A CHIMERIC RECEPTOR WITH NKG2D SPECIFICITY ENHANCES NATURAL KILLER CELL ACTIVATION AND KILLING OF TUMOR CELLS

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Supplementary Methods, Figure S1, Movie, and Table S1

SUPPLEMENTARY METHODS

Virus production and gene transduction

To generate RD144-pseudotyped retrovirus, we transfected using fuGENE 6 (Roche, Indianapolis, IN) 2.5×10^6 293T cells, maintained in 10-cm tissue culture dishes for 16 h, with 3.5 μg of cDNA encoding NKG2D constructs, 3.5 μg of pEQ-PAM3(-E), and 3 μg of pRDF (1, 2). After replacing the medium with RPMI-1640 with 10% FCS and antibiotics at 24 hours, we harvested the conditioned medium containing retrovirus at 48, 72 and 96 hours and added it to RetroNectin (Takara, Otsu, Japan)-coated polypropylene tubes, which were centrifugated at 1400 g for 10 min and incubated at 37°C for 4 hours. After additional centrifugation, and removal of the supernatant, expanded NK cells (5×10^5) were added to the tubes and left in at 37°C for 24 h. This procedure was repeated on two other successive days. Cells were then maintained in RPMI-1640 with FBS, antibiotics and 50 IU/ml of IL-2 until the time of the experiments, 3-10 days after transduction.

Analysis of NKG2D-DAP10-CD3ζ expression

Surface expression of NKG2D was analyzed by flow cytometry using an anti-human NKG2D antibody conjugated to peridinin chlorophyll protein (R&D, Minneapolis, MN). Expression of DAP10-FLAG was visualized with an anti-FLAG antibody conjugated to phycoerythrin (Abcam, Cambridge, MA). For Western blotting, NK cells were incubated with 0.1 μM sodium orthovanadate and 0.034% H_2O_2 at 37°C for 10 minutes. Cells were lysed in Cellytic M lysis Buffer (Sigma, St Louis, MO) containing 1% protease inhibitor cocktail (Sigma) and 1% phosphatase inhibitor cocktail 2 (Sigma). After centrifugation, lysate supernatants were boiled with an equal volume of LDS buffer (Invitrogen, Carlsbad, CA) with or without reducing buffer

(Invitrogen) and then separated by NuPAGE Novex 12% Bis-Tris Gel (Invitrogen). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was incubated with a rabbit anti-human CD3ζ phospho (pY83) antibody (clone EP776(2)Y; Epitomics, Burlingame, CA). Membranes were then washed, incubated with a goat anti-rabbit IgG horseradish peroxidase-conjugated second antibody (Cell Signaling, Danvers, MA), and developed by using the Amershan ECL Prime detection reagent (GE Healthcare).

mRNA electroporation

The pCMV6-XL5 vector (Origene, Rockville, MD) was used as a template for *in vitro* mRNA transcription. The NKG2D-CD3ζ and DAP10 cDNA were subcloned into EcoRI and XbaI sites of pCMV6-XL5. The corresponding mRNA were transcribed *in vitro* with T7 RNA polymerase using Ambion mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, Tx) (3).

For electroporation we used the MaxCyte GT system (MaxCyte, Gaithersburg, MD), as previously described (3). Briefly, expanded NK cells (4×10^6) were washed once with EP buffer (MaxCyte), mixed with 400 μg/ml mRNA, transferred into the processing chamber, and transfected using the program "NK#2" (3). Immediately after electroporation, cells were transferred from the processing chamber to a 96-well plate, incubated for 20 minutes at 37^{o}C, and then cultured in RPMI-1640 with FBS, antibiotics and 50 IU/mL IL-2.

References

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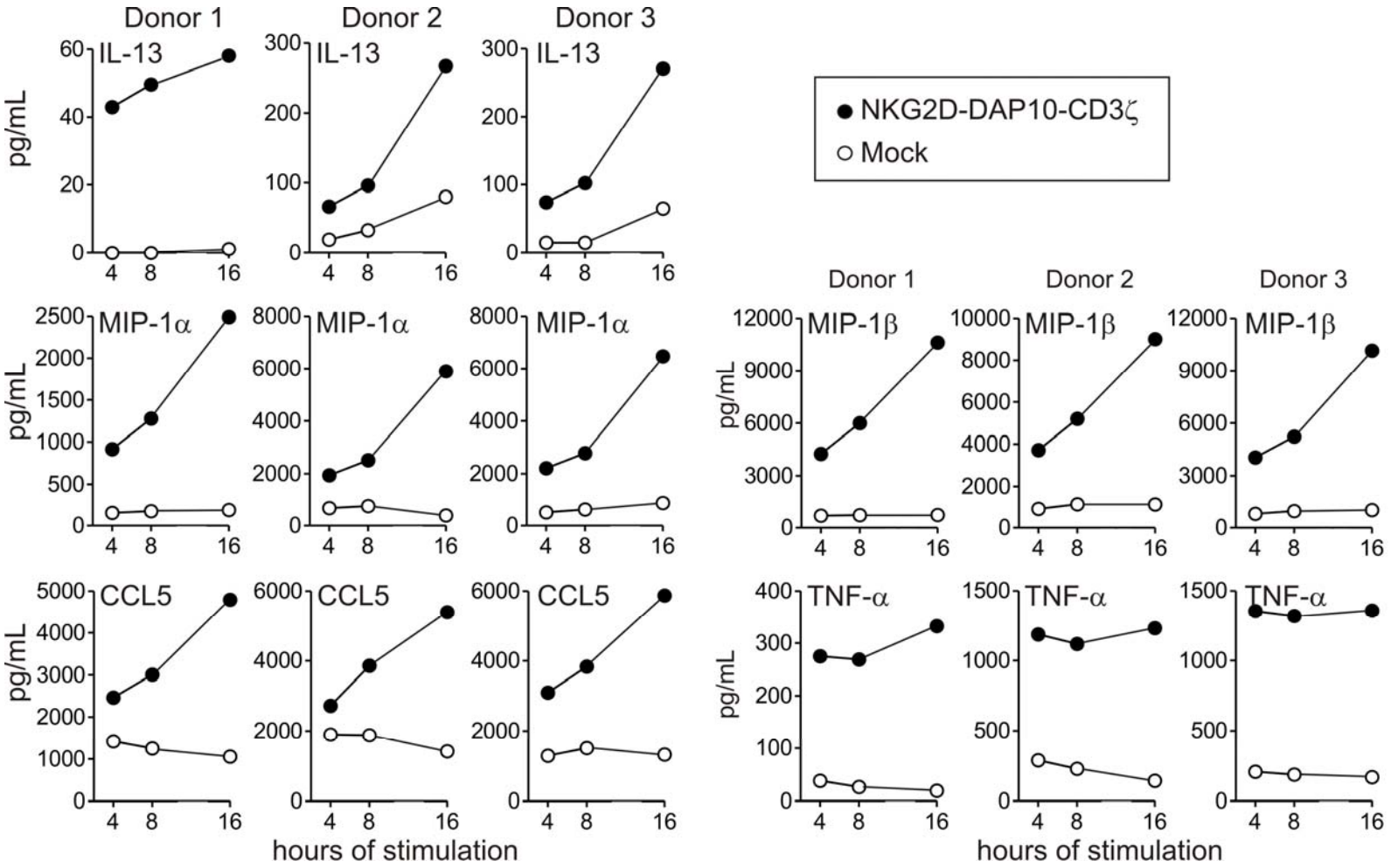
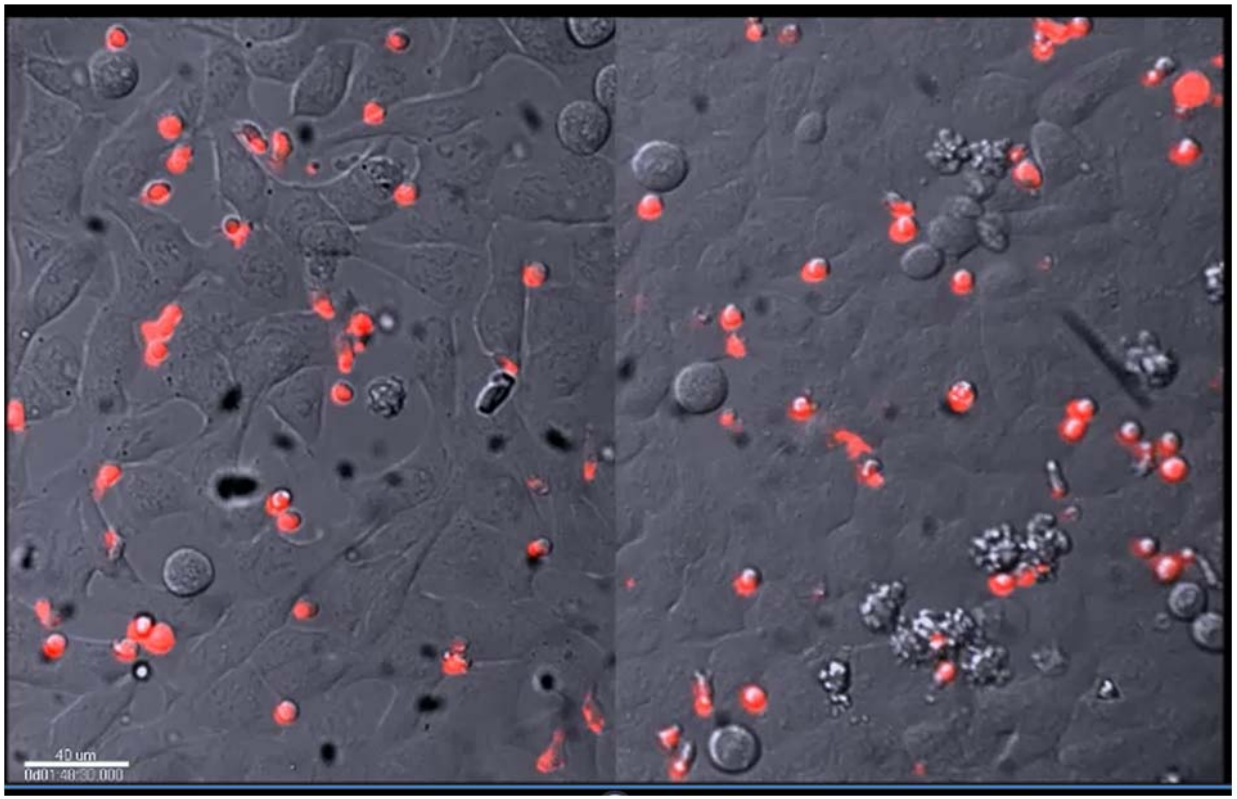
3. Shimasaki N, Fujisaki H, Cho D, Masselli M, Lockey T, Eldridge P, et al. A clinically adaptable method to enhance the cytotoxicity of natural killer cells against B-cell malignancies. Cytotherapy. 2012;14:830-40.



Supplementary Figure S1. Cytokine/chemokine secretion of mock- and NKG2D-DAP10-CD3ζ-transduced NK cells from 3 donors after incubation with a biotin-conjugated anti-NKG2D agonistic antibody (clone 1D11; eBioscience) and anti-biotin beads (MACSiBeads; Miltenyi Biotec) (see also Fig. 4 and Supplementary Table S1).



Supplementary Movie. Live cell confocal photography of mock- (left panel) and NKG2D-DAP10-CD3ζ-transduced NK cells (right panel) co-cultured U2 OS osteosarcoma cells. NK cells were labeled with PKH26 (Sigma) before culture and appear red. Microscopy was performed with a Nikon TE2000E2 microscope equipped with a Nikon C1Si confocal using 488nm and 561nm DPSS lasers for excitation. Temperature was maintained at 37^{o}C and CO2 at 5% using an environmental control chamber. Images were acquired with a Nikon 40x 1.3 NA DIC objective every 30s for 2hr using Nikon EZC1 software. Imaris (Bitplane Scientific Software) was used to analyze speed and displacement of NK cells.

Supplementary Table S1. Cytokine/chemokine secretion (mean ± SD pg/mL) after stimulation of mock- or NKG2D-DAP10-CD3ζ-transduced NK cells with anti-NKG2D[a]

Cytokine/ chemokine	No stimulation		Stimulation	
	Mock	NKG2D-DAP10-CD3ζ	Mock	NKG2D-DAP10-CD3ζ
GM-CSF	20±17	18±16	71±50	750±310
IFN-γ	9±8	12±8	81±62	717±466
IL-2	606±15[b]	580±27[b]	521±48[b]	375±75[b]
IL-13	< 5	< 5	49±42	198±121
MIP-1α	126±88	233±147	482±349	4952±2151
MIP-1β	178±67	346±88	956±198	9931±827
Rantes	310±47	500±37	1267±188	5354±541
TNF-α	13±11	12±10	110±80	974±559
Eotaxin	< 5	< 5	< 5	7±7
FGF-2	12±4	14±10	15±7	32±29
Flt-3L	< 5	9±1	10±3	24±22
Fractalkine	24±17	9±16	43±7	55±37
GRO	5±9	< 5	< 5	< 5
IFN-α2	< 5	< 5	< 5	10±15
IL-1rα	< 5	< 5	8±7	< 5
IL-5	< 5	< 5	< 5	5±4
IL-8	< 5	< 5	< 5	10±8
MCP-1	< 5	< 5	< 5	7±5
PDGF-AA	< 5	< 5	< 5	6±2
TNF-β	< 5	< 5	< 5	15±9
VEGF	12±11	20±18	12±11	9±16
sCD40L	10±3	14±13	17±4	34±34

For EGF, G-CSF, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-7, IL-9, IP-10, MCP-3, MDC, PDGF-BB and TGF-α, all the measurements were <5 pg/mL.

[a]NK cells expanded from 3 donors, either transduced with NKG2D-DAP10-CD3ζ or mock-transduced, were incubated with anti-Biotin MACSiBeads (Miltenyi Biotec, Auburn, CA) coated with biotin-anti NKG2D (clone 1D11; eBioscience; San Diego, CA) antibody (ten beads for one NK cell). Supernatants were collected after 16 hours at 37°C and analyzed using the Luminex human cytokine/chemokine panel I (41 human cytokines/chemokines) (Merck Millipore; Billerica, MA). Supernatants collected from cells cultured without anti-NKG2D beads were also studied.

[b]Exogenous IL-2 (50 IU/ml) was present in the tissue culture medium.