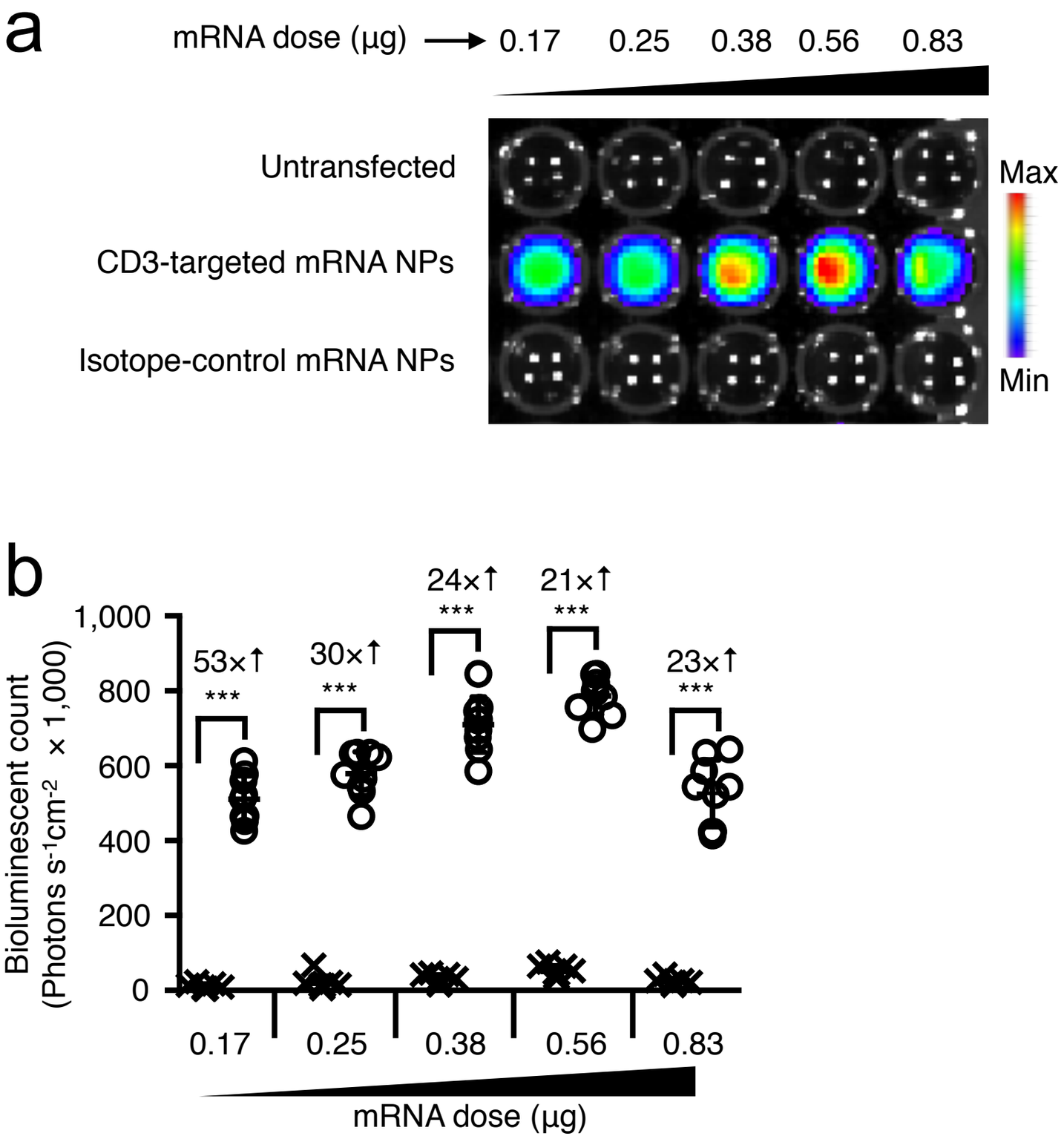
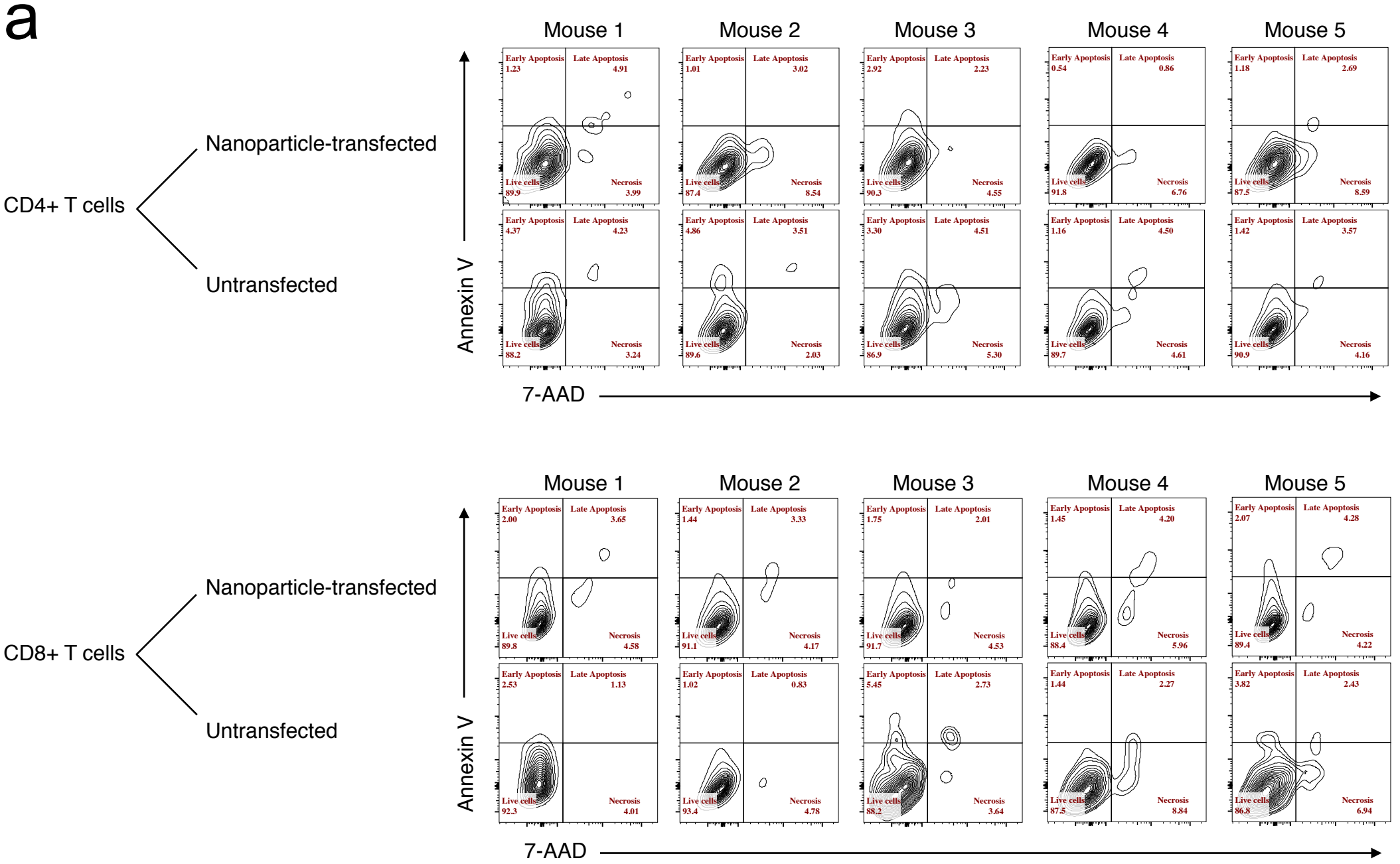
**Supplementary Information**

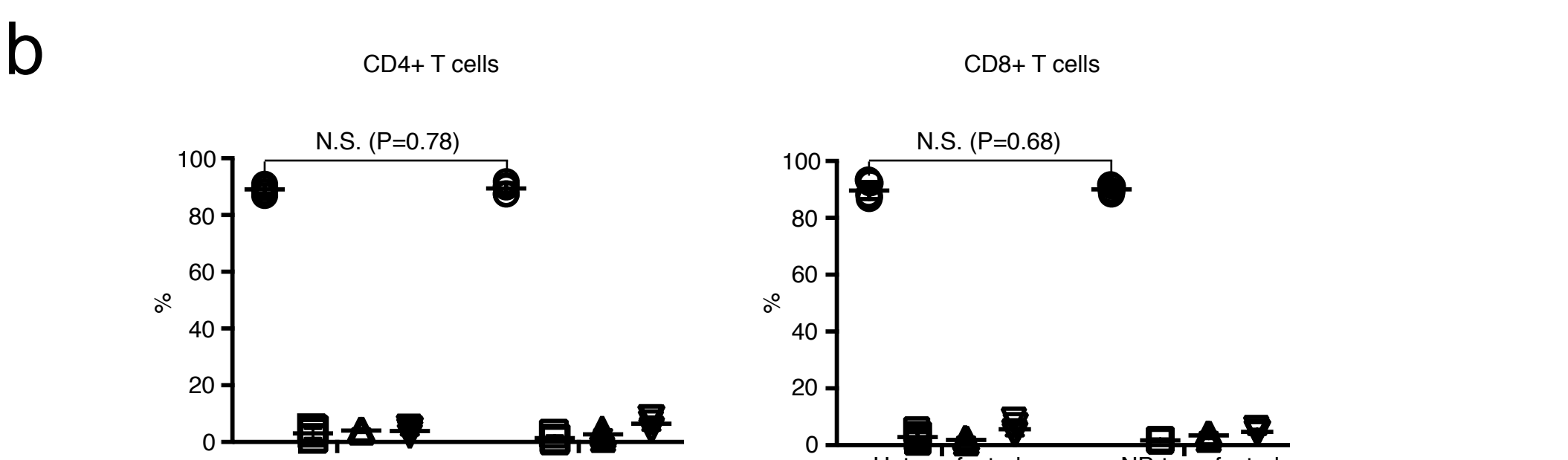
# In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo

**Parayath, et al**.



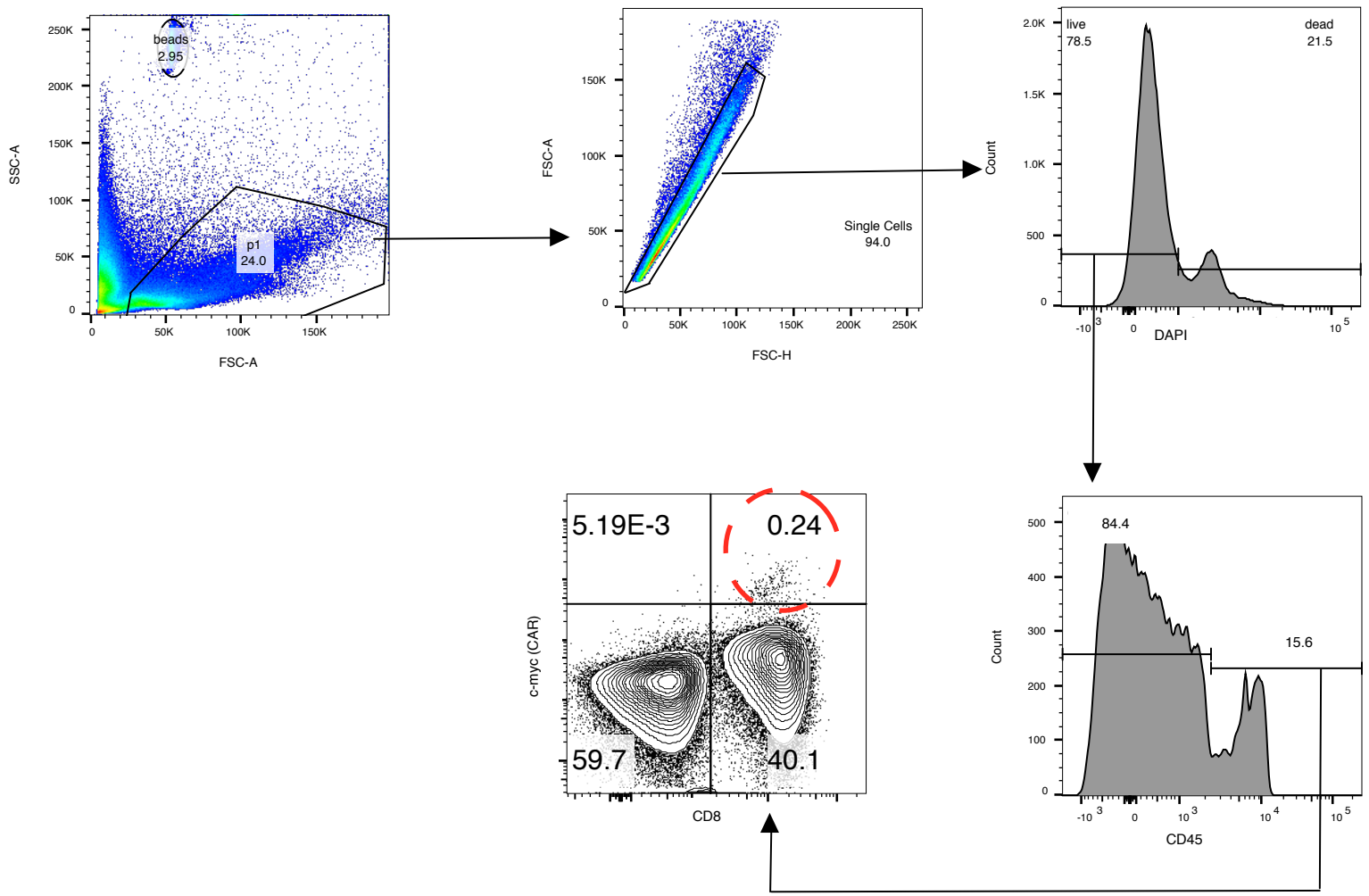

**Supplementary Figure 1: Selective transfection of human T cells with CD3-targeted mRNA nanoparticles.** PGA conjugates were formed with either anti-human CD3 (Bio X Cell, clone OKT3, cat#BE00001-2) or isotype control (Miltenyi mouse IgG2, clone S43.10, cat#130-106-546). Nanoparticles were formed by vortexing firefly luciferase mRNA, PBAE and PGA conjugate (1:60:2.5) in 25 mM NaOAc, pH 5.2. Nanoparticles were then directly added to purified human T cells at the indicated concentrations and luciferase expression was measured 24 hours later. (**a**) Bioluminescent signals emanating from transfected T cells. (**b**) Graphs showing mean photon counts. ***; significant, P<0.0001. N=9 biologically independent samples pooled from three independent experiments.



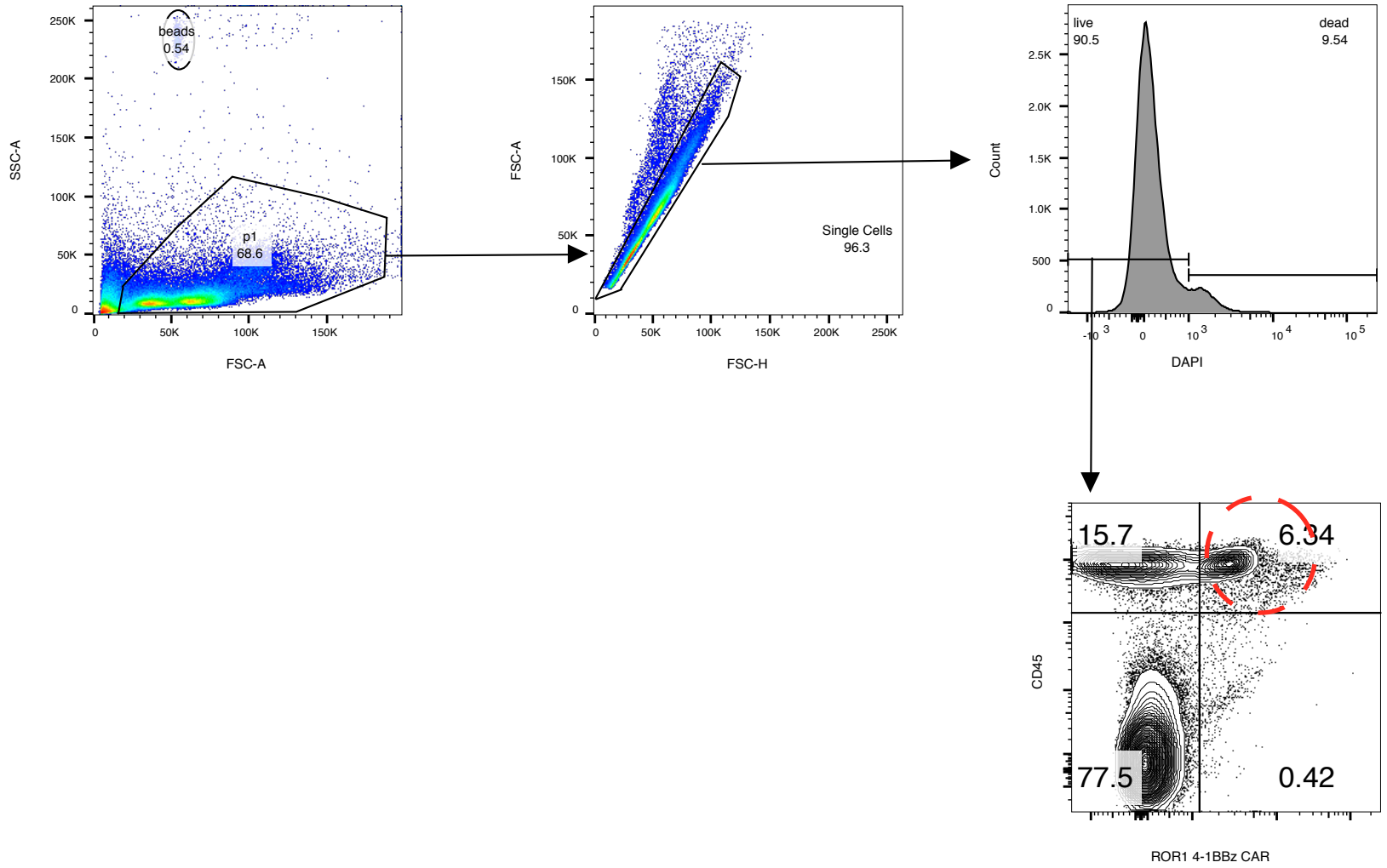




**Supplementary Figure 2: *In situ* transfection of T cells does not compromise their viability**. Ai14 reporter mice (n=5) were injected intravenously with three daily doses (day 1-3) of nanoparticles loaded with 15 µg mRNA encoding nuclear localization signal (NLS)-Cre. Nanoparticles were targeted to mouse T cells using a full-length anti-CD3 MuIgG2a designed as LALAPG variants to ablate Fc receptor binding and complement activation. Forty-eight hours after the final injection (day 5), single-cell suspensions of spleens were labeled with antibodies against CD45 and T cell markers (CD4+ or CD8+), washed twice with cold cell staining buffer (Biolegend catalog # 420201) and then resuspended in Annexin V binding buffer (Biolegend catalog # 422201) at a concentration of $1\times10^6$ cells/ml. The apoptosis/necrosis markers used were Annexin A5 (Biolegend catalog # 640953, 1:20) and 7-AAD (Biolegend catalog # 420404, 1:100). Data were collected using a BD FACSymphony analyzer running FACSDIVA software (Beckton Dickinson) and analyzed using FlowJo 10.0 software. (**a**) FACS profiles of Annexin V/7-AAD staining of splenocytes using the following gates:
CD4+ T cells, nanoparticle-transfected: CD45+ dtTomato+ CD4+
CD4+ T cells, untransfected: CD45+ dtTomato- CD4+
CD8+ T cells, nanoparticle-transfected: CD45+ dtTomato+ CD8+
CD8+ T cells, untransfected: CD45+ dtTomato- CD8+

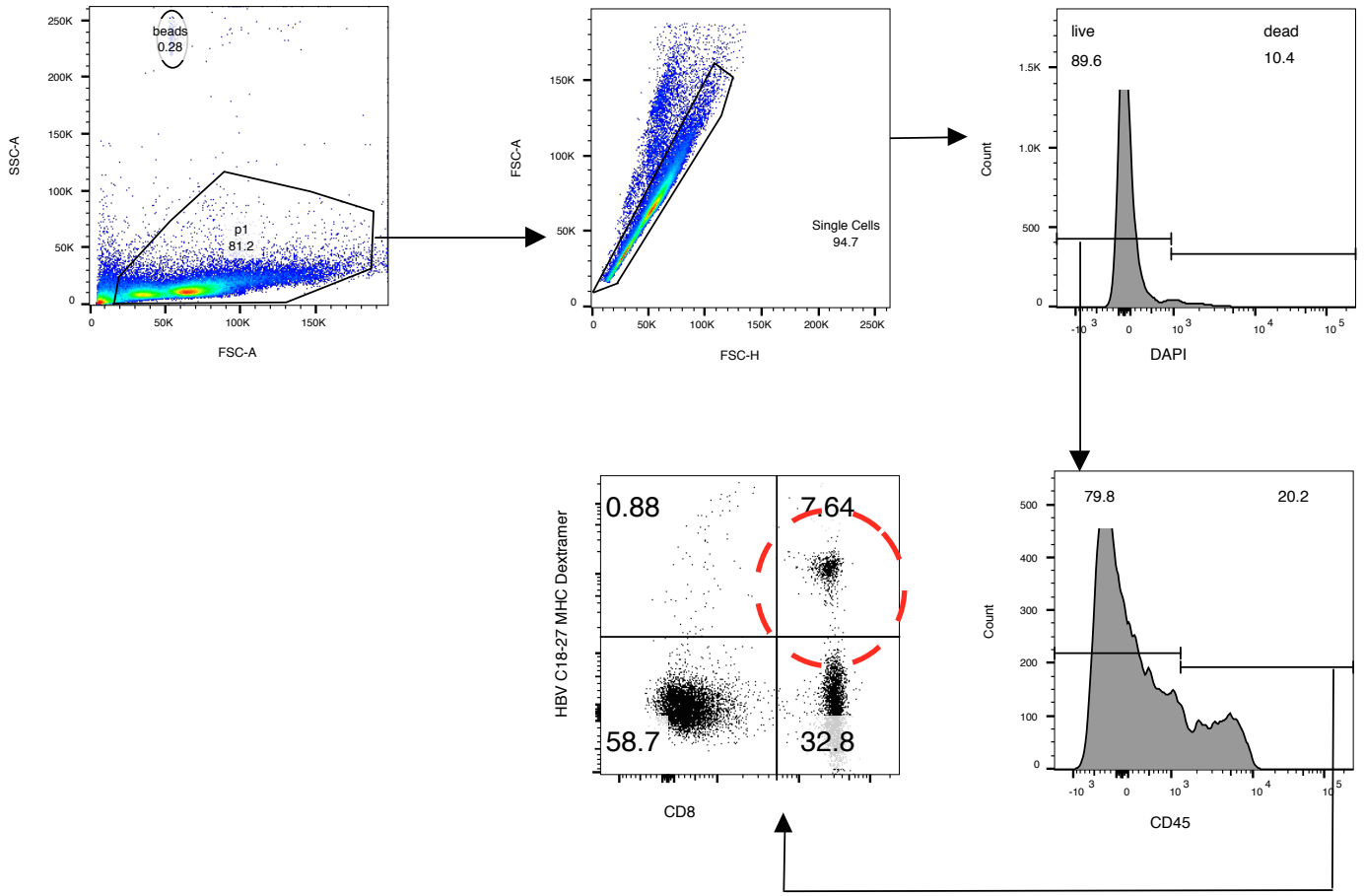
(**b**) Graphs displaying the mean % ± SD of live, early apoptotic, late apoptotic, and necrotic cells. Pairwise differences were statistically analyzed by two-tailed Student's t-test. N=5 biologically independent samples.




**Supplementary Figure 3:** Gating strategy for multicolor flow cytometry analysis shown in Figure 4d. Peripheral blood was stained with antibodies against cell surface markers as indicated and followed with flow cytometry (see methods). Cells were first gated on size and singularity followed by DAPI exclusion to identify live cells for further analysis. Live cells were gated on CD45 before identifying CD8+ c-myc+ (CAR+) cells.




**Supplementary Figure 4:** Gating strategy for multicolor flow cytometry analysis shown in Figure 7g. Tumors were digest and single cell suspensions were stained with antibodies against cell surface markers as indicated and followed with flow cytometry (see methods). Cells were first gated on size and singularity followed by DAPI exclusion to identify live cells before identifying CD45+ROR1 4-1BBz CAR+ cells.




**Supplementary Figure 5:** Gating strategy for multicolor flow cytometry analysis shown in Figure 8d. Livers were digest and single cell suspensions were stained with antibodies against cell surface markers as indicated and followed with flow cytometry (see methods). Cells were first gated on size and singularity followed by DAPI exclusion to identify live cells for further analysis. Live cells were gated on CD45 before identifying CD8+ HBV C18-27 MHC Dextramer+ cells.

**Supplementary Table 1** List of primary antibodies and other staining reagents used for flow cytometry

| Specificity | Clone | Isotype | Dilution | Dye | Supplier | Catalog# |
|---|---|---|---|---|---|---|
| Human CD8 | 3B5 | IgG2a | 1:400 | APC-Alexa Fluor 750 | ThermoFisher | MHCD0827 |
| Human CD3 | UCHT1 | IgG1, kappa | 1:400 | Alexa Fluor 532 | ThermoFisher | 58-0038-42 |
| Human CD45 | HI130 | IgG2a | 1:400 | PE-Cyanine 7 | ThermoFisher | 25-0459-42 |
| c-Myc | 9B11 | IgG2a | 1:50 | | Cellsignal.com | 3739S |
| Human ROR-1 | | IgG | 1:200 | Donkey anti-Goat IgG secondary antibody, Alexa Fluor Plus 647 (ThermoFisher, Cat# A32849) | Antibodies-online.com | ABIN4899817 |
| PSMA | | IgG | 1:300 | FITC | LifeSpan BioSciences | LS-C317473-50 |
| Human PSCA | 7F5 | IgG2b kappa | 1:200 | PE | Santa Cruz Biotechnology | Sc-80654 |
| HLA-A201-HBV core 18-27-PE pentamer | - | - | 1:200 | PE | Proimmune | F023-2B - 23 - A*02:01 - FLPSDFFPSV - **Pentamer** - 150 test R-PE |
| Live/Dead Fixable Green | - | - | 1:800 | FITC | Life Technologies | L23101 |
| Mouse CD45 | 30-F11 | IgG2b, κ | 1:100 | BUV661 | BD Biosciences | 612975 |
| Mouse CD11b | M1/70 | IgG2b, κ | 1:100 | BUV395 | BD Biosciences | 563553 |
| Mouse CD11c | HL3 | IgG1, λ2 | 1:100 | APC-Cy™7 | BD Biosciences | 561241 |
| Mouse LY6G | 1A8 | IgG2a, κ | 1:100 | BUV737 | BD Biosciences | 741813 |
| Mouse CD19 | 1D3 | IgG2a, κ | 1:100 | BUV805 | BD Biosciences | 749027 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Mouse F4/80 | BM8 | IgG2a, κ | 1:200 | FITC | Biolegend | 123108 |
| Mouse CD4 | RM4-5 | IgG2a, κ | 1:100 | Brilliant Violet 421 | Biolegend | 100563 |
| Mouse CD8a | 53-6.7 | IgG2a, κ | 1:50 | PerCP/Cyanine5.5 | Biolegend | 100734 |
| Mouse CD44 | IM7 | IgG2b, κ | 1:100 | BUV496 | BD Biosciences | 741057 |
| Mouse CD62L | MEL-14 | IgG2a, κ | 1:50 | BV650 | Biolegend | 104453 |
| Mouse CD69 | H1.2F3 | IgG1, λ3 | 1:100 | BUV563 | BD Biosciences | 741234 |
| Live/dead stain - Zombie Aqua | - | - | 1:400 | BV510 | Biolegend | 423101 |
| Annexin A5 | - | - | 1:20 | APC/Fire™ 750 | Biolegend | 640953 |
| 7-AAD | - | - | 1:100 | - | Biolegend | 420404 |

## Supplementary Methods

**Anti-CD3 murine IgG2a LALA-PG and nonbinder control (used only for in vivo experiments in immunocompetent mice/ Figure 6)**

Heavy Chain: anti-CD3 MuIgG2a LALA-PG:

**MELGLCWVFLVAILEGVQC**EVKLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWVRQAPGRGLESVAYITSSSINIKYLDAVKGRF
TVSRDNAKNLLFLQMNILKSEDTAMYYCARFDWDKNYWGQGTMVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTL
TWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKI**EPRGPTIKPCPPCKCGAPNAAGGPSVFIFPP
KIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLP
APIERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVE
KKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPG**

Light Chain: anti-CD3 murine constant Kappa (VK):

**MDMRVPAQLLGLLLLWLPGAKC**DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNWYQQKPGKAPKLLIYYTNKLADGVPSRFSGS
GSGRDSSFTISSLESEDIGSYYCQQYYNYPWTFGPGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQ
NGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

As a specificity control, the following 2C11-based nonbinder protein was designed:

Nonbinder Heavy Chain with LALA-PG:

**MELGLCWVFLVAILEGVQC**EVKLVESGGGLVQPGKSLKLSCEASGFTFSGYGMHWVRQAPGRGLESVAYITSSSINIKYLDAVKGRF
TVSRDNAKNLLFLQMNILKSEDTAMYYCARGDSDGNSGGQGTMVTVSSAKTTAPSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLT
WNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKI**EPRGPTIKPCPPCKCGAPNAAGGPSVFIFPPKI
KDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAP
IERTISKPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEK
KNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGK**

Nonbinder light kappa chain variable chain region (VK):

**MDMRVPAQLLGLLLLWLPGAKC**DIQMTQSPSSLPASLGDRVTINCQASQDISNYLNWYQQKPGKAPKLLIYYTNKLADGVPSRFSGS
GSGRDSSFTISSLESEDIGSYYCQQGSNGPWTFGPGTKLEIKRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQ
NGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

Both proteins were expressed and purified by ATUM Biosciences (Newark, California, USA).