Supplemental Data

Supplemental Methods

Evaluation of B cells, monocytes, and T cell memory subsets in non-human primates following NKTR-255 administration

Male cynomolgus monkeys (3 to 4 per group) received a single IV dose of NKTR-255 at dose levels of 0.001, 0.01, or 0.1 mg/kg. Blood samples were collected from each animal before treatment (days -5 and -2) and at multiple intervals following treatment to evaluate absolute numbers of B cells ($CD3^{-}CD20^{+}$) and monocytes ($CD45^{+}CD3^{-}CD20^{-}CD8^{-}SSC^{mid}$) by flow cytometry. Naïve ($CD45RA^{+}CCR7^{+}CD95^{-}$), stem cell memory ($CD45RA^{+}CCR7^{+}CD95^{+}$), central memory ($CD45RA^{-}CCR7^{+}$), and effector memory ($CD45RA^{-}CCR7^{-}$) subsets were examined for Ki-67 expression. Data was acquired using a LSRFortessa X-20 or FACSCanto II (BD Biosciences) and analyzed using FlowJo software (BD Life Sciences).

Evaluation of cytokines *in vitro* in human CAR-T

CAR-T cells were co-cultured with irradiated (15,000 cGy) $K562\text{-}CD19^{+}$ cells at different effector to target ratios and various concentrations of NKTR-255. Supernatants were collected after 24 hours and levels of IFN-γ and TNF-α were measured by Luminex multiplex assay (Luminex Corporation) according to manufacturer's instructions and read on a Luminex 200 instrument.

Intracellular cytokine staining in human CAR-T

Mouse bone marrow were co-cultured with irradiated (12,000 cGy) $K562\text{-}CD19^{+}$ cells at 1:1 effector to target (E:T) ratio for 24 hours. Cells were co-cultured with GolgiStop (BD Bioscience) for five hours and washed with PBS prior to immunophenotyping. Cells were stained with live dead fixable AmCyan (Life Technologies) in PBS at 4°C. Cells were washed with staining buffer (PBS/2% FBS) then blocked with human FcR block (Miltenyi Biotec) and/or mouse FcR block (BioLegend). Surface staining (CD45, CD3, CD8, CD4, Erbitux) was performed with brilliant stain buffer (BD Biosciences). For intracellular staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm Solution Kit (BD Biosciences) and stained with IFN-γ (BioLegend) and TNF-α (BD Biosciences) antibodies. Cells were washed then resuspended in Perm/Wash buffer with liquid counting beads (BD Biosciences). Data was acquired using a LSR II, FACSymphony A5 (BD Biosciences) and analyzed using FlowJo software (BD Life Sciences).

Figure S1

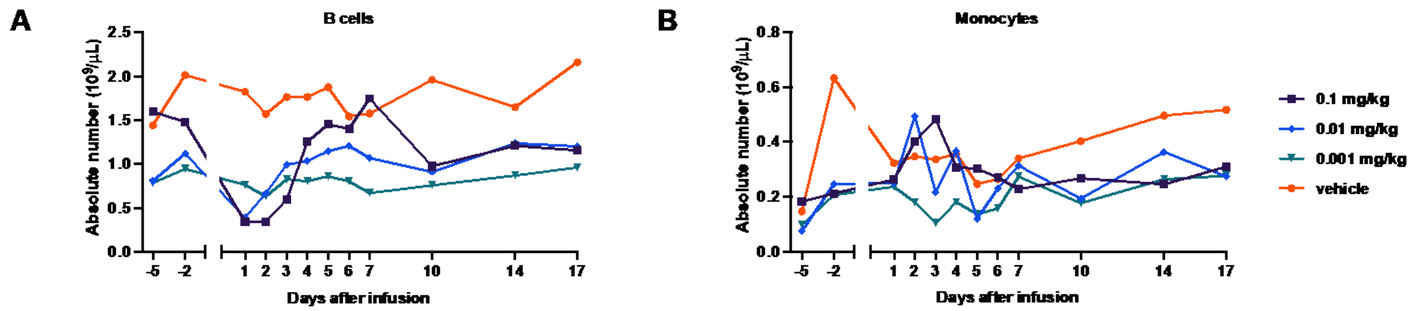


Figure S1. A single dose of NKTR-255 does not increase absolute numbers of B cells and monocytes in non-human primates. Cynomolgus monkeys (n = 3-4 per group) received a single IV dose of 0.001, 0.01 or 0.1 mg/kg of NKTR-255 or vehicle. Blood samples were collected pre-infusion and at indicated timepoints to assess the absolute numbers of B cells (A) and monocytes (B) by flow cytometry. Figures show mean values.

Figure S2

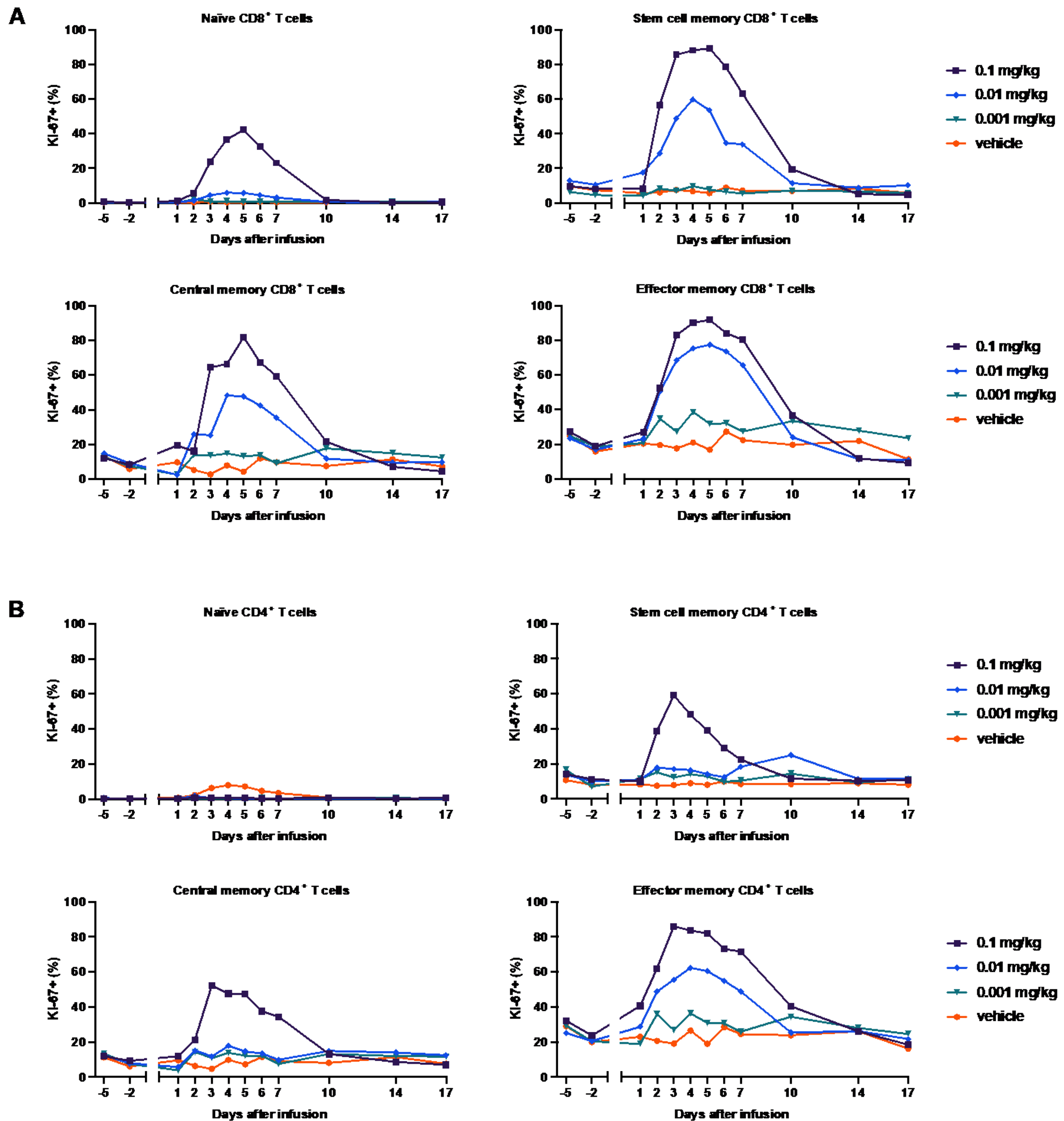


Figure S2. NKTR-255 increases proliferation in naïve and memory CD8⁺ and CD4⁺ T cells in non-human primates. Cynomolgus monkeys (n = 3-4 per group) received a single IV dose of 0.001, 0.01 or 0.1 mg/kg of NKTR-255 or vehicle. Blood samples were collected pre-infusion and at indicated timepoints to assess the percent of Ki-67 positive cells in $CD8^+$ (A) and $CD4^+$ (B) naïve ($CD45RA^+CCR7^+CD95^-$), stem cell memory ($CD45RA^+CCR7^+CD95^+$), central memory ($CD45RA^-CCR7^+$), and effector memory ($CD45RA^-CCR7^-$) T cell subsets. Figures show mean values.

Figure S3

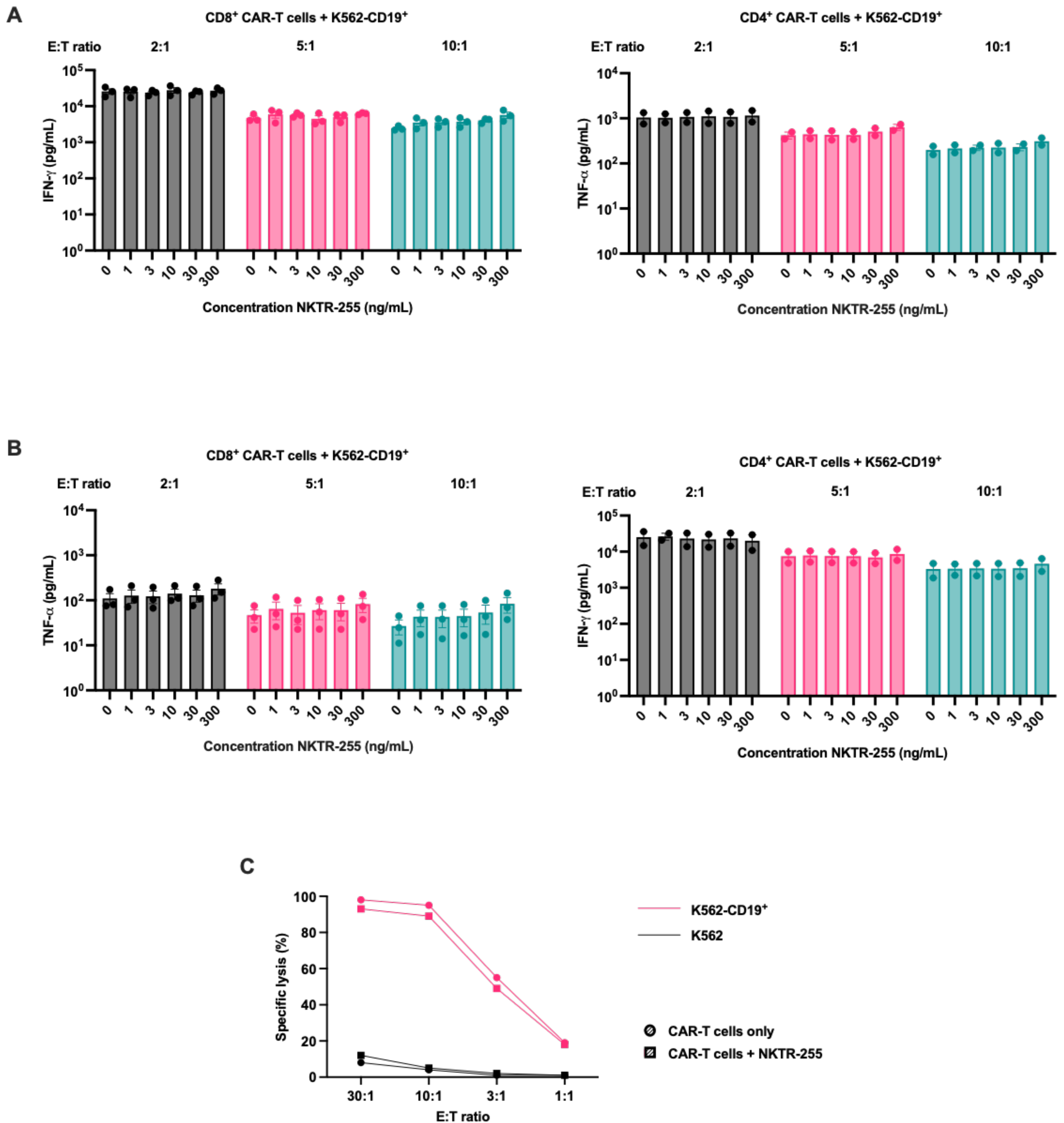
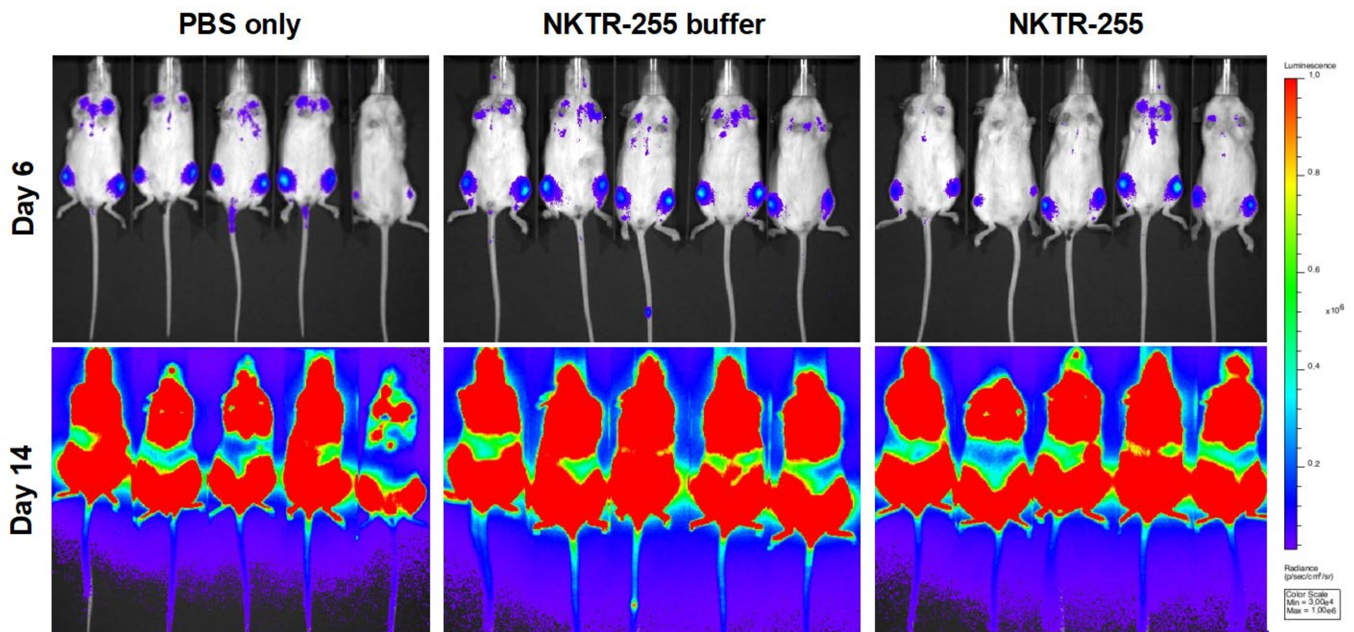
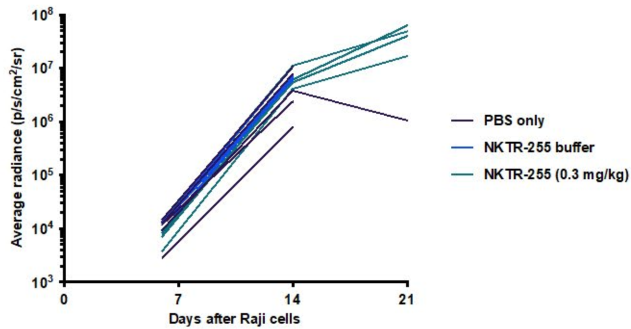


Figure S3. NKTR-255 does not increase antigen-dependent cytokine production and cytolytic activity from CAR-T cells. Human CD19 CAR-T cells were generated from healthy donors (n = 2-4) and assayed on day 14-16 after start of manufacturing. (A-B) $CD8^+$ (A) and $CD4^+$ (B) CAR-T cells were independently co-cultured for 4 days with CD19-expressing K562 cells at the indicated effector to target ratios and indicated concentrations of NKTR-255. Concentrations of IFN-γ (left) and TNF-α (right) in the supernatant were measured by Luminex. Figures show mean +/- standard error of the mean (SEM). (C) Cytolytic activity of $CD8^+$ CD19 CAR-T cells co-cultured with or without NKTR-25 against ^{51}Cr-labeled CD19-expressing K562 cells and control K562 target cells at the indicated effector to target ratios analyzed by a standard 4-hour chromium release assay (data representative of two independent experiments).

Figure S4





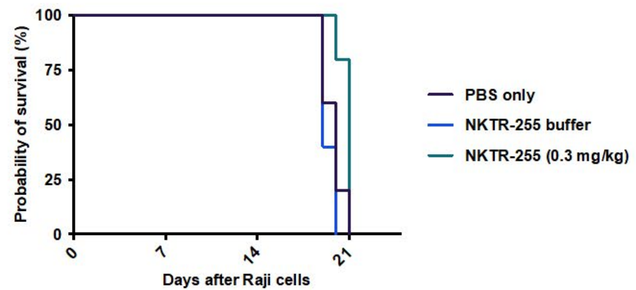


Figure S4. NKTR-255 does not affect Raji tumor growth kinetics. NSG mice were injected with Raji tumor cells IV. Six days later, following tumor engraftment, mice received either PBS, NKTR-255 buffer, or NKTR-255 IV weekly. (A) Bioluminescence imaging of Raji tumor burden indicating timepoints. (B) Average radiance of bioluminescence. (C) Kaplan-Meier survival curve.

Figure S5

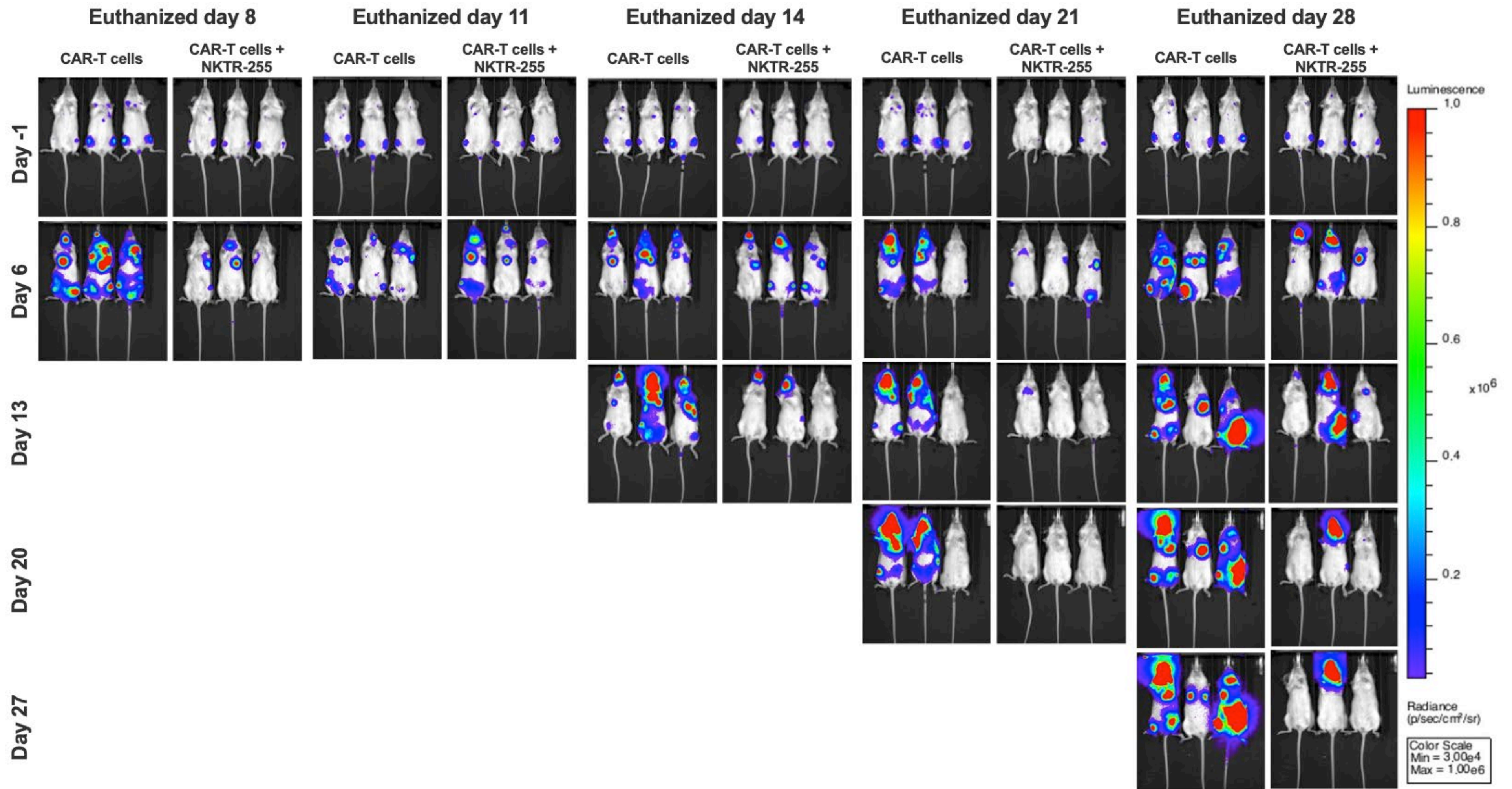


Figure S5. NKTR-255 increases antitumor efficacy of human CD19 CAR-T cells *in vivo*. NSG mice were intravenously injected with 5 x 10^5 Raji cells. Seven days later, tumor bearing mice received 0.8 x 10^6 CD19 CAR-T cells (1:1 $CD8^+$:$CD4^+$) IV. Cohorts of mice (n = 3 per group) received either buffer or NKTR-255 0.3 mg/kg IV weekly starting on day 7 and euthanized at the indicated timepoints. Mice euthanized on day 14, 21, and 28 did not receive 2nd, 3rd, and 4th dose of NKTR-255, respectively. Bioluminescence imaging of Raji tumor burden was obtained at indicated timepoints.

Figure S6

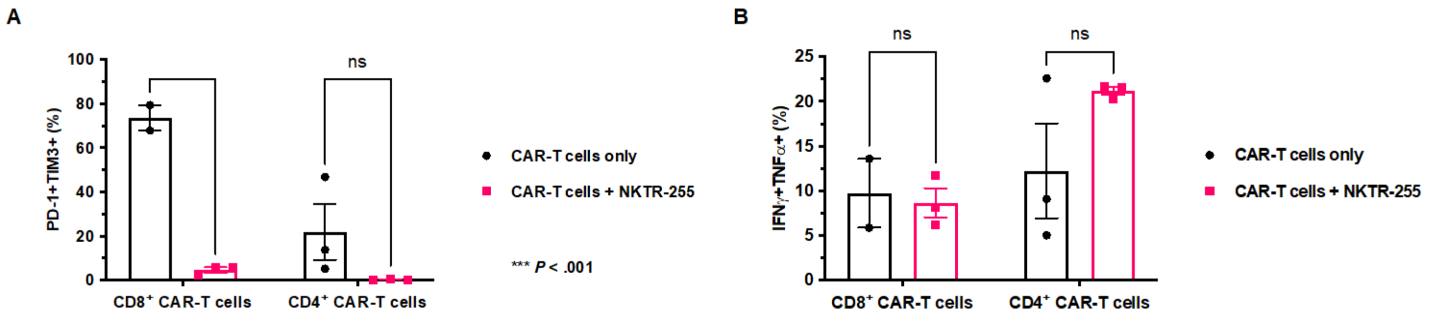


Figure S6. Persistent human CD19 CAR-T cells are less activated in NKTR-255-treated mice. NSG mice were intravenously injected with 5 x 10^5 Raji cells. Seven days later, tumor bearing mice received 0.8 x 10^6 CD19 CAR-T cells (1:1 $CD8^+$:$CD4^+$) IV. Cohorts of mice (n = 3 per group) received either buffer or NKTR-255 0.3 mg/kg IV weekly starting on day 7 and were euthanized on day 28 after CAR-T cell infusion. Single cell suspensions from bone marrow were analyzed by flow cytometry. (A) Percentage of PD-1 and TIM3 double-positive $CD8^+$ and $CD4^+$ CAR-T cells in bone marrow. (B) Percentage of IFN-γ and TNF-α double-positive $CD8^+$ and $CD4^+$ CAR-T cells in bone marrow. One mouse in the CAR-T only group did not have persistent detectable $CD8^+$ CAR-T cells. Figures show mean +/- standard error of the mean (SEM). Unpaired t test with false discovery rate of 1% by two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli were used to compare differences between groups. ns, not significant.