

Supplementary Methods

Materials and Methods

1. Cell lines

The K562 and NCI-H2087 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The K562 (ATCC CCL-243) cells and NCI-H2087 (ATCC CRL-5922) cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and maintained at 37°C under a humidified atmosphere containing 5% CO₂. The K562 cells were sub-cultured by the replacement of fresh medium every 2 to 3 days under standard conditions as recommended by the supplier, and the NCI-H2087 cells were sub-cultured every 2 to 3 days by trypsin-EDTA treatment at a 1:2 to 1:4 ratio as recommended by the supplier.

2. NK cell isolation and expansion

Briefly, the CD56⁺ cells were isolated from PBMCs using CliniMACS CD56 microbeads (Miltenyi Biotech GmbH, Galdbach, Germany) according to the manufacturer's instructions. The isolated CD56⁺ cells were then cultured in RPMI-1640 medium (WELGENE Inc., Gyeongsan, Korea) supplemented with 10% FBS (Hyclone, Tauranga, New Zealand), 20 µg/mL gentamicin (GIBCO, Grand Island, NY), γ -irradiated (100 Gy) KL-1 and LCL feeders, 500 IU/mL interleukin (IL)-2 (PROLEUKIN, Novartis, Basel, Switzerland), and 50 ng/mL IL-21 (NKMAX Co.). The growing NK cells were sub-cultured every 3-4 days using fresh RPMI-1640 medium containing IL-2. After 17-18 days of culture, the cells were harvested, washed twice with phosphate-buffered saline (PBS; WELGENE Inc.) and once with Hartmann's solution (DAI HAN PHARM., Ansan, Korea), and formulated in 100 mL Hartmann's solution containing 1% human serum albumin (Albumin inj.; GREEN CROSS, Cheongju, Korea) and IL-2 (500 IU/mL) with the cell number of 2×10^9 cells. The criteria for the release of NK cell products included the absence of microbial contamination (bacteria, fungus, virus, and mycoplasma), $\geq 80\%$ viability in a trypan blue exclusion assay, $\geq 50\%$ cytotoxicity against K562 target cells at the effector to target cell ratio of 10:1, ≤ 0.5 EU/mL endotoxin level, and immune phenotyping via flow cytometric analysis proving the expression of the NK cell markers (CD56⁺/CD3⁻) ($\geq 80\%$) and the absence of CD14, CD3, and CD20 ($\leq 5\%$ each). Only NK cell products that met all the criteria for release were shipped to the clinic at 2-8°C and then injected into the subjects via intravenous

administration over 45 ± 15 min/100 mL (2×10^9 cells).

3. Immunostaining and flow cytometric analysis

The phenotype of the culture-expanded NK cells was assessed flow cytometrically. The following monoclonal antibodies were used to stain every batch (from 1st to 6th) of culture-expanded NK cells: anti-CD56-FITC, anti-CD3-PE, anti-CD20-PerCP/Cy5.5, and anti-CD14-APC (BD Biosciences, San Jose, CA). Moreover, the following monoclonal antibodies were used to stain the 1st batch of culture-expanded NK cells: anti-NKG2D-APC, anti-NKp46-PE (BD Biosciences), anti-CD16-APC, anti-DNAM-1-PerCP/Cy5.5, anti-NKp30-PerCP/Cy5.5, anti-NKp44-PE, anti-CD158b-PerCP/Cy5.5, anti-CXCR3-PerCP/Cy5.5, anti-CX3CR1-PE (BioLegend, San Diego, CA), anti-NKG2A-PE, anti-CD158a-PE, and anti-CD158e-APC (Miltenyi Biotech GmbH). The cells were stained with the antibodies or their corresponding isotype control antibodies for 30 minutes at 4°C . For intra-cellular staining, the NK cells were stained with FITC conjugated anti-CD56 mAb. The cells were then fixed and permeabilized by incubation in fixation buffer (BioLegend) and labelled with anti-perforin-PerCP/Cy5.5 (BD Bioscience) and anti-granzyme-PE (BioLegend) antibodies. The flow cytometric data was acquired on a FACS flow cytometer (BD FACSVerse) and analyzed using the BD FACSuite v1.2 software.

4. Cytotoxicity assay

The cytotoxicity of the NK cells against the target cells (K562 and NCI-H2087 cell lines) was assessed using a fluorometric cytotoxicity assay. Each cell line was stained with 4 mM calcein-AM solution (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C followed by washing with RPMI 1640 medium. The NK cells and target cells were mixed at the E:T ratio of 10:1, 3:1, 1:1, and 0.5:1 in 96-well U-bottom plates. After a 4-hour incubation in a humidified incubator with 5% of CO_2 , 80 μL of supernatant was transferred to a new 96-well flat-bottom black plate. Fluorescence signal was determined using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA), exciting at 485 nm and detecting emission at 525 nm. The percent specific lysis was calculated using the formula: $(\text{Test release} - \text{Spontaneous release}) / (\text{Maximum release} - \text{Spontaneous release}) \times 100$.

5. Degranulation assay

For the NK cell degranulation assay, the culture-expanded NK cells were incubated

in media containing the anti-CD107a-APC antibody (BioLegend) or isotype control. Degranulation was induced by adding K562 target cells (E:T ratio=1:1) or phorbol 12-myristate 13-acetate plus ionomycin as a positive control, respectively (both from Sigma). In the negative control, complete culture medium was added instead of degranulation stimuli. The NK cells were incubated for 2 hours, and subsequently monensin (Sigma) was added to the medium before the next 3-hour incubation to prevent the degradation of internalized CD107a. Then, the cells were washed and stained with the anti-CD56-FITC antibody and analyzed using the FACS flow cytometer (BD FACSVerse).

6. Mouse xenograft experiment

All animal studies were performed in accordance with the guidelines of the Korean Food and Drug Administration. The protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Medical School. The C57BL/6 and SCID/g (*NOD-Prkdc^{scid}tm1Baek*) mice were obtained from GemBiosciences (Cheongju, Korea).

To test the in vivo efficacy of the PD-1 blockade with immune cell depletion, 5-week-old C57BL/6N mice were injected subcutaneously in the dorsal flank with LLC-1 mouse lung carcinoma cells and LLC-1 cells with stably expressing empty vector or PD-L1 vector (2×10^5 cells/mice) in 50 μ L of ice-cold PBS (Gibco). Five days after the injection of LLC-1, the mice were randomly divided into two groups to receive either 200 μ g of monoclonal PD-1 antibody (#BE0146, Invivogen) or control IgG1 (#BE0089, Invivogen) via intraperitoneal (i.p.) injection (n=4). The tumor volume was monitored for 20 days. The antibodies were treated via i.p. injection 4 times (2- or 3-day interval).

To deplete the CD8⁺ T cells, the mice were injected i.p. with 250 μ g CD8 α monoclonal antibodies on days 1 and 2 relative to tumor injection. The CD4⁺ T cells were depleted via the i.p. injection of 500 μ g of GK1.5 monoclonal antibodies on days 1 and 3. To deplete the NK cells in C57BL/6 mice, 250 μ g of PK136 (specific for NKR-P1C) was injected i.p. on days 1 and 2.

To test the in vivo efficacy of SNK cells and the PD-1 blockade, 5-week-old *NOD-Prkdc^{scid} tm1Baek* mice (n=6) were injected subcutaneously in the dorsal flank with H460 or H460-PD-L1 knockout human lung adenocarcinoma cells (5×10^5) in 50 μ L of ice-cold PBS (Gibco) i.p. Four days after injection, the mice were randomly divided into two groups to receive either 200 μ g of monoclonal PD-1 antibody (#BE0193, Invivogen) or control IgG1

(#BE0297, Invivogen) via i.p. injection. The tumor growth was monitored for 20 days. The monoclonal PD-1 or control IgG antibodies were i.p. injected 5 times (2- or 3-day interval). The SNK cells were injected into the tail vein 6 times (2- or 3-day interval).

7. Immunohistochemistry (IHC)

Four- μ m paraffin-embedded tissue samples were incubated with 10mM sodium citrate buffer (pH 6.0) and autoclaved for 15 minutes for antigen retrieval. The samples were then blocked with a PBS-based mixture of 5% bovine serum albumin (Affymetrix, Santa Clara, CA) and 1% normal horse serum (NGS, Vector Laboratories, Burlingame, CA) for 30 minutes. After blocking, the sections were incubated with anti-human NKp46 (1:100, MAB1850, R&D Systems, Minneapolis, MN) primary antibody overnight at 4°C, followed by incubation using the Anti-Rabbit IgG polymer kit (MP-7401, Vector Laboratories) or anti-goat IgG polymer kit (MP-7405, Vector Laboratories) for 1 hour at room temperature. The tissues were stained with 3,3'-diaminobenzidine (DAB; SK-4100, Vector Laboratories) and then counterstained with Mayer's Hematoxylin (ab220365, Abcam) and mounted using Permount medium (SP15-100, Fisher Chemical, Pittsburgh, PA). All incubations were conducted in wet chambers. The positive signal was visualized using a TE-2000U microscope (Nikon, Tokyo, Japan).