Supplementary Materials and Methods

Flow cytometry

For analyzing cell surface markers, cells were first resuspended in fluorescence-activated cell sorting (FACS) buffer composed of phosphate-buffered saline (PBS; Gibco), 2 mM EDTA (Gibco), and 2% fetal bovine serum (FBS; Gibco), followed by staining with an antibody. Resuspended cells were incubated with antibodies at 4°C in the dark for 30 minutes, washed two times with FACS buffer, and then subject to flow cytometric analysis (BD, Fortessa X-20). All flow antibodies were listed in **Supplementary Table 1**.

For detecting intracellular proteins, cells were resuspended with FACS buffer and stained with antibodies against surface markers prior to fixation and permeabilization using the Fixation/Permeabilization Solution Kit (BD). Fixed/permeabilized cells were incubated with antibodies resuspended in the BD Perm/Wash buffer™ for 30 minutes at 4°C in the dark. The cells were washed twice with BD Perm/Wash buffer and then subjected to flow cytometric analysis.

Journal Pre-proof

Enzyme-linked immunosorbent assay

1×10^6 NK cells were co-cultured with Capan-1 or PANC-1 cells, gifts from Dr. Yuan Chen (formerly City of Hope and now University of California-San Diego), for 24 hours in a 1:3 ratio of effector: target cells (E:T ratio). The supernatants were collected and subjected to quantification of IFN-γ or IL-15 using an ELISA kit. The basal level of IFN-γ or IL-15 from NK cells was determined by engineered NK cells cultured with media only (no tumor cells were added).

Bioluminescence imaging

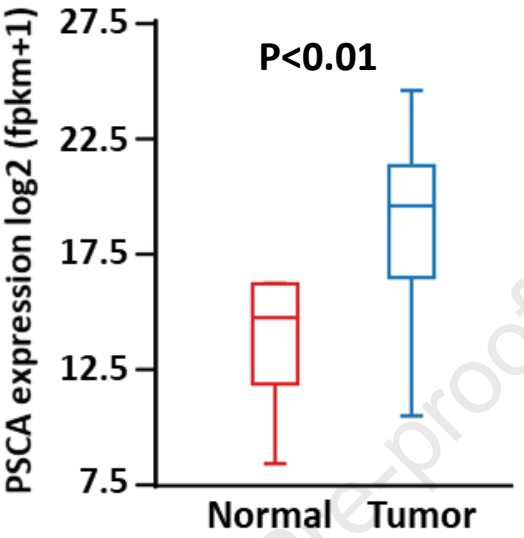
D-luciferin potassium salt (GoldBio) was dissolved in sterile water following the manufacturer's instructions and given to the Capan-1_luc-engrafted NSG mice by *i.p.* injection (150 mg/kg). The mice were anesthetized with 2% isoflurane and oxygen (1 L/min) in an imaging chamber and luminescence images were captured by Lago-X (Spectral Instruments Imaging) following the manufacturer's instructions and quantified by Aura Imaging Software (Version 2.2.1.1).

Journal Pre-proof

Supplementary Table 1. Primary antibody for FACS

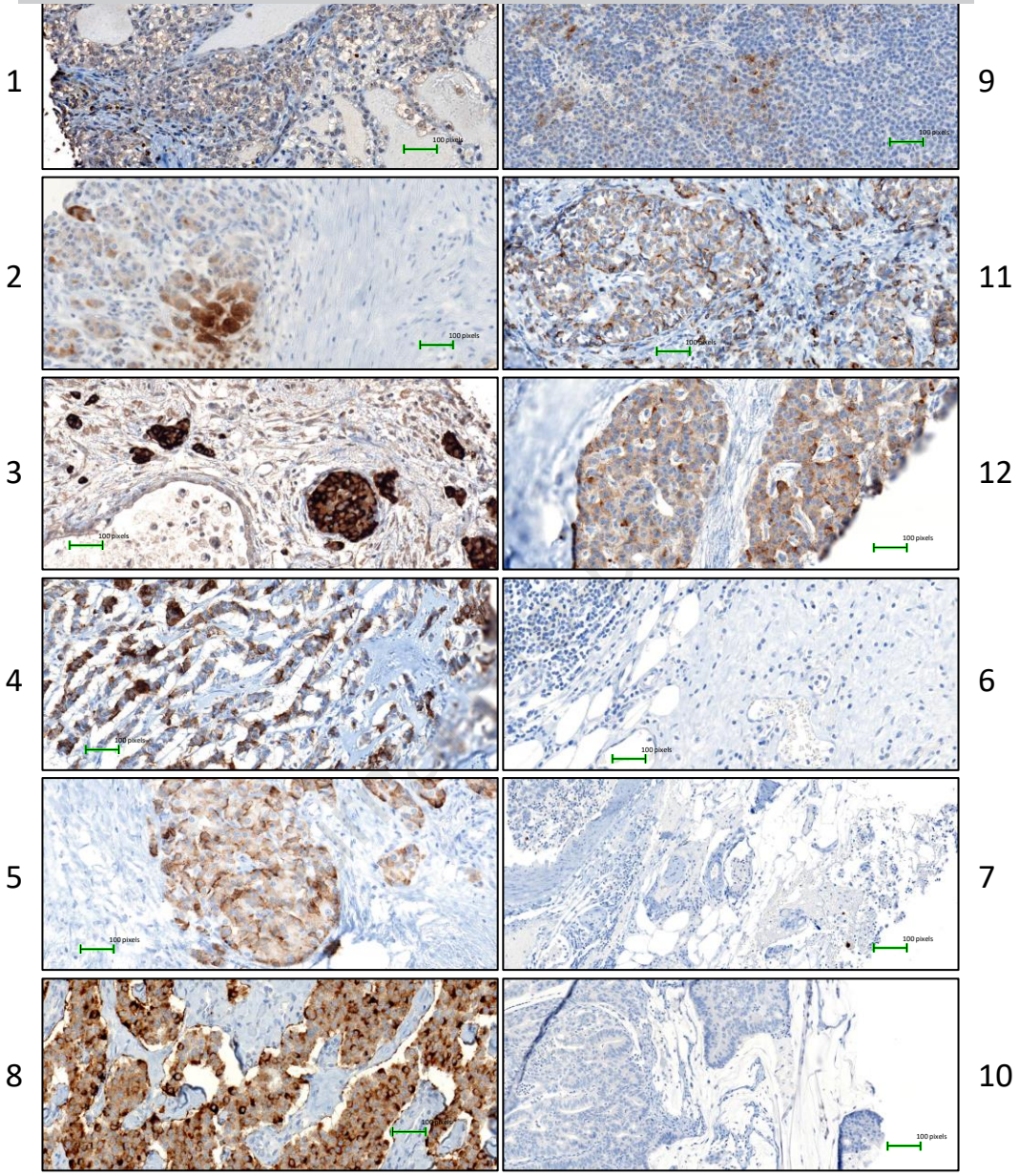
Antibody	Manufacturer	Clone #
CD3	BD	Clone UCHT1
CD16	BD	Clone 3G8
TRAIL	BD	Clone RIK-2
CD62L	BD	Clone DREG-56
NKp46	BD	Clone 9E2
CD69	BD	Clone FN50
CD25	BD	Clone M-A251
CD94	BD	Clone HP-3D9
NKG2D	BD	Clone 1D11
KIR-NKAT2	BD	Clone DX27
DNAM-1	BD	Clone DX11
NKp44	Miltenyi	Clone 2.29
NKG2A	Miltenyi	Clone REA110
CD45	Biolegend	Clone 2D1
NKp30	Biolegend	Clone P30-15
EGFR	Biolegend	Clone AY13
Anti-human IgG F (ab')2	Jackson ImmunoResearch	Polyclonal

Journal Pre-proof

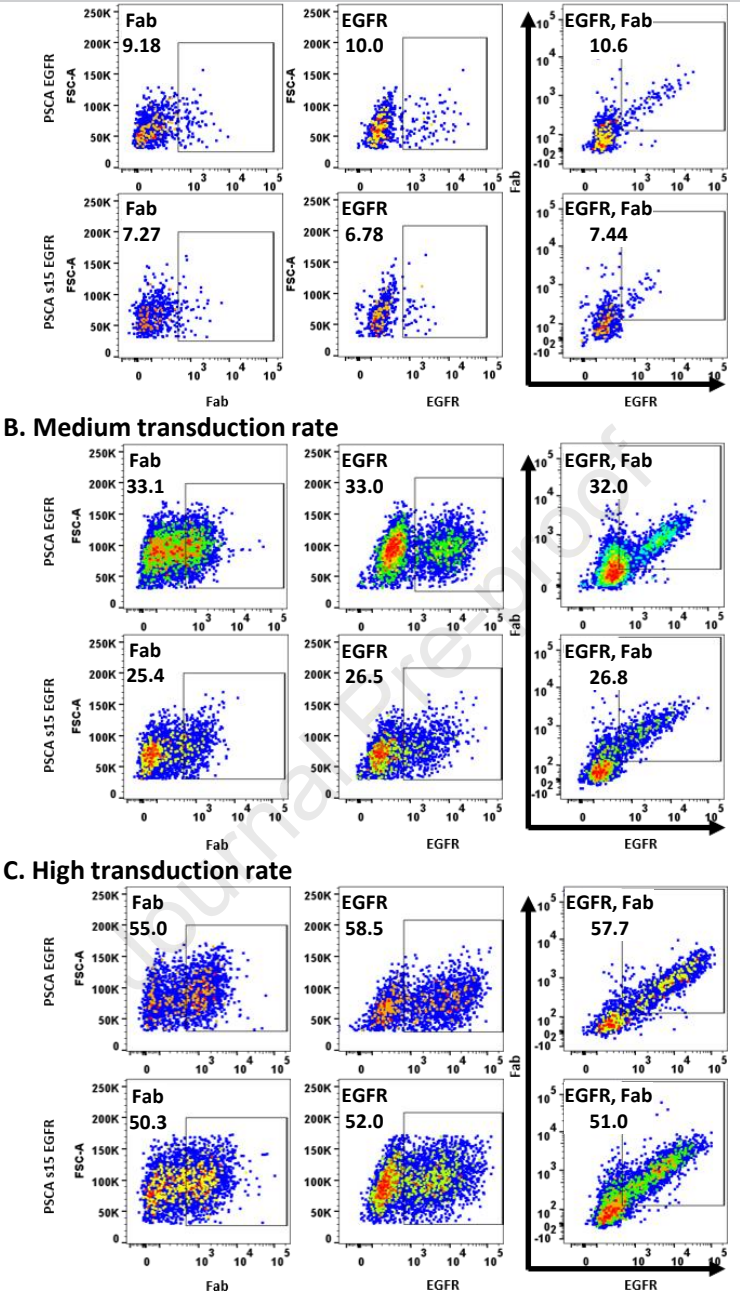


Supplementary Figure 1. PSCA expression in adjacent normal and primary tumors.
RNA sequencing data for PSCA in the normal solid tissue (n=4) or primary tumors (n=177) from pancreas. Data were retrieved from Genomic Data Commons (GDC) and The Cancer Genome Atlas (TCGA) and graphed using UCSC Xena (https://xena.ucsc.edu/).

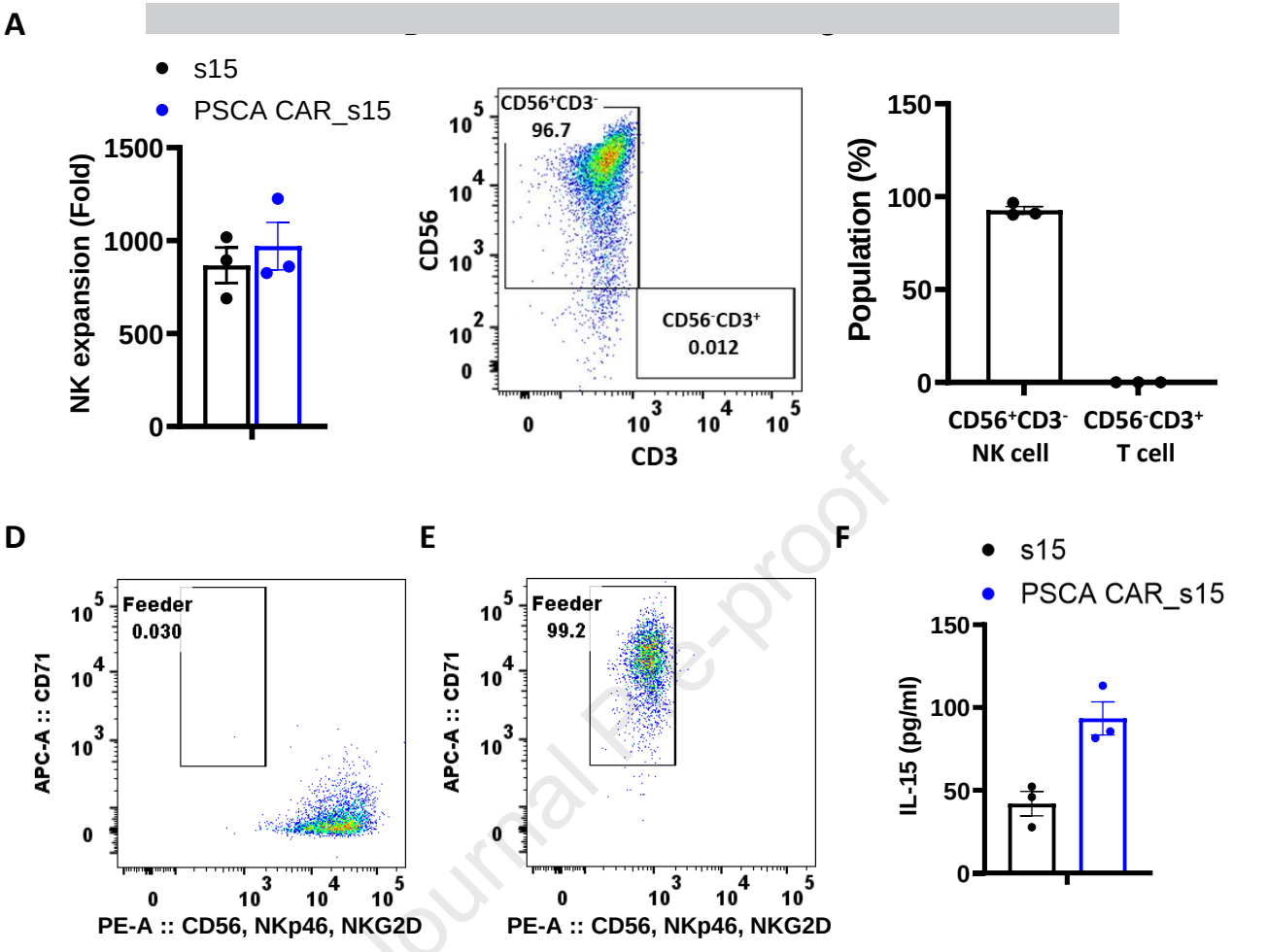
Journal Pre-proof



Supplementary Figure 2. PSCA protein expression from pancreatic cancer patients. Immunohistochemistry (IHC) of PSCA was performed on the primary pancreatic tumors (n=12). The brown color indicates PSCA expression, mostly on the membrane of the cells. All images were taken under 200X magnification. Patient 1: Pancreatic metastatic renal cell carcinoma; Patient 2, 3: Pancreatic ductal adenocarcinoma; Patient 4, 5, 8, 9, 11: Pancreatic neuroendocrine neoplasm; Patient 6: Pancreatic solid pseudopapillary neoplasm, Patient 7: Pancreatic serous papillary ovarian adenocarcinoma; Patient 10, 12: Pancreatic adenocarcinoma

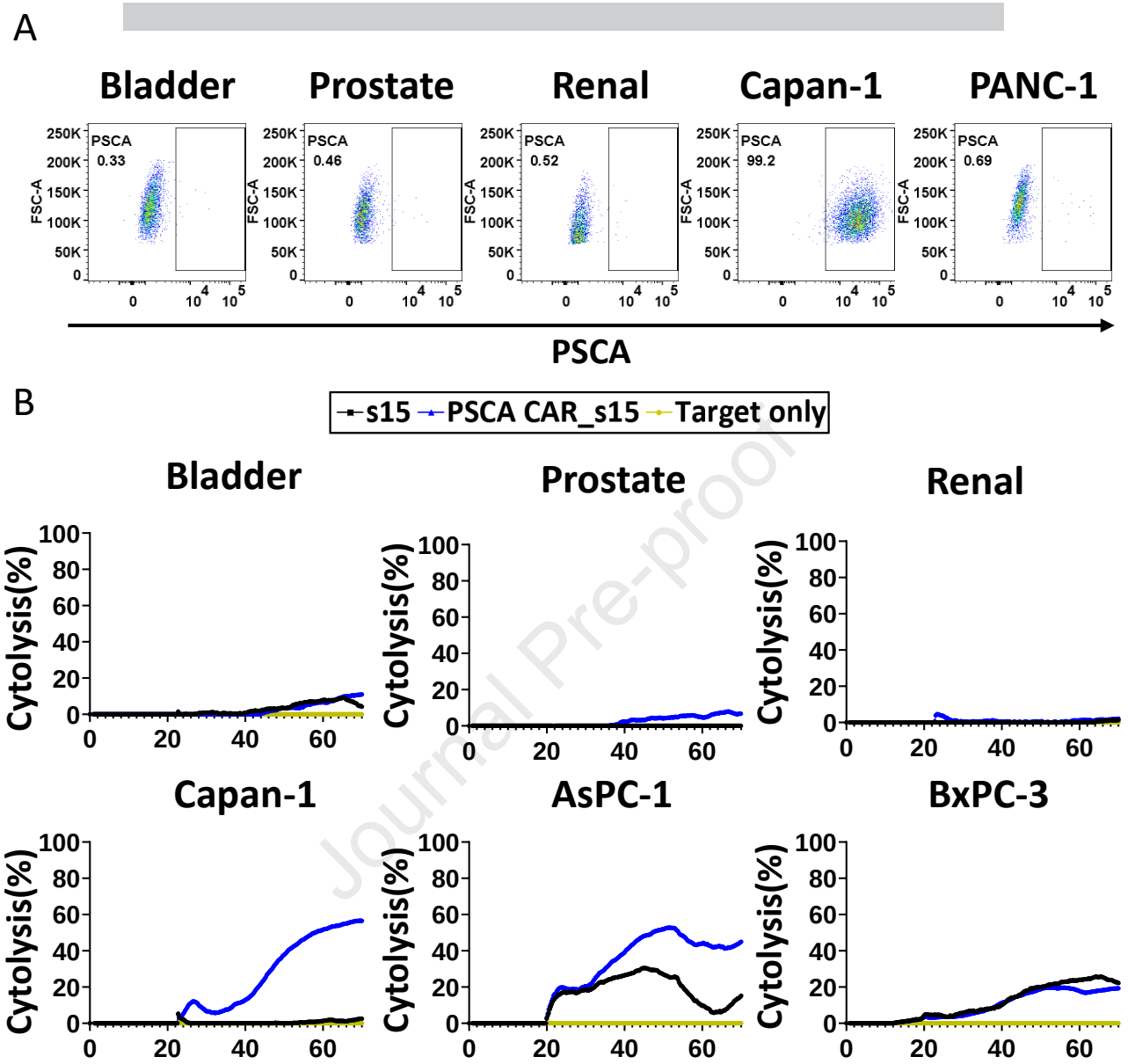


Supplementary Figure 3. Evaluation of PSCA CAR and tEGFR expression in the engineered NK cells. NK cells transduced with PSCA CAR_tEGFR or PSCA CAR_s15_tEGFR at (A) low, (B) medium, or (C) high levels of transduction rates. Both constructs were evaluated by the expression of CAR (Fab+) and tEGFR. NK cells were treated with IdeS protease enzyme prior to staining with anti-human IgG Fab antibody and anti-EGFR antibody. The expression of CAR and tEGFR were assessed by flow cytometry.



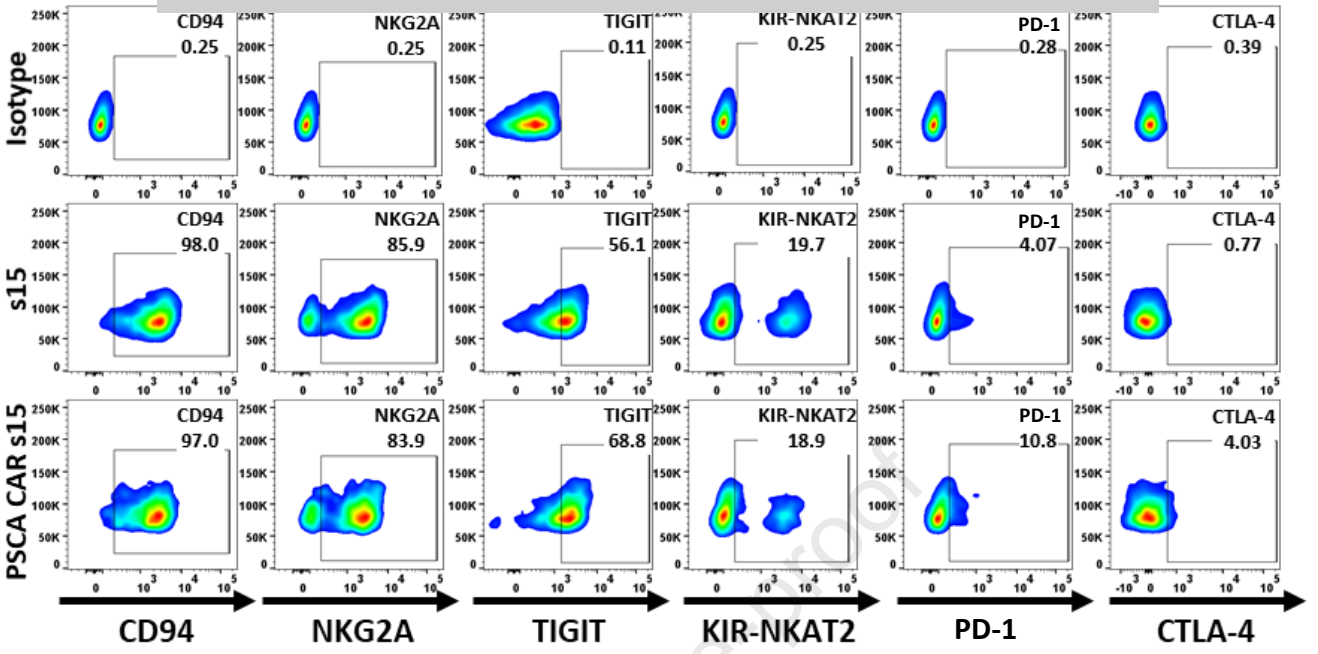
Journal Pre-proof

Supplementary Figure 4. Primary NK cell expansion and IL-15 production. (A) NK cells (n=3) engineered with s15 or PSCA CAR_s15 were expanded with K562 feeder cells expressing membrane-bound IL-21 and CD137L for 15 days and the cell number was determined by the MUSE cell analyzer. The fold change was normalized to the initial seeding numbers and presented as fold ± SEM. (B) The purity of expanded NK cells was determined by CD56(+)CD3(-) staining. (C) Quantification of CD56(+)CD3(-) NK cells or CD56(-)CD3(+) T cells after expansion. (D) Representative flow data of the residual feeder cells (defined as CD71(+)CD56(-)NKp46(-)NKG2D(-) in the final product of expanded NK cells with (E) a positive control of feeder cells staining in a parallel experiment. The gate was set on the isotypes. (F) IL-15 was quantified using ELISA from the supernatants of engineered NK cells (1×10^6) 48 hours after incubation.



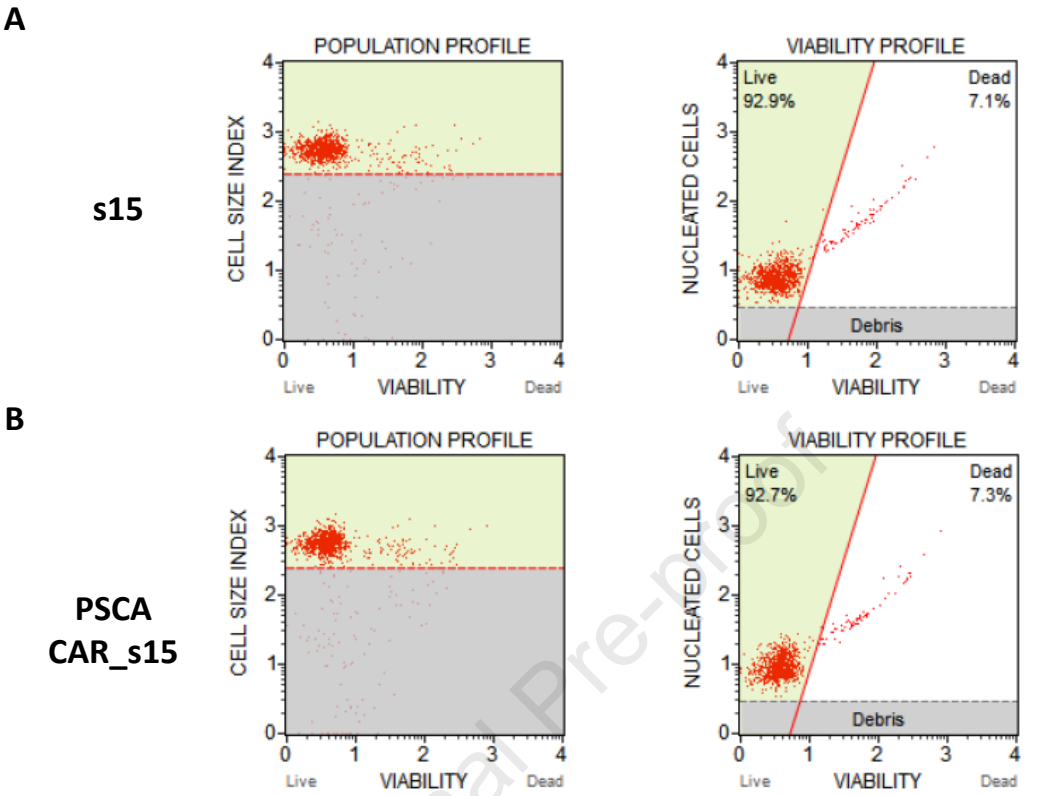
Journal Pre-proof

Supplementary Figure 5. Cytotoxicity of the "off-the-shelf" PSCA CAR NK cells against normal primary cells vs. pancreatic cancer cells. (A) PSCA expression in the normal primary bladder epithelial cells, prostate epithelial cell, and renal proximal tubule epithelial cells. PANC-1 is a negative control pancreatic cancer cell line while Capan-1 is a positive control pancreatic cancer cell line for assessing the expression of PSCA. (B) Functional validation of the frozen PSCA CAR_s15 NK and s15 NK against normal primary bladder epithelial cells, prostate epithelial cell, and renal proximal tubule epithelial cells or pancreatic cancer cell lines PSCA(+) AsPC-1, PSCA(+) Capan-1, and PSCA(-) BxPC-3 using RTCA assay at a E/T ratio of 1:1.



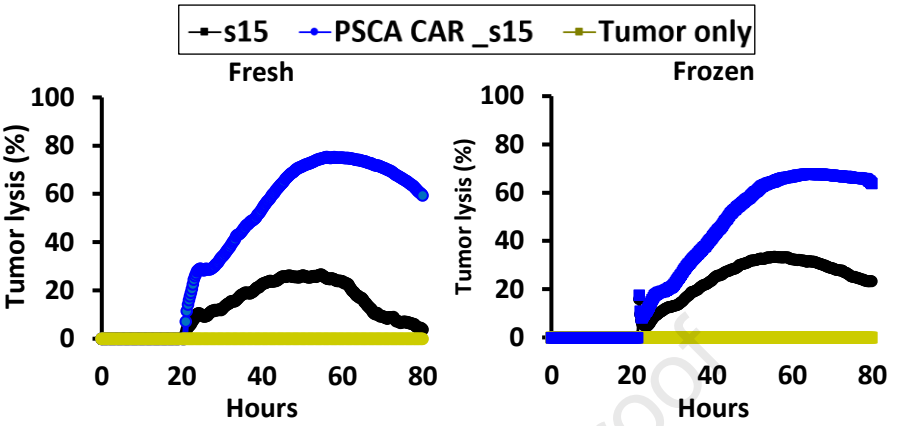
Supplementary Figure 6. Inhibitory receptors or markers of NK cell exhaustion for sIL-15 NK and PSCA CAR_sIL15 NK cells. NK cells engineered with s15 and PSCA CAR_sIIL-15 were co-cultured with PSCA(+) Capan-1 cells at an E/T ratio= of 1:1 for 12 hours. The percentage of cells expressing an inhibitory marker is shown after the CD56(+) living singlet cells are gated as total cells, based on staining of a corresponding isotype as a baseline.

Journal Pre-proof



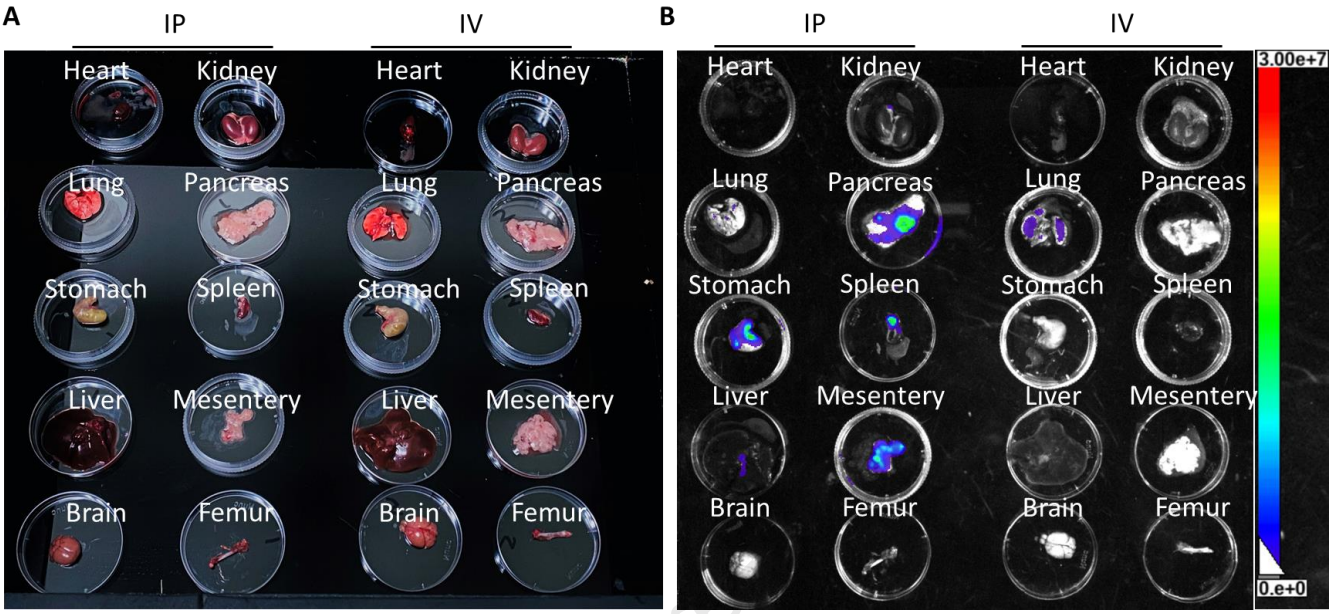
Journal Pre-proof

Supplementary Figure 7. Cell analysis of NK cells recovered from cryo-freezing status. NK cells engineered with (A) s15 or (B) PSCA CAR_s15 were expanded and cryopreserved in the liquid nitrogen. The NK cells shown here were thawed after 6 months of cryopreservation and analyzed with the MUSE cell analyzer. The viability of engineered NK cells was determined with the MUSE cell analyzer with the Muse® Count & Viability Kit (Luminex).



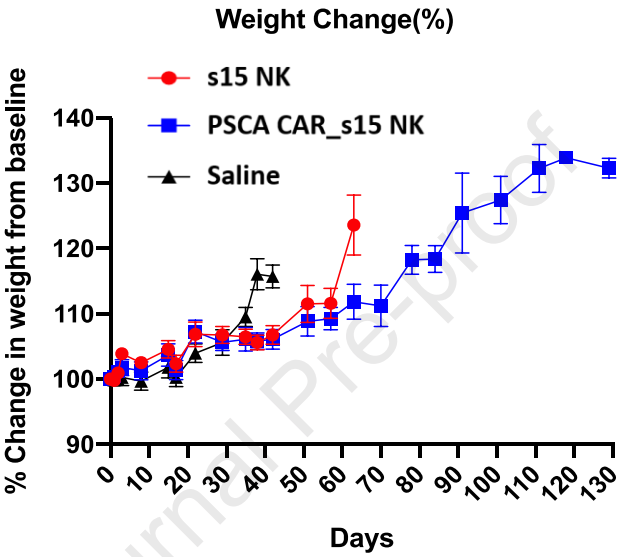
Supplementary Figure 8. A comparison of cytotoxicity between fresh and two-week cryopreserved engineered NK cells. Cytotoxicity of engineered NK cells before and after a freeze-thaw cycle (n=4) against Capan-1 tumor cells as assessed by RTCA (E:T ratio=1:3) in the presence of IL-2. NK cells used in this assay were from the same donors and cryopreserved for 2 weeks.

Journal Pre-proof



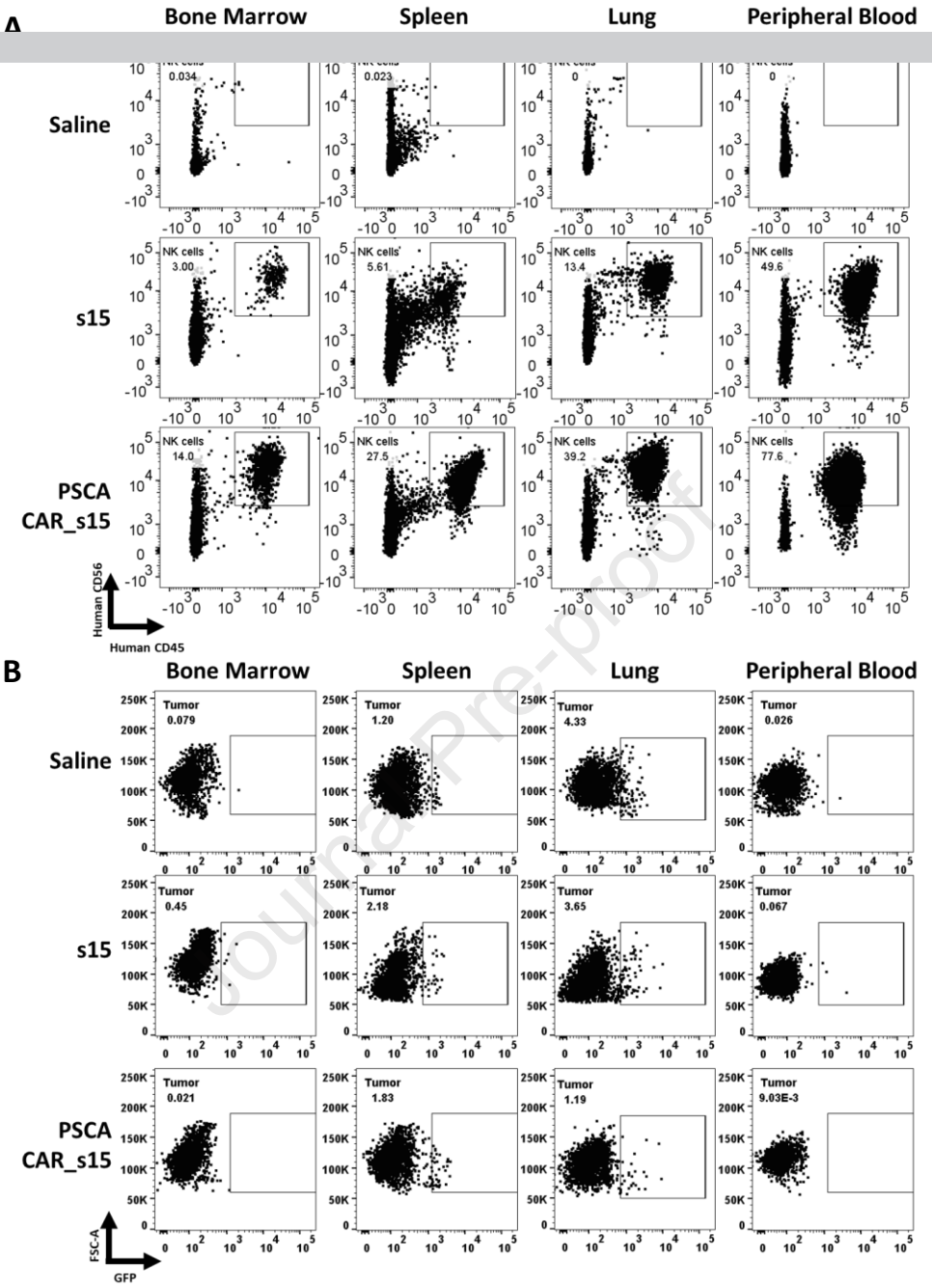
Supplementary Figure 9. Comparison of NK cell distribution in mice after *i.p.* or *i.v.* delivery by luciferase-based imaging. PSCA CAR_s15 NK cell co-expressing a luciferase_ZsGreen reporter were injected to NSG mice (5 million/ mouse) either by *i.p.* or *i.v.* Tissues or organs were harvested 7 days post CAR NK infusion. (A) Normal white light images of the organs grouped by the two delivery routes. (B) Luminescence images of the organs grouped by the two delivery routes.

Journal Pre



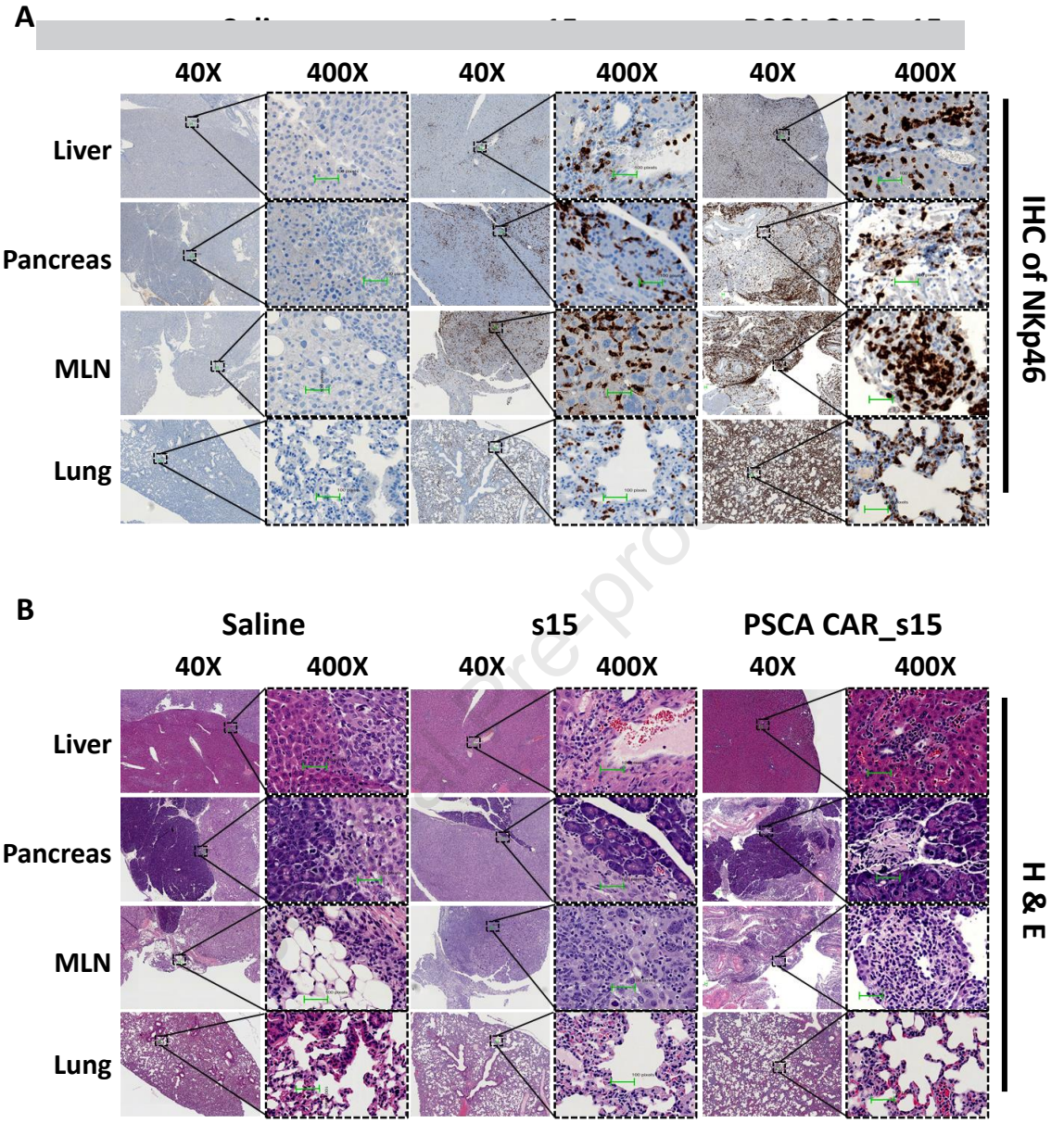
Journal Pre-proof

Supplementary Figure 10. Body weight changes over time in the metastatic pancreatic cancer model. The body weights of Capan-1_luc engrafted mice with the indicated treatments were recorded each week and normalized to their initial body weight. Body weight increases at the point of early removal criteria were attributed to ascites caused by the metastatic tumors. Data are presented as mean ± SEM.

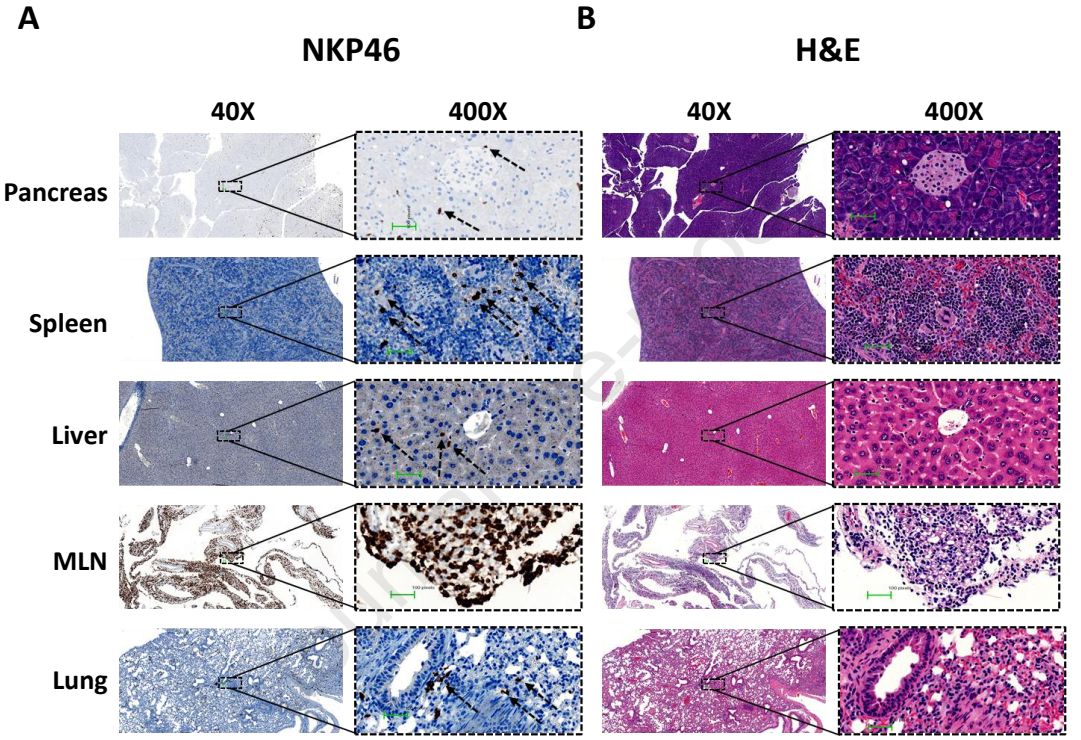


Supplementary Figure 11. FACS analysis of infused NK and tumor cells in the metastatic pancreatic cancer model. Cells collected from the bone marrow, spleen, lung, and peripheral blood after the indicated treatments were subjected to flow cytometric analyses. Tumors were defined by the expression of ZsGreen detected in the GFP channel. NK cells were defined by human CD45 and human CD56. Both tumor and NK cells were gated on the singlets and live ($SYTOX^-$) lymphocyte gate.

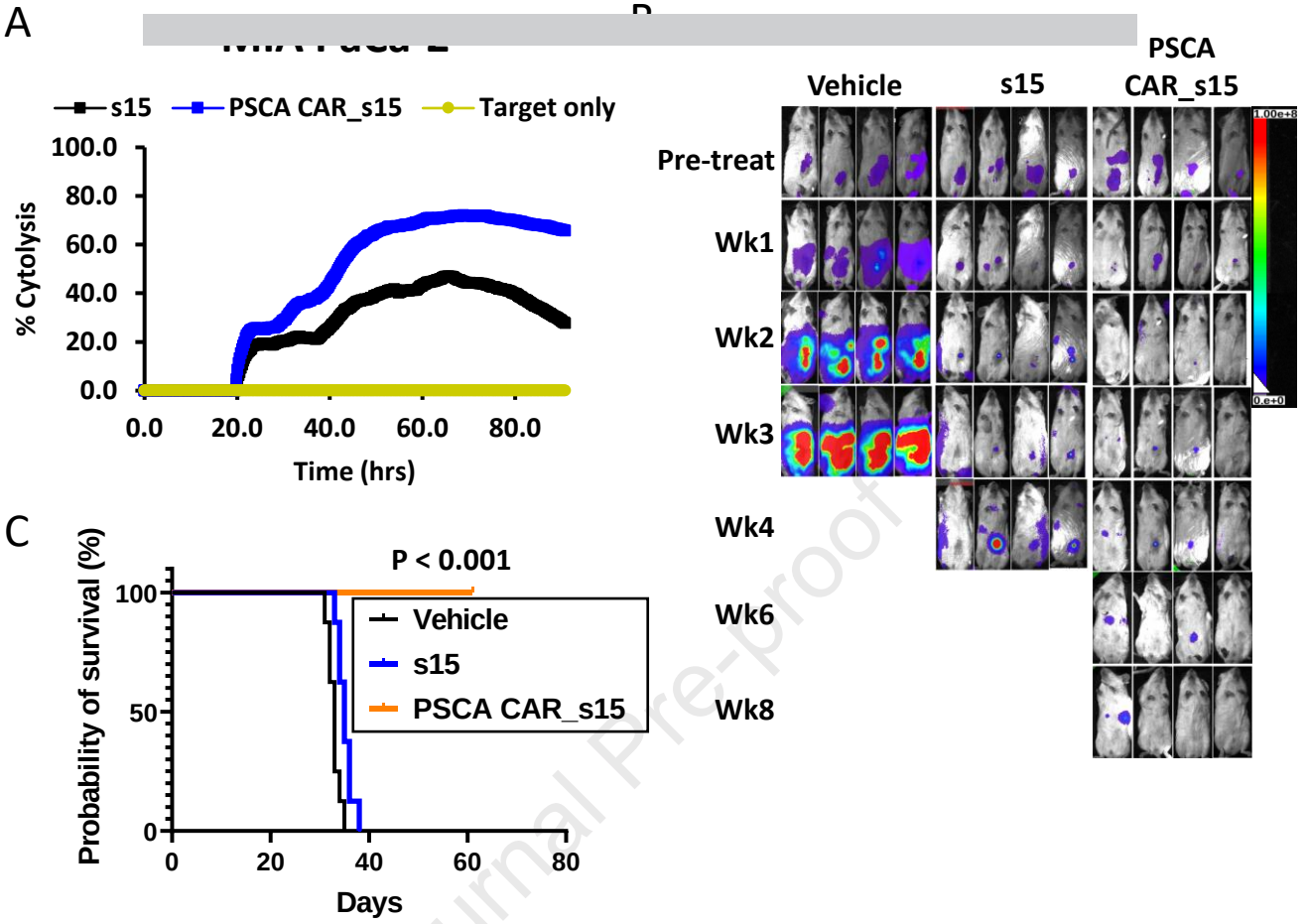
Journal Pre-proof



Supplementary Figure 12. Immunohistochemistry of human NKp46 and H&E staining in the metastatic pancreatic cancer model. Tissue sections of the liver, pancreas, mesenteric lymph node (MLN), and lung from the vehicle-, s15-, or PSCA CAR_s15 NK-treated mice were stained with human (A) NKp46 or (B) H&E. Representative images were taken under the indicated magnification and scale bar is 100 pixels.

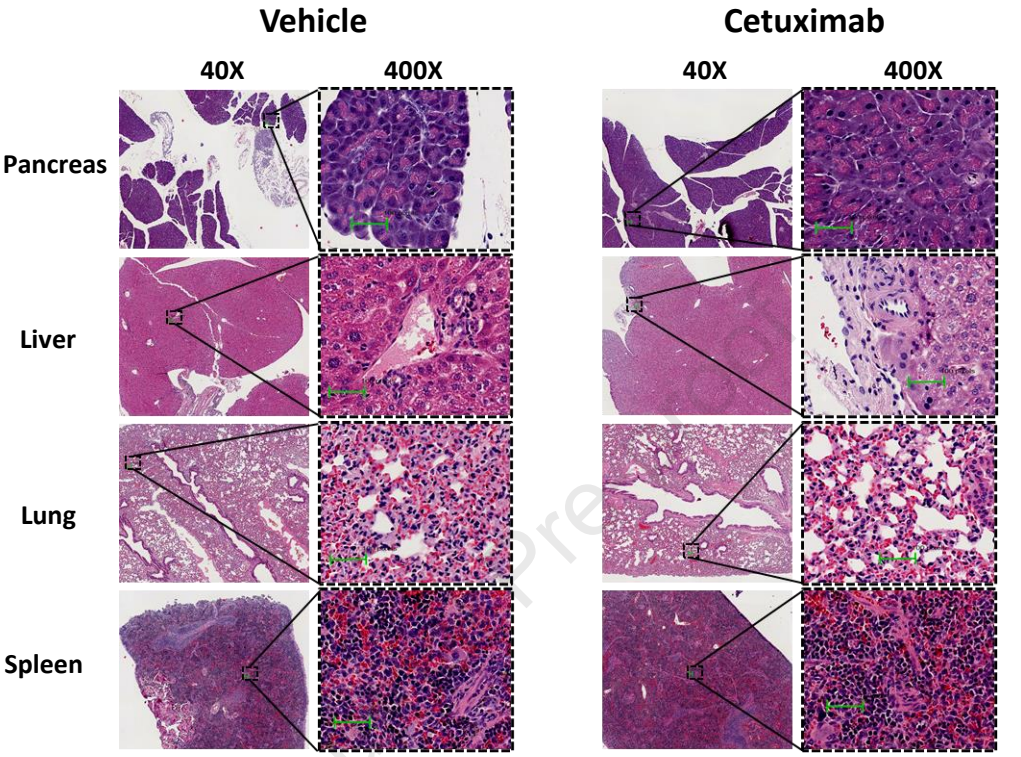


Supplementary Figure 13. Immunohistochemistry of human NKp46 and H&E staining in the PSCA CAR_s15 NK-treated mice >90 days following the last injection. Tissue sections of pancreas, spleen, liver, MLN, and lung from PSCA CAR_s15 NK-treated mice were stained with human (A) NKp46 or (B) H&E. Representative images were taken under the indicated magnification and scale bar is 100 pixels.



Journal Pre-proof

Supplementary Figure 14. PSCA CAR_sIL-15 NK cells suppress MIA PaCa-2 *in vitro* and *in vivo*. (A) Functional validation of the off-the-shelf PSCA CAR_sIL-15 NK cells and s15 NK cells with PSCA(+) MIA PaCa-2 cell line using the RTCA assay under E/T ratio= 1:3. (B) Metastatic pancreatic cancer model established with the MIA PaCa-2 cell line (0.2 million/mouse). Mice were treated with *i.v.* (1 million transduced cell/dose) and *i.p.* (1 million transduced cell/dose) injections every two weeks (n=7 per group) for total 6 doses every two weeks. Time-lapse luciferase imaging of the MIA PaCa2_luc PC tumor in mice treated as indicated. (C) Survival analysis of the pancreatic metastatic model showed in (B) by log-rank test. *** denotes p< 0.001.



Supplementary Figure 15. Histology of PSCA CAR_s15 NK cells in vehicle- and cetuximab-treated mice. H&E staining of the pancreas, liver, lung, and spleen from PSCA CAR_s15 NK cell-treated mice after the indicated treatment. Representative images were taken under the indicated magnification and scale bar is 100 pixels.