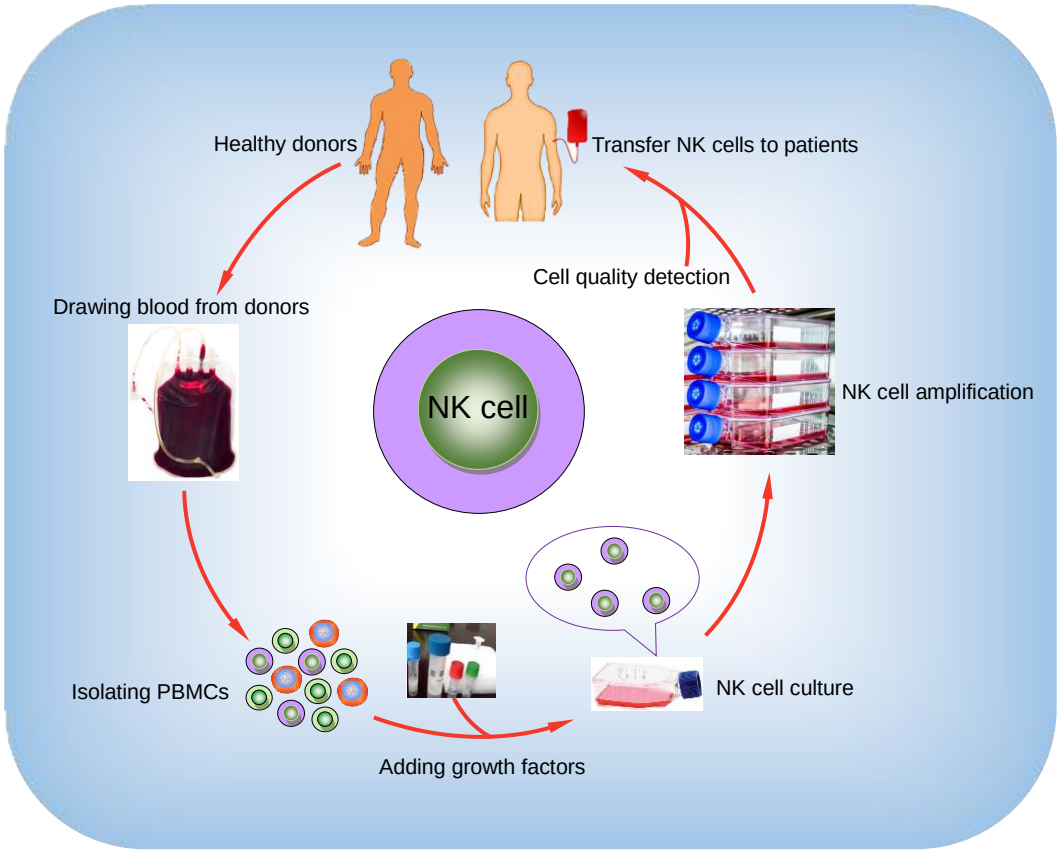
Supplementary Figure 4. PD-1 expression on human NK cells and T cells. The proportions of PD-1$^+$ cells among CD56$^+$ cells and CD3$^+$ cells obtained from healthy donors and patients were determined by flow cytometry. Data are shown as Box and Whiskers graph (bottom: 25%; top: 75%; line: median; whiskers: minimum to maximum. *P<0.05, **P<0.01, compared with before treatment. $^{\#}P$<0.05, compared with group B patients. n = 109. Two-sided student's t test.



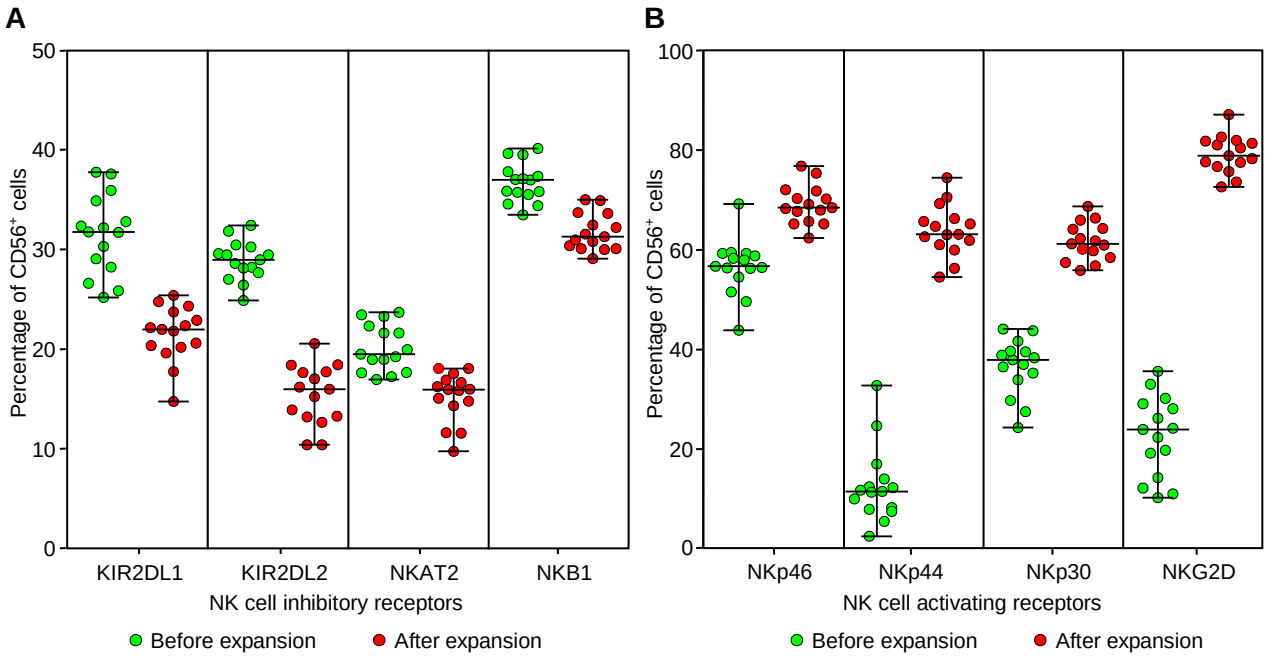
Supplementary Figure 5. Cytotoxicity of the expanded NK cells. The effector cells were NK cells derived from donor PBMCs and the target cells were K562 cells; the effector:target (E/T) ratios were 20:1, 10:1, and 5:1. The cells were incubated together at 37°C for 4 hours. All experiments were performed in triplicate. Data are shown as scatter plots with median and range. *n* = 15.



Supplementary Figure 6. The total PBMCs and proportion of NK cells before expansion and on day 12 after expansion. (A) The representative result from a donor. (B) Total cell number from 55 donors before and after expansion. (C, D) Percentage of $CD56^{+}CD3^{-}$ cells and $CD56^{-}CD3^{+}$ cells from 55 donors before and after expansion. Data are shown as scatter plots with median and range. *n* = 55.



Supplementary Figure 7. Flow chart of preparation and infusion of NK cells from healthy donors into NSCLC patients. *n* = 55.



Supplementary Figure 8. The activity of NK cells before expansion and on day 12 after expansion. The expanded NK cells were stained with PE- or FITC-conjugated antibodies against (A) cell-inhibitory receptors (KIR2DL1, KIR2DL2, NKAT2, and NKB1) or (B) cell-activating receptors (NKp46, NKp44, NKp30, and NKG2D), then analyzed by flow cytometry. Data are shown as scatter plots with median and range. *n* = 15.

Supplementary Table 1. The percentage of $CD3^+$ T cells, $CD8^+$ T cells, $CD4^+$ T cells, and NK cells before treatment and 90 days after treatment

Group / Cell type	Group A		Group B	
	Before treatment	After treatment	Before treatment	After treatment
$CD3^+$ T cells	73.31±7.19	66.55±7.59	72.42±8.85	72.55±8.74
$CD8^+$ T cells	32.49±4.19	29.15±4.65	30.81±3.13	32.31±4.32
$CD4^+$ T cells	39.86±4.38	36.7±3.5	40.71±6.48	38.98±4.77
NK cells	8.76±4.06	20.67±5.31	9.41±4.8	11.64±4.29

Supplementary Table 2. The quality control criteria of HANK cells on day 9 of culture

Indicators	Criteria
Live cells	≥90%
$CD56^{+}CD3^{-}$ cells	≥70%
Endotoxin	≤1.0 EU/ml
Cytotoxicity	≥80% (K562 cells were used as target cells, E:T=20:1)
Sterile test	Negative for bacterial, fungal and mycoplasma contamination

Supplementary Table 3. The release criteria of HANK cells on day 12 of culture

Indicators	Criteria
Live cells	≥90%
$CD56^{+}CD3^{-}$ cells	≥80%
Endotoxin	≤1.0 EU/ml
Sterile test	Negative for bacterial, fungal and mycoplasma contamination

Supplementary Table 4. Genotyping determined KIR receptors and their MHC class I ligands

Receptors	Ligands
KIR2DL1	HLA-C2 (Cw2, Cw4, Cw5, Cw6, Cw15, Cw17, Cw18)
KIR2DL2/KIR2DL3	HLA-C1 (Cw1, Cw3, Cw7, Cw8, Cw12, Cw13, Cw14)

Supplementary Table 5. Mismatch information of KIR from donors and HLA-Cw from patients

Patient no.	KIR genotypes	HLA-Cw genotypes	Blast result
1	2DL1, 2DL3, 3DL1	Cw3, Cw14	Mismatched
2	2DL1, 2DL2/2DL3, 3DL1	Cw4, Cw6, Cw15	Mismatched
3	2DL1, 2DL3, 3DL1	Cw3	Mismatched
4	2DL1, 2DL3, 3DL1	Cw3, Cw7	Mismatched
5	2DL2, 3DL1, 3DL1	Cw3, Cw12, Cw14	Mismatched
6	2DL1, 2DL2/2DL3, 3DL1	Cw13, Cw14	Mismatched
7	2DL1, 2DL3, 3DL1	Cw3, Cw12	Mismatched
8	2DL1, 2DL3, 3DL1	Cw7, Cw12	Mismatched
9	2DL1, 2DL3, 3DL1	Cw4, Cw15	Mismatched
10	2DL1, 2DL2/2DL3, 3DL1	Cw2, Cw17	Mismatched
11	2DL1, 2DL3, 3DL1	Cw5	Mismatched
12	2DL1, 2DL3, 3DL1	Cw1, Cw13	Mismatched
13	2DL1, 2DL3, 3DL1	Cw1	Mismatched
14	2DL1, 2DL2/2DL3, 3DL1	Cw7, Cw8	Mismatched
15	2DL1, 2DL2, 3DL1	Cw7, Cw13	Mismatched
16	2DL1, 2DL2/2DL3, 3DL1	Cw4, Cw6	Mismatched
17	2DL1, 2DL3, 3DL1	Cw7, Cw14	Mismatched
18	2DL1, 2DL3, 3DL1	Cw7	Mismatched
19	2DL1, 2DL3, 3DL1	Cw6, Cw17	Mismatched
20	2DL1, 2DL2/2DL3, 3DL1	Cw8, Cw13	Mismatched
21	2DL1, 2DL3, 3DL1	Cw3, Cw8, Cw14	Mismatched
22	2DL1, 2DL3, 3DL1	Cw1, Cw12	Mismatched
23	2DL1, 2DL2/2DL3, 3DL1	Cw14	Mismatched
24	2DL1, 2DL3, 3DL1	Cw1, Cw7	Mismatched
25	2DL1, 2DL3, 3DL1	Cw15	Mismatched
26	2DL1, 2DL2/2DL3, 3DL1	Cw15, Cw18	Mismatched
27	2DL1, 2DL3, 3DL1	Cw5, Cw6	Mismatched
28	2DL2, 3DL1	Cw7, Cw8, Cw13	Mismatched
29	2DL1, 2DL3, 3DL1	Cw8, Cw12	Mismatched
30	2DL1, 2DL2, 3DL1	Cw8, Cw12, Cw14	Mismatched
31	2DL1, 2DL2/2DL3, 3DL1	Cw12, Cw13	Mismatched
32	2DL1, 2DL3, 3DL1	Cw1, Cw3	Mismatched
33	2DL1, 2DL3, 3DL1	Cw3, Cw13	Mismatched
34	2DL1, 2DL2/2DL3, 3DL1	Cw1, Cw8	Mismatched
35	2DL1, 2DL3, 3DL1	Cw2, Cw4	Mismatched
36	2DL1, 2DL3, 3DL1	Cw3, Cw8	Mismatched
37	2DL1, 2DL3, 3DL1	Cw4, Cw18	Mismatched
38	2DL1, 2DL2/2DL3, 3DL1	Cw5, Cw17	Mismatched
39	2DL1, 2DL3, 3DL1	Cw17, Cw18	Mismatched
40	2DL1, 2DL3, 3DL1	Cw1, Cw8, Cw14	Mismatched
41	2DL1, 2DL3, 3DL1	Cw12, Cw14	Mismatched
42	2DL1, 2DL3, 3DL1	Cw2, Cw5	Mismatched
43	2DL1, 2DL3, 3DL1	Cw4, Cw17	Mismatched
44	2DL1, 2DL3, 3DL1	Cw2, Cw5, Cw18	Mismatched
45	2DL1, 2DL3, 3DL1	Cw5, Cw18	Mismatched
46	2DL1, 2DL2/2DL3, 3DL1	Cw6, Cw15	Mismatched
47	2DL1, 2DL3, 3DL1	Cw15, Cw17	Mismatched
48	2DL1, 2DL3, 3DL1	Cw12	Mismatched
49	2DL1, 2DL3, 3DL1	Cw2, Cw18	Mismatched
50	2DL1, 2DL2/2DL3, 3DL1	Cw4, Cw5	Mismatched
51	2DL1, 2DL3, 3DL1	Cw6, Cw18	Mismatched
52	2DL1, 2DL3, 3DL1	Cw2, Cw15	Mismatched
53	2DL1, 2DL3, 3DL1	Cw1, Cw14	Mismatched
54	2DL1, 2DL2, 3DL1	Cw17	Mismatched
55	2DL1, 2DL3, 3DL1	Cw6	Mismatched

References

1. Zhang M, et al. Anti-West Nile virus activity of in vitro expanded human primary natural killer cells. *BMC Immunol.* 2010;11:3.
2. Girart MV, et al. Engagement of TLR3, TLR7, and NKG2D regulate IFN-γ secretion but not NKG2D-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. *J Immunol.* 2007;179(6):3472-3479.
3. Xie S, et al. Iodine-125 seed implantation and allogenic natural killer cell immunotherapy for hepatocellular carcinoma after liver transplantation: a case report. *Onco Targets Ther.* 2018;11(7345-7352.
4. Leung W, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol.* 2004;172(1):644-650.
5. Delgado DC, et al. Genotypes of NK cell KIR receptors, their ligands, and Fcgamma receptors in the response of neuroblastoma patients to Hu14.18-IL2 immunotherapy. *Cancer Res.* 2010;70(23):9554-9561.