YMTHE, Volume *27*

Supplemental Information

Adoptive Transfer of NKG2D CAR mRNA-Engineered Natural Killer Cells in Colorectal Cancer Patients

Lin Xiao, Dongzhi Cen, Haining Gan, Yan Sun, Nanqi Huang, Hanzhen Xiong, Qiongmei Jin, Liqun Su, Xuejuan Liu, Kejian Wang, Guangrong Yan, Tianfa Dong, Shangbiao Wu, Pengzhi Zhou, Jinshan Zhang, Weixiang Liang, Junlan Ren, Yaoshu Teng, Can Chen, and Xue Hu Xu

Adoptive Transfer of NKG2D CAR mRNA-Engineered Natural Killer Cells in Colorectal Cancer Patients

SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS

Construction of chimeric NKG2D CAR vectors and generation of CAR-NK cells

To construct NKG2D mRNA CAR vectors, the basal backbone vector pFBCMV-T7 was generated by inserting into pFastbac1 vector (Life Technologies, Carlsbad, CA) a synthetic sequence containing the T7 promoter, a 5'UTR with Kozak sequence, a multiple cloning site with *EcoRI*, *SphI*, *SalI*, *Hind III* and *ClaI*, the GM-CSF signal peptide encoding sequence (SP) and the alpha-globin 3'UTR sequence. The extracellular domain of human NKG2D (NKG2D-ED, uniprot P26718-1, amino acids 83-216) was amplified by PCR from a PBMC cDNA library using the primers 5'-gcgcgcatgccttcaaccaagaagttcaaattcc-3' (forward primer with *SphI* site) and 5'-acgaagctagccacagtcctttgcatacagatgtacgtatttggag-3' (reverse primer with *NheI* site). NKG2D CAR vector was generated by fusing NKG2D-ED to the CD8α hinge and transmembrane region (CD8 H-TM, uniprot P01732, amino acids 128-210) and CD3ζ signaling moiety (P20963, amino acids 52-164) (NKG2Dz), or DAP12 (uniprot O43914, amino acids 62-113) (NKG2Dp), and then subcloned into pFBCMV-T7 with *EcoRI* and *SalI*. The control vector was generated by replacing the NKG2D ED part of the NKG2Dz vector with EGFP encoding sequence (the start codon removed) by *SphI* and *NheI*.

To generate mRNA molecules encoding the NKG2D CARs, PCR was performed using the above pFBCMV-T7 vectors as DNA templates, a forward primer CMV-F (5'-atccgctcgagtagttattaatagt aatcaattacggggtc-3'), and reverse primer T150-R. Capped mRNA was generated through *in vitro* transcription of the PCR DNA templates using the mMESSAGE mMACHINE T7 ULTRA transcription kit (Invitrogen) or the mScript™ RNA system (Epicentre, Madison, WI). For mRNA CAR NK cell generation, NK cells were mixed with NKG2D CAR mRNA and electroporated in a 2 or 4-mm cuvette (Bio-Rad, Hercules, CA) using a NEPA21 electroporator (Nepagene, Chiba, Japan) or a BTX electroporator (AgilePulse, Holliston, MA). The electroporated NK cells were rested overnight before use.

Flow cytometric analysis and cytotoxicity assay

For flow cytometric analysis, 5 x 10^5 to 1 x 10^6 cells were suspended in 100 µL cold MACS buffer (Miltenyi Biotech, Germany). Antibodies were added according to the manufacturer's recommendations and incubated at 4°C for 15 min. After washing with MACS buffer, the cells were re-suspended in 500 µL MACS buffer for flow-cytometry analysis with Accuri C6 cytometer (BD Biosciences, Franklin Lakes, NJ). To detect the NKG2D expression on NK cells, phenotyping was analyzed by single-color staining with APC-anti-NKG2D (Miltenyi). To detect NKG2D ligand expression

on tumor cells, phenotyping was analyzed by single-color staining with anti-human MICA/MICB FITC (Miltenyi), human ULBP-1 PE (R&D Systems, Minneapolis, MN), ULBP2/5/6 APC (R&D), ULBP-3 APC (R&D), and ULBP-4/RAET1E APC (R&D).

The cytolytic activity of CAR-modified NK cells was examined with a non-radioactive method (DELFIA EuTDA Cytotoxicity Reagents kit, PerkinElmer, MA). Time-resolved fluorescence was measured in Victor3 multilabel plate counter (Perkin Elmer). The effector to target (E:T) ratios used ranged from 5:1 to 0.5:1. Control groups were set up to measure spontaneous release (only target cells added), maximum release (target cells added with 10 µL lysis buffer), and medium background (no cell added). Killing efficacy was calculated by using the following formula:

$$\% \text{ Specific release} = \frac{\text{Experimental release (counts)-Spontaneous release (counts)}}{\text{Maximum release (counts)-Spontaneous release (counts)}} \times 100$$

Animal experiments

Non-obese diabetic/severe combined immunodeficiency/IL-2Rγcnull (NSG) mice (6-8 weeks old, female) were used in the current study. Mice were inoculated via intraperitoneal (i.p.) injection of 1 x 10^7 HCT116-luc. On day 7 post-tumor inoculation, tumor engraftment was confirmed by live bioluminescence imaging (BLI) monitored using an IVIS Spectrum Imaging platform with Living Image software (PerkinElmer). Mice with similar BLI signal intensity were randomly divided into different treatment groups, 5 mice per group. To investigate *in vivo* anti-tumor effects, 1 x 10^7 of immune effector cells electroporated with NKG2Dp RNA CAR were i.p. injected into tumor-bearing mice twice a week for 3 weeks. Mice treated with PBS or mock NK cells were used as controls. The mice were followed with serial weekly imaging to assess the tumor burden. All luminescent signals and images were acquired and analysed with the Xenogen living imaging software v2.5. Behaviour and survival of the mice were monitored closely. Humane endpoints were used and mice were euthanized by cervical dislocation under sodium pentobarbital anesthesia upon signs of distress such as swollen belly due to tumor ascites formation, seizures, tremors, labored or difficulty in breathing, significant weight loss (> 15% body weight), signs of emaciation (i.e., prominent skeletal structures), impaired ambulation, inability to remain upright, or evidence of moribund condition. The survival curves were established based on the dates when mice were found dead or euthanized.

Clinical study design

A one-way, open-label, pilot study (NCT03415100) was designed to evaluate the manufacturing feasibility and safety of mRNA transfected NKG2D-CAR NK cells in patients with advanced colorectal cancer. The study was performed according to the principles of the Declaration of Helsinki. The

research protocol was reviewed and approved by the Ethical Committee of the Third Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). To meet inclusion criteria, patients aged between 18 - 70 years had to have histologically or cytologically proven metastatic colorectal cancer, an expected survival of at least 3 months, an Eastern Cooperative Oncology Group performance status (PS) of 0-3, normal kidney, liver, and bone marrow function, and be resistant to standard therapies (failed at least two lines of systemic therapy). Exclusion criteria were acute or chronic infections, relevant concomitant hematological, cardiovascular, pulmonary, hepatic, renal, pancreatic or endocrinal disease, with immune deficiency, autoimmune diseases, severe allergic disorders, or exposure to cell-based therapy in the preceding 3 months. Those receiving systemic steroid therapy or who were pregnant or lactating were also excluded.

NK cells expanded from a patient (auto-NK) or haploidentical family donors (allo-NK) were used to prepare NKG2Dp-CAR NK cells. After patients received an i.p. catheter placed under ultrasound guidance in local anesthesia, freshly prepared CAR-NK cells met the releasing criteria (Table 1) were intraperitoneally infused via the i.p. catheter into patients. Patient1001 received 2×10^7 NKG2Dp-CAR NK cells administered on day 0, followed by a second infusion with 1×10^8 NKG2Dp-CAR NK cells on day 7. The patient was withdrawn from the study after two rounds of NK cell infusion due to disease progression although promising signs of immunological reactivity reflected by the reduction of tumor cells in ascites were observed. Patient1002 received 4 infusions of NKG2Dp-CAR NK cells on days 0 (1×10^8), 7 (3×10^8), 14 (5×10^8), and 21 (7×10^8), respectively. Patient1003 was treated with 6 infusions of allogeneic haploidentical CAR-NK cells: 5×10^8 CAR-NK cells administered 2x/week for the 1st week, 1×10^9 CAR-NK cells 2x/week in the 2nd week, and 2×10^9 CAR-NK cells 2x/week in the 3rd week. Specifically, the patient received 5×10^8 CAR-NK cells administered intraperitoneally on day 0. Starting from the 2nd infusion on day 3 and guided by ultrasound, half of each dose of CAR-NK cells were percutaneously injected into cancer lesions with high metabolic activity in the liver segment VI, i.e. 2.5×10^8, 5×10^8, 5×10^8, 1×10^9, 1×10^9 CAR-NK cells on days 3, 7, 10, 14 and 17, respectively. The rest CAR-NK cells were i.p. infused at each time point. Tumor response was assessed by computed tomography (CT) and ^{18}F-FDG-PET/CT imaging before and after therapy.

Feasibility to manufacture CAR-NK cells was determined by measuring NK cell purity, expansion fold, T cell depletion efficiency, sterility, electroporation efficiency, NK cell viability, and cancer cell killing activity of CAR-NK cells. Safety assessments included incidence of treatment-related adverse events (AEs), according to the National Cancer Institute Common Terminology Criteria of Adverse Events version 4.0. Dose limiting toxicity (DLT) was defined as toxicity following dosing that was at least “possibly related” to CAR-NK cell infusion and met one of the following criteria: 1) any grade 3 or higher toxicity; 2) grade 3 or higher autoimmune toxicity including pericarditis, peritonitis, or

pleuritis; or 3) grade 3 or higher allergic reaction or grade 2 allergic reactions in which symptoms reappear after repeat infusion.

Allogeneic NK cell preparation

Allogeneic NK cells were prepared with two hundred mL of blood from haploidentical family donors. After NK cell expansion for 10 days as described above, αβ T-cell depletion was performed. Briefly, the cells were incubated with anti-CD3 microbeads (Miltenyi) at 4°C for 15 min. After washing with PBS supplemented with 2% AB serum (Gemini, USA), the cells were re-suspended in 500 μL of the corresponding buffer and passed through LS column (Miltenyi). After depletion, NK cells were co-cultured with γ-irradiated K562 feeder cells at 1:1 NK:K562 ratio for another 7 days.

Ascites fluid analysis

For patients 1001 and 1002, ascites was collected as part of the standard of care by large volume paracentesis in sterile disposable collection bag containing anti-coagulant heparin. The bags were delivered to the laboratory at 4°C and photos were taken to record the appearance of the ascites. The ascites was transferred to 50 mL centrifuge tubes and centrifuged at 400 g for 6 min. The supernatant was discarded and the red blood cells were removed through ACK lysing buffer (Life Technologies). After washing once with PBS, the total cell number in the ascites was counted by manual counting. The ascites cells were stained with PE anti-EpCAM antibody (R&D) at 4°C for 30 min and washed. The EpCAM positive cells were detected using a BD Fortessa (BD Bioscience) and analyzed through FlowJo.

Immunohistochemistry and hematoxylin and eosin (H&E) analysis

Tissue samples of patient1003 were collected from CAR-NK cell injection sites and tumor tissues away from the treatment sites under ultra-sound guidance. Samples were sent to Department of Pathology, the Third Affiliated Hospital of Guangzhou Medical University for histological examination. Briefly, samples were preserved in 10% neutral buffer formalin and embedded by paraffin. Sections of 5-micron thickness were prepared from all specimens, stained with H&E and anti-MICA/B (R&D), Villin (Genetech, Shanghai), CDX2 (Genetech, Shanghai) antibodies according to manufacturer's instructions, and examined microscopically.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Schematic illustration of the procedure for NK cell expansion from human PBMCs and CAR mRNA electroporation. The main assays used to evaluate the quality of clinical NK cell samples are listed.

Supplementary Figure 2. Flow cytometric analysis to detect the surface expression of EGFP and NKG2D on electroporated NK cells. (A) The electroporation efficiency as demonstrated with EGFP mRNA transfection. The flow cytometric analysis was performed 24 hours after mRNA electroporation. The percentage of positive cells and mean fluorescence intensity (MFI) from two different donors (ID#A and ID#B) are shown. (B) Flow cytometric analysis data from a representative human sample to demonstrate NKG2D MFI increase after electroporation of NKG2D CAR mRNA into NK cells. Electroporated NK cells were stained with an anti-NKG2D antibody 24 hours after mRNA electroporation. Significant increased surface expression of NKG2D on NK cells electroporated with two NKG2D CARs, as compared with controls, indicates CAR expression. (C) Time lapse analysis of NKG2Dp CAR expression on NK cells. *Left:* Histograms to show the surface expression of NKG2D on NK cells derived from one healthy donor and electroporated with mRNA encoding NKG2Dp CAR. MFI values of cells positive for NKG2D staining over an isotype control are indicated. *Right:* Change in MFI values over time. The data represent means ± S.D. of three samples from different healthy donors. By day 7, the MFI value went down to levels often observed on non-modified NK cells.

Supplementary Figure 3. Change in cancer cell killing activity of NKG2D CAR-modified NK cells over time. Human HCT116 hepatocellular carcinoma cells were used as a target. NK cells were electroporated with NKG2Dp RNA CARs and used at days post-electroporation as indicated for co-culturing with target tumor cells for 2 hours. Mock NK cells served as negative controls. Delfia EuTDA cytotoxicity assay was performed to assess tumor cell lysis efficiency. Although the tumor cell killing activity of CAR-NK cells decreases over time, the differences between CAR-NK cells and mock NK cells at E:T=5:1 are statistically significant at all 4 tested time points ($p < 0.001$).

Supplementary Figure 4. Quality control of generated CAR-NK cells to ensure that release criteria are met for patient infusion. Representative results from each patient are shown here. The results are detailed in Table 1. (A) More than 90% of the CAR-NK cells are CD56+CD3-. (B) The increase of NKG2D MFI after NKG2D CAR mRNA electroporation is > 1.2 fold of the original level. (C) Cancer cell killing activities of the CAR-NK cells against HCT116 colorectal cancer cells are > 60% at E:T ratio of 10:1.

Supplementary Figure 5. Effects of electroporation on the survival of NK cells used for patient infusion. Two, four and six rounds of electroporation were performed for NK cells for patients 1001, 1002, and 1003, respectively. NK cells were harvested and electroporated with NKG2D CAR mRNA. After overnight recovery, the numbers of survived NK cells were counted and % survival rates was calculated.

Supplementary Figure 6. CAR-NK dose escalation test design. To establish the recommended dose and schedule of NKG2Dp CAR-NK cells for clinical trials, the listed doses and schedules were tested in 3 patients. Numbers of injected CAR-NK cells and injection times are labelled. Fold changes relative to the first dose in the first patient are indicated in round brackets.

Supplementary Figure 7. Body temperature changes in patient1001 after CAR-NK cell infusion. A low fever of 38.4°C was observed on day 6 post-the 1st CAR-NK cell infusion and was back to normal without treatment.

Supplementary Figure 8. Body temperature changes in patient1002 after CAR-NK cell infusion. A fever of 39.5°C was observed on day 2 post-the 2nd CAR-NK cell infusion. Pharmacologic treatment with Radix Bupleuri injection and Metamizole Sodium reduced fever. Fever appeared again in the next 3 afternoons and was back to normal after ice pack application or without treatment.

Supplementary Figure 9. Body temperature changes in patient1003 after CAR-NK cell infusion. Fever was observed after the 2nd, 4th, 5th and 6th CAR-NK cell infusion and reduced with ice pack application or without treatment.

Supplementary Figure 10. IL-6 and CRP concentrations during CAR-NK treatment. Patient blood samples were collected for IL-6 (A) and CRP (B) concentration measurement. The IL-6 concentrations of the three patients were first measured before CAR-NK treatment. For P1001 and P1002, it was measured again 2-3 days after CAR-NK treatment, and for P1003, IL-6 concentration was measured on day 1 after 4th infusion and day 1 after 6th infusion. Given repeated high fevers in the third patient, CRP concentrations were measured only in this patient 4 times as indicated after the 3rd infusion.

Supplementary Figure 11. Characterization of EpCAM-positive cells in patient's ascites. Ascites samples from patient P1001 and P1002 were collected before CAR-NK treatment at indicated times. Flow cytometric analysis with an anti-EpCAM antibody demonstrates the presence of EpCAM-positive cells in the ascites of the two patients.

Supplementary Figure 12. Axial CT scanning of three patients with metastatic colorectal cancer treated with CAR-NK cells. Pre-and post-treatment CT scans are shown.

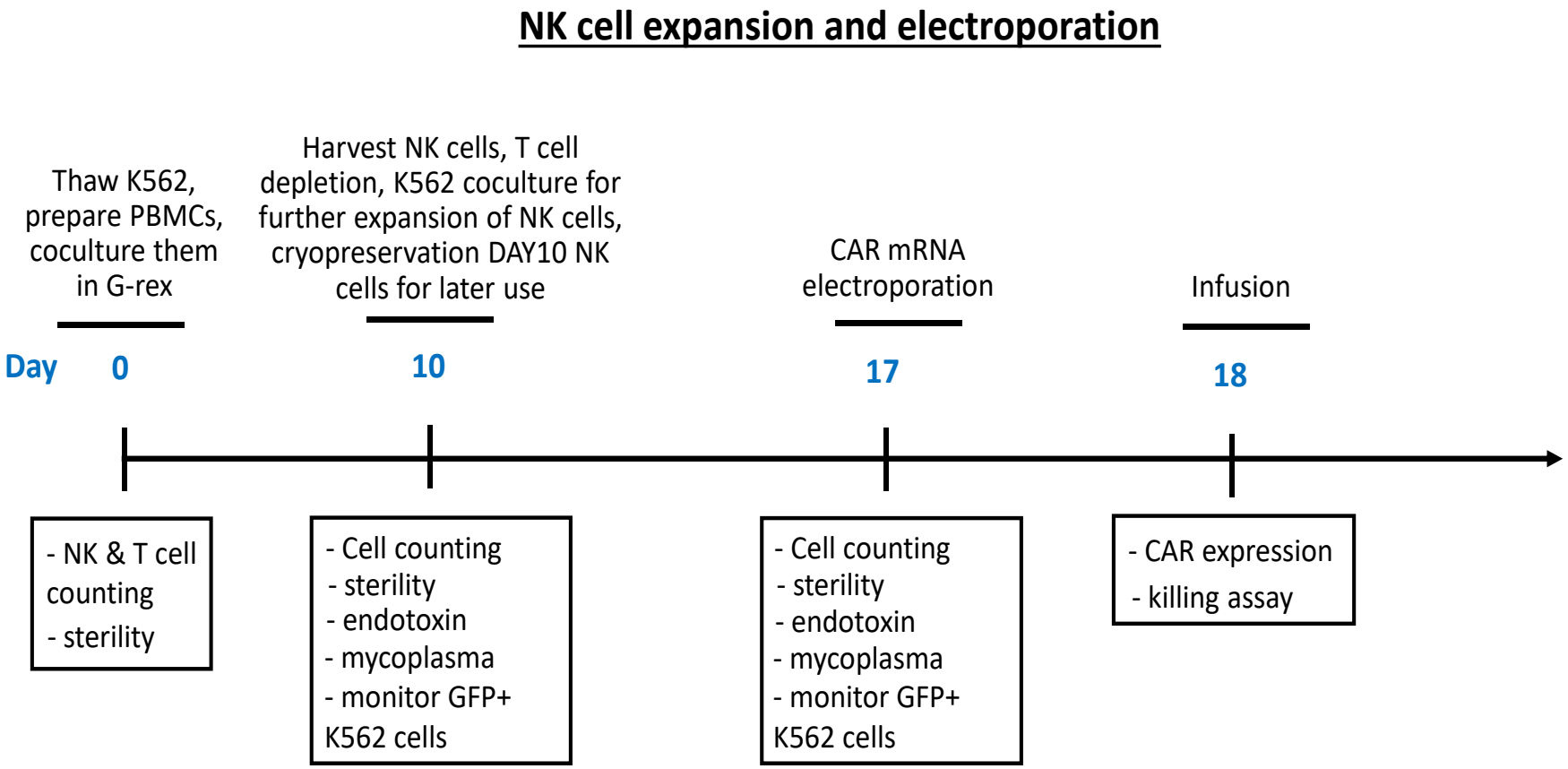
Supplementary Figure 13. Ultrasound-guided fine-needle biopsy to obtain tissue samples for histological examination. (A) in the CAR-NK injected site. (B) in residual tumor tissues left.

Supplementary Figure 14. Immunohistochemical staining of needle biopsy specimens. Antibodies against NKG2D ligand MICA/B and two highly sensitive and specific markers of adenocarcinomas of intestinal origin Villin and CDX2 were used.

SUPPLEMENTARY VIDEO

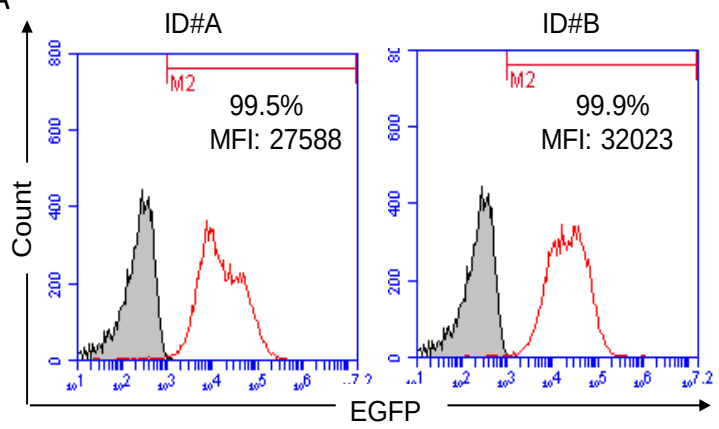
Supplementary video to show ultrasound-guided CAR-NK cell infusion. After injection of 10 mL of saline into the periphery of a big liver tumor (66 x 28 mm in size) to generate a liquid cavity, a needle with a tubing connected to an infusion syringe pump was inserted into the cavity, followed by continuous infusion of 100 mL of CAR-NK cells. Tiny bubbles are visible during infusion.

Supplementary Figure 1

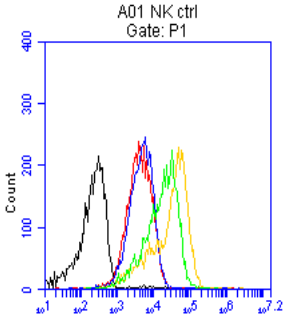


Supplementary Figure 2

A



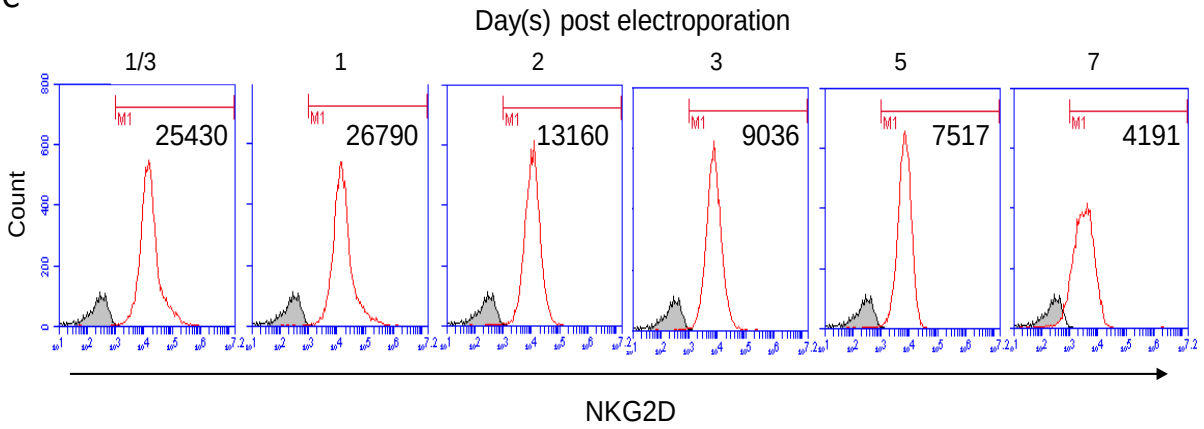
B

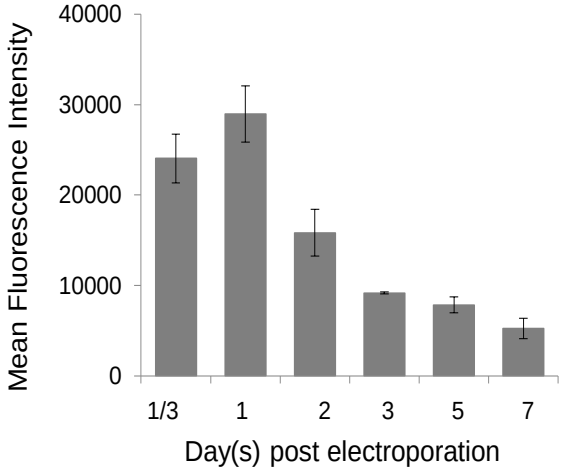


isotype ctrl+WT NK
aNKG2D+WT NK
aNKG2D+ctrlCAR-NK
aNKG2D+NKG2Dp-NK
aNKG2D+NKG2Dz-NK

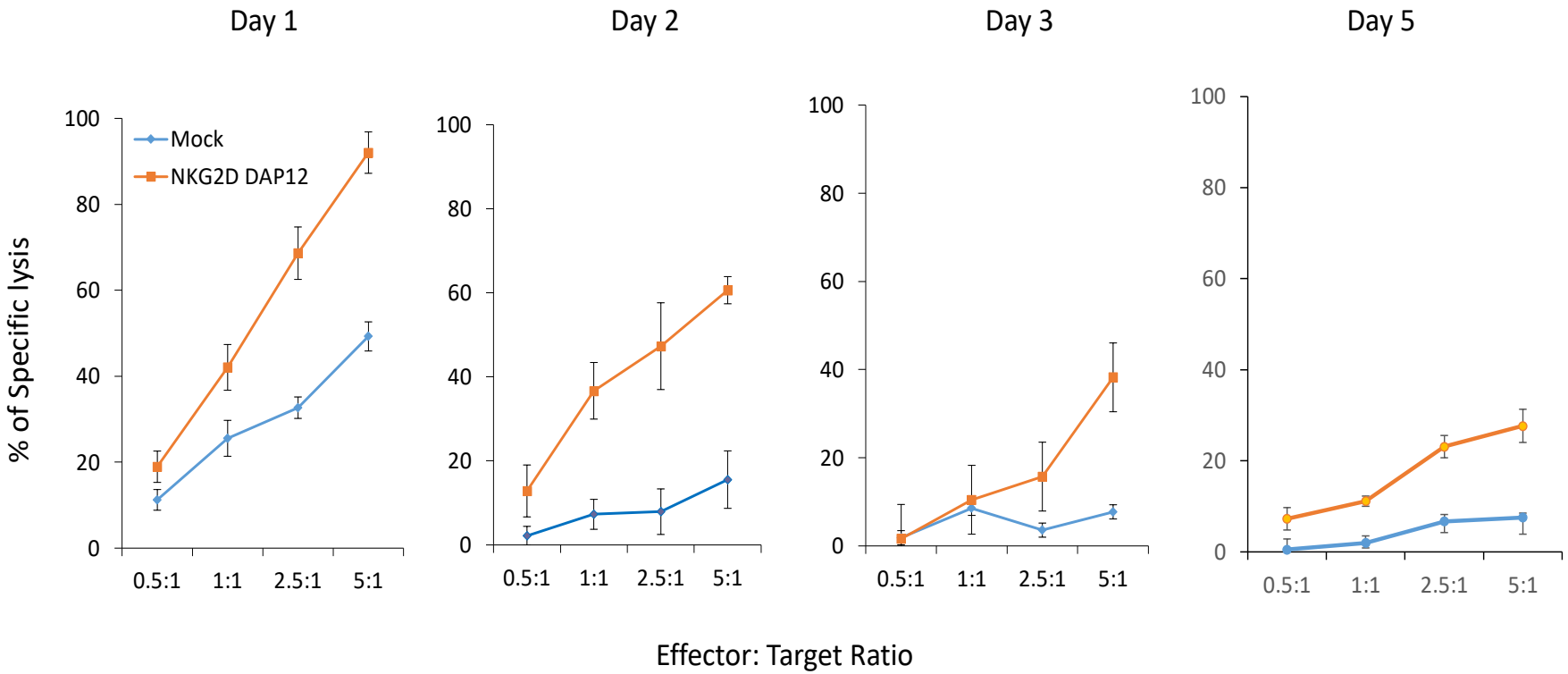
Plot 5: Multiple Samples Gated on P1	Count	Volume (µL)	% of This Plot	% of All	Mean FL4-A	CV FL4-A	Median FL4-A
A01 NK ctrl : This Plot	5,268	29.5	100.00%	30.38%	385.66	730.24%	165.0
A02 NK NKG2D : This Plot	5,069	32.4	100.00%	27.41%	6,538.85	563.47%	4,027.0
A05 NK GFP NKG2D : This Plot	5,000	13.6	100.00%	26.91%	7,556.20	732.93%	4,666.0
A06 NK DAP12 NKG2D : Thi...	5,000	19.3	100.00%	28.54%	43,060.12	129.50%	36,441.5
B01 NK CD3Z NKG2D : This ...	5,000	15.2	100.00%	34.56%	23,552.43	94.45%	18,611.0

C

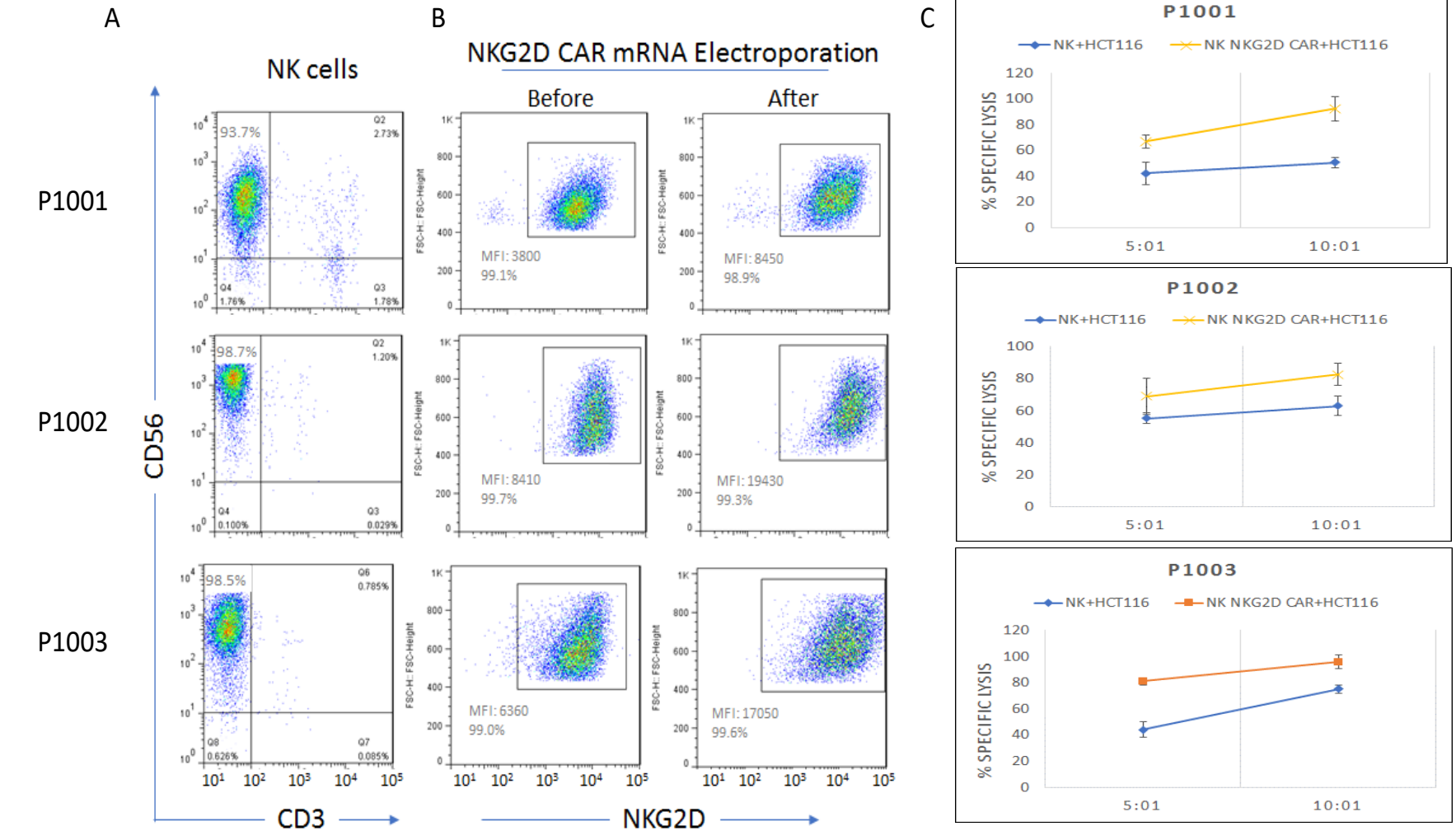




Supplementary Figure 3

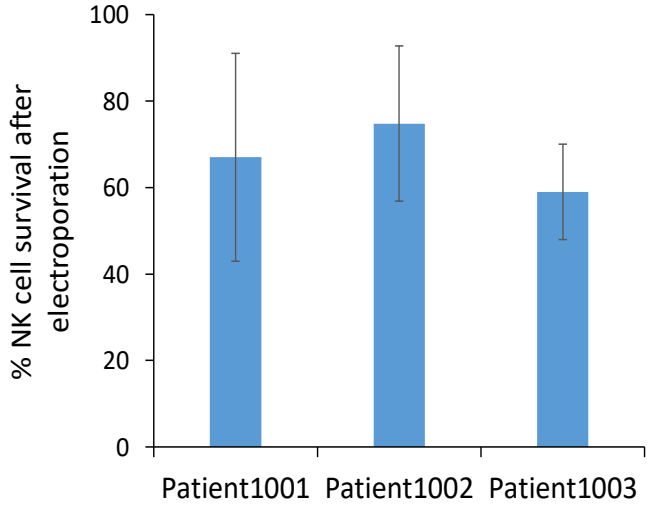


Supplementary Figure 4

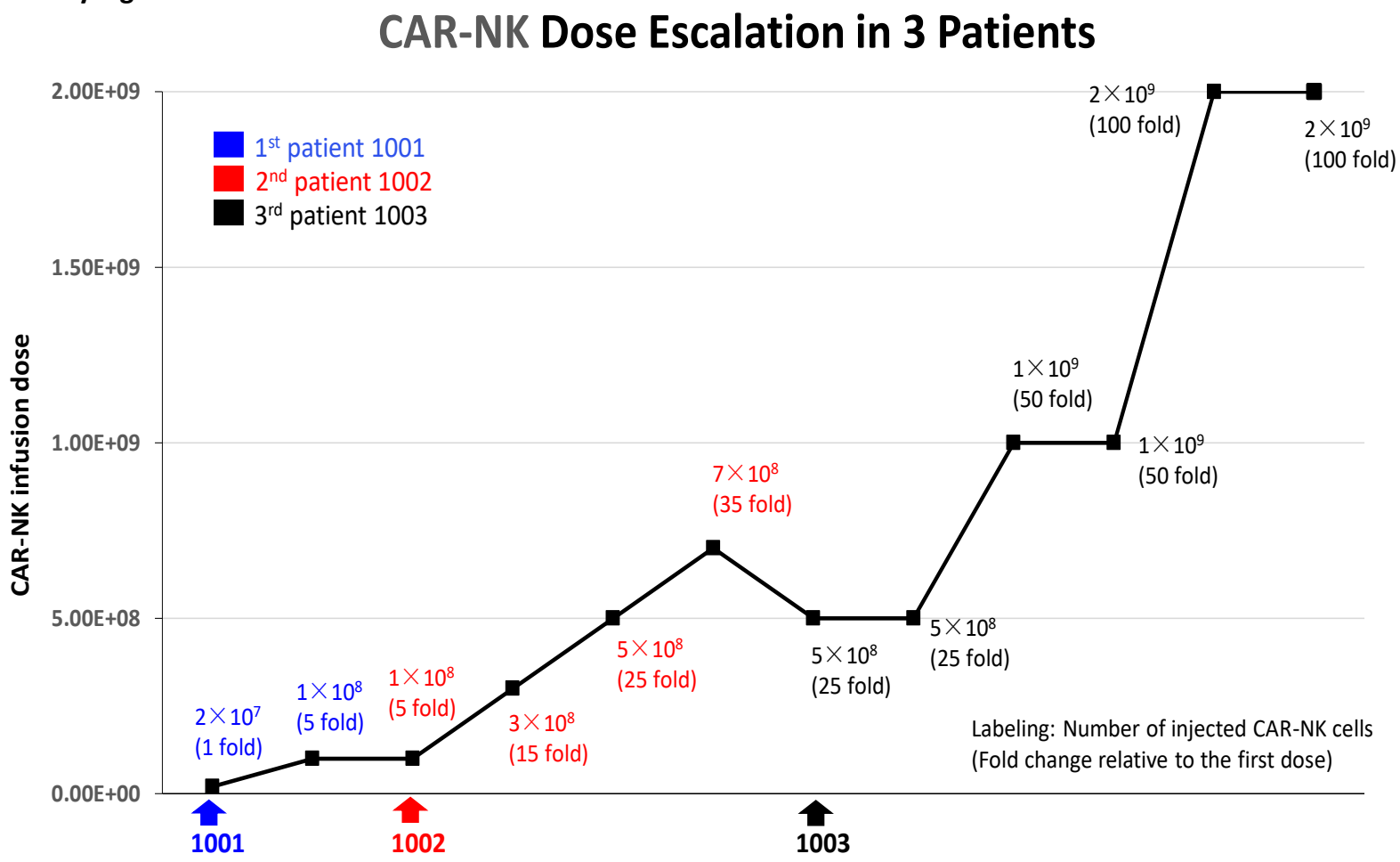


Supplementary Figure 5

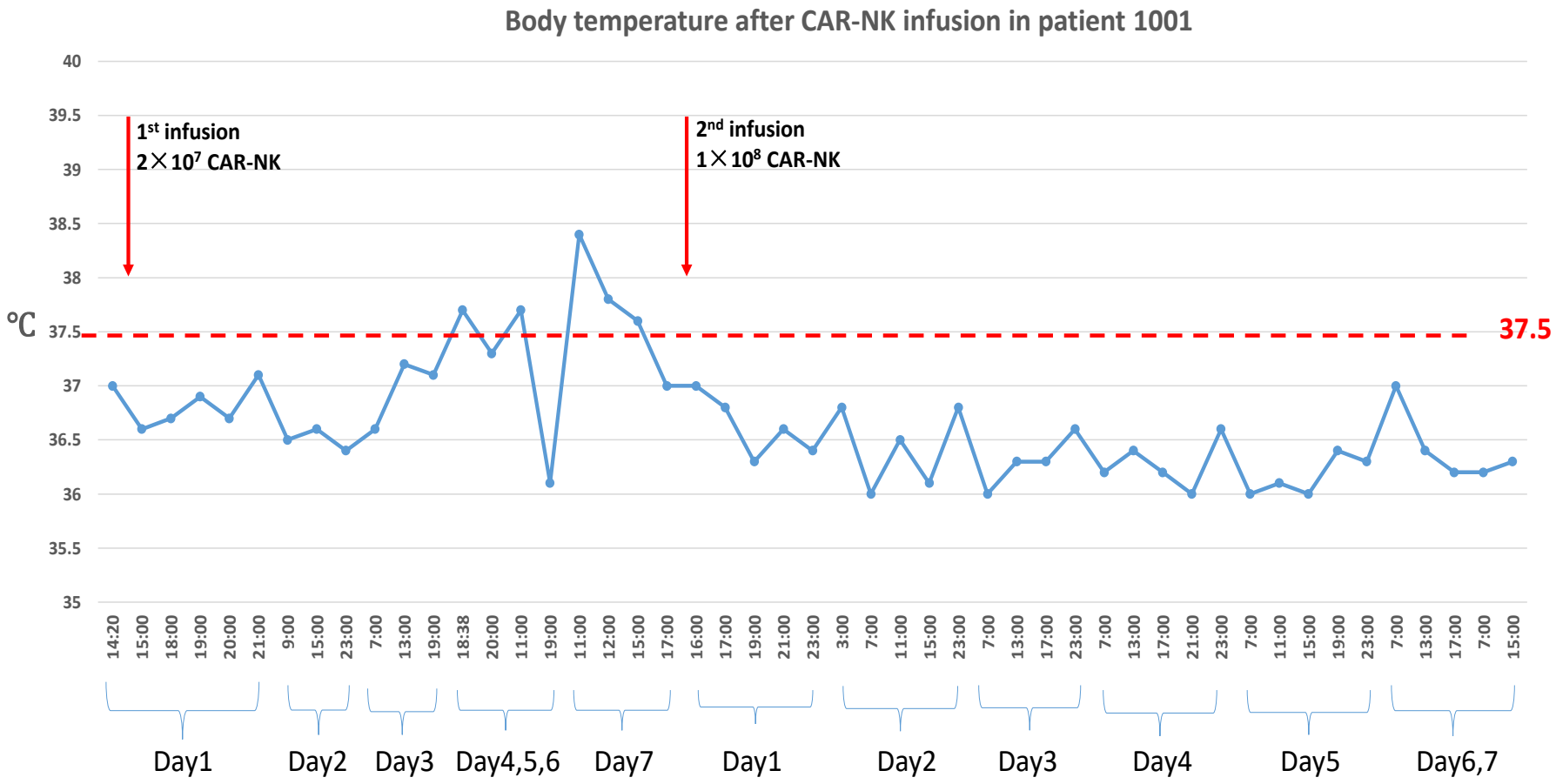
% NK cells after electroporation (EP)	1st EP	2nd EP	3rd EP	4th EP	5th EP	6th EP	Average	STD
Patient1001	50.0	84.0					67.0	24.04
Patient1002	69.5	68.8	101.0	60.0			74.8	17.95
Patient1003	57.5	68.8	59.8	53.0	42.0	72.6	59.0	11.03



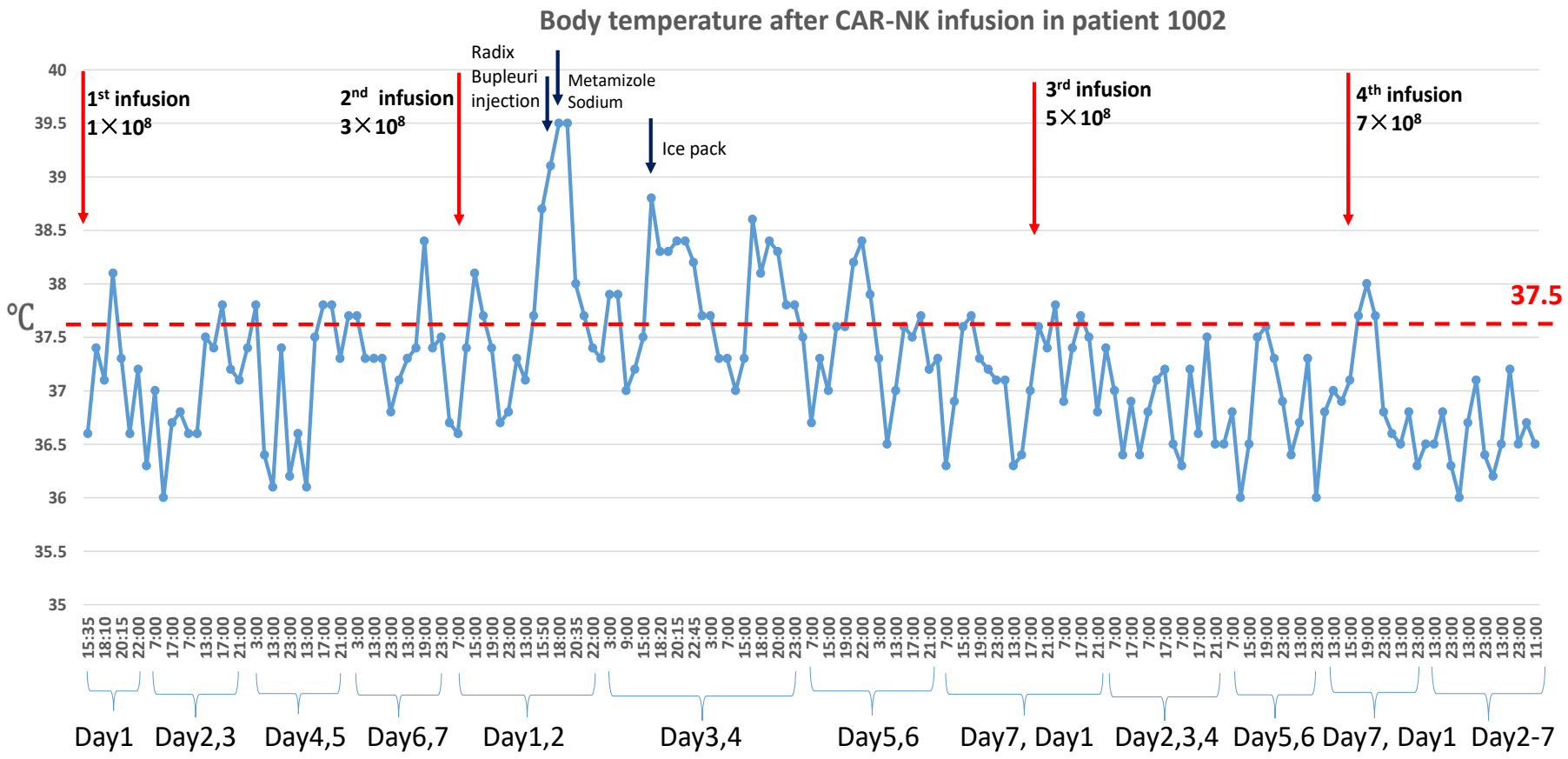
Supplementary Figure 6



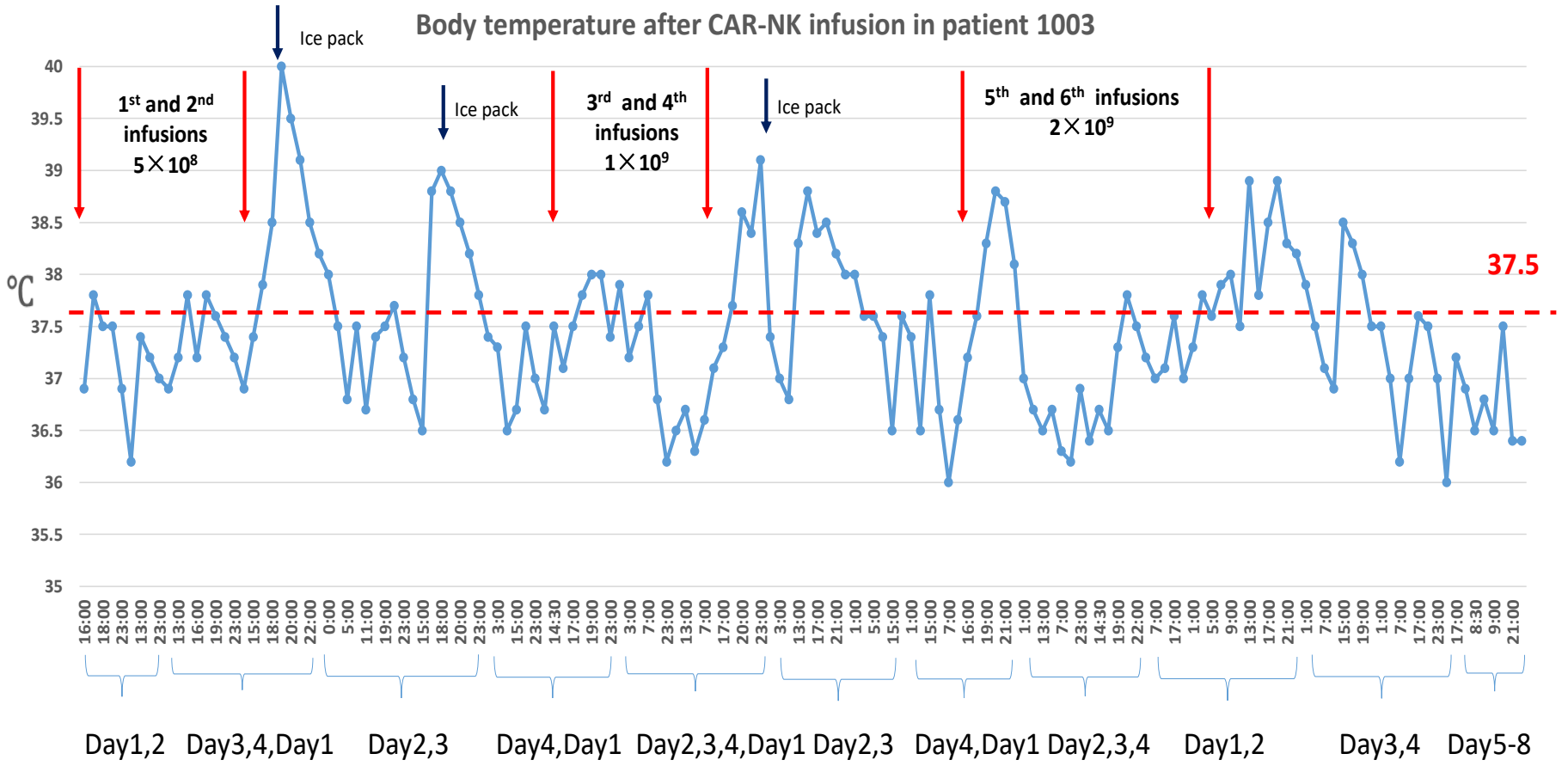
Supplementary Figure 7



Supplementary Figure 8

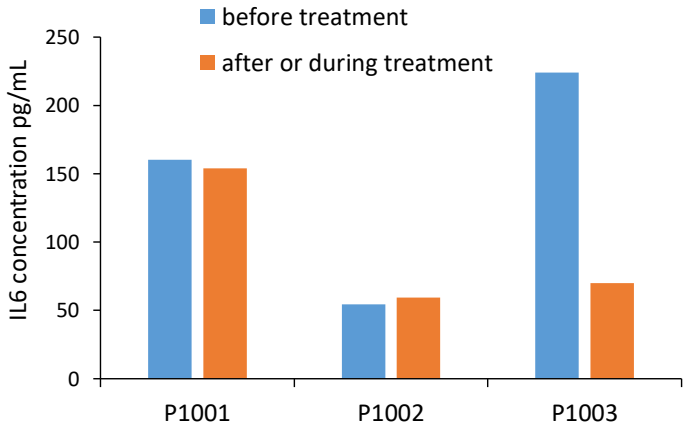


Supplementary Figure 9

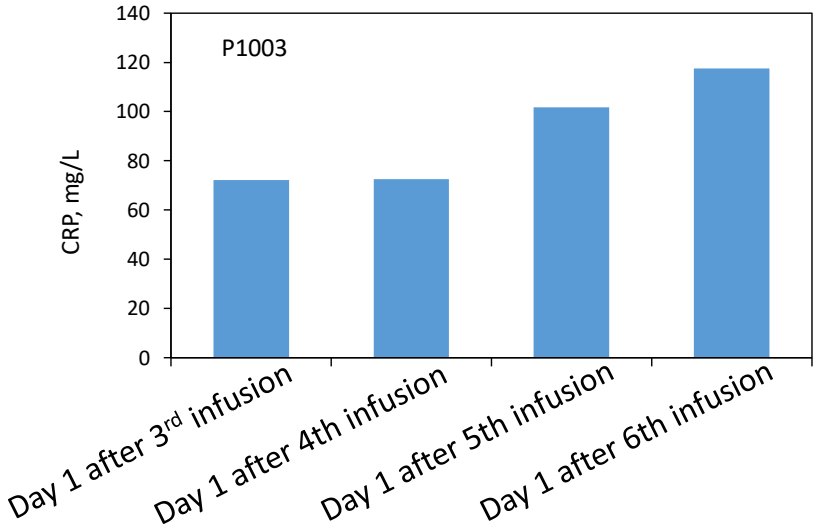


Supplementary Figure 10

A

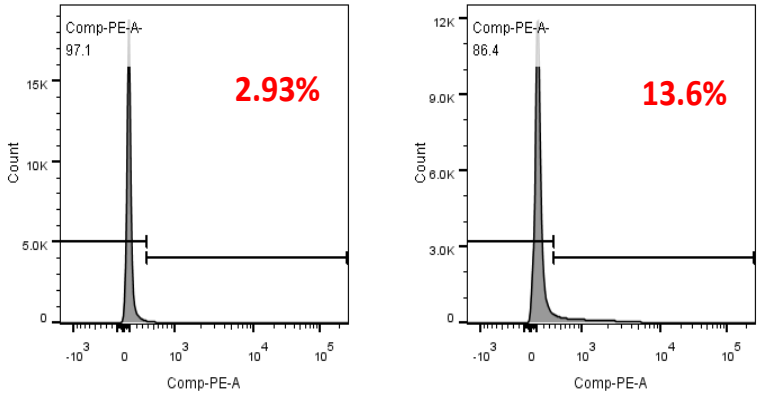


B



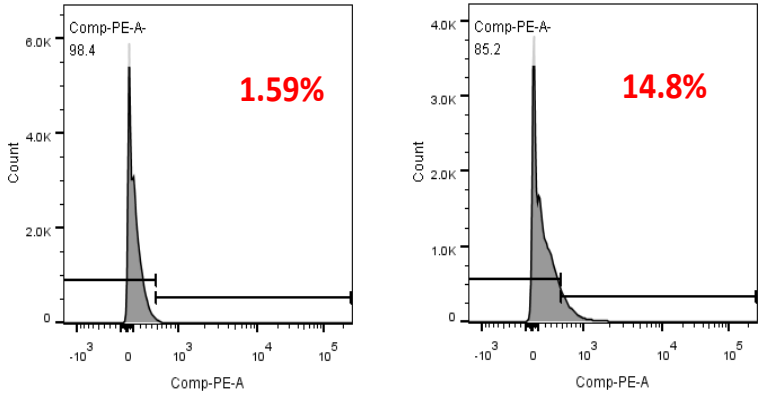
Supplementary Figure 11

P1001 before infusion （Day 0）



Isotype control　　anti-EpCAM

P1002 before infusion （Day -1）



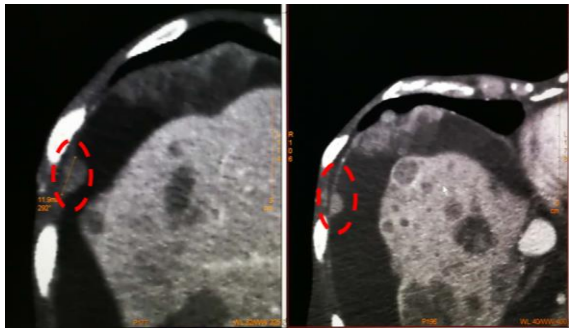
Isotype control　　EpCAM

Supplementary Figure 12

Patient 1001

Before therapy

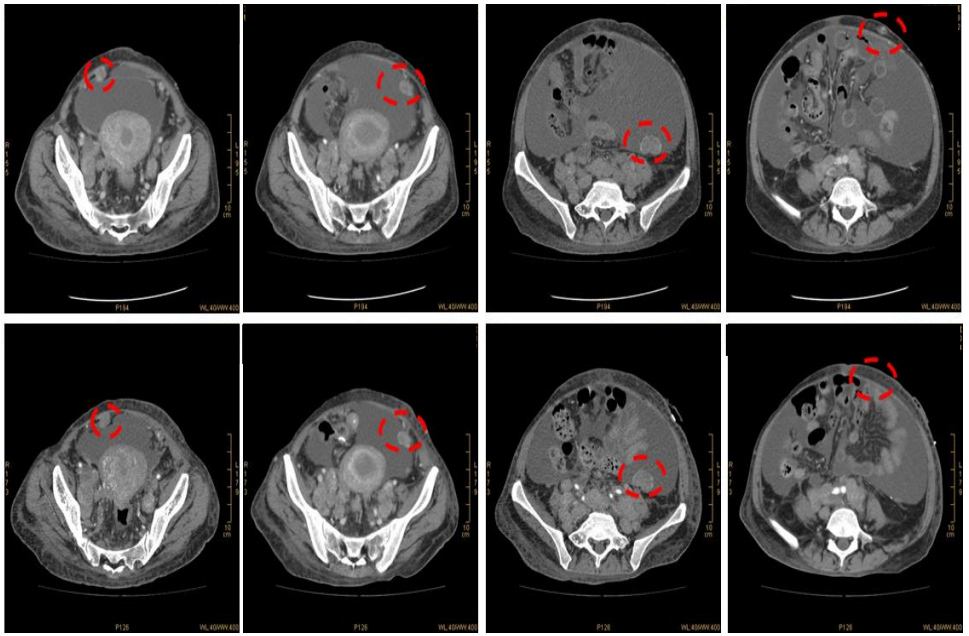
2 weeks after therapy



Patient 1002

Before therapy

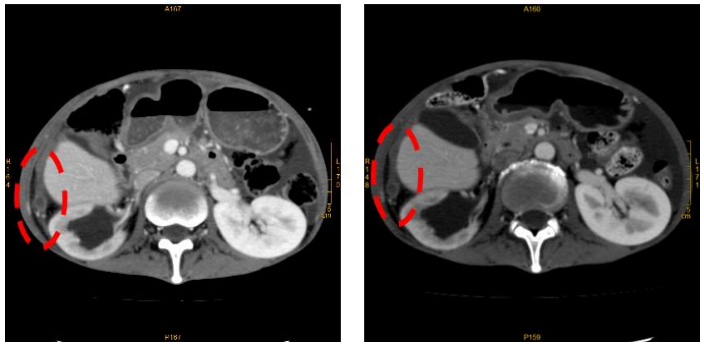
4 weeks after therapy



Patient 1003

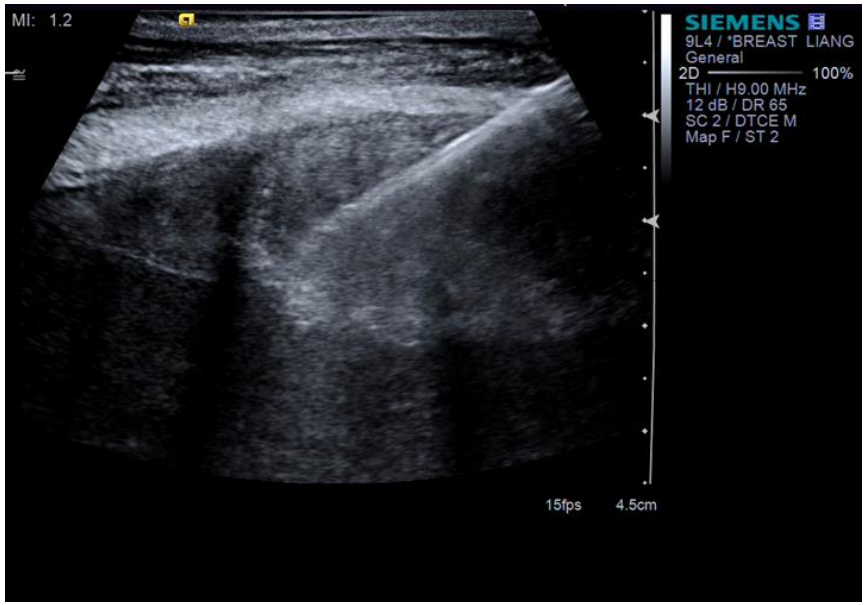
Before therapy

4 weeks after therapy

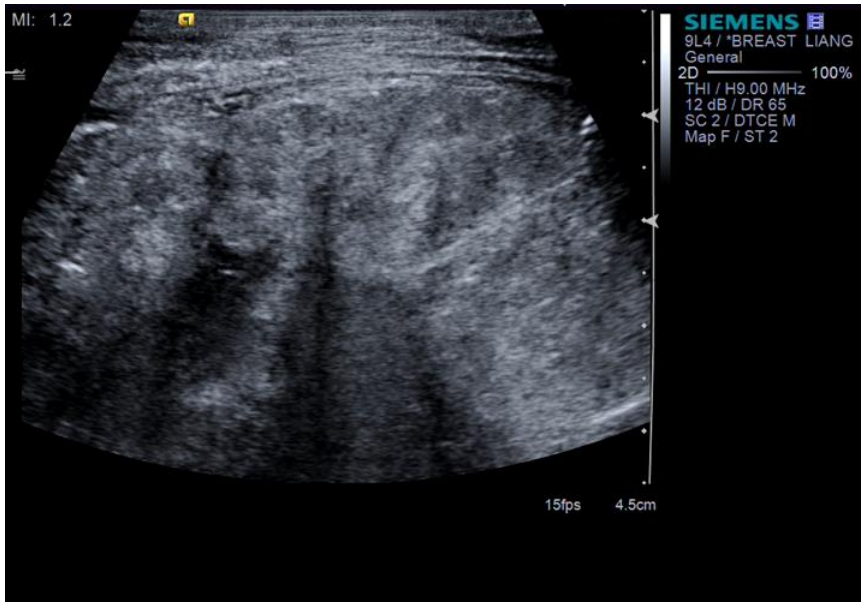


Supplementary Figure 13

A



B



Supplementary Figure 14