Tyrosinase-mediated synthesis of nanobody-cell conjugates

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Materials and Methods

General Methods and Instrumentation

All reagents were obtained from commercial sources and used without any further purification. Tyrosinase isolated from *Agaricus bisporus* (abTYR, both 25 kU [SKU = T3824-25KU] and 50 kU [SKU = T3824-50KU] were used in these studies) from Sigma-Aldrich. FITC-5-maleimide, CFSE, MitoTracker Red, Calcein AM dye, and propidium iodide were all purchased from ThermoFisher. FITC-labeled HER2 was purchased from AcroBiosystems. Alkyne-tyramide probe was purchased from MedChemExpress. Spin concentrators with 10 and 100 kDa molecular weight cutoffs (MWCO) and sterile spin filters with 0.22 µm pores were purchased from Millipore (Billerica, MA). Doubly distilled water (ddH₂O) was obtained from a Millipore purification system. Antibodies used in this paper are as follows: anti-hNKG2D (clone 1D11, BioXCell); anti-hMICA/B (clone 6D4, ThermoFisher); mouse isotype control (clone MOPC-21; BioXCell).

**Liquid chromatography mass spectrometry (LC-MS) analysis.** Acetonitrile (Optima grade, 99.9%, Fisher, Waltham, MA), formic acid (1 mL ampules, 99+%, Pierce, Rockford, IL), and water purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA) were used to prepare mobile phase solvents for LC-MS. Electrospray ionization mass spectrometry (ESI-MS) of protein bioconjugates was performed using an Agilent 1260 series liquid chromatograph outfitted with an Agilent 6224 time-of-flight (TOF) LC-MS system (Santa Clara, CA). The LC was equipped with a Proswift RP-4H (monolithic phenyl, 1.0 mm × 50 mm) analytical column. Solvent A was 99.9% water/0.1% formic acid and solvent B was 99.9% acetonitrile/0.1% formic acid (v/v). For each sample, approximately 15 to 30 picomoles of analyte were injected onto the column. Following sample injection, a 5-100% B elution gradient was run at a flow rate of 0.30 mL/min over 8 min. Data was collected and analyzed by deconvolution of the entire elution profile in order to provide reconstructed mass spectra that are representative of the entire sample using Agilent MassHunter Qualitative Analysis B.05.00. Percent modification was determined through integration of MS peaks using open-source Chartograph software (www.chartograph.com). The integration of the completely unmodified protein peak served as an internal standard in determining the percent modification.

**UV-VIS measurements.** A NanoDrop 1000 (Thermo) was used to quantify the samples in this work based on absorbance values at 280 nm (or 488 nm for sfGFP).

**Flow cytometry.** A ThermoFisher Attune NxT flow cytometer was used for flow analysis. For every dye used, the optimal laser was selected based on the ThermoFisher fluorophore selection guide. Voltage settings were determined using single color and unlabeled samples. For all experiments, 10,000 events were collected on an initial gate in the FSC-A vs SSC-A. The gating scheme employed involved an initial gate on the cell population in the FSC-A vs SSC-A, then a doublet discrimination in the FSC-A vs FSC-H, then a live-dead discrimination in the propidium iodide channel (BL3-H), to produce a histogram of the corresponding sfGFP or FITC signal (BL1-H).

**Statistical analyses.** For relevant data, a two-tailed T-test was performed to determine statistical significance. A p-value less than 0.001 is denoted with ***, a p-value of less than 0.01 is denoted with **, and a p-value of less than 0.05 is denoted with *.

### Synthetic Procedures

**Synthesis of FITC-labeled nbGFP_{A77C} (nbFITC).** A 1 mL labeling reaction was performed by combining 211 µL of 474 µM nbGFP_{A77C} ([f] = 100 µM) with 250 µL of 10 mM FITC-maleimide ([f] = 2.5 mM, ThermoFisher, dissolved in 50% DMF:H₂O) and diluted to 1 mL via the addition of 539 µL of DPBS. The whole reaction mix was wrapped in tinfoil and rotated end-over-end for 2 h at RT. The reaction was purified using a NAP-10 column (GE Healthcare) and the collected labeled protein was concentrated using a 10 kDa MWCO spin filter (Corning). The reaction was analyzed using an ESI-QTOF mass spectrometer.

**Synthesis of nbHER2_{SG4Y}-sfGFP conjugate.** Protein-protein conjugation reactions were prepared at a 100 µL scale by combining 12.6 µL of a 478 µM solution of nbHER2_{SG4Y} ([f] = 60 µM) and 10 µL of a 100 µM solution of sfGFPY200C ([f] = 10 µM) with 2.4 µL of a 16.7 µM solution of abTYR ([f] = 400 nM) in 55 µL of ddH₂O with 20 µL of 100 mM phosphate buffer at pH 6.5 ([f] = 20 mM). The reaction was allowed to proceed for 45 min at RT, after which the activity of abTYR was halted via the addition of 2 µL of 100 mM tropolone. To help remove some of the excess nanobody, the reaction was spun four times against 30 kDa MWCO spin filters (Corning) using DPBS, and the reaction was then analyzed using an ESI-QTOF mass spectrometer.

### Protein Cloning and Expression Procedures

Sequences for nbGFP-SG4Y (nbGFP_{A77C}) and nbHER2-SG4Y (nbHER2_{SG4Y}) were purchased as gene blocks from International DNA Technologies, Inc. (IDT), designed to have the open reading frame (ORF) inserted into a pET28b(+) expression vector using Gibson assembly. Thus, a pET28b(+) vector was linearized by digestion with XbaI and XhoI for 3 h at 37 °C and purified by column (QiAquick PCR Purification Kit, QIAGEN).
The gene blocks contained 21 and 27 base pairs of homology with the linearized pET28b vector (underlined) that contained the XbaI/Xhol cut sites (bold). The sequences for the nanobodies (UPPERCASE) contained a C-terminal His6 tag for purification (red) and an SG4Y handle for tyrosinase chemistry (blue):

\[
\begin{align*}
\text{nbGFP-SG4Y} & \quad \text{nbGFP}_{\text{Cys}} \quad \text{nbGFP}_{\text{Tyr} A76C} \\
\text{nbHER2-SG4Y} & \quad \text{nbHER2}_{\text{Cys}} \quad \text{nbHER2}_{\text{Tyr} A76C}
\end{align*}
\]

Which corresponds to the following amino acid sequence:

\[
\begin{align*}
\text{MADVQLVESGGALVQPGGSLRSLSCAASGFPVNRYSNRRQAPGKEREWVAGMSAGDRSSEDSV}
\end{align*}
\]

Both with insert and vector ready for Gibson assembly, 50 ng of linearized vector was combined with insert in a 3:1 insert:vector molar ratio in a volume of 10 μL, and added to 10 μL of 2X Gibson Assembly® Master Mix (New England Biolabs). The resulting mixture was incubated for 1 hr at 50 °C and then transformed into XL1-Blue competent cells via a 45 s, 42 °C heat shock and plated on LB/kanamycin. Resulting colonies were used to inoculate 6 mL LB/kanamycin cultures (50 μg/mL) and grown overnight at 37 °C, 225 RPM. Plasmid DNA was isolated using a Qiagen Qiaprep Spin Miniprep Kit and sequenced using the pET primer: 5'-CTCGATCCCGCGAAATTA-3' to confirm insertion of the desired protein sequence.

The nbGFP-SG4C (\text{nbGFP}_{\text{Cys}}) and the 

\[
\begin{align*}
\text{nbGFP}_{\text{Tyr} A76C} & \quad \text{nbGFP}_{\text{Tyr} A76C} \\
\end{align*}
\]

The plasmids were then sequenced using the pET primer to confirm cysteine point mutations (pink):
Culturing of human PBMCs. Five million isolated human PBMCs from AllCells were cultured for 3 days in 5 mL of complete media (RPMI-1640 containing 10% FBS, 2 mM L-glutamine, and 30 U/mL recombinant human IL-2), at 37 °C, 5% CO₂ in a T-25 flask prior to modification with tyrosinase.

Culturing of SK-BR-3 and MDA-MB-468 cells. Cells were cultured in phenol-red containing DMEM supplantsed with 10% FBS and grown at 37 °C, 5% CO₂. After thawing and initial recovery in 5 mL of complete media for 3-4 days in a T-25 flask (Falcon), cells were diluted down to a concentration of 0.2E6 cells/mL in either T-75 or T-125 flasks (Falcon). Cells were allowed to reach a maximal density of ~2E6 cells/mL, typically at day 4 after passage, after which they were trypsinized and passaged at a 1:4 split ratio in complete DMEM.

Culturing of Jurkat cells. Jurkat cells were acquired from ATCC and cultured in RPMI 1640 supplemented with 10% FBS and grown at 37 °C, 5% CO₂. After thawing and initial recovery in 5 mL of complete media for 3-4 days in a T-25 flask (Falcon), the cells were collected by centrifugation at 8000 rpm for 15 min at 4 °C, then frozen at -80 °C for 20 min. The cells were thawed and resuspended in 20 mL of Ni-NTA equilibration buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4) with 0.1 mM - 1 mM PMSF. They were then sonicated for 6.67 min on ice, with 2 s bursts followed by 4 s rest time (20 min total time per sample) at 85% amplitude. Lysed cells were centrifuged at 12,000 g at 4 °C for 8 min. Next, the supernatant was loaded onto a spin column with 3 mL Ni-NTA resin and rotated for 30 min at 4 °C. The resin was washed 10x with 6 mL of Ni-NTA wash buffer (20 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole, pH 7.4), and the protein was eluted 3x with 3 mL Ni-NTA elution buffer (20 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.4). The fractions were collected, buffer-exchanged into PBS, and concentrated using Amicon Ultra 10 kD MWCO centrifugal concentrators (MilliporeSigma).

Experimental Procedures

In all protocols, DPBS refers to Dulbecco’s phosphate buffered saline without Mg²⁺ or Ca²⁺. These divalent cations should be avoided as they can interfere with abTYR activity.

Culturing of NK-92MI cells. NK-92MI cells were acquired from ATCC and stored on liquid N₂ until use. Cells were gently thawed at 37 °C for 1-2 min until no ice was visible in the tube, after which the cells were gently transferred into ~ 5 mL of chilled Myelocult H5100 media (StemCell Technologies). The cells were spun at ~ 125 g for 5 min and the supernatant was gently distributed into a fresh T-75 flask (Falcon) and allowed to recover for 3-4 days at 37 °C, 5% CO₂, and the viability diminished.

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Culturing of SK-BR-3 and MDA-MB-468 cells. Cells were cultured in phenol-red containing DMEM supplantsed with 10% FBS and grown at 37 °C, 5% CO₂ in either T-25 or T-75 flasks (Falcon). Cells were allowed to reach a maximal confluency of ~80%, typically at day 4 after passage, after which they were trypsinized and passaged at a 1:4 split ratio in complete DMEM.

Culturing of human PBMCs. Five million isolated human PBMCs from AllCells were cultured for 3 days in 5 mL of complete media (RPMI-1640 containing 10% FBS, 2 mM L-glutamine, and 30 U/mL recombinant human IL-2), at 37 °C, 5% CO₂ in a T-25 flask prior to modification with tyrosinase.

Sequenced plasmids were then transformed into BL21(DE3)Star competent cells via a 45 s, 42 °C heat shock and plated on LB/kanamycin cultures (50 μg/mL) and grown overnight at 37 °C, 225 RPM. A 10 mL portion was then added to 1 L of LB media and grown at 37 °C to an OD600 of 0.6-0.8. Expression was induced with a final concentration of 0.1 mM IPTG, switching the temperature to 18 °C. After 18-24 h the cells were collected by centrifugation at 8000 rpm for 15 min at 4 °C, then frozen at -80 °C for 20 min. The cells were thawed and resuspended in 20 mL of Ni-NTA equilibration buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4) with 0.1 mM - 1 mM PMSF. They were then sonicated for 6.67 min on ice, with 2 s bursts followed by 4 s rest time (20 min total time per sample) at 85% amplitude. Lysed cells were centrifuged at 12,000 g at 4 °C for 8 min. Next, the supernatant was loaded onto a spin column with 3 mL Ni-NTA resin and rotated for 30 min at 4 °C. The resin was washed 10x with 6 mL of Ni-NTA wash buffer (20 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole, pH 7.4), and the protein was eluted 3x with 3 mL Ni-NTA elution buffer (20 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.4). The fractions were collected, buffer-exchanged into PBS, and concentrated using Amicon Ultra 10 kD MWCO centrifugal concentrators (MilliporeSigma).

Which corresponds to the following amino acid sequence:

\[
\text{(M)ADVQLVESGALVQPGSRLSCLASAGFPVRASMRWYRQAPGKER}
\]

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Culturing of human PBMCs. Five million isolated human PBMCs from AllCells were cultured for 3 days in 5 mL of complete media (RPMI-1640 containing 10% FBS, 2 mM L-glutamine, and 30 U/mL recombinant human IL-2), at 37 °C, 5% CO₂ in a T-25 flask prior to modification with tyrosinase.

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\[
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\]
To thaw these cells, in brief, a cryovial containing the cells was incubated in a 37 °C water bath for 10 minutes. The cells were transferred to a 50 mL tube, and warm complete media (19 mL) was added at a rate < 5 mL/min. The cells were then spun down at 125 xg, and washed with 1 mL of complete media, two times. Five million cells were then transferred to 5 mL of complete media in a T-25 flask.

Attachment of nanobodies to cell-surfaces. The following general protocol has been found to yield reproducible levels of cell labeling with tyrosine-tagged proteins:

1. Start by counting cells - each reaction will require ~1 - 2 million cells (technical note: if more cells are required in downstream applications prepare additional replicates of each sample, do not scale the reaction beyond 2 million cells).
2. Pool the required number of cells and spin down at ~300 xg for 2 min.
3. Remove supernatant and resuspend cells in ~ 1 mL of DPBS. Repeat the centrifugation step and remove the supernatant to wash the cells. Repeat until a total of three washes have been completed. On the final wash, resuspend the cells in 1 mL of DPBS and count the cells.
4. Aliquot the cells so there are ~ 1 million cells per tube. Add 1 mL of DPBS to each tube so there is extra volume and perform a final spin down at ~ 300 g for 2 min.
5. Remove the supernatant and perform a labeling reaction with 10 µM of tyrosine-tagged nanobody, 400 nM abTYR all in DPBS and at a final volume of 400 µL.
6. Oxidation controls should be prepared in the absence of abTYR, and depending on the downstream application, isotype controls should be prepared using 10 µM of a different tyrosine-tagged nanobody and abTYR. Untreated controls, consisting of just 400 µL of DPBS, should also be prepared.
7. Allow all reactions to proceed for 10 min at 37 °C, 5% CO₂. After the 10 min, add 1 mL of DPBS to each tube and spin the tube at ~300xg for 2 min (be sure no FBS or media is present at this step as the abTYR in the reaction will form reactive intermediates with free amino acids and proteins in these mixtures).
8. Wash the cells until a total of two washes have been performed and move on to any downstream applications.

Secondary Labeling of nanobody-cell conjugates with sfGFP. The following general protocol was used to verify successful cell conjugation:

1. Follow the general protocol for the attachment of nanobodies to cell-surfaces as above, being sure to use 10 µM of nbGFP₉ in the reaction.
2. After the 10 min labeling step is done, add 1 mL of DPBS to each tube and spin the tube at ~300xg for 2 min (be sure no FBS or media is present at this step as the abTYR in the reaction will form reactive intermediates with free amino acids and proteins in these mixtures).
3. Remove the supernatant and resuspend the cells in 1 mL of warm cell binding buffer (5% FBS with 1% w/v of NaN₃ all in DPBS).
4. Perform a second spin down and remove the supernatant. Bring each sample up in 200 µL of 1 µM sfGFP in DPBS and allow the samples to sit on ice for 30 min.
5. After 30 min, add 1 mL of cell binding buffer and spin down the cells at ~ 300xg for 2 min.
6. Remove the supernatant and repeat the wash steps until a total of three washes have been completed.
7. Resuspend the cells in 1 mL of cell binding buffer supplemented with 2 µL of propidium iodide stock solution (1 mg/mL).
8. Store all cells on ice and analyze using an NxT Attune flow cytometer (ThermoFisher).

Secondary Labeling of nanobody-cell conjugates with FITC-HER2.

1. Follow the general protocol for the attachment of nanobodies to cell-surfaces as above, being sure to use 10 µM of nbHER2₉ in the reaction. As an isotype control, label some cell with 10 µM of nbGFP₉ using 400 nM abTYR.
2. After the 10 min labeling step is done, add 1 mL of DPBS to each tube and spin the tube at ~300xg for 2 min (be sure no FBS or media is present at this step as the abTYR in the reaction will form reactive intermediates with free amino acids and proteins in these mixtures).
3. Remove the supernatant and resuspend the cells in 1 mL of warm cell binding buffer (5% FBS with 1% w/v of NaN₃ all in DPBS).
4. Perform a second spin down and remove the supernatant. Bring each sample up in 200 µL of 10 µg/mL of FITC-HER2 (AcroBiosystems) in DPBS and allow the samples to sit on ice for 30 min. After 30 min, add 1 mL of cell binding buffer and spin down the cells at ~ 300 x g for 2 min.

5. Remove the supernatant and repeat the wash steps until a total of three washes have been completed.

6. Resuspend the cells in 1 mL of cell binding buffer supplemented with 2 µL of propidium iodide stock solution (1 mg/mL).

7. Store all cells on ice and analyze using an Nxt Attune flow cytometer (ThermoFisher).

**Protocol for determining density of nanobodies on cell surface.** Cells were pooled and centrifuged at ~ 300 x g for 3 min. The supernatant was removed and the cells were resuspended in 1 mL portion of warm DPBS two times. After resuspening, cells were resuspended in 1 mL of DPBS and a cell density measurement was taken. Approximately 1E6 cells were transferred to each tube and then diluted with 1 mL of DPBS. A final spin down at ~ 300 x g for 2 min was performed and the supernatant was removed. Labeling reactions were performed using 10 µM, 5 µM, or 1 µM final concentrations of nbFITC with 400 nM abTYR in 400 µL of DPBS. A control reaction was run using 10 µM final concentration of nbFITC with no abTYR in 400 µL DPBS, as well as an untreated control consisting of just 400 µL of DPBS. The labeling reactions were allowed to proceed for 10 min at 37 °C, 5% CO₂. After the reaction was complete, the reactions were diluted with 1 mL of warm DPBS and centrifuged at ~ 300 x g for 2 min. The supernatant was removed and the cells were resuspended in 1 mL of warm cell binding buffer (DPBS supplemented with 5% FBS and 1% NaN₃ w/v) two times. The cells were resuspended in 1 mL of warm cell binding buffer with the 2 µL of propidium iodide stock solution (1 mg/mL). All cells were stored on ice and analyzed using an Nxt Attune flow cytometer (ThermoFisher). For FITC density measurements, the protocol form the Quantum FITC-5 MESF kit from Bangs Laboratories was used.

**Protocol for determining half-life of nanobodies on cell surface.** Approximately 10 million NK-92MI cells were labeled using nbGFP_, as described above in batches of 1 million NK-92MI cells per reaction. As a control, 10 million NK-92MI cells were incubated with DPBS. After modification cells were resuspended in Myelocult H5100 to a final density of ~0.5e6 cells/mL, and ~1.5 mL of this solution was pipetted into 6 well TC treated plates. At the indicated time points the full 1.5 mL volume of cells was removed from the plate and subjected to a 2º labeling with 0.5 µM of sfGFP as described above. After labeling and rinsing, cells were taken up in 0.5 mL of 2% para-formaldehyde in DPBS and allowed to fix at room temp for 8 mins. Immediately after fixing, cells were diluted with 1 mL of DPBS, spun down at 300xg for 2 min, and washed with another 1 mL of DPBS. After the final rinse, the fixed cells were resuspended in 1 mL of DPBS, wrapped in foil, and stored at 4 °C until analysis using flow cytometry. After analysis, MFI were plotted vs time and the data was fit to an exponential decay in GraphPad Prism.

**Protocol for imaging subcellular location of nanobody attachment.** Cells were modified with 10 µM nbFITC in the presence or absence of 400 nM abTYR according to the above protocol. After attachment, cells were washed two times with 1 mL portions of cell binding buffer (5% FBS with 1% w/v NaN₃ in DPBS) and then resuspended in 1 mL of cell binding buffer. To this was added 1 µL of CellMask Deep Red (ThermoFisher) cell membrane dye stock solution, and the cells were stained for 10 mins at 37 °C. After cell membrane staining, cells were washed two times with 1 mL portions of cell binding buffer and then fixed with 1 mL of 4% PFA (v/v) in DPBS for 10 min at RT. After fixing, cells were imaged using a Zeiss LSM880 confocal microscope.

**Small molecule proteomics experiment.** Jurkat cells were grown to a density of 2e6 cells/mL in T-125 flasks (Falcon) in ~50-100 mL of complete RPMI 1640 media and then ~10 million Jurkat cells were pelleted at 300 g for 5 min. Cell pellets were washed with 50 mL of DPBS two times. Jurkat cells were labeled in a 10 mL reaction in pre-warmed DPBS with alkynyl-tyramide probe (+100 µL of 10 mM alkynyl-tyramide probe, [f] = 10 µM) and abTYR (+240 µL of a 16.7 µM abTYR stock [~2mg/mL], [f] = 400 nM). The labeling reaction was allowed to proceed for 20 min at 37 ºC with shaking at 220 rpm. After 20 min, the reaction was diluted with 40 mL of DPBS and pelleted at 300 g for 5 min. The supernatant was removed and the pellet was washed two more times with 50 mL of DPBS. Cells were resuspended in 1 mL of RIPA lysis buffer (ThermoFisher) supplemented with protease inhibitor cocktail (ThermoFisher). Cells were lysed using probe ultrasonication (20% amplitude, 10 cycles of 5 sec on/1 sec off). The probe-labeled proteome sample was diluted to a final concentration of 2 mg/mL of protein in solution in 2 mL of DPBS. The diluted proteome sample was then aliquoted at 500 µL volumes into four separate 2 mL eppendorf tubes. To each tube was then added 10 µL of a 5 mM N₇-TEV-biotin tag (9.24 mg/mL). To this mix was then added 10 µL of freshly prepared 50 mM TCEP solution (in water) as well as 30 µL of TBTA ligand solution (0.9 mg/mL in DMSO:t-butanol = 1:4) and the mixture was vortexed. To this was added 10 µL of 50 mM Cu(II) Sulfate (12.5 mg/mL in water) and the sample was vortexed. The reaction was incubated at RT for 1.5 hr with vortexing every 15 min (note: at this stage the proteins will start to precipitate and the reaction will grow cloudy). After the reaction, the tubes were centrifuged at 6500 g for 4 min. The supernatant was removed from the pelleted proteins, and to the pellet in each tube was added 500 µL of pre-chilled MeOH. The pellets were then resuspended using probe ultrasonication (20% amplitude for 10 sec). All four resuspended pellets were then combined into a single 2 mL eppendorf tube, and the combined samples were centrifuged at 6500 g for 4 min at 4 ºC. The supernatant was removed, and the combined pellet was washed two more times with 500 µL
then centrifuged at 1400 g for 3 min and the supernatant was removed. The beads were washed via the addition of 5 mL of PBS. Using a 200 µL pipette tip with its end cut off, 170 µL of washed beads were transferred to a 15 mL conical containing 5 mL of PBS. The supernatant was then removed from the centrifuged proteomic sample and applied to the 15 mL conical containing the beads ([f] of SDS in the sample is 0.2%). The labeled proteome sample rotated overnight at 4 °C and was allowed to bind to the streptavridin resin.

After incubating overnight, the proteome samples were rotated for 2-4 hr at RT to resolubilize the SDS. The conical was then centrifuged at 1400 g for 3 min and the supernatant was removed. The beads were washed via the addition of 5 mL of 0.2% SDS/PBS (w/v), placed on a rotator for 10 min, and then spun at 1400 g for 3 min and the supernatant removed. The beads were transferred to Micro Bio-Spin columns using two washes of 500 µL of PBS and washed on a vacuum manifold using three 1 mL portions of PBS. The beads were then washed with three 1 mL portions of water. The beads were transferred to a new eppendorf tubes using two 250 µL washes of freshly prepared 6 M urea/PBS (1.8g/5mL). To the tube was added 25 µL of freshly prepared DTT solution (30 mg/mL in water) and the tube was incubated at 65 °C for 20 min. The tube was then allowed to cool to RT before adding 25 µL of freshly prepared 400 mM iodoacetamide solution (74 mg/mL in water). The tube was incubated with agitation at 37 °C for 30 min. The reaction was diluted by adding 950 µL of PBS and then spun at 1400 g for 2 min to remove the supernatant. To the tube was added a premixed solution of 200 µL of 2 M urea/PBS, 2 µL of 100 mM calcium chloride in water, and 4 µL of trypsin solution (20 µg of lyophilized trypsin in 40 µL of trypsin buffer). The tube was allowed to incubate overnight in a shaking incubator at 37 °C, being sure that the reaction did not proceed for longer than 21 hr.

The next day, the beads were transferred to a Bio-Spin column and the supernatant was removed. The beads were washed with three portions of 500 µL PBS followed by three portions of 500 µL of water. The beads were then transferred to a new eppendorf tube using 2 portions of 500 µL of water and spun down at 1400 g for 2 min. The beads were then washed with 1 x TEV buffer (141 µL water, 7.5 µL of 20 x TEV buffer, 1.5 µL of 100 mM DTT) and spun down again. The supernatant was removed, and to the beads was added the premixed solution of TEV buffer as above, this time containing 5 µL of Ac-TEV protease. The TEV elution step was allowed to proceed for at least 24 hr at 29 °C with mild agitation, after which the beads were transferred to a new Bio-Spin column and the released peptides were eluted into a new eppendorf tube via centrifugation at 1400 g for 2 min. The beads were washed with two portions of 75 µL of water and the washes were combined with the initial eluent. To the eluted peptide sample was added 16 µL of LC-MS grade formic acid, and the samples were stored at -80 °C until mass spectrometric analysis.

Data were extracted in the form of MS1 and MS2 files using Raw Extractor v.1.9.9.2 (Scripps Research Institute) and searched against the Uniprot human database using ProLuCID search methodology in IP2 v.3 (Integrated Proteomics Applications, Inc.). Cysteine residues were searched with a static modification for carboxyaminomethylation (+57.02146) and the TEV tag probe modification +615.3129. Lysine residues were searched for the TEV tag probe modification +613.29724. Histidine residues were searched for the TEV tag probe modification +613.29724. Peptides were required to be fully tryptic peptides and to contain the TEV modification. ProLuCID data were filtered through DTASelect to achieve a peptide false-positive rate below 5%. After filtering assigning modifications, all peptides were filtered based on the presence of the TEV tag probe modification and any duplicates were removed and the sum of observed cysteine, lysine, and histidine modifications were extracted. A subcellular protein localization analysis was performed using code previously reported.4

Protein-"BAIT" proteomics experiment. Jurkat cells were grown to a density of 266 cells/mL in T-125 flasks (Falcon) in ~50-100 mL of complete RPMI 1640 media and then ~100 million Jurkat cells were pelleted at 300 g for 5 min. Cell pellets were washed with 50 mL of DPBS two times. Jurkat cells were labeled in a 25 mL reaction in pre-warmed DPBS with nbBAIT (+3 µL of 83 µM biotinylated nbGFP[52], A76C, [f] = 10 µM) and abTYR (+600 µL of a 16.7 µM abTYR stock [~2mg/mL], [f] = 400 nM). The labeling reaction was allowed to proceed for 10 min at 37 ºC with shaking at 220 rpm. After 10 min, the reaction was diluted with 40 mL of DPBS and pelleted at 300 g for 5 min. The supernatant was removed and the pellet was washed two more times with 50 mL of DPBS. Cells were resuspended in 1 mL of RIPA lysis buffer (ThermoFisher). Cells were lysed using probe ultrasonication (20% amplitude, 10 cycles of 5 sec on/1 sec off). Proteins were precipitated by 20% cold TCA dissolved in water, and the tubes were centrifuged at max speed for 1 min. The supernatant was removed from the pelleted proteins, and each pellet was washed with 1 mL of pre-chilled MeOH three times. After the last wash step, the supernatant was removed and the pellet was resuspended in 1 mL of 1.2% SDS/PBS (w/v). The resuspended pellet was then heated at 80-90 ºC for 5 min and then centrifuged at 6500 g for 5 min. Meanwhile, streptavidin agarose beads were washed with 1 mL portions of PBS three times and then resuspended in their original volume wih PBS. Using a 200 µL pipette tip with its end cut off, 170 µL of washed beads were transferred to a 15 mL conical containing 5 mL of PBS. The supernatant was then removed from the centrifuged proteomic sample and applied to the 15 mL conical containing the beads ([f] of SDS in the sample is 0.2%). The labeled proteome sample rotated overnight at 4 °C and was allowed to bind to the streptavridin resin.

After incubating overnight, the proteome samples were rotated for 2-4 hr at RT to resolubilize the SDS. The conical was then centrifuged at 1400 g for 3 min and the supernatant was removed. The beads were washed via the addition of 5 mL of of pre-chilled MeOH. After the last wash step, the supernatant was removed and the pellet was resuspended in 1 mL of 1.2% SDS/PBS (w/v) and sonicated for several seconds until the solution turned clear. The resuspended pellet was then heated at 80-90 ºC for 5 min and then centrifuged at 6500 g for 5 min. Meanwhile, streptavidin agarose beads were washed with 1 mL portions of PBS three times and then resuspended in their original volume with PBS. Using a 200 µL pipette tip with its end cut off, 170 µL of washed beads were transferred to a 15 mL conical containing 5 mL of PBS. The supernatant was then removed from the centrifuged proteomic sample and applied to the 15 mL conical containing the beads ([f] of SDS in the sample is 0.2%). The labeled proteome sample rotated overnight at 4 °C and was allowed to bind to the streptavridin resin.
0.2% SDS/PBS (w/v), placed on a rotator for 10 min, and then spun at 1400 g for 3 min and the supernatant removed. The beads were transferred to Micro Bio-Spin columns using two washes of 500 µL of PBS and washed on a vacuum manifold using three 1 mL portions of PBS. The beads were then washed with three 1 mL portions of water. The beads were transferred to eppendorf tubes using two 250 µL washes of freshly prepared 6 M urea/PBS (1.8g/5mL). To the tube was added 25 µL of freshly prepared DTT solution (30 mg/mL in water) and the tube was incubated at 65 °C for 20 min. The tube was then allowed to cool to RT before adding 25 µL of freshly prepared 400 mM iodoacetamide solution (74 mg/mL in water). The tube was incubated with agitation at 37 °C for 30 min. The reaction was diluted by adding 950 µL of PBS and then spun at 1400 g for 2 min to remove the supernatant. To the tube was added a premixed solution of 200 µL of 2 M urea/PBS, 2 µL of 100 mM calcium chloride in water, and 4 µL of trypsin solution (20 µg of lyophilized trypsin in 40 µL of trypsin buffer). The tube was allowed to incubate overnight in a shaking incubator at 37 °C, being sure that the reaction did not proceed for longer than 21 hr.

The next day, the eluted peptides were collected by spinning through a 0.2 µm spin filter to remove the resin. Proteins were desalted using a C18 ZipTip and dried by vacuum centrifugation. Peptides were resuspended and acidified using LC-MS grade formic acid and then submitted for LC-MS/MS analysis.

**Experiment for evaluating nbHER2\textsubscript{\textgamma} sfGFP conjugate binding specificity.** T-25 flasks (Falcon) were grown to 80% confluency containing either the HER2+ cell line SK-BR-3 or the HER2- cell line MDA-MB-468. Cells were trypsinized and resuspended in 1 mL of warm cell binding buffer (DPBS suplanted with 5% FBS and 1% Na\textsubscript{2} SO\textsubscript{4} w/v). Cells were spun down at ~ 300xg for 2 min and the supernatant removed, then washed with 1 mL portions of warm cell binding buffer an additional two times. Cells were brought to a final concentration of 2.0E6 cells/mL in warm cell binding buffer. To a 100 µL aliquot of either the SK-BR-3 or MDA-MB-468 cells was added 20 µL of 10 µM nbHER2\textsubscript{\textgamma}-sfGFP ([f]) = 2 nM). As negative controls, 10 µL of DPBS were added to separate 100 µL aliquots of the cells. Samples were allowed to incubate on ice for 1 h, were diluted with 1 mL of warm cell binding buffer and spun down at ~ 300xg for 2 min. The supernatant was removed and samples were then washed an additional two times with 1 mL of warm cell binding buffer for a total of three washes. Finally, samples were resuspended in 1 mL of warm cell binding buffer and placed on ice until analysis via flow cytometry.

**Cell-cell binding study.** To begin, the HER2+ cell line SK-BR-3 was loaded with CFSE dye (ThermoFisher). A T-75 flask with SK-BR-3 cells grown to 80% confluency was lifted using 1 mL of trypsin solution at 37 °C, 5% CO\textsubscript{2} for 90 sec. Once the cells were free, the trypsin was diluted with 9 mL of warm complete DMEM. The cell suspension was collected and spun at ~ 300xg for 3 min. The supernatant was carefully removed and the cells were rinsed in 1 mL of warm DPBS three times. The cells were resuspended in 1 mL of warm DMEM and a cell density measurement was measurement. To the cells in 1 mL of DMEM was added 0.5 µL of CFSE stock solution (10 mM) per 1 million SK-BR-3 cells. The cells were allowed to incubate at 37 °C, 5% CO\textsubscript{2} for 10 min. After the incubation was complete, the cells were centrifuged at ~ 300xg for 3 min, the supernatant was removed, and the cells were rinsed three times with 1 mL of portion of warm, complete DMEM (phenol-red free). After rinsing, the CFSE-labeled SK-BR-3 cells were diluted to 40,000 cells/mL and stored on ice until use.

Next, the NK-92MI cells were pooled and spun down at ~ 300xg for 3 min. The supernatant was removed and the cells were rinsed with 1 mL portion of warm DPBS for a total of two washes. After the washes, the cells were counted and ~ 1 - 2 million NK cells were aliquoted into four tubes for labeling reactions. Labeling reactions were performed with 10 µM nbHER2\textsubscript{\textgamma} and 400 nM abTYR in 400 µL of DPBS. A control was performed in the absence of abTYR, as well as an isotype control which involved labeling the cells with 10 µM nbGFPTyr and 400 nM abTYR in 400 µL of DPBS. A no treatment control was also prepared, which just involved diluting the cells in 400 µL of DPBS only. Labeling reactions were allowed to proceed for 10 min at 37 °C, 5% CO\textsubscript{2}, after which the cells were diluted with 1 mL of warm DPBS and spun down at ~ 300xg for 2 min. Cell were rinsed with 1 mL portions of DPBS two times and then resuspended in 1 mL of warm DPBS. Cell density measurements were performed, and 0.5 µL of a Mitotracker Red stock solution (1 mM) were added per 1 million cells to each tube. The cells were incubated at 37 °C, 5% CO\textsubscript{2} for 10 min and then rinsed three times with 1 mL of warm, complete DMEM (phenol-red free). The samples were brought up in 0.5 mL of warm, complete DMEM (phenol-red free) and brought to a density of 80,000 cells/mL in this same medium.

To a black-walled 24 well plate (Vision Plate, Brooks Life Sciences) was added 0.5 mL of the SK-BR-3 cell solution (at 40,000 cells/mL) and 0.5 mL of the labeled NK cell solution (at 80,000 cells/mL) for a final ratio of 1:2 SK-BR-3 to NK cells. Cell-cell binding samples were prepared in quadruplicate and the cells were allowed to sit at 37 °C, 5% CO\textsubscript{2} for 2 h. The cells were then imaged using a high throughput fluorescence microscope (ImageXpress Micro, Molecular Devices), collected 25 unique regions from every well. A nearest neighbor analysis between the CFSE-labeled SK-BR-3 cells and the MitoTracker Red-labeled NK cells was performed in CellProfiler (Broad Institute) and the results were exported into Excel (Microsoft). A SK-BR-3 cell was marked as "bound" if it was found to be neighbored by 2 or more NK cells. The number of "bound" SK-BR-3 cells was then divided by the total number of SK-BR-3 cells detected to determine the proportion of SK-BR-3 cells bound by NK cells in each condition.

**General protocol for cell killing study.** Adapted from (5).

1. Start by loading the HER2+ cell line SK-BR-3 with calcein AM dye (ThermoFisher). Lift a T-75 flask with SK-BR-3 cells grown to 80% confluency with 1 mL of trypsin solution at 37 °C, 5% CO\textsubscript{2} for 90 s. Once the cells are free, dilute the trypsin with 9 mL of warm complete DMEM. Collect the cell suspension and spin at ~ 300xg for 3 min. Carefully
aspirate off the supernatant and resuspend the cells in 1 mL of warm complete DMEM.

2. Take a cell count measurement, and for every million SK-BR-3 cells add 4 µL of a 1 mg/mL calcein AM stock solution (prepared according to ThermoFisher specifications). Incubate the cells for ~ 30 min at 37 °C, 5% CO₂. While the cells are labeling, move on to the abTYR-mediated synthesis of the nanobody-NK cell conjugates, detailed below - it is okay if the calcein AM incubation goes over 30 min while the NK cells are being labeled.

3. After the SK-BR-3 cells are done labeling with calcein AM, spin down the cells at ~300xg for 2 mins. Rinse the cells twice with 1 mL of warm DPBS, and after the second wash resuspend the cells in warm Myelocult H5100.

4. Keep the cells off to the side until ready to mix with NK cells.

5. While the SK-BR-3 cells are incubating with the calcein AM dye, move on to labeling the NK-92MI cells. Pool all the NK-92MI cells and spin down at ~ 300xg for 3 min. Remove the supernatant and wash the cells twice with warm DPBS. After the second wash, resuspend the cells in 1 mL of warm DPBS and take a cell count measurement.

6. Aliquot out ~ 1 million NK-92MI cells into separate tubes (n.b. if more labeled NK-92MI cells are required in downstream applications set up multiple reaction tubes in parallel as opposed to scaling up the labeling reaction). Rinse the aliquoted NK-92MI cells one final time with 1 mL of warm DPBS and remove the supernatant.

7. Prepare labeling reactions at 400 µL volume in DPBS with 10 µM nbHER2Tyr and 400 nM abTYR, as detailed above. Set up a negative control in the absence of abTYR and an isotype controls using 10 µM nbGFPTyr and 400 nM abTYR, as well as an untreated controls consisting of 400 µL of warm DPBS. Allow the reactions to incubate for 10 min at 37 °C, 5% CO₂.

8. After incubation, add 1 mL of warm DPBS and spin down the reactions at ~ 300xg for 2 min. Remove the supernatant and rinse the cells with 1 mL portions of DPBS another two times (n.b. at this point any replicates of a particular condition can be combined into a single tube for washing).

9. After washing, bring the cells up in 1 mL of warm Myelocult H5100 and take cell density measurements.

10. Once all the cells are labeled, combine the different NK samples with the calcein-AM labeled SK-BR-3 cells in a 96 well plate at 100 µL volumes containing 5,000 SK-BR-3 cells and the desired ratio of NK cells (i.e. for a 5-fold excess of NK cells use 25,000 NK cells per well). To do so, prepare master mixes of each sample containing 250,000 labeled NK cells and 50,000 labeled SK-BR-3 cells all in 1 mL. Immediately after preparing a particular master mix sample, aliquot out 100 µL of the mixed cells into a black walled 96-well plate (Corning).

11. Aliquot out all the samples in at least quadruplicate. These constitute the experimental samples. Be sure to include at least eight replicates of Myelocult H5100 media only samples and SK-BR-3 only samples. These will serve, respectively, as a media correction control and a spontaneous dye release correction control.

12. Allow the whole plate to sit at 37 °C, 5% CO₂ for 4 h.

13. Forty-five min before the endpoint of the experiment, add 10 µL of Lysis solution (Promega) to half of the Myelocult H5100 media only samples and SK-BR-3 only samples. These will serve, respectively, as a volume correction control and a maximum dye release control.

14. At 4 h, spin down the plate of cells at ~ 300xg for 3 min, then collect 50 µL of supernatant from all wells and transfer to a new black-walled 96 well plate, being sure not to transfer any cell material at the bottom of the wells.

15. Analyze the supernatant in a plate reader (Tecan), exciting at 490 nm and reading fluorescence at 530 nm from the bottom.

16. With the fluorescence readings in hand, first perform a background correction using the average fluorescence values from the Myelocult H5100 only samples for all samples except the lysed SK-BR-3 cells, which should be corrected using the average fluorescence values from the Myelocult H5100 plus lysis buffer samples.

17. To determine percent specific cytotoxicity of each sample, use the following equation:

\[
\text{% Specific Cell Lysis} = \frac{\text{Experimental} - \text{Spontaneous Release}}{\text{Maximal Release} - \text{Spontaneous Release}} \times 100
\]

Protocol for exploring the role of NKG2D in mediating NK-conjugate cell killing. Adapted from 6. The cell killing assay was set-up as described above, being sure to prelabel the NK-92MI cells with nbHER2Tyr, and preload the SK-BR-3 cells with Calcein AM dye. After these initial labeling steps, the nbHER2Tyr-NK conjugates were preincubated with either an anti-hNKG2D antibody or a mouse isotype control at a final concentration of 10 µg/mL in 1 mL of Myelocult H5100 for 5 min at room temp. In parallel, an aliquot of SK-BR-3 cells was preincubated with an anti-hMICA/B antibody at a final concentration of 10 µg/mL in 1 mL of Myelocult H5100 for 5 min at room temp. After these incubations, NK-cell conjugates and SK-BR-3 cells
were mixed at a ratio of 5:1 (25,000 NK cells to 5,000 SK-BR-3 cells) in 100 µL of Myelocult H5100. Cells were incubated for 4 hr at 37 ºC with 5% CO₂, and specific cell cytotoxicity was determined as above.

**Supporting Information References**

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Figure S1. Nanobody oxidation tests. (a) To determine the optimal amount of time required for tyrosine oxidation, a time-course experiment was performed on 10 μM nbGFP<sub>a</sub> with 400 nM abTYR in DPBS at 37 °C. Oxidation of the parent nanobody (MW = 14289) was complete by 10 min, as indicated by a MW shift of +14 Da via ESI-QTOF (MW = 14303). (b) As a control, nbGFP<sub>cys</sub> which has a C-terminal Ser-Gly<sub>2</sub>-Cys tag as opposed to a Ser-Gly<sub>2</sub>-Tyr tag, was subjected to the same conditions. After 10 min, no change in mass was observed (MW = 14228), indicating that abTYR was acting on the installed tyrosine residue in the C-terminal tyr-tag.
**Figure S2.** Evaluation of NK-92MI cell viability following modification with nanobodies and abTYR. No increase in propidium iodide signal (a live-dead stain) was detected via flow cytometry after cells were modified with n6GFP, +ab and abTYR (red trace) over negative controls (blue and orange traces), indicating no change in viability following the reaction.
**Figure N:** The half-life of the conjugated nanobodies to natural killer cells. NK cells were modified with nbGFP_{np} and incubated at 37°C. At various time points, aliquots of the modified or untreated NK cells were removed and their ability to bind GFP was determined using flow cytometry. Plotting the results shows that the modified NK cells have a half-life of ~9 h.
Figure S1. Synthesis of a HTC-labeled nanobody. The mbGFP_{α, A76C} mutant (MW = 14321) was modified with HTC-maleimide following the protocol above. The modification went to completion, as indicated by the shift of the nanobody MW to 14748 (mbHTC pdt) by ESI-QTOF.
Figure S5. Synthesis of nanobody-Jurkat cell conjugates. (a) To test if abTYR can mediate the attachment of nanobodies to the surface of Jurkat cells, 1 million Jurkat cells were modified with 10 mM of nbfITC and 400 nM abTYR (red trace). After 10 min at 37 °C, 5% CO₂, the cells showed an increase in FITC fluorescence via flow cytometry over no abTYR and untreated controls (blue and orange traces). (b) Following nanobody attachment, Jurkat cells were also evaluated for changes in cell viability. No increase in propidium iodide signal was observed for cells treated with nbfITC and abTYR over controls, indicating no change in cell viability following modification. (c) After attachment of 10 mM nbfITC to the Jurkat cell surfaces using tyrosinase, comparison against FITC calibration beads determined that a median value of ~210,000 copies of the nanobodies were linked to the cells. Data are represented as box plots, with the top of the box representing the 75th percentile of the data, the middle line representing the median of the data, and the bottom of the box representing the 25th percentile of the data.
**Figure S6.** Modification of human PBMCs using tyrosinase. PBMCs from human samples were modified with nbGFP$_{16}$ in the presence or absence of 400 nM abTYR. Only samples exposed to both nanobody and abTYR were able to bind GFP in solution, as indicated by an increase in GFP signal over no abTYR and untreated PBMC controls.
**Figure S7.** Imaging of subcellular location of abTYR-mediated nbfITC attachment. Jurkat cells were labeled with 10 µM nbfITC in the presence or absence of 400 nM abTYR. Cell membranes were then labeled with CellMask Deep Red (ThermoFisher). Imaging with a confocal microscope revealed distinct halos of FITC signal only in cells treated with nbfITC and abTYR (top images, nbfITC and merge images). Some nonspecific binding of nbfITC was observed in the absence of abTYR (middle images), but no clear halo was observed.
Figure S8. Small molecule proteomic analysis of tyrosinase-modified Jurkat cells. (a) Schematic representation of proteomics experiment. To 10x10⁵ Jurkat cells in 10 mL of DPBS was added an alkynyl-tyramide probe to a final concentration of 10 μM and ahYR to a final concentration of 400 μM. The labeling reaction was allowed to proceed for 20 min at 37 °C, after which cells were lysed and probe tagged proteins were reacted with a N-TEV-biotin peptide via Cu(I)AAC chemistry. After this reaction, labeled proteins were pulled down onto streptavidin-agarose resin and then trypically digested. Probe labeled peptides were eluted from the streptavidin resin using TEV protease and then analyzed for sites of modification using mass spectrometry. (b) Following analysis, 437 uniquely modified peptide sequences were identified. Analyzing for sites of modification revealed that the probe modified cysteine, lysine, and histidine residues, with cysteine accounting for 19.2% of observed modifications, lysine accounting for 50.6% of observed modifications, and histidine accounting for 30.2% of observed modifications. (c) The subcellular locations of the identified proteins were also determined. Of the proteins that were modified, 23% had cell membrane or secreted annotations.
Figure S9. Proteomic analysis of nBBAIT-modified Jurkat cells. (a) Jurkat cells were modified using a nGFP$_{bb}$ that bore a single biotin molecule at an engineered thiol (nBBAIT). After modification with nBBAIT, cells were lysed and bound to a streptavidin resin. Tryptic digest released peptides that then underwent MS analysis. (b) Of the peptides identified in this nBBAIT proteomic experiment, nine overlapped with entries found in our small molecule proteomics experiment. Seven of these had known annotations to the cell membrane or extracellular space, and represented identified modifications on cysteine, lysine, and histidine residues.
Figure S10. Synthesis and binding evaluation of nanobody-siGFP conjugates. (a) To synthesize a nbHER2\textsubscript{Cys}siGFP conjugate, 60 \textmu M of nbHER2\textsubscript{Cys} was mixed with 10 \textmu M of siGFP Y290C mutant and 400 \textmu M abTYR all in 20 \textmu M phosphate buffer, pH 6.5. The mix was allowed to react for 45 min at RT, and then evaluated for successful conversion using ESQ100. Conversion of the siGFP Y290C mutant (MW = 27549) to the corresponding nbHER2\textsubscript{Cys}siGFP conjugate (MW = 42023) was observed (MW = 27555 corresponds to a capped glutathione adduct that is inert to modification). (b) To verify that attachment of nbHER2\textsubscript{Cys} to proteins would not perturb the binding ability of the nanobody, 1 \textmu M nbHER2\textsubscript{Cys}siGFP conjugate was exposed to either HER2\textsuperscript{+} (SK-BR-3) or HER2\textsuperscript{−} (MDA-MB-468) cells. The conjugate was allowed to bind for 1 h on ice, and the cells were evaluated for GFP fluorescence signal using flow cytometry. Only HER2\textsuperscript{+} cells showed an increase in GFP fluorescence when exposed to the nbHER2\textsubscript{Cys}siGFP conjugate, validating that attachment of the nanobody to proteins did not perturb its antigen binding.
**Figure SII.** Influence of the NKG2D receptor on NK-conjugate mediated cell death. Blocking the NKG2D receptor on nbHER2neo-modified NK cells did not lead to a reduction in specific cytotoxicity over isotype controls. Interestingly, blocking the ligand of NKG2D, MIC-A/B, lead to a statistically significant reduction in nbHER2neo-modified NK cell killing over isotype controls. This suggests that nanobody modification of the NK cell surface prevents antibody binding to the NKG2D receptor but still allows its functional engagement and thus cell killing.