Highly activated and expanded natural killer cells for multiple myeloma immunotherapy

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Online Supplementary Design and Methods

Cell lines, samples, and cell selection

K562, OPM2 and U266 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The K562-mb15-41BBL cell line was obtained from St. Jude Children's Research Hospital (Memphis, TN, USA).[1,2] OPM2 and U266 cells were transfected with luciferase (OPM2-Lu/U266-Lu) as previously described.[3] Informed consent was obtained from subjects according to the Declaration of Helsinki. The University of Arkansas for Medical Sciences Institutional Review Board approved this study. $CD138^+$ multiple myeloma (MM) and $CD34^+$ progenitor cells were isolated from bone marrow or apheresis products, respectively, using magnetic bead selection following the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). For gene expression profiling (GEP), non-expanded (non-exp-) and expanded natural killer (exp-NK) cells were purified using the EasySep kit according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada) resulting in >95% $CD56^+/CD3^-$ NK cells. Prior to injection into mice exp-NK cells were >95% $CD56^+CD3^-$. Patients' phytohemagglutinin-blast cells were generated by stimulating peripheral blood mononuclear cells with 2 μg/mL phytohemagglutinin and 50 IU/mL interleukin (IL)2 for 1 week.

Natural killer cell expansion

Peripheral blood was centrifuged over Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ, USA). Peripheral blood mononuclear cells were harvested and washed in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen). The peripheral blood mononuclear cells were then co-cultured with irradiated (100 Gray) K562-mb15-41BBL cells at a ratio of 1.5:1 in RPMI 1640 + 10% FBS + 300 IU/mL IL2 (Prometheus, San Diego, CA, USA). One half of the medium was changed every other day with fresh medium containing IL2. The NK cells were re-stimulated with K562-mb15-41BBL cells on day 7 and collected on days 10-14 for assays. The number of viable cells was based on counts using trypan blue exclusion. Fold expansion was calculated by dividing the number of viable cells at day 7 or day 10-14 by the number of cells at the beginning of the culture (day 0).

Gene expression profiling

Purified non-exp-NK cells and exp-NK cells from healthy donors (HD) and MM patients were subjected to GEP, performed with the Affymetrix U133 Plus 2.0 microarray platform (Santa Clara, CA, USA).[4,5] Microarray data have been deposited in the NIH Gene Expression Omnibus available at http//www.ncbi.nlm.nih.gov/geo under accession number GSE27838. Gene expression data were normalized with MAS5 using default parameters in Affymetrix GeneChip operating software. All statistical analyses were performed with the statistics software R (Version 2.12.0; available from *http://www.r-project.org*) and R packages developed by the BioConductor project (available from *http://www.bioconductor.org*). The reported fold change was defined as the ratio of geometric means of expression data for exp-NK cells *versus* non-exp-NK cells or MM NK values *versus* HD NK values. The paired *t* test and independent two-sample *t* test were used on log-transformed GEP intensity values for exp-NK *versus* non-exp-NK comparisons and MM *versus* HD comparisons, respectively. The Q-value[6] was used to estimate the false discovery rate and identify differentially expressed genes. The false discovery rate cutoff was set at < 0.05.

^{51}Cr release cytotoxicity assays

Target cells were incubated for 1.5 h with 100 μCi sodium chromate 51 (PerkinElmer Inc., Billerica, MA, USA), washed, and then incubated with effectors at the indicated E:T ratios in RPMI 1640 media + 10% FBS in 96-well V-bottom plates. Effector cells were whole NK cell cultures since there was little contamination by NKT cells, T-cells and γδ T-cells. After incubation for 4 h, supernatants were collected to determine target cell lysis. Controls for spontaneous release and maximum release were included in replicates of six while each E:T ratio

was performed in triplicate. Specific lysis percentage was calculated as [(test release - spontaneous release)/(maximal release - spontaneous release) x 100].

Time-lapse microscopy

K562-mb15-41BBL cells with high expression of enhanced green fluorescence protein (GFP) or primary MM cells were seeded in poly-l-lysine coated glass bottom Petri dishes (MatTek Corp., Ashland, MA, USA). For time-lapse microscopy, Petri dishes were placed on a temperature controlled stage with a 5% CO_2 chamber fitted with a Zeiss Axiovert 100M inverted microscope (Carl Zeiss, Germany). Patients' exp-NK cells were introduced into the dish prior to imaging with a 2:1 E:T ratio. Images were collected with a Zeiss 20X differential interference contrast (DIC) objective, using 520/18 excitation, 565/20 emission, 545DRLP dichroic and XF203 filter set, every 12 sec for 120 min using a Scion CG-7 frame grabber (Scion Corp., Frederick, MD, USA). The images were processed with Image J software (NIH, Bethesda, MD, USA).

Cytokine analysis by cytometric bead array

In vitro cytokine secretion of exp-NK cells was assessed by culturing $1x10^5$ purified NK cells and $5x10^4$ K562 cells in a 96-well U-bottom tissue culture plate (BD Falcon) in 200 μL of medium for 72 h. The supernatant content of tumor necrosis factor-α, interferon-γ, IL4, IL6 and IL10 was assayed using a cytometric bead array according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA).

Murine model

For live-animal imaging, mice were anesthetized with ketamine plus xylazine and injected intraperitoneally with D-luciferin firefly potassium salt (150 mg/kg; Caliper Life Sciences, Hopkinton, MA, USA). Luciferase activity was detected using the Xenogen IVIS 200 imaging system (Caliper Life Sciences). For enzyme-linked immunosorbent assays (ELISA), mice were bled from the tail weekly to obtain 100 μL of blood in EDTA-coated tubes. The specimens were spun for 20 min and both plasma and cellular fractions were harvested. We have validated the use of murine plasma for human immunoglobulin ELISA, performed as previously described.[7,8] The peripheral blood cellular fraction was then used for flow cytometry determination of persistence, phenotyping and *in vivo* expansion assays.

Histology and immunohistochemistry

All reactions were performed using an automated immunostainer (DakoCytomation, Carpenteria, CA, USA) in conjunction with the Envision™-HRP or –AP detection systems (Dako Cytomation) using either diaminobenzidine or Fast Red as the chromogens for dual staining. Endogenous peroxidase was inhibited with methyl alcohol containing 0.01% H_2O_2 with additional blocking carried out using Background Sniper™ (Biocare Medical, Concord, CA, USA) and/or Background Terminator™ (Biocare Medical). Nuclei were counterstained with hematoxylin and sections were evaluated by light microscopy using an Olympus BH-2 microscope (Olympus, Melville, NY, USA) fitted with a SPOT2 digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

References

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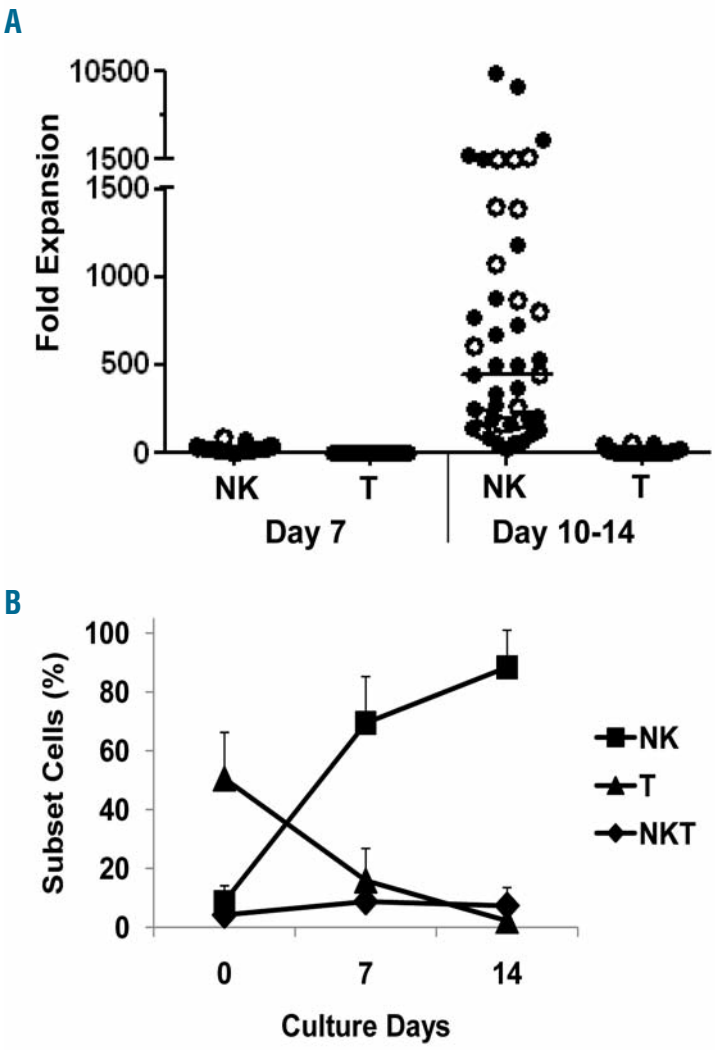
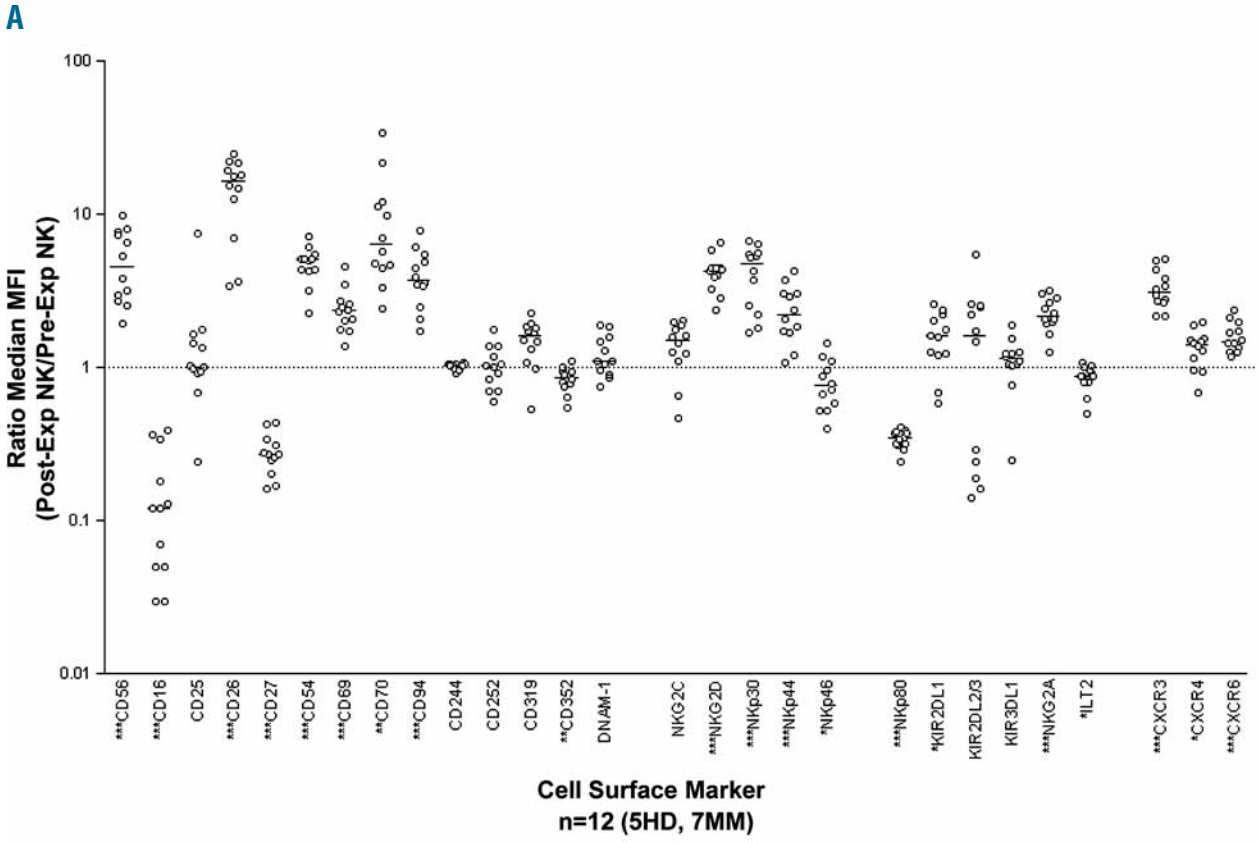
Online Supplementary Table S1. Antibodies and antigens used in the study.

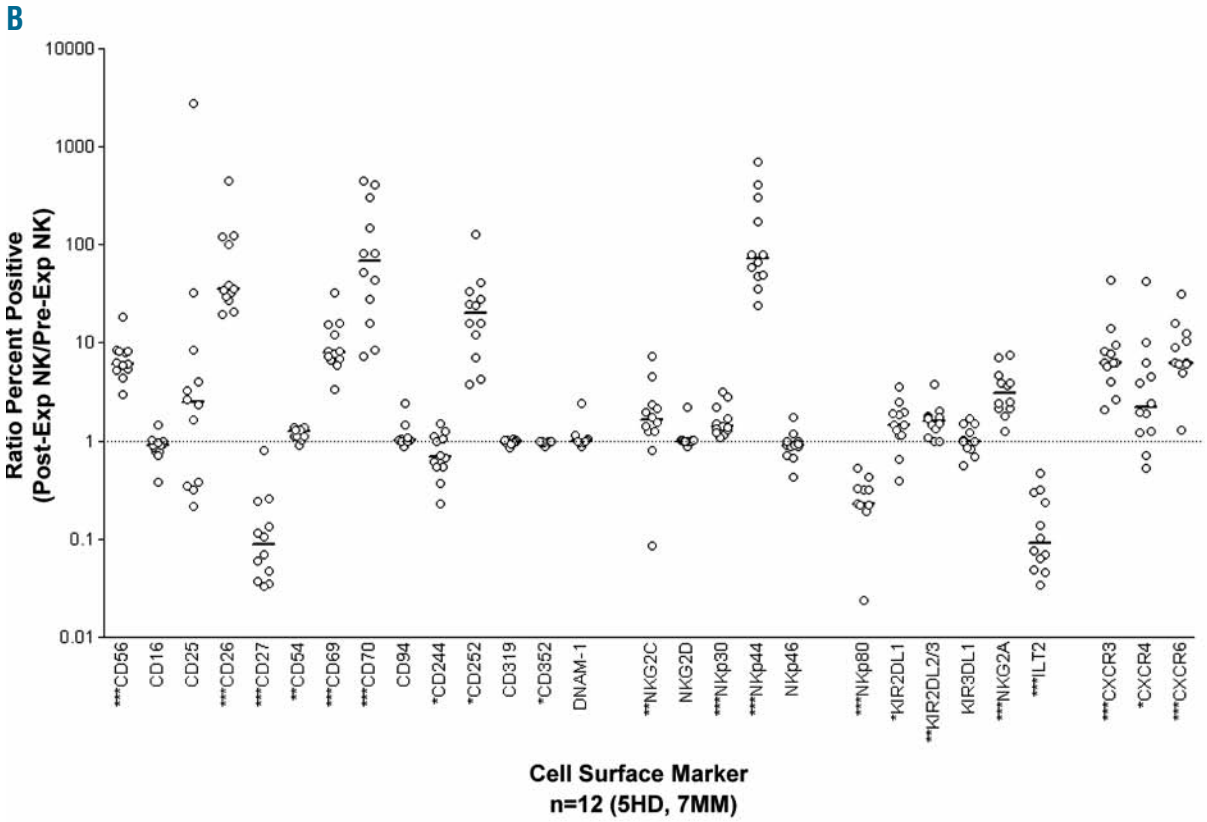
Antigen[a]	Clone/Format	Fluorochrome[b]	Source[c]	Antigen[a]	Clone/Format	Fluorochrome[b]	Source[c]
CD3	Leu-4	FITC	BD	FAS-L (CD178)	NOK-1	Unconjugated	BD Pharmingen
CD3	MRQ-39	Unconjugated	Cell Marque, Inc.	Granzyme A	MOPC-21	PE	BD
CD14	M0P9	PE	BD	Granzyme B	GB11	PE	BD
CD16	3G8	PE	BD	IgG1 Fc Chimera	rh	N/A	R&D Systems
CD19	J3-119	PE	Beckman Coulter	IgG, Rabbit	pAb	Unconjugated	Sigma
CD25	2A3	PE	BD	IgG1, Mouse	pAb	Unconjugated	Sigma
CD26	4EL-1C7 (Ta1)	PE	Beckman Coulter	ILT2 (CD85j/LIR-1)	GHI/75	PE	R&D systems
CD27	M-T271	PE	BD	KIR2DL1 (CD158a)	HP-3E4	PE	BD
CD27	M-T271	Unconjugated	BD Pharmingen	KIR2DL2/3 (CD158b)	CH-L	PE	BD
CD33	906	PE	Beckman Coulter	KIR3DL1 (NKB1, CD158E)	DX9	PE	BD
CD38	HB7	PE	BD	NKG2A	131411	PE	R&D Systems
CD54	JB-2	PE	BD	NKG2C	134591	PE	R&D Systems
CD56	N901 (NHK-1)	APC	Beckman Coulter	NKG2D	149810	PE	R&D Systems
CD56	NCAM16.2	FITC	BD	NKG2D Fc Chimera	rh	N/A	R&D systems
CD57	NK1	Unconjugated	Thermo Scientific	NKG2D-L (MICA)	pAb	Unconjugated	R&D Systems
CD69 (CLECB)	FN50	PE	BD	NKG2D-L (MICB)	6D4	Unconjugated	Serotec
CD69 (CLECB)	FN50	Unconjugated	BD Pharmingen	NKG2D-L (ULBP-1)	170818	Unconjugated	R&D systems
CD70 (CD27L)	Ki-24	PE	BD	NKG2D-L (ULBP-2)	165903	Unconjugated	R&D systems
CD70 (CD27L)	Ki-24	Unconjugated	BD	NKG2D-L (ULBP-3)	166510	Unconjugated	R&D systems
CD94	HP-3D9	PE	BD	NKp30	Z25	PE	Beckman Coulter
CD138 (Syndecan 1)	B-A38	Unconjugated	Biocare Medical	NKp30	210845	Unconjugated	R&D Systems
CD178 (FAS ligand)	NOK-1	PE	eBioscience	NKp30 Fc Chimera	rh	N/A	R&D Systems
CD244 (2B4)	550816	PE	BD	NKp44	253415	Unconjugated	R&D Systems
CD252 (OX40L)	IK-1	PE	BD	NKp44	Z231	PE	Beckman Coulter
CD253 (TRAIL)	RIK-2	PE	BioLegend	NKp44 Fc Chimera	rh	N/A	R&D Systems
CD319 (CS1, SLAMF7)	235614	PE	R&D systems	NKp46	1850NK	Unconjugated	R&D Systems
CD352 (NTB-A)	NT-7	PE	BioLegend	NKp46	BAB281	PE	Beckman Coulter
CXCR3	1C6	PE	BD	NKp46 Fc Chimera	rh	N/A	R&D Systems
CXCR4	12G5	PE	BD	NKp80	pAb	Unconjugated	R&D systems
CXCR6	56811	PE	R&D Systems	NKp80/KLRF1	239127	PE	R&D Systems
DNAM-1	DX11	PE	BD	Perforin	δG9	PE	BD
DNAM-1	102511	Unconjugated	R&D Systems	Osteocalcin	OCG4	Unconjugated	QED Biosciences Inc.
DNAM1-L (CD155/PVR)	pAb	Unconjugated	R&D Systems	TRAIL (CD253)	RIK-2	Unconjugated	BioLegend

[a] All antibodies and isotypes are mouse anti-human monoclonal antibodies unless otherwise specified; [b] FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin; [c] BD (Becton Dickinson, San Jose, CA); Beckman Coulter, Miami, FL; BioLegend, San Diego, CA; Invitrogen, Carlsbad, CA; Serotec, Raleigh, NC; Sigma, St. Louis, MO; R&D Systems, Minneapolis, MN; Biocare Medical, Concord, CA; Thermo-Scientific, Waltham, MA; Novus Biologicals, Littleton, CO; Abnova, Walnut, CA; QED Biosciences; San Diego, CA; eBioscience, San Diego, CA; [d] pAb (polyclonal antibody); [e] rh (recombinant human protein)

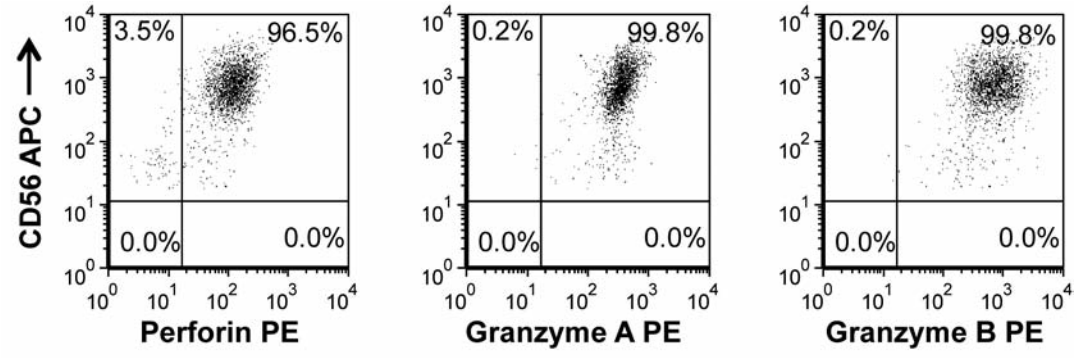


Online Supplementary Figure S1. NK cells expand dramatically after stimulation with K562-mb15-41BBL transfectants. (A) Fold expansion of NK and T-cells for 45 samples (30 MM patients, closed circles, and 15 HD, open circles) and median (–) are depicted. (B) The median percent NK cells in cultures increased from 8.70% on day 0 to 69.4% on day 7 and 88.3% at harvest, while the numbers of T-cells and NKT-cells were low on day 7 and at harvest.

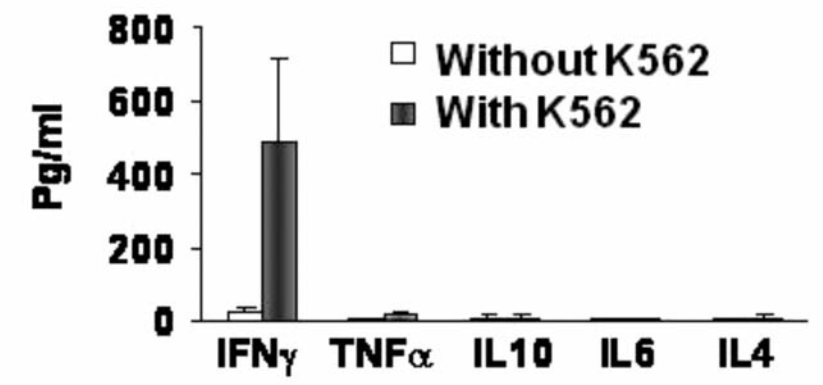




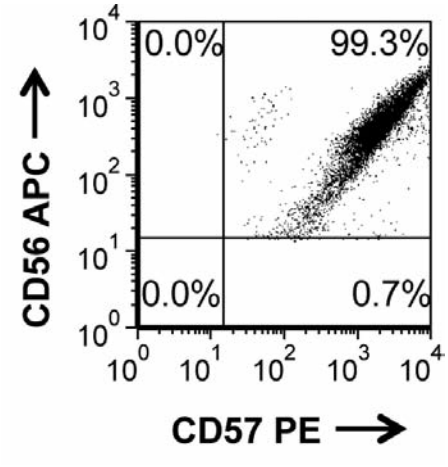
Online Supplementary Figure S2. Stimulation with K562-mb15-41BBL transfectants leads to changes in expression of receptors on exp-NK cells. (A) Post-/pre-expansion MFI ratios for the cell surface receptors. (B) The percent positive ratio for post-/pre-expansion of NK cells for each receptor is shown. Each symbol depicts the value for a HD or MM patient with medians (—). The dashed line represents a ratio = 1 (unchanged).



Online Supplementary Figure S3. Exp-NK cells have high cytoplasmic granular contents. Flow cytometric analysis confirms the presence of cytoplasmic granules in appreciable amounts in exp-NK cells. Representative FACS plots are shown. The numbers in each quadrant are the percentages based on total $CD3^{-}CD56^{+}$ NK cells. (A) Perforin. (B) Granzyme A. (C) Granzyme B.



Online Supplementary Figure S4. Exp-NK cells release interferon-γ (IFN-γ) but not tumor necrosis factor-α (TNF-α), interleukin(IL)10, IL6 or IL4 upon incubation with non-modified target K562 cells.



Online Supplementary Figure S5. Exp-NK cells have high CD57 expression. Flow cytometry was performed on total exp-NK cells, and $CD3^{-}CD56^{+}$ cells were gated and analyzed for expression of CD56 and CD57. A representative FACS plot is shown. The numbers in each quadrant are the percentages based on total $CD3^{-}CD56^{+}$ NK cells.

Online Supplementary Movie. Time lapse microscopy showing patient-derived exp-NK cells killing K562-mb15-41BBL cells in real time. K562-mb15-41BBL cells lose GFP expression almost immediately after being targeted by effectors, co-cultured at a 2:1 E:T ratio. A 42 sec compilation playing at a rate of 9.5 frames per sec is shown; frames were captured at 12 sec intervals for 79 min. SEE MOVIE FILE