***In Vivo* PET Imaging of ^{89}Zr-Labeled Natural Killer Cells and the Modulating Effects of a Therapeutic Antibody**

Supplemental Data

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MATERIALS AND METHODS

RESULTS

MATERIALS AND METHODS

1. Human NK cell isolation and *ex vivo* expansion

Experiments using human blood received approval from KCL-REC (Study Reference HR/DP-20/21-24483). All donors provided written informed consent.

Typically, a sample of 25 mL of freshly isolated human peripheral blood was carefully layered on top of 15 mL of Ficoll-Paque® PLUS (GE Healthcare Life Sciences USA) density centrifugation medium in a 50 mL Falcon conical centrifugation tube. The samples were centrifuged for 20 min at room temperature (RT) at 1200 *g*. The mononuclear layer (PBMC) (located between the Ficoll-Pague® medium and the separated plasma) was collected. The PBMCs were then resuspended in 50 mL phosphate buffered saline (PBS) and centrifuged at 300 *g* for 10 min. After removing the supernatant, the PBMCs were resuspended in 2 mL of the same donor's peripheral blood and mixed with 150 µL of RosetteSepTM Human NK Cell Enrichment Cocktail (STEMCELL Technologies, #15065) and incubated for 20 min at RT. The mixture was then diluted to 15 mL with PBS and NK cell-enriched mononuclear cells were purified using the Ficoll-Paque® gradient method. The NK cells were washed twice with PBS and then cultured in NK MACS expansion medium (Miltenyi Biotec, #130-114-429) supplemented with 500 IU/mL of recombinant human IL-2 (rhIL-2, Peprotech, #200-02) and 140 IU/mL of recombinant human IL-15 (rIL-15, Miltenyi Biotec, #130-095-764) and 5% human serum AB (Sigma-Aldrich, #H4522). The expansion protocol was performed as per manufacturer's instructions (*21*). The cells were incubated at 37°C in a humidified 5% CO2 incubator and harvested on day 14-16 for further experiments.

2. Cell lines

The HER2-positive (HER2+) human breast cancer cell lines SKBR3 and HCC1954 were kindly gifted by Dr Chenoweth (KCL, UK). HER2-negative (HER2-) MDA-MB-231 cell line was gifted by the Imaging Sciences Department (KCL, UK). The cells were cultured in either RPMI-1640 media (for HCC1954 and MDA-MB-231) or DMEM high glucose (for SKBR3), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. The cells were passaged twice weekly and kept in an incubator at 37°C in a humidified 5% CO_2 environment.

3. NK cell radiolabeling with ^{89}Zr-oxine

The oxine kit and ^{89}Zr-oxine radiolabeling were prepared in accordance with the previously established method (*10*). ^{89}Zr-Oxine was reproducibly synthesized, with a labeling yield of 90.0 ± 5.8% (*n* = 16).

Freshly prepared ^{89}Zr-Oxine was added to *ex vivo* expanded NK cells, which had been washed and resuspended in PBS at 15-20 x 10^6 cells/mL in a 50 mL centrifuge tube, at circa 45 kBq/10^6 cells. The cells were then incubated at RT for 15 minutes, with gentle swirling every 5 minutes. The tube was topped up to 50 mL with PBS and centrifuged at 400 g for 10 minutes. The supernatant was transferred to a new 50 mL centrifuge tube, while the pellet was resuspended in:

1. NK MACS complete medium (1 x10^6 cells/mL), supplemented with 500 IU/mL of rhIL-2, for ADCC and degranulation assays,
2. NK MACS complete medium (0.5 x 10^6 cells/mL), interleukin free, for cell growth, viability and efflux studies, or
3. in serum and interleukin free medium (1 x10^6 cells/mL) for migration assays.

Unlabeled NK cells were kept under similar conditions as controls. The amount of radioactivity in the supernatant and the cells was then measured using a CRC®-25R dose calibrator (Capintec Inc, USA) to determine the cell labeling efficiency (%) as 100 x ($Activity_{pellet}$)/($Activity_{pellet}$ + $Activity_{supernatant}$). Finally, the cells were incubated at 37°C in a humidified 5% CO_2 incubator for subsequent assessments. Over the course of this study, cell radiolabeling efficiencies of 42.2 ± 8.0% (*n* = 44) were measured. The final amount of ^{89}Zr per cell measured 16.7 ± 4.7 kBq/10^6 cells (*n* = 34).

4. ADCC assays on HER2+ cancer cells with trastuzumab

The CyQUANT™ LDH Cytotoxicity Assay kit (Invitrogen, #C20300) was used to perform ADDC assays. ^{89}Zr-labeled (^{89}Zr-) and unlabeled NK cells were harvested, resuspended in culture media containing L-glutamine and 2% FBS, at a concentration of 4 x 10^6 cells/mL. At the same time, cancer cell lines SKBR3 or HCC1954 were harvested and resuspended at 0.4 x 10^6 cells/mL. 50 µL of solution containing each cell type was then added to each well of a 96-well plate in triplicate, together with 10 µL of solution containing trastuzumab (Herceptin) with the final antibody concentrations titrated at 1:3

from 10 µg/mL. The plates were incubated at 37°C, 5% CO_2 for 4 hours, with 10 µL of 10X lysis buffer added to the positive control wells 30 minutes before the end of the incubation period. Afterwards, the plate was centrifuged at 400 *g* for 1 minute before 50 µL of the supernatant was transferred to a new flat-bottom 96-well plate, followed by the addition of 50 µL of reaction mixture. The plate was then incubated in the dark for 10 minutes before 50 µL of stop solution was added to all wells. The absorbance was measured at 490 nm and 680 nm using the microplate reader. In accordance with the instructions, after subtracting the 680 nm absorbance value from the corresponding 490 nm absorbance value, % cell lysis was expressed as = 100 x ($Absorbance_{Sample}$ – $Absorbance_{spontaneous}$)/($Absorbance_{max}$ – $Absorbance_{spontaneous}$).The data was subsequently analyzed and fitted to a four-parameter logistic curve using GraphPad Prism 9 software.

5. Cell migration assays

The QCM Chemotaxis Cell Migration Assay (96-well (5 µm), fluorimetric (Millipore, #ECM512)) was utilized to perform chemotaxis assays, as per the manufacturer's instructions. After 18-24 hours of radiolabeling, interleukin and serum depletion, the NK cells were washed twice with PBS and then resuspended in serum-free medium at a concentration of 2 x 10^6 cells/mL. 150 µL of serum-free medium or 50% FBS medium was added to each well in the migration feeder tray. 100 µL of solution containing NK cells were added to each migration chamber well. The plate was then covered and incubated for 4 hours at 37°C in a humidified 5% CO_2 incubator. After discarding the cells/media from the top side of the insert, the migration chamber plate was placed onto a new 96-well feeder tray containing 150 µL of prewarmed Cell Detachment Solution in the wells and incubated at 37°C for another 30 minutes. In a new 96-well plate, 50 µL of Lysis Buffer/Dye Solution was added to each well that contained 75 µL of cell detachment solution with the migrated cells, along with an additional 75 µL from the first feeder tray. Following 15 minute incubation at room temperature, 150 µL of the mixture was transferred to a new 96-well fluorescent compatible plate (Greiner bio-one, #655090) that was then read with a GloMax® fluorescence plate reader (Promega) using 482/520 nm filter set. The data were expressed as relative fluorescence units (RFU) and analyzed with GraphPad Prism 9 software.

6. CD107a degranulation assay to evaluate NK cell antitumor activity against HER2+ and HER2- cell lines

NK cells and cancer cells (HCC1954 or MDA-MB-231) were resuspended in complete growth medium at concentrations of 10^6 cells/mL and 10^5 cells/mL, respectively, and were added in 100 µL volume each per well in a 96 well-plate. Wells containing only NK cells were used to measure the baseline degranulation state, while NK cells with an activation cocktail of 12-*O*-tetradecanoylphorbol 13-acetate (PMA) (50 ng/mL)/ionomycin (1 µg/mL) served as a positive control. The final volume was adjusted to 210 µL/well using growth medium. 5 µL of a solution containing antihuman CD107a-PE-Cy7 antibody (BD Pharmingen, clone H4A3, #561348) was added to each well and incubated at 37°C and 5% CO_2 for 1 hour. 5 µL of freshly prepared 40X monensin solution (Bio-Rad Laboratories Ltd, #BUF074) was then added per well, and the well contents mixed thoroughly, followed by an extra 3-hour incubation at 37°C and 5% CO_2. The entire content of each well was then transferred to a 5 mL FACS tube, and the cells were washed twice with PBS and centrifuged at 400 *g* for 5 min at room temperature. Finally, the cells were stained with anti-human antibodies CD3-PE (Biolegend, clone HIT3a, #300308), CD56-FITC (Biologend, clone HCD56, #318304), and CD16-APC (Biolegend, clone 3G8, #302012) for flow cytometry analysis with BD FACSMelody cell sorter (BD Life Sciences, USA). The data was then analyzed using FlowJo V10 software (BD Life Sciences, USA).

7. *In vivo* tracking of ^{89}Zr-NK cells

Animal studies

All animal experiments were ethically reviewed by an Animal Welfare & Ethical Review Board at King's College London and carried out in accordance with the Animals (Scientific Procedures) Act 1986 UK Home Office regulations governing animal experimentation.

Mammary fat pad breast cancer tumor establishment

Eight to ten-week-old female NOD scid gamma (NSG) (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice (Charles River, Margate, UK) were subcutaneously inoculated with 1.5 x 10^6 HCC1954 cells in 100 µL of 1:1 mixture of PBS/Matrigel (Corning™, #356234) in the left mammary fat pad between the 4th and 5th pairs of nipples. Experiments were conducted when the tumor volume reached ~100-150 mm^3. Tumor growth

was measured using caliper measurements and the tumor volume was calculated using the formula: V = ($L*W^2$)/2 with V = volume, L = length (the long axis of the tumor), W = width (the short axis of the tumor).

HCC1954 tumor-bearing NSG mice were randomized into three groups and intravenously administered 1 x 10^7 freshly radiolabeled NK cells (150-200 kBq) and rhIL-15 (2500 IU) together with i) PBS sham, ii) isotype control (5 mg/kg) in PBS, or iii) trastuzumab (5 mg/kg) (~200 µL PBS). NK cells from three healthy human volunteers were employed. Additional doses of rhIL-15 (2500 IU/dose) were given on days 3 and 6 via intra-peritoneal injection to support the *in vivo* survival and expansion of NK cells (*12*).

PET/CT imaging and reconstruction

Static PET/CT images were acquired using a NanoScan PET/CT scanner (Mediso, Budapest, Hungary) with mice under 0.8-1.5% isoflurane in oxygen anesthesia and warmed to 37 °C for the duration of the experiment on days 1, 3 and 7 after cell injection. Animals were anaesthetized using 2 – 2.5 % isoflurane in 100% oxygen and transferred to an air-heated single-mouse bed. Respiration was monitored throughout the scan. A 1 hour PET acquisition scan was performed (400-600 keV energy window, 4 ns coincidence window and 1-5 coincidence mode) followed by a CT scan for anatomical visualization (480 projections; helical acquisition; 55 kVp; 600 ms exposure time).

PET/CT datasets were reconstructed using a Monte Carlo-based full-3D iterative algorithm (TeraTomo, Mediso) with 4 iterations, 6 subsets, and 0.4 mm isotropic voxel size. Isotropic voxel size was set at 0.4 mm^3, images were corrected for scatter attenuation and decay corrected to the time of cell injection. Images were co-registered and analyzed using VivoQuant v.3.0 (Invicro). Regions of interest (ROIs) were delineated for PET activity quantification in specific organs. Uptake in each ROI was expressed as a percentage of injected dose per gram of tissue (%ID.g^{-1}).

8. *Ex vivo* biodistribution study

Mice from imaging studies were employed for biodistribution studies on day 7 and extra animals were used for the studies on day 3. After euthanizing, organs were dissected, washed, gently dried, weighed,

and γ-counted together with standards prepared from a sample of injected material. The percentage of injected dose per gram (%ID.g^{-1}) of tissue was calculated.

9. *Ex vivo* flow cytometric study

Samples of mouse liver and spleen tissues were collected three days after the injection of ^{89}Zr-NK cells. The tissue was cut into small pieces and passed through a 100 µm nylon mesh strainer, followed by rinsing with 2 mL of ice-cold PBS/EDTA and sequential filtration through 70 µm and 40 µm cell strainers to produce single cell suspensions. The cells were then washed twice with PBS and fixed with 4% PFA for 15 minutes at room temperature. Subsequently, the cells were resuspended at 2 x 10^6 cells/mL in 0.5 mL PBS (without EDTA) and stained with antihuman antibodies including CD3-PE, CD56-FITC, CD16-APC, and CD45-PE-Cy7. After the incubation period, the cells were washed twice with PBS before the cell pellets were resuspended in 2% BSA/PBS for flow cytometry analysis with the BD FACSMelody cell sorter (BD Life Sciences, USA). CD45+ and CD45- populations were sorted and collected for γ-counting and the results were normalized as counts per minute (cpm) per 10^6 cells. Flow cytometric data was analyzed using FlowJo V10 software (BD Life Sciences, USA).

10. Dual ^{89}Zr-NK cell PET/CT and [^{111}In]In-CHX-A”-DTPA-trastuzumab SPECT/CT imaging

Conjugation of trastuzumab to *p*-SCN-Bn-CHX-A”-DTPA was undertaken according to standard procedures (doi:10.1038/nprot.2006.49). Briefly, a solution of trastuzumab was isolated in HEPES buffer (0.1 M, pH 8.8) by ultracentrifugation using Vivaspin 2 ultracentrifugation tubes. A solution of *p*-SCN-Bn-CHX-A”-DTPA (0.5 mg, 20 equiv. in 5 mL DMSO) was added to the antibody solution (0.5 mL) and the mixture was allowed to react at room temperature overnight with constant agitation.

The reaction solution was applied to a sephadex PD-10 size exclusion column (GE Healthcare Life Sciences, UK), and eluted with aqueous ammonium acetate solution (0.2 M). The fourth eluate fraction (1 mL) contained the desired immunoconjugate. The CHX-A”-DTPA-trastuzumab immunoconjugate was then further purified (in ammonium acetate solution, 0.2 M, pH 6) by successive ultracentrifugation cycles (5 cycles with Vivaspin 2 ultracentrifugation tubes).

To radiolabel CHX-A"-DTPA-trastuzumab, a solution of immunoconjugate (5 mg mL^{-1}, 0.6 mg, 120 mL) was combined with an aqueous solution of ammonium acetate (0.2 M, 200 mL) and a solution of $^{111}InCl_3$ (170 MBq in 200 mL of 0.1 aqueous HCl solution, Mallinckrodt Medical B.V., Petten, Netherlands). After 30 min incubation at 37 °C, an aliquot was applied to a HPLC BioSep SEC s2000 size exclusion column (Phenomenex, Macclesfield, UK) (isocratic mobile phase, PBS containing 2 mM EDTA, 1 mL min^{-1}, UV detector: λ = 280 nm). Radiochemical yield and purity was > 99%.

Each HCC1954 tumor-bearing NSG mouse (n=4) was injected intravenously with 20-30 MBq of ^{111}In-CHX-A"-DTPA-trastuzumab (100 mg of immunoconjugate) alongside 1 x 10^7 freshly radiolabeled NK cells (150-200 kBq) and rhIL-15 (2500 IU) (~200 µL PBS). 3-day post injection, the animals were subjected to sequential SPECT/CT and PET/CT imaging. Animals were anaesthetized using 2 – 2.5 % isoflurane in 100% oxygen and transferred to an air-heated single-mouse bed. Respiration was monitored throughout the scan. SPECT/CT scan was acquired on a dedicated small animal SPECT system, NanoSPECT/CT Silver Upgrade (Mediso Ltd., Budapest, Hungary), calibrated for indium-111. The whole-body SPECT scan time was 1 h with a frame time of 60 s (using a 4-head scanner with 4 × 9 [1.4 mm] pinhole collimators in helical scanning mode) followed by a helical CT (45 kVP X-ray source, 1000 ms exposure time in 180 projections over 7.5 min). After that, the animal and the bed were transferred to the PET/CT system. A 1.5 hour PET acquisition scan was performed (400-600 keV energy window, 4 ns coincidence window and 1-5 coincidence mode) followed by a CT scan (a 55 kVp X-ray source, 1,000 ms exposure time, 1:4 binning, in 180 projections over approximately 9 min).

SPECT/CT images were reconstructed in a 256 × 256 matrix using HiSPECT (ScivisGmbH), a reconstruction software package. PET/CT datasets were reconstructed using a Monte Carlo-based full-3D iterative algorithm (TeraTomo, Mediso) with 4 iterations, 6 subsets, and 0.4 mm isotropic voxel size. Isotropic voxel size was set at 0.4 mm^3, images were corrected for scatter attenuation and decay corrected to the time of cell injection. Images were fused using proprietary VivoQuant v.3.5, patch 1 software (InVicro LLC., Boston, USA) software.

11. Confocal microscopy of CMFDA-labeled NK cells in lung, liver spleen and tumor sections

Ex vivo expanded NK cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CMFDA) for *ex vivo* histological tracking purposes. Initially, NK cells were resuspended at a concentration of $5x10^7$ cells/mL in pre-warmed serum-free (SF) medium. CMFDA stock solution (10 mM) was added at a dilution of 1:1000 to achieve a final concentration of 10 µM CMFDA, followed by a 30-minute incubation period at 37°C. Subsequently, the labeled NK cells were washed twice with PBS and resuspended in sterile PBS at a concentration of $10x10^7$ cells/mL for injection. HCC1954 tumor-bearing NSG mice were randomized into two groups and intravenously administered ~$5x10^6$ CMFDA-labeled NK cells and rhIL-15 (2500 IU), together with either PBS sham or trastuzumab (5 mg/kg) (~200 µL PBS).

Mice were culled 72 h post-injection by cervical dislocation. The harvested organs (tumor, liver, spleen, lungs, muscle) were fixed in 4% paraformaldehyde overnight, processed and paraffin embedded. Sections (5 µm thickness) were cut and mounted on PolysineTM Adhesion Microscope Slides (Epredia, Netherlands) for further evaluation.

The paraffin sections were dewaxed in xylene and rehydrated through a decreasing series of ethanol concentrations, before a final PBS wash. The sections were mounted using mounting medium with DAPI-Aqueous (Fluoroshield) and imaged on the confocal microscope.

High-resolution confocal fluorescence images were captured with a Leica TCS SP5 II confocal (Leica Microsystems Ltd) at 40x magnification (oil-based). Excitation/emission wavelengths were 488/525 nm and UV 358/461 nm for AlexaFluor488 and DAPI, respectively, alongside brightfield images. Images were processed using LAS AF Lite (ver 2.6.3, build 8173, Leica Microsystems Ltd), and final figures were produced using Microsoft PowerPoint.

RESULTS

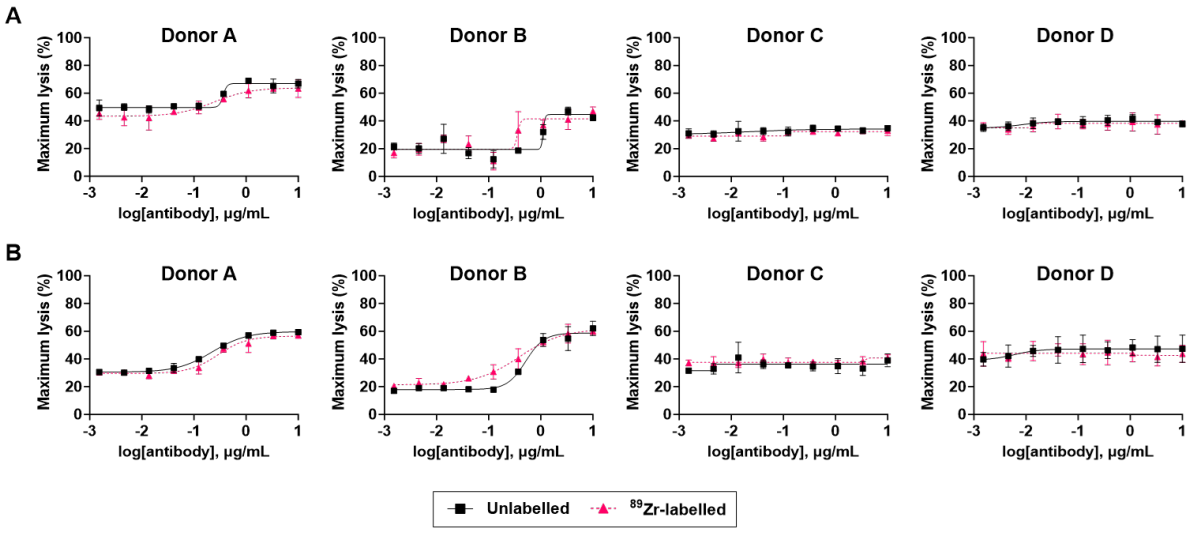

Figure S1. ADCC functionality in ^{89}Zr-NK cells. ADCC assays using ^{89}Zr-NK (24 hours after radiolabeling) and unlabeled NK cells were performed against HER2-expressing breast cancer cell lines (HCC1954 and SKBR3) (10:1 effector:target ratio) at a range of trastuzumab concentrations. ^{89}Zr-NK cells exhibited a similar ADCC response compared with that of unlabeled NK cells. The ADCC response varied between human NK cells from different healthy volunteers. Data is shown as percentage of percentage of maximum lysis and has not been adjusted for baseline cytotoxicity. The data was fitted to a four-parameter logistic curve.

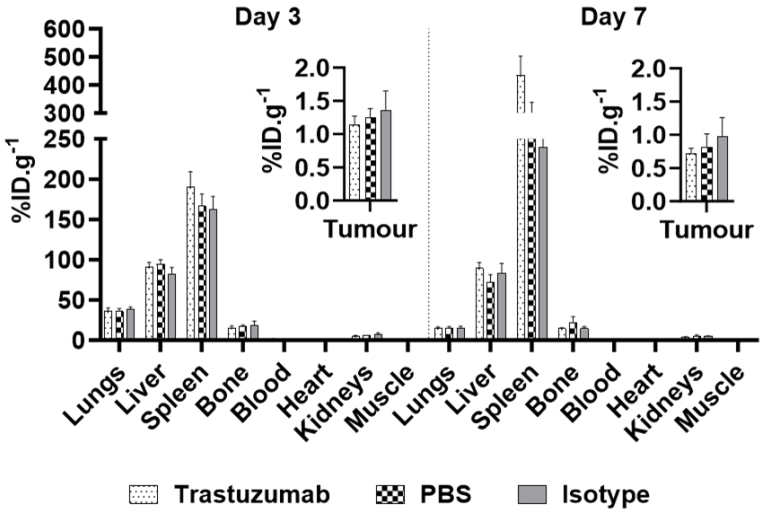

Figure S2. Ex vivo biodistribution of ^{89}Zr-NK cells in HER2+ tumor bearing mice. *Ex vivo* biodistribution of ^{89}Zr radioactivity in organs and tumors at day 3 and 7 post-injection of ^{89}Zr-NK cells (10^7 cells, ~150-200 kBq) in combination with trastuzumab (5 mg/kg), or phosphate buffered saline (PBS) sham, or anti-NIP IgG1 isotype control antibody (5 mg/kg) (mean ± SD, n = 5-9/group).

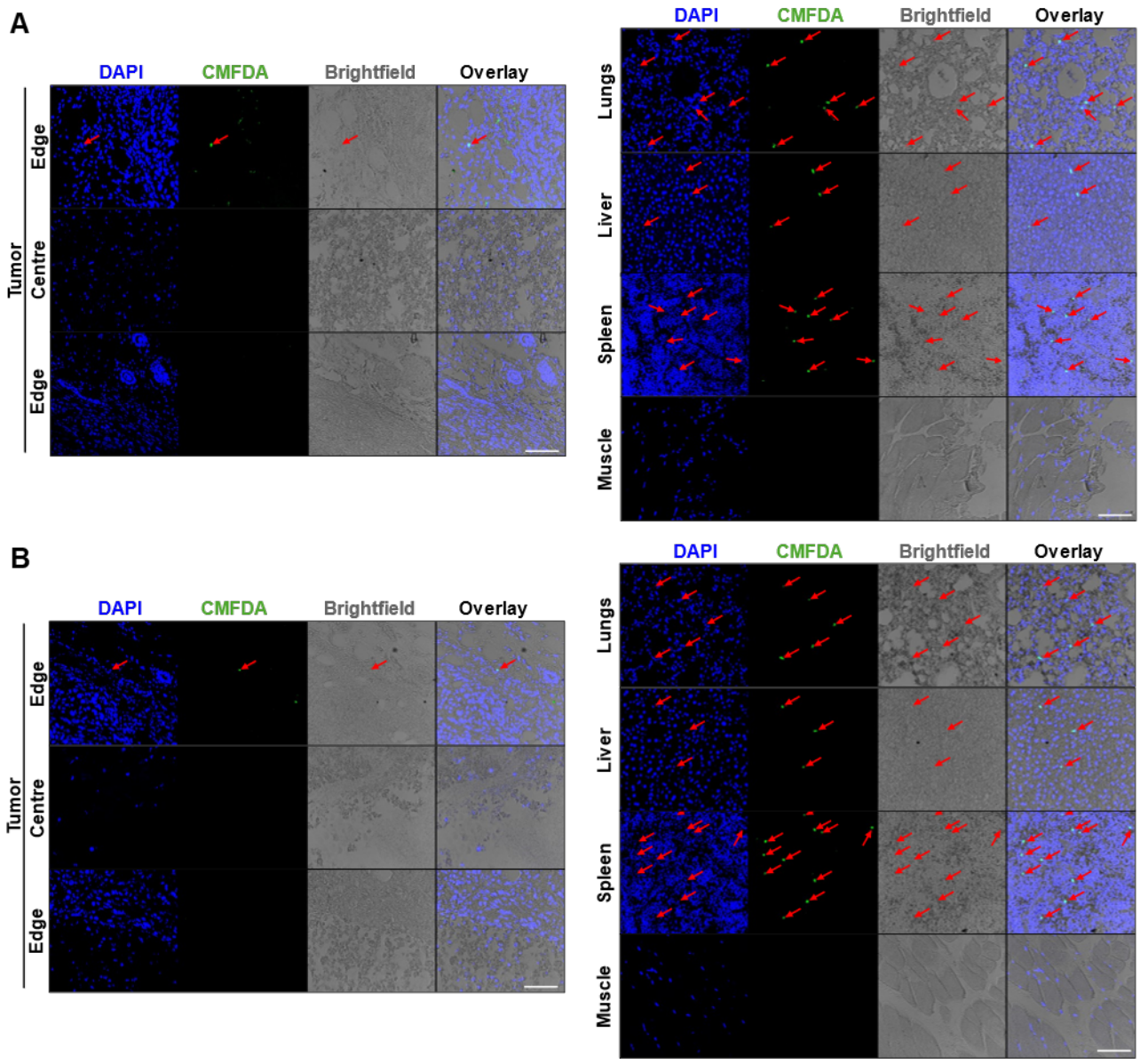

Figure S3. Fluorescence microscopy of CMFDA-labeled NK cells. Female NSG mice bearing orthotopic HER2-expressing HCC1954 tumors were administered CMFDA-labeled NK cells with either **(A)** PBS sham or **(B)** trastuzumab antibody (5 mg/kg). Confocal microscopy images (40× magnification) of murine tumor, lung, spleen and liver sections, co-stained with DAPI), from 3 days PI of NK cells, revealed the presence of CMFDA-labeled NK cells. Scale bar = 100 μm.

Table S1. Summary of whole-body imaging studies tracking NK cell migration in both preclinical and clinical settings

	Primary human NK cells, in NSG mice	Lungs	Liver and bone marrow for up to 21 days	(*7*)
	Human NK cells, in NSG mice bearing MDA-MB-231 breast cancer tumors	Lungs and tumor	n/a	(*6*)
	UCB-NK cells in NOD/SCID/IL2Rg^{null} mice	Lungs	Liver, spleen and bone marrow	(*11*)
	Autologous NK cells in rhesus macaques	Lungs	Liver, spleen and bone marrow	(*12*)
	Autologous NK cells in human patients with colon carcinoma	i.v. injection: lungs	i.v. injection: spleen, liver	(*10*)
	Allogenic NK cells in human patients with renal cell carcinoma	Lungs	Liver, spleen and bone marrow 2 out of 4 tumor metastases	(*9*)