Supplementary Materials

Bispecific Immune Cell Engager Enhances the Anticancer Activity of CD16+ NK Cells and Macrophages In Vitro, and Eliminates Cancer Metastasis in NK Humanized NOG Mice

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Supplementary Method 1: *Cell culture and handling*

CD16+ natural killer cells from peripheral blood (PB-NK) were purchased (Lonza, Cat# 2W-501) and maintained in X-VIVOTM 15 Serum-free Hematopoietic Cell Medium (Lonza, 02-053Q) supplemented with 100 U/mL hIL-2 and hIL-15. THP-1-CD16A cells, while in a monocytic state, were maintained in RPMI1640 (ThermoFisher), supplemented with 10% FBS (ThermoFisher) and 2 mM Glutamine-alanine (ThermoFisher). They were seeded at a density of 0.2 million per ml complete media in a low adherent flask and passaged every 3 days. SKOV-3 cells (ATCC, HTB-77) were maintained in McCoy's 5A (Modified) Medium supplemented with 10% FBS. OVASC-1 cells were maintained in RPMI 1640 media supplemented with 15% FBS and 2.5 µg/ml insulin. JIMT-1 cells were cultured in DMEM/F-12, GlutaMAX™ supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, and 10 µg/ml insulin. MDA-MB-231 and BT-474 cells were purchased from ATCC and cultured as per provided protocol.

Supplementary Method 2: *Expression and purification of BiKE:E5C1*

To express BiKE, 1 liter of LB media containing 50 μg/ml kanamycin was inoculated with 20 ml of overnight culture. Once the OD_{600} reached 1.6, 0.5 mM IPTG was added to induce protein expression. The expression continued at 25°C for 18 hours and then bacterial culture was centrifuged at 10,000 g for 10 min at 4°C. The pellet was resuspended in 70 ml of lysis buffer **(Table 1)** and incubated at room temperature (RT) for 30 min while shaking. The bacterial suspension was then sonicated (5s on, 3s off, 70% amplitude) for 3 x 15 min on ice. Next, the cell debris was pelleted via centrifugation (40,000 g, 4°C, 45 min), the supernatant was loaded onto a Ni-NTA column and washed with 30 ml of wash buffer I and wash buffer II **(Table 1)**. Before eluting the target protein, the column was washed twice with 25 ml of dulbecco's phosphate buffer saline (DPBS). To elute BiKE, 100 U/mL of WELQut protease (Thermo Scientific, Cat#EO0861) was added to the resin and incubated overnight at 4˚C. The purity and concentration of the eluted protein was measured using SDS-PAGE and BCA assay, respectively.

Table 1. The compositions of buffers that were used for the purification of the BiKE:E5C1 from bacterial lysate.

Reagents/Buffers	**Lysis buffer**	**Wash Buffer I**	**Wash Buffer II**	**WELQut**
MiliQ water	Yes	Yes	Yes	Yes
Tris	50mM (6.057g/L)	50mM (6.057g/L)	50mM (6.057g/L)	--
Na_2HPO_4	100mM (7.1g/L)	100mM (7.1g/L)	100mM (7.1g/L)	--
NaCl	250mM (14.6g/L)	500mM (29.2g/L)	1M (58.4g/L)	250mM (14.6g/L)
KCl	--	--	--	250mM

				(18.64g/L)
Imidazole	5 mM 0.33g/L	10 mM 0.66g/L	10 mM 0.66g/L	--
EDTA	1mM (0.292g/L)	--	--	--
Glycerol	5% (v/v)	5% (v/v)	5% (v/v)	--
PBS (solvent)	--	--	--	Yes
Triton X-100	1% (10mL/L)	0.2% (2mL/L)	--	--
PMSF	1mM	--	--	--
Lysozyme	0.2mg/mL	--	--	--
RNase A	10 ug/mL	--	--	--
DNase I	5 ug/mL	--	--	--
pH	7.0	7.5	7.5	7.5

Supplementary Method 3: *Measurement of the degranulation of laNK92 cells using surfaced CD107a*

SKOV-3 cells were seeded in a 96-well plate at the density of 10^4 cells per well and incubated overnight. The next day, a serial dilution of BiKE, spanning from 0 to 100 nM, was added into the wells and incubated for 30 minutes at 37°C. Then, laNK92 (GFP+) cells were added to SKOV-3 cells at an E:T ratio of 4. This cell combination was then incubated for 3 hours at 37°C. Following this stage, a human LAMP-1/CD107a APC-conjugated Antibody (R&D Systems, Cat#IC4800A) was added to the mix and incubated for 1 h. Subsequently, the plate was centrifuged, the supernatant was removed, and a double washing step was executed to eliminate excess antibody. A Beckman Coulter CytoFLEX Cytometer was then used for data acquisition. For data analysis, the GFP+ (laNK92) subset was gated to distinguish between the effector and target cells. In the final analytical step, the mean fluorescent intensity (MFI) of surfaced CD107a molecules within the GFP+ cell population was quantified.

Supplementary Method 4: *Evaluation of the impact of hIL-2 and hIL-15 on laNK92 cytotoxicity*

One day prior to the assay, 10,000 SKOV-3 cells were seeded. The next day, the cells were incubated with BiKE:E5C1 (100 nM) for 30 minutes at 37°C. This was followed by the addition of laNK92 cells at E:T ratios of 0, 1, 2, and 4. In the case of the BiKE:E5C1 kill curve, SKOV-3 cells were seeded and then treated with varying BiKE:E5C1 concentrations ranging from 0 to 100 nM for 30 minutes at 37°C. After this, laNK92 cells were added at an E:T ratio of 4. Following a 4-hour incubation period, we assessed the cell viability using alamarBlue cytotoxicity kit and protocol.

Supplementary Method 5: *Measurement of cytokine release*

SKOV-3 cells were seeded in a 96-well plate at the density of 10^4 cells per well. The next day, 100 nM BiKE:E5C1 was added to the plate and incubated at 37˚C for 30 min. Next, PB-NK or laNK92 cells were added at E:T ratio of 4. After 24 h, plates were centrifuged at 2000 g for 10 min to pellet the cells. Then, the supernatant was transferred into a non-treated 96-well plate. The amounts of TNF-α and IFN-γ cytokines were measured using Duoset ELISA Kit (R&D Systems, USA). The data are presented as mean ± SD (n=3).

In in vivo study, the concentrations of IFN-γ within murine blood were measured. Blood samples were collected one week after treatment, using heparinized microhematocrit tubes (Fisher Scientific, Cat#22-362566). Following collection, blood samples were centrifuged at 10,000 g for 10 minutes at 4˚C. The concentration of IFN-γ was measured using the Duoset ELISA Kit as mentioned above. The data are presented as mean±s.d. (n=3).

Supplementary Method 6: *Genetic engineering and characterization of NK92-nLuc cells*

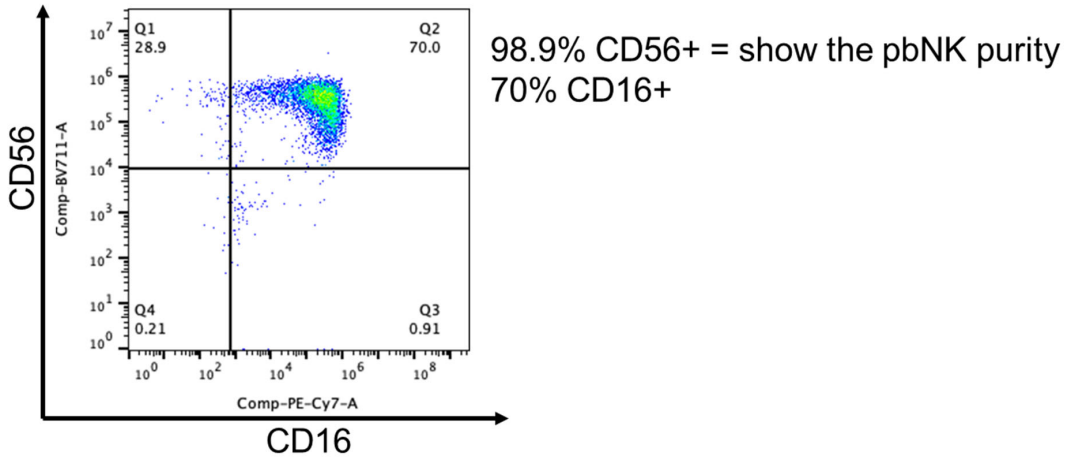
To engineer laNK92-nluc cells, laNK92 cells were passaged at a low density of 50,000 cells/ml supplemented with 600 IU/ml of human interleukin 2 (hIL-2) (Peprotech) and then processed using the buffers from the kit associated with Neon™ NxT electroporation system (ThermoFisher Scientific). On the day of electroporation, cells were harvested and subjected to three consecutive washes with Opti-MEM (Invitrogen). Following the final wash, cells were resuspended in a buffer solution at a concentration of 4×10^7 cells in 850 µl of O buffer containing 0.1% CD buffer. Subsequently, 85 µl of this cell suspension was mixed with 15 µl of plasmids, consisting of 5 µg of piggybac transposase and 10 µg of pb-nLuc. Electroporation was carried out using the Neon™ NxT electroporation system with two pulses: an initial pulse of 1650 V for 20 ms followed by a second pulse of 500 V for 100 ms (please see PMID: 31114587). Immediately post-electroporation, cells were resuspended in NK92 culture media supplemented with 600 IU/ml of hIL-2 and incubated for three days. Then, 200 µg/ml of hygromycin B (Gibco) was added the culture media to select for nanoluciferase-expressing laNK92 cells (laNK92-nLuc). The selection process was maintained for a duration of three weeks.

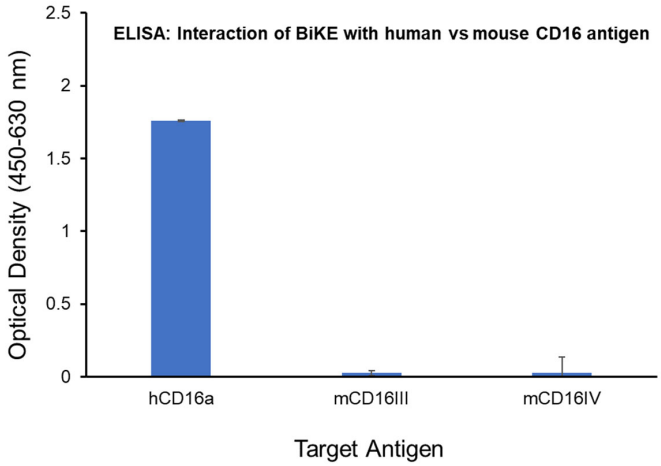
To compare the proliferation rate of the laNK92 cells with laNK92-nluc cells, they were collected and washed with DPBS. Subsequently, cells were fixed using a 3.7% formaldehyde solution for a duration of 15 minutes, followed by a dual washing step with DBPS. The fixed cells were then rendered permeable through treatment with 1% Triton X-100 for 15 minutes followed by a dual washing sequence. The resultant permeabilized cells were resuspended in an intracellular staining solution comprising DPBS, 2% FBS, and 0.1% Triton-X100. To stain the cells, they were incubated overnight with APC-conjugated anti-Ki-67 antibody (Biolegend) at 4°C. Upon

completion of the staining, cells were washed three times and then analyzed using a Beckman Coulter Cytoflex flow cytometer at Rutgers Flow Cytometry Core Facility.

To compare the proliferation rates of laNK92 and laNK92-nLuc cells, the Ki-67 proliferation index was measured using flow cytometry and established protocols (please see PMID: 28446615).

Supplementary Fig. 1: Characterization of the PB-NK cells in terms of CD56 and CD16 receptor expression levels using flow cytometry. The data show the purity of the PB-NK cells is ~99%, and 70% of the PB-NK cells used in this study expressed CD16 receptors.





Supplementary Fig. 2: Characterization of BiKE:E5C1 and its interaction with mouse CD16 antigen (mCD16III and mCD16IV) using ELISA. In this experiment, a 96-well ELISA plate was coated with human CD16a, mouse CD16 III and CD16 IV proteins at concentrations of 1 µg/mL in coating buffer. After an overnight incubation at 4°C, the coating solutions were discarded, and the plates were washed once with DPBS containing 0.05% Tween-20 (PDBS-T). Nonspecific binding sites were blocked by adding 200 µL of blocking buffer (1% BSA in DPBS) per well and incubating at room temperature for 2 hours. Next, BiKE:E5C1, prepared at concentration of 1 µg/ml in 0.5% BSA in DPBS, was added (100 µL/well). After 1 hour of incubation at room temperature while shaking at 700 rpm, the plate was washed thrice with PBS-T. Then, 100 µL of anti-cMyc HRP-conjugated secondary antibody was added and incubated for 1 hour while shaking at 700 rpm. After additional PBS-T washing, 50 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added to initiate the colorimetric reaction, which was allowed to proceed for 15 minutes at room temperature. The reaction was stopped by adding 50 µL of 2M sulfuric acid to each well. Absorbance measurements were taken at 450 nm and 630 nm (as a reference) wavelength using Tecan Infinite® M200 PRO plate reader.