Natural killer cell lines preferentially kill clonogenic multiple myeloma cells and decrease myeloma engraftment in a bioluminescent xenograft mouse model

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Online Design and Methods

Cell growth conditions

The U266 human MM cell line was purchased from the American Type Culture Collection (ATCC) and cultured as recommended. The RPMI 8226 and NCI-H929 human MM cell lines were provided by Dr. Suzanne Trudel (Princess Margaret Hospital, Toronto, ON, Canada) and were both cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) with 10% FBS (Sigma-Aldrich). The K562 leukemia cell line (ATCC) was cultured in IMDM with 10% FBS. K562 does not express HLA class I and is, therefore, killed well by NK cell lines. The NK-92 human natural killer cell line was provided by Dr. Hans Klingemann (at the time at Rush University Medical Center, Chicago, IL, USA) and cultured in GM1 medium with 450 IU/mL of IL-2 (Novartis). The KHYG-1 natural killer cell line was purchased from The Human Science Research Resources Bank (JCRB0156; Tokyo, Japan) and cultured in RPMI 1640 medium with 10% FBS and 100-200 IU/mL of IL-2. All cell lines were negative for mycoplasma as tested by PCR.

Cytotoxicity mechanism

Antigen expression on NK and MM cells were evaluated using the following fluorescently conjugated antibodies: DNAM-1-PE, NKp30-APC, NKp46-PE, MICA/MICB-PE, TRAIL DR5-APC, TRAIL DR4-biotin, CD155-PE, CD112-PE (BioLegend), NKp44-PE (Beckman Coulter), NKG2D-PE, ULBP-1-purified, ULBP-2-purified, ULBP-3-purified (R&D Systems). When primary antibodies were not directly conjugated to fluorescent molecules, a secondary fluorescent antibody was used: Streptavidin-PE-Cy7 (eBioscience) and anti-mouse secondary FITC (Sigma-Aldrich). The mean fluorescence intensity (MFI) shown is the ratio of the geometric MFI of the selected monoclonal antibody to the geometric MFI of the isotype control.

Methylcellulose clonogenic assays

After 1-2 weeks, colonies of greater than 50 cells were enumerated on each plate. Low density control samples included separate incubation of target and effector cells in the 96-well plate followed by injecting the samples into one tube of methylcellulose to control for any impact of the NK cells on MM colony formation in methylcellulose. Target and effector cells were also injected separately into methylcellulose to identify the baseline number of MM and NK cell colonies. Pictures of representative methylcellulose plates were taken on a Leica Stereomicroscope at 1.5X magnification. The clonogenic inhibition was calculated according to Equation (1):

$$\%\ 1^{\circ}\ \text{clonogenic inhibition} = \left[\frac{(\#\ \text{colonies}_{LDC}) - (\#\ \text{colonies}_{Treatment})}{(\#\ \text{colonies}_{LDC})}\right] x\ 100\% \quad (1)$$

where #Colonies$_{LDC}$=the number of colonies in the low density control.

Secondary replating of methylcellulose colonies

For replating individual colonies, each colony was plucked, washed and resuspended in 0.2 mL of methylcellulose and plated in one well of a 24-well plate. After two weeks of incubation at 37°C, 5% CO_2 and 100% humidity, colony formation was enumerated. A total of 10 colonies were selected from each sample.

The additional percentage of secondary clonogenic inhibition was determined by multiplying the percentage of residual primary colony growth in Equation (2) by the percentage of secondary clonogenic inhibition in the secondary assay (3). The percentage of cumulative clonogenic inhibition was calculated by adding the percentage of clonogenic inhibition from the primary culture with the additional percentage of clonogenic inhibition defined by colonies that could not self-renew in a secondary methylcellulose culture (4):

$$\%\ \text{residual primary colony growth} = [100 - \%\ 1^{\circ}\ \text{clonogenic inhibition}] \quad (2)$$

$$\%\ \text{additional } 2^{\circ}\ \text{clonogenic inhibition} = [\%\ \text{Residual primary clonogenic growth}]\ x \left[\frac{(\#\ 2^{\circ}\ \text{colonies}_{LDC}) - (\#\ 2^{\circ}\ \text{colonies}_{Treatment})}{(\#\ 2^{\circ}\ \text{colonies}_{LDC})}\right] \quad (3)$$

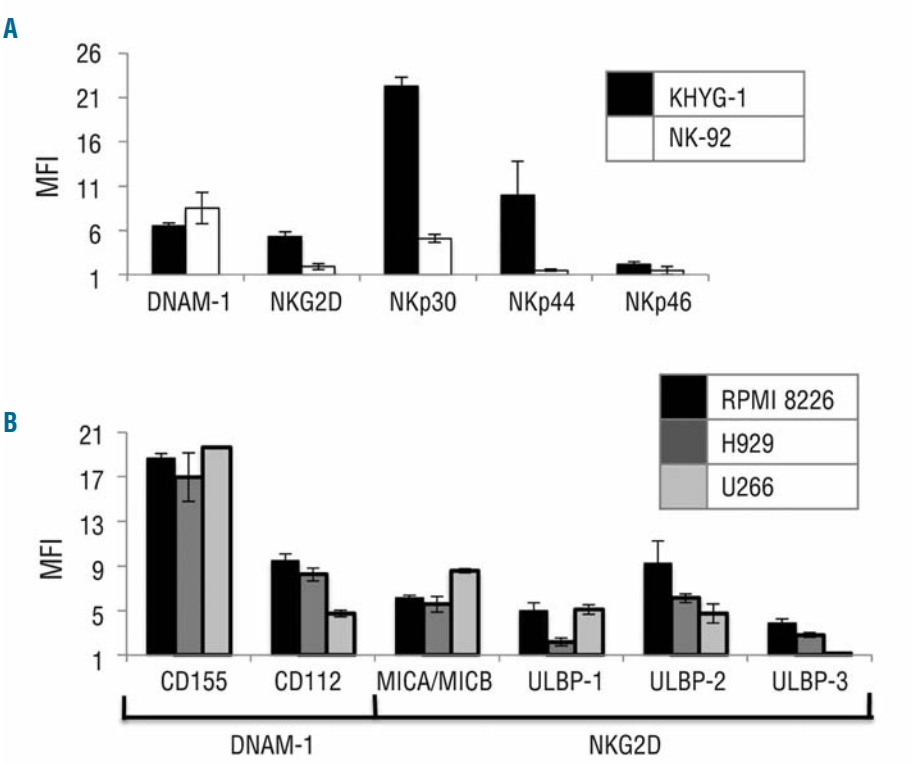
$$\%\ \text{cumulative clonogenic inhibition} = \%\ 1^{\circ}\ \text{clonogenic inhibition} + \%\ 2^{\circ}\ \text{additional clonogenic inhibition} \quad (4)$$

In vivo bioluminescence imaging

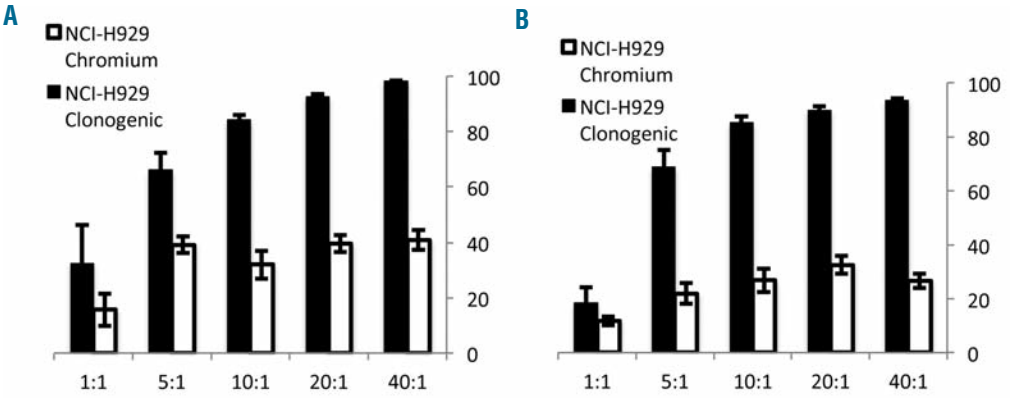
The U266 MM cell line was transduced with a previously developed bicistronic lentiviral vector encoding the cDNAs for enhanced green fluorescent protein and luciferase (eGFPluc) expression.[1] D-Luciferin (Caliper Lifesciences) was injected intraperitoneally at 75 mg/kg 15-20 min prior to imaging. Mice were anesthetized using 5% isofluorane until loss of consciousness then maintained at 2% isofluorane for the duration of imaging. The IVIS® Imaging System and LivingImage™ Software (Caliper Lifesciences) were used to acquire images and quantify bioluminescence, respectively.

References

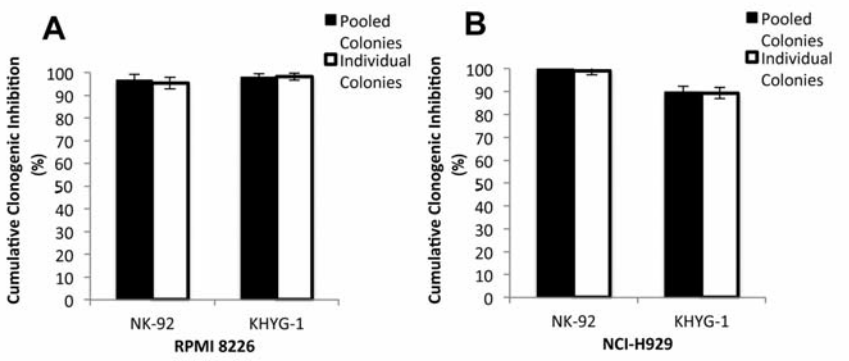
1. Siegers GM, Felizardo T, Mathieson AM, Kosaka Y, Wang X, Median JA, et al. Anti-Leukemia Activity of In Vitro-Expanded Human Gamma Delta T Cells in a Xenogeneic Ph+ Leukemia Model. PLoS ONE. 2011;6(2):1-13.



Online Supplementary Figure S1. (A) Expression of activating receptors on NK cell lines. (B) Expression of DNAM-1 and NKG2D ligands on MM cell lines.



Online Supplementary Figure S2. (A) Preferential killing of clonogenic NCI-H929 cells by NK-92. (B) Preferential killing of clonogenic NCI-H929 cells by KHYG-1.



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	NK-92		KHYG-1	
	Clonogenic Inhibition (Primary)	Cumulative Clonogenic Inhibition	Clonogenic Inhibition (Primary)	Cumulative Clonogenic Inhibition
RPMI 8226	93%	96%	96%	98%
NCI-H929	93%	99%	89%	89%

Online Supplementary Figure S3. Cumulative cytotoxicity representing the sum of the primary cytotoxicity and the number of colonies that cannot self-renew in a secondary replating. The self-renewal was evaluated by pooling primary colonies (black) and replating individual colonies (white). (A) Cumulative cytotoxicity of RPMI 8226 by NK-92 and KHYG-1. (B) Cumulative cytotoxicity of NCI-H929 by NK-92 and KHYG-1. (C) Comparison of cumulative cytotoxicity to the cytotoxicity of primary colonies.