Supplementary Materials for

Inability of granule polarization by NK cells defines tumor resistance and can be overcome by CAR or ADCC mediated targeting

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SUPPLEMENTARY METHODS

Image analysis

To analyze target cell death, we measured the mean fluorescence intensity (MFI) of SYTOX Blue-positive target cells at each time-point. Background intensity was assessed by measuring the MFI of multiple positions without cells at each time-point, and subtracted from the corresponding experimental intensity. Calculated data were used to determine the ratio to the maximum MFI detected for the respective cell, and presented as a percentage. If the target cells were not killed, we used the mean of all maximum MFIs of the corresponding target cell line.

Only conjugates that remained stable for more than 15 minutes were considered to exclude random contacts. Conjugates were further confirmed in 3D analysis, based on the cell membrane labeling and interference contrast using Imaris software.

For granule movement studies in experiments evaluating clustering and polarization, lytic granule segmentation was conducted using the spots function (Imaris) and the automatic threshold based on LysoTracker Red fluorescence intensity, to automatically detect individual granule positions. Granule movement was calculated as previously described (1). Granule clustering was considered as the distance between individual granules and the mean position of all granules within the cell (hereafter referred to as the granules cluster center). The absolute distance of each granule G(xi|yi|zi) to the granules cluster center M(mx|my|mz) was calculated and weighted by the respective number of voxels (V) corresponding to their size. Weighting was performed to reduce bias caused by clustered granules that are difficult to separate by image segmentation. Granule distance (|GM|) and subsequent voxel-weighted granule distance (VW|GM|) were calculated as follows.

$$\left|\overrightarrow{GM}\right|(\mu m) = \frac{\left(\sum_{i=1}^{n}\sqrt{(m_x - x_i)^2 + (m_y - y_i)^2 + (m_z - z_i)^2}\right)}{n}$$

$$VW\left|\overrightarrow{GM}\right|(\mu m) = \sum_{i=1}^{n}\left|\overrightarrow{GM}\right|_i \times \left[\frac{V_i}{\sum_{i=1}^{n} V}\right]$$

Granule polarization was measured as the distance between individual granules and the E/T conjugation point M(mx|my|mz), using the above-described equations.

For “pre-stimulation”, a time point at the beginning of the acquisition or at least one or two frames (3 to 6 minutes) before cell contact was taken. For “post-stimulation”, we monitored each cell over time until polarization was first established (typically between 6 to 15 minutes after cell

contact). For NK-92+MDA-MB-453 we analyzed the time points 6 minutes, 9 minutes and 15 minutes after cell contact, with no differences observed.

To analyze the dynamics, time-points for the respective steps of granule-mediated cytotoxicity for each NK cell were assessed. Specifically, the time-points with the first visible signs of conjugation, polarization, or SYTOX Blue uptake were noted. For presentation of the images, contrast was uniformly adjusted, and the CellMask channel was bleach-corrected in Fiji using the bleach correction function.

Flow cytometry

Cells were stained for 30 min on ice using antibodies specific for CD16 (3G8), LFA-1 (HI111), and ICAM-1 (MOPC-21). ErbB2-CAR detection was performed as previously described (2). Live cells were discriminated using 7-AAD (BD Biosciences). For phosphorylation experiments, cells were fixed using BD Cytofix, permeablized with BD Perm Buffer III (BD Biosciences), and stained with rabbit anti-pAkt, anti-PLCγ1 and anti-pERK1/2 antibodies (Cell Signaling), followed by goat anti-rabbit secondary antibody (Thermofisher Scientific). Samples were acquired using a BD FACSCanto II flow cytometer, and data analyzed using FlowJo software (FlowJo).

Flow cytometry-based conjugation assay

NK and cancer cells were differentially labeled with PKH-67 or PKH-26 membrane dyes (Sigma-Aldrich) according to the manufacturer protocol. Cells were washed 4x times with serum-containing medium and kept further for 1h in complete Xvivo-10 medium at 37°C to wash out residual PKH to protect cross-staining. Samples were next adjusted for the final concentration in

fresh complete Xvivo-10 medium. 1*10^5 NK cells were mixed with 2*10^5 cancer cells in final volume of 200ul, centrifuged (50 x g, 1min) and incubated for 20min at 37°C. The reaction was stopped by brief vortexing and the addition of 300ul of 0.5% paraformaldehyde (PFA, Sigma-Aldrich). Samples were directly acquired by BD FACSCanto II flow cytometer and conjugates were determined as double-positive events.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1

Detachment of CAR- and ADCC- retargeted NK-92. Details of rarely observed detachment events of haNK (A) or NK-92/5.28.z (B) from MDA-MB-453, showing torn pieces of plasma membrane from target cells retained by the NK cells (arrows and enlarged fields).

Supplementary figure 2

Conjugation properties of NK-92 or its retargeted versions with K562 and MDA-MB-453, measured with a standard flow cytometry-based conjugation assay. (A) NK-92, NK-92/5.28.z or haNK+Herceptin were labeled with PKH-67 while K562 or MDA-MB-453 were labeled with PKH-26. NK cells were incubated with targets for 20 min at 37°C as indicated. 0min control was used to exclude unspecific binding or cross-staining. Cells were fixed and analysed by flow cytometry. Percentage of NK cells in conjugates was calculated from all NK cells. Pooled data from 3 independent experiments performed in triplicates are shown. Data represent mean $\pm$SEM. (B) Dot plots show gating strategy where NK cells are identified as PKH-67+ PKH-26-, target

cells as PKH-67- PKH-26+ and conjugates as PKH-67+ PKH-26+. (C) Dot plots show overlay of conjugates and all acquired events, demonstrating position of conjugates in doublet region.

Supplementary figure 3

Gating strategy for protein phosphorylation analysis by flow cytometry (belongs to **Figure 7**). Singlets were selected based on FSC and SSC parameters. NK cells were identified by CD56 staining and further plotted for anti-phospho proteins as shown in **Figure 7A**. NK-92/5.28.z, MDA-MB-453 and 20min or 60min co-culture are shown as representative examples.

SUPPLEMENTARY REFERENCES

1. Mentlik AN, Sanborn KB, Holzbaur EL, Orange JS. Rapid lytic granule convergence to the MTOC in natural killer cells is dependent on dynein but not cytolytic commitment. Mol Biol Cell. 2010;21(13):2241-56.
2. Nowakowska P, Romanski A, Miller N, Odendahl M, Bonig H, Zhang C, et al. Clinical grade manufacturing of genetically modified, CAR-expressing NK-92 cells for the treatment of ErbB2-positive malignancies. Cancer immunology, immunotherapy : CII. 2017.