

# Preclinical proof of concept for VivoVec, a lentiviral-based platform for in vivo CAR T-cell engineering

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## ABSTRACT

**Background** Chimeric antigen receptor (CAR) T-cell therapies have demonstrated transformational outcomes in the treatment of B-cell malignancies, but their widespread use is hindered by technical and logistical challenges associated with ex vivo cell manufacturing. To overcome these challenges, we developed VivoVec, a lentiviral vector-based platform for in vivo engineering of T cells. UB-VV100, a VivoVec clinical candidate for the treatment of B-cell malignancies, displays an anti-CD3 single-chain variable fragment (scFv) on the surface and delivers a genetic payload that encodes a second-generation CD19-targeted CAR along with a rapamycin-activated cytokine receptor (RACR) system designed to overcome the need for lymphodepleting chemotherapy in supporting successful CAR T-cell expansion and persistence. In the presence of exogenous rapamycin, non-transduced immune cells are suppressed, while the RACR system in transduced cells converts rapamycin binding to an interleukin (IL)-2/IL-15 signal to promote proliferation.

**Methods** UB-VV100 was administered to peripheral blood mononuclear cells (PBMCs) from healthy donors and from patients with B-cell malignancy without additional stimulation. Cultures were assessed for CAR T-cell transduction and function. Biodistribution was evaluated in CD34-humanized mice and in canines. In vivo efficacy was evaluated against normal B cells in CD34-humanized mice and against systemic tumor xenografts in PBMC-humanized mice.

**Results** In vitro, administration of UB-VV100 resulted in dose-dependent and anti-CD3 scFv-dependent T-cell activation and CAR T-cell transduction. The resulting CAR T cells exhibited selective expansion in rapamycin and antigen-dependent activity against malignant B-cell targets. In humanized mouse and canine studies, UB-VV100 demonstrated a favorable biodistribution profile, with transduction events limited to the immune compartment after intranodal or intraperitoneal administration. Administration of UB-VV100 to humanized mice engrafted with B-cell tumors resulted in CAR T-cell transduction, expansion, and elimination of systemic malignancy.

**Conclusions** These findings demonstrate that UB-VV100 generates functional CAR T cells in vivo, which could expand patient access to CAR T technology in both

## WHAT IS ALREADY KNOWN ON THIS TOPIC

Chimeric antigen receptor (CAR) T cells have demonstrated unprecedented success in the treatment of B-cell malignancies, but their widespread use is hindered by the logistic complexities of ex vivo manufacturing, a prolonged wait and hospital stay for the patient, efficacy challenges due to lack of in vivo expansion and persistence, and safety challenges related to lymphodepletion and cytokine release syndrome.

## WHAT THIS STUDY ADDS

In this report, we demonstrate successful engineering of a lentiviral vector which transduces functional CAR T cells in vivo and demonstrates a favorable biodistribution profile. We also demonstrate the use of a rapamycin-activated cytokine receptor system to use rapamycin to selectively drive proliferation of CAR T cells.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

These findings demonstrate the feasibility of an in vivo engineering approach, a successful strategy to armour CAR T cells for enhanced proliferation, and proof-of-concept data for a clinical approach.

hematological and solid tumors without the need for ex vivo cell manufacturing.

## INTRODUCTION

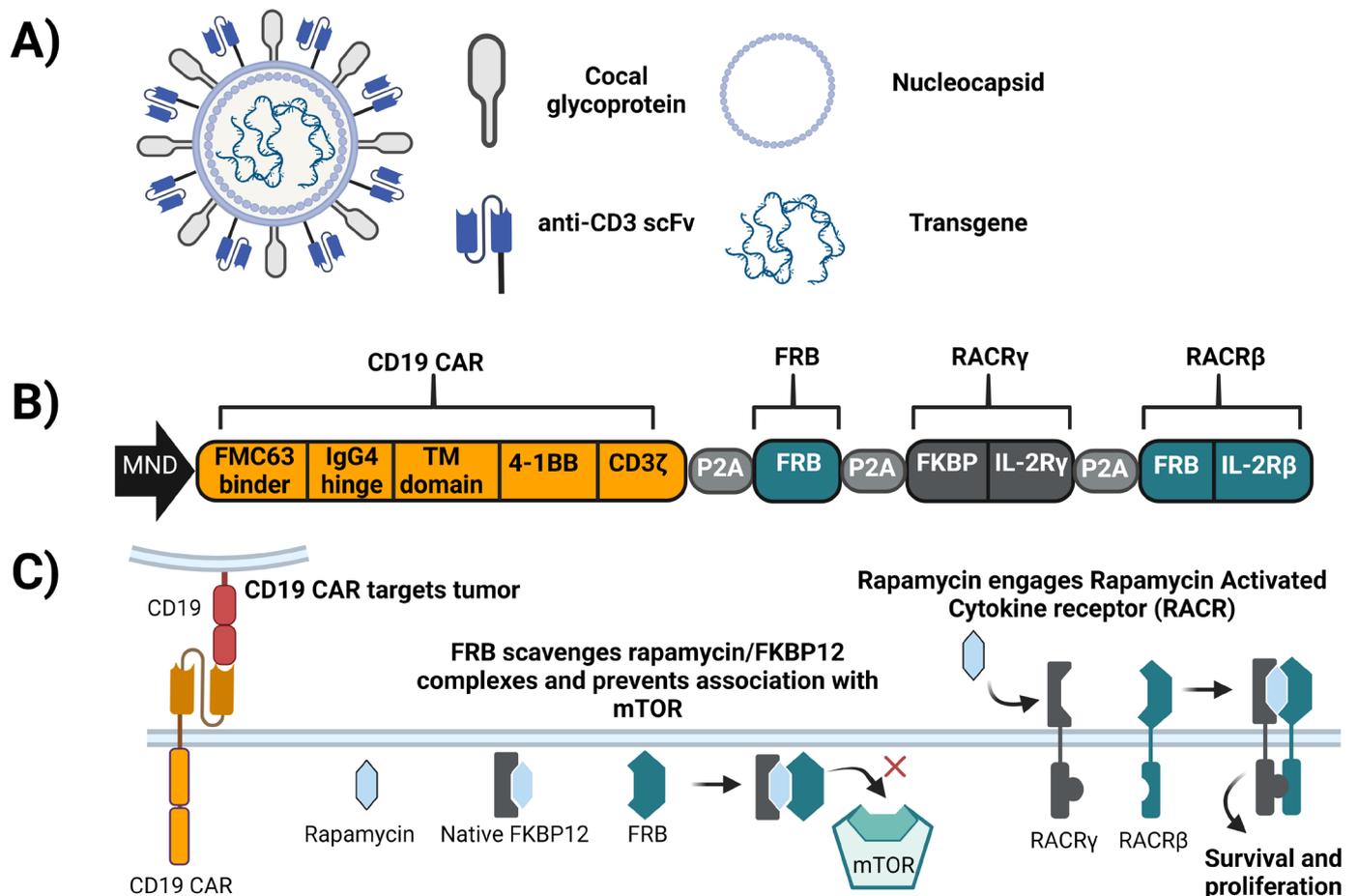
Chimeric antigen receptor (CAR) T-cell therapies have demonstrated unprecedented success in the treatment of relapsed/refractory B-cell malignancies, which has generated substantial investment in engineered T cells as a therapeutic modality.<sup>1–3</sup> Current autologous CAR T-cell manufacturing requires cell collection from the patient, genetic manipulation, expansion, and quality release before

starting treatment. In addition, patients undergo preconditioning with lymphodepleting chemotherapy before receiving CAR T-cell treatment as a requirement for successful engraftment. The complexity of the manufacturing process, logistical challenges required to maintain this supply chain, and toxicity related to lymphodepleting chemotherapy and CAR T cells results in prolonged waiting periods to receive therapy and greatly limits the number of facilities where these products can be administered.<sup>45</sup> Another consideration is the high cost associated with approved autologous CAR T-cell products which introduces challenges related to hospital payer and insurance coverage.

In vivo engineering to generate CAR T cells is a potential solution to the difficulties associated with ex vivo cell manufacturing as demonstrated successfully in preclinical models.<sup>6–8</sup> Viral vector manufacturing is well established in the context of ex vivo CAR T-cell manufacturing and can be further engineered to enable direct administration to the patient. Viral vectors are ‘off-the-shelf’ products

that, in contrast to off-the-shelf allogeneic cell therapies, generate autologous CAR T cells that are compatible with the patient’s immune system.

To enable in vivo T-cell engineering, we developed VivoVec, a surface-engineered third-generation self-inactivating replication-incompetent lentiviral vector (LVV) platform designed to generate CAR T cells without ex vivo cell manufacturing or lymphodepletion. UB-VV100, the first VivoVec platform-derived clinical candidate drug product, targets CD19 for the treatment of B-cell malignancies (figure 1A). UB-VV100 particles (comprising VivoVec particles packaging the UB-VV100-specific payload) are manufactured at clinical scale using a suspension process to enable in vivo administration. UB-VV100 particles are pseudotyped with the coccal fusion glycoprotein, a glycoprotein with a close structural relationship to that of VSV-G (71.5% shared amino acid sequence).<sup>9</sup> Compared with VSV-G, coccal is resistant to serum inactivation in humans, improving in vivo persistence and enabling direct administration



**Figure 1** Overview of UB-VV100. (A) UB-VV100 is a third-generation replication-incompetent lentiviral vector. UB-VV100 is pseudotyped with coccal glycoprotein and displays anti-CD3 scFv moieties to mediate T-cell activation and transduction. (B) The UB-VV100 transgene encodes a polycistronic payload driven by the MND promoter and separated by P2A peptide fragments. The resulting proteins are a second-generation anti-CD19 CAR, FRB, and the RACR. RACR is a chimeric heterodimer consisting of FKBP extracellular unit attached to an IL-2R $\gamma$  signaling domain and an FRB extracellular unit attached to an IL-2R $\beta$  signaling domain. (C) The components of UB-VV100 work together to mediate tumor killing and promote cell survival. CAR, chimeric antigen receptor; FRB, FKBP12–rapamycin-binding protein; IL, interleukin; RACR, rapamycin-activated cytokine receptor; scFv, single-chain variable fragment.

to patients.<sup>9,10</sup> UB-VV100 is designed to express an anti-CD3– single-chain variable fragment (scFv) on the viral envelope to mediate T-cell binding, activation, and efficient T-cell transduction.

The UB-VV100 payload encodes a polycistronic sequence with an MND promoter (a synthetic promoter with constitutive expression in the hematopoietic lineage)<sup>11</sup> driving the expression of four proteins separated by P2A sequences (figure 1B). These include a second-generation anti-CD19 CAR with the FMC63 scFv, CD3 $\zeta$ , and a 4-1BB costimulatory endodomain, and the components of the rapamycin-activated cytokine receptor (RACR) system. In the presence of rapamycin, the RACR system provides pro-survival and expansion signals to CAR T cells *in vivo* and confers resistance to rapamycin-mediated mammalian target of rapamycin (mTOR) suppression. The RACR system is composed of

- ▶ An FKBP12–rapamycin-binding protein (FRB) domain which is intended to act as a sink for intracellular rapamycin-FKBP12 complexes and prevent their inhibition of mTOR.
- ▶ A synthetic chimeric cytokine receptor consisting of an FKBP-interleukin (IL)-2R $\gamma$  fusion and FRB-IL-2R $\beta$  fusion which heterodimerize on engagement with rapamycin, resulting in intracellular signaling that mimics IL-2 and IL-15 to promote growth and proliferation (figure 1C).<sup>12</sup>

Here we demonstrate that UB-VV100 activates and transduces T cells *in vitro* and *in vivo* in a dose-dependent manner, generating CAR T cells that mediate tumor control. We demonstrate RACR-mediated expansion and enrichment in the presence of rapamycin, and characterize the biodistribution of UB-VV100 following intranodal and intraperitoneal administrations to evaluate the safety of this approach.

## METHODS

### Vector production

Lentiviral particles were generated by polyethylenimine-mediated transient transfection of in-house suspension adapted HEK293Ts (American Type Culture Collection (ATCC), Manassas, Virginia, USA) or viral production cells (Thermo Fisher Scientific, Waltham, Massachusetts, USA) grown in a shaking flask incubator in FreeStyle or LV-Max medium (Thermo Fisher Scientific), respectively. For larger preparations of suspension culture-generated lentiviral particles, single-use bioreactors replaced shake flasks for bulk production. The lentiviral particles were further purified, concentrated, and formulated through a series of filtration steps to remove cell debris, host cell proteins, host cell DNA, and residual plasmid DNA. The final particles were then sterilized via 0.2  $\mu$ m filtration and frozen. Lentiviral lots were titered on SUP-T1 cells by flow cytometry against surface CAR expression and ddPCR against lentiviral Psi integration element using the following primer sets: forward 5'-ACT TGA AAG CGA AAG GGA AAC-3', reverse 5'-CACCCATCTCTC

TCCTTCTAGCC-3, Taqman probe PSI-FAM: 5'-/56-FAM/ AGCTCTCTC/ZEN/GACGCAGGACTCGG-C/3IABkFQ/-3'. Samples were analyzed on the QX200 automated droplet generator and reader system (Bio-Rad, Hercules, California, USA). Vector formulations were evaluated for particle count by p24 ELISA (ZeptoMetrix, Buffalo, New York, USA) and evaluated by western blot to confirm anti-CD3scFv incorporation (Abcam ab40844, Cambridge, UK).

### Cell lines

Cryopreserved peripheral blood mononuclear cells (PBMCs) were purchased from AllCells (Alameda, California) and Bloodworks (Seattle, Washington, USA). Nalm-6 and Raji parental lines, and lines carrying mCherry and GFP::fluc marker genes were obtained from Seattle Children's Therapeutics. CD19 KO Nalm6 lines were gifted from Seattle Children's Therapeutics. HEK-293T (CRL-11268) and SUP-T1 (CRL-1942) cells were obtained from the ATCC. Cryopreserved human patient samples were acquired from ProteoGenex (Inglewood, California, USA).

### Assessment of UB-VV100-mediated PBMC transduction

Donor PBMCs were cultured in RPMI 1640 (Gibco) with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) and 50 IU IL-2 (R&D Systems, Cambridge, Massachusetts, USA). PBMCs were transduced at a density of 2E+06 cell/mL by adding vector directly to the well. Unless otherwise noted, PBMCs were transduced at a multiplicity of infection of 5 for 3 days for all *in vitro* studies. Rapamycin (1–40 nmol) was added to the indicated cultures on day 3. Activation and transduction were assessed on days 3 and 7 by flow cytometry using surface detection of FMC63 to identify CAR T cells unless otherwise noted (ACROBiosystems, Newark, Delaware, USA). Cells were washed with PBS, stained with a fixable viability dye, washed with FACS buffer (2% FBS in PBS), and then stained for 30 min in FACS buffer (online supplemental table S1). Cells were enumerated with CountBright beads for all flow cytometry studies (Thermo Fisher Scientific). Data were acquired on a Cytoflex (Beckman Coulter, Brea, California, USA).

### Assessment of CAR T-cell function in healthy PBMCs

Healthy donor PBMCs were transduced with UB-VV100. In indicated studies, PBMCs were activated with CD3/CD28 Dynabeads (Thermo Fisher Scientific) but not transduced. Ten days post transduction, PBMCs were plated with CD19+ or CD19KO Nalm-6 tumor cells for 24 hours at the indicated effector-to-target ratio. Nalm-6 viability was assessed by flow cytometry. Supernatant cytokines were analyzed using Meso Scale Discovery multiplex detection with the Proinflammatory Panel 1 (human) V-plex Kit (Rockville, Maryland, USA). T-cell degranulation was assessed in cultures of 1:2 CAR T:Nalm-6 for 4 hours in the presence of 1 $\times$  monensin, 1 $\times$  brefeldin A

(BioLegend) and 1:200 diluted CD107a- PE antibody (BioLegend).

### Cotransduction of Nalm-6 tumor and PBMCs

PBMCs from eight healthy donors were plated at a density of  $5 \times 10^5$  cells per well with an equal number of Nalm-6 GFP+tumor cells. The cocultures were transduced with UB-VV100 or a vector encoding a control CAR. Transduction was assessed by intracellular detection of P2A in both T cells and Nalm-6 GFP cells. Detection of CD19 was assessed by flow cytometry (online supplemental table S1).

### Transduction of B-cell malignancy PBMCs from patient samples

PBMCs from B-cell acute lymphoblastic leukemia (B-ALL) and relapsed/refractory (R/R) diffuse large B-cell lymphoma (DLBCL) patient samples were transduced with UB-VV100 (online supplemental table S2). From day 7 to day 20, UB-VV100-transduced PBMCs were expanded in 10 nM rapamycin. On day 20, rapamycin-expanded CAR T cells were assessed for cytokine release target killing in Nalm-6 cultures as previously described.

### Depletion of B cells in CD34 humanized NSG mice

Studies were conducted at Fred Hutch Cancer Research Center under approval of the Institutional Animal Care and Use Committee (IACUC) protocol Proto202000003 (online supplemental table S3). Female Nod.Cg-*Prkdc*<sup>scid</sup>-*IL2rg*<sup>tm1Wjl</sup>/SzJ (NSG) mice were irradiated and engrafted with CD34+ human hematopoietic stem cells at Jackson Laboratories (Bar Harbor, Maine, USA). UB-VV100 formulation was thawed on the day of treatment, diluted in PBS, and injected via intraperitoneal injection. Blood was collected via retroorbital sinus into sodium-EDTA coated microtainers and centrifuged at  $4000 \times g$  for 10 min to collect plasma. Blood was lysed in 1× Pharmlyse (BD Biosciences, San Diego, California, USA) and washed in PBS (Life Technologies, Rockville, Maryland, USA). Cells were stained for viability and surface antibody (online supplemental table S1). Samples were acquired on a 4-laser Cytotflex S (Beckman Coulter) and analyzed using FlowJo V.10 (Ashland, Oregon, USA). Data were graphed in Microsoft Excel and plotted on GraphPad Prism V.9 (San Diego, California, USA).

### Biodistribution of UB-VV100 in CD34-humanized NCG mice

Studies were conducted at Charles River under the approval of IACUC protocol Umoja 2021–3225. All vector formulations were thawed day of administration. Female CD34-humanized mice of NOD-*Prkdc*<sup>em26Cd52</sup> *Il2rg*<sup>em26Cd22</sup> / NjuCrl (NCG) mice were acquired at Charles River Laboratories and were accepted for enrollment based on >45% humanization (online supplemental table S4). UB-VV100 formulation was thawed the day of treatment, diluted in PBS, and injected via intraperitoneal injection once on study day 1. Blood was prepared for flow cytometry and acquired on FACS Lyric flow cytometer (BD Biosciences). Formalin-fixed, paraffin embedded (FFPE) tissues were

stained with H&E and analyzed by a board-certified pathologist at Charles River Laboratory.

### Biodistribution of αCD3 coccal pseudotyped vector encoding enhanced green fluorescent (eGFP) in canines

Studies were conducted at Charles River Laboratories under the approval of IACUC protocol Umoja 2021–3232 (online supplemental table S5). Male and female beagles 6–7 months of age were sourced from Marshall Biore-sources (North Rose, New York, USA). The test article evaluated (CD3-cocal-GFP) consisted of VivoVec particles containing an *EGFP* payload under an MND promoter. Vector was administered via ultrasound-guided inguinal lymph node injection or intraperitoneal injection once on study day 1. FFPE tissues were stained with H&E and analyzed by a board-certified pathologist at Charles River Laboratory.

### Quantification of vector integration events in mouse and canine

Genomic DNA was extracted from flash-frozen whole blood using the Nucleospin Blood QuickPure kit (Macherey-Nagel) and from flash-frozen tissues using the DNeasy Blood and Tissue kit (Qiagen). DNA was quantified using the NanoDrop Lite (Thermo Fisher Scientific). The qPCR reaction was performed in triplicate using 200 ng of DNA per well on a TaqPath ProAmp™ Master Mix (Applied Biosystems) against the viral *PSI* element of the UB-VV100 payload as previously described. QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) was used for acquisition. RNA in situ hybridization (ISH) against the desired cellular targets (online supplemental table S6) was performed using the RNAscope LS Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, Newark, California, USA). Briefly, 5 μm FFPE sections were pretreated with heat and protease prior to hybridization with the target oligo probes. Pre-amplifier and amplifier were hybridized sequentially, followed by tyramide signal amplification (TSA) fluorophore reaction. Specific RNA staining signal was identified as fluorescent, punctate dots. Samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were acquired using Panoramic SCAN II digital slide scanner (3DHitech) under  $\times 40$  magnification. Sections were manually examined to quantify and identify transduced cells (online supplemental table S7-S9).

### PBMC humanized mouse model

Studies were conducted at Seattle Children's Research Hospital under the approval of IACUC protocol ACUC00654. Female NSG mice, 6–12 weeks of age, lacking expression of MHC I and II molecules (NSG DKO, stock number 025216) were purchased from the Jackson Laboratories (online supplemental table S10). Animals were engrafted with  $0.25 \times 10^6$  Nalm-6 tumor cells expressing green fluorescent protein (GFP) and firefly luciferase (GFP::*ffluc*) via tail vein injection on study day –4. Animals were injected with 15 mg/kg of XenoLight D-Luciferin

(Perkin Elmer, Waltham, Massachusetts) and evaluated after 25 min via non-invasive In Vivo Imaging System (IVIS) Spectrum instrument (Perkin Elmer). Animals were humanized with 20E+06 PBMCs via intraperitoneal injection on day -1, and then dosed intraperitoneal with vehicle (10 mM Tris, 10% sucrose, 0.1% poloxamer 188 (v/v), pH 7.1), UB-VV100 diluted in vehicle, or a coccal pseudotyped vector encoding an FMC63-RACR transgene (coccal control) on day 0. Blood was collected for flow cytometry as previously described (online supplemental table S1).

## RESULTS

### UB-VV100 anti-CD3 scFv surface engineering enables targeted activation and transduction of T cells in a dose-dependent manner, generating functional CAR T cells

We first evaluated UB-VV100-mediated activation and transduction of unstimulated T cells. We incubated PBMCs from healthy human donors with UB-VV100 or a control LVV containing the same payload but lacking anti-CD3 scFv surface engineering (figure 2A). We found UB-VV100 treatment resulted in dose-dependent activation and transduction of CD4 and CD8 T cells. In contrast, the LVV lacking anti-CD3 scFv surface engineering did not activate or efficiently transduce T cells, consistent with previous observations that unstimulated T cells are inefficiently transduced by VSV-G and coccal-pseudotyped lentiviruses<sup>9,13</sup> (figure 2B). We did not observe any transduction events in the CD3-negative population, as compared with cells treated with empty vector particles (online supplemental figure 1A,B).

UB-VV100-transduced CAR T cells were incubated with CD19+ and CD19-deficient (CD19KO) Nalm-6 B-ALL target cells. CAR T cells lysed Nalm-6 cells and secreted effector cytokines in an antigen-specific and dose-dependent manner (figure 2C,D). CAR T-cell degranulation, measured as CD107 $\alpha$  positivity, was readily observed when CAR T cells were cocultured with CD19+ but not with CD19KO Nalm-6 target cells (figure 2E). Together these results demonstrate the expected functionality and specificity of UB-VV100-transduced CAR T cells.

Although UB-VV100 is intended to activate and transduce T cells, transduction of malignant B cells in vivo poses theoretical safety and efficacy risks to patients due to the potential for CD19 epitope masking. This phenomenon occurred in a pediatric B-ALL patient treated with ex vivo manufactured anti-CD19 CAR T-cell product, CTL019, which induced resistance to CTL019 therapy.<sup>14</sup> To evaluate the risk of epitope masking by UB-VV100, CAR+Nalm-6 cells were generated with UB-VV100 and characterized for surface CD19 epitope detection and resistance to CAR T cytotoxicity.

CAR+Nalm-6 cells demonstrated reduced, but not absent, surface detection of CD19 as measured by median fluorescence intensity (MFI), while total CD19 levels were similar to that of non-transduced Nalm-6 cells (figure 2G). This data suggests that the surface CD19 epitope of

CAR+Nalm-6 cells is only partially masked. Despite diminished surface detection of CD19 on CAR+Nalm6 cells, anti-CD19 CAR T cells were able to kill CAR+Nalm6 cells in an antigen-dependent manner. Importantly, the percentage of lysis was similar between CAR+Nalm6 cells and non-transduced Nalm6 cells with normal surface CD19 expression (figure 2G). These results were corroborated in a separate in vitro study in which CAR T cells completely eliminated CAR+Nalm-6 cells when a 1:1 mixture of PBMCs and Nalm6 cells were transduced with UB-VV100 (online supplemental figure S2). The CD19-CAR encoded by UB-VV100, encodes a CD19-CAR with a short IgG4 hinge (14 amino acids) that would be predicted to inefficiently interact with the membrane-distal CD19 epitope on the cell surface, in contrast to CTL019, which encodes a longer CD8a hinge (45 amino acids) and cis-masks CD19 from CTL019 recognition.<sup>15,16</sup>

### Rapamycin-induced RACR activation drives UB-VV100-transduced T-cell expansion and enrichment

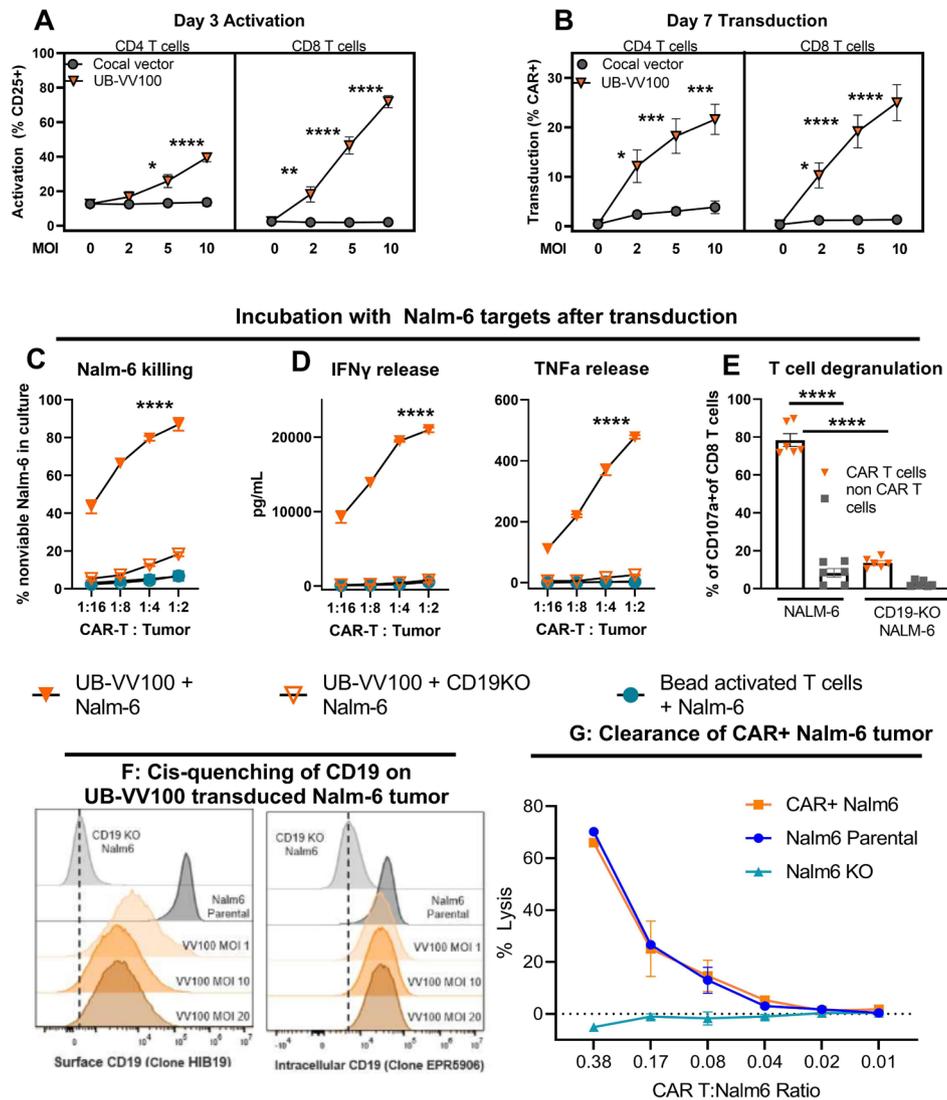
To evaluate CAR T-cell expansion driven by the RACR system, we treated UB-VV100-transduced PBMCs with 0, 1, 4, 10, and 40 nM of rapamycin. Treatment with 1–40 nM of rapamycin resulted in a concentration-dependent expansion of CAR+CD4 and CD8 T cells compared with PBMC cultures without rapamycin, and compared with CAR-negative cells within the same well. Peak expansion was evident at 10 nM for most, but not all, individual donors (figure 3A). Together, the data demonstrate effective engagement of the RACR system along a range of rapamycin concentrations that are consistent with clinical rapamycin dosing troughs (5–20 ng/mL), depending on indication.<sup>17</sup> Notably, we observed that T cells were the only cell type to reliably survive in culture under all tested conditions.

To characterize the kinetics of RACR-driven expansion and enrichment, CAR T-cell frequency and total number were measured over time with and without 10 nM rapamycin treatment. Rapamycin treatment led to absolute expansion of both CD4+ and CD8+ CAR T cells between days 7 to 21 (figure 3B). These findings demonstrate that RACR system engagement selectively expands transduced T cells at clinically relevant concentrations of rapamycin.

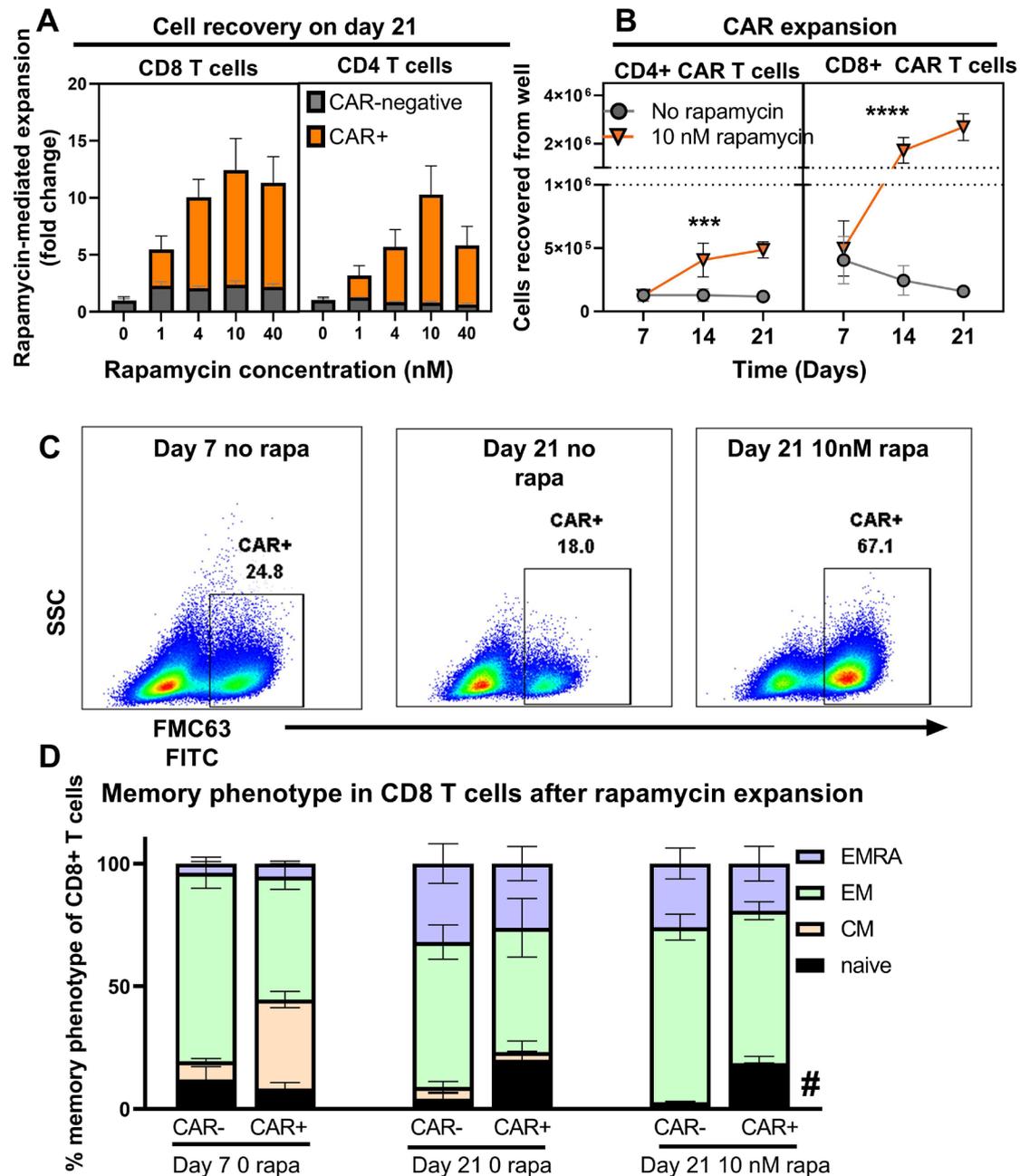
To determine the impact of prolonged RACR signaling on T-cell memory differentiation state, we assessed the memory phenotype (CD45RA and CCR7) of the CAR+ and CAR-negative T cells from wells treated with 10 nM rapamycin for 18 days. On day 21, the CAR+ T cells displayed increased frequencies of phenotypically naïve T cells (CD45RA+CCR7+) than the CAR-negative fraction, but we did not find evidence that adding rapamycin for RACR engagement drove terminal differentiation of CAR T cells in culture (figure 3D).

### UB-VV100 generates functional CAR T cells from PBMCs collected from a heavily pretreated patient population

Compared with healthy donors, patients with B-cell malignancies can have a heightened inflammatory state

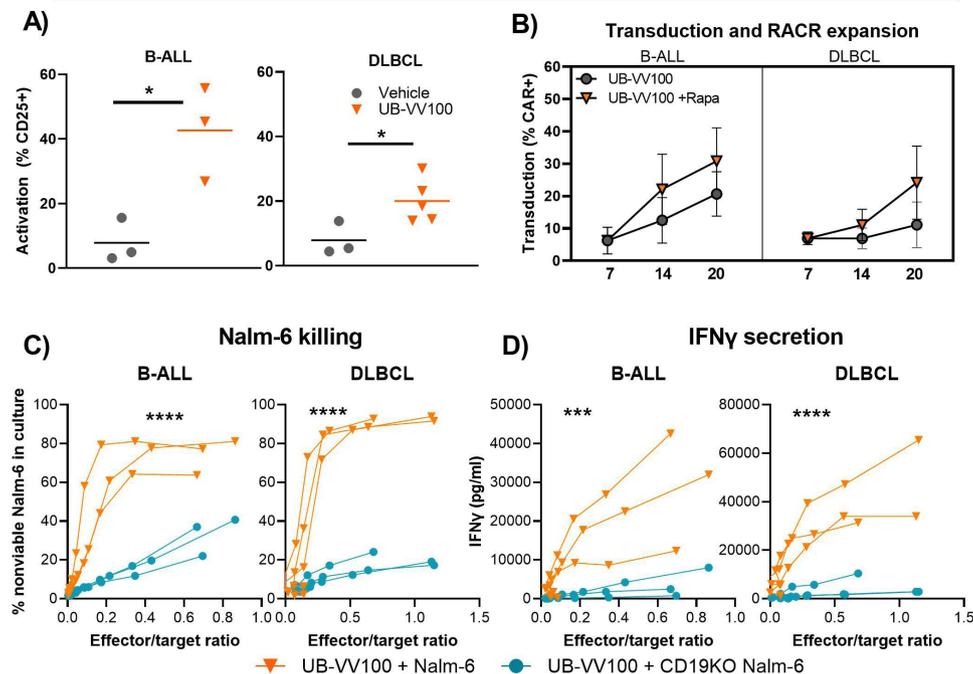


**Figure 2** UB-VV100 activates and transduces unstimulated T cells. UB-VV100 is added directly to cultured PBMCs in the presence of IL-2 and no additional stimulation. Activation, transduction, and rapamycin-mediated expansion are evaluated in the wells. (A) T-cell activation, measured by expression of CD25 (IL-2 receptor alpha), 3 days after vector addition. (B) T-cell transduction frequency, measured by surface FMC63 CAR expression, 7 days after vector addition at the indicated MOI. Data points represent mean $\pm$ 1 SEM. Data represent pooled results of n=5 for three unique donors analyzed in two independent experiments. The symbol \* indicates significance using two-way ANOVA full interaction model with Tukey's tests for multiple comparison on the indicate time point. (C) PBMCs transduced with UB-VV100 were incubated with CD19 KO Nalm-6 or parental Nalm-6 at varying E:T cell ratios as indicated. PBMCs activated with CD3/CD28 beads and incubated with parental Nalm-6 were used as an additional negative control. Cocultures were assessed for detection of dead Nalm-6 cells by flow cytometry and (D) release of IFN- $\gamma$  and TNF- $\alpha$  into the culture supernatant. The symbol \* denotes values indicating significance for ordinary two-way ANOVA, comparison of UB-VV100+Nalm-6 compared with the other groups at the indicated time point by Tukey's postcomparison test. Partial results are related for simplicity. n=3 unique donors per group, combined the result of two independent experiments originally performed in technical duplicate. (E) CAR T cells were cultured with Nalm6 cells at an E:T of 1:2 for 4 hours, and degranulation was measured in P2A+CAR+ CD8+ T cells or P2A-negative non-CAR CD8+ T cells by flow cytometric detection of extracellular CD107a. The symbol \* indicates significance for one-way ANOVA. Data represent two donors pooled from two independent experiments in technical duplicates. Nalm-6 cells were transduced with UB-VV100 at MOIs 1, 10, and 20. On day 10, CAR+Nalm-6 cells were stained with an anti-CD19 antibody (clone HIB19) to assess (F) surface CD19 expression levels and an anti-CD19 antibody that binds to an intracellular CD19 epitope (clone EPR5906) to determine the total CD19 protein level. (G) CAR T cells (VV100, MOI 5) from three healthy donors were cocultured with CAR+Nalm6 cells (VV100, MOI 10), non-transduced Nalm6 parental cells, or CD19 KO Nalm6 cells (Nalm6 KO) at multiple CAR T to Nalm6 ratios. After 24 hours of coculture, CAR+Nalm6 cells were identified based on P2A transgene expression, and the frequency of dead Nalm6 cells was determined by viability dye staining via flow cytometry. The percentage of lysis was calculated as the frequency of dead CAR+Nalm6 cells normalized to lysis of Nalm6 cells cocultured with mock transduced PBMCs. Data points represent mean $\pm$ SEM. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001 for all data panels for the indicated analysis. ANOVA, analysis of variance; CAR, chimeric antigen receptor; E:T, effector-to-target; IFN- $\gamma$ , interferon gamma; KO, knockout; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; TNF- $\alpha$ , tumor necrosis factor alpha.



**Figure 3** Rapamycin promotes selective expansion of CAR T cells. (A) PBMCs were transduced with UB-VV100 at an MOI of 5 for 3 days before vector removal. Cells were transferred to wells containing the indicated concentration of rapamycin and cultured for an additional 18 days. On study day 21, CAR+ and CAR-negative T-cell expansion was enumerated by flow cytometry.  $n=5$ , pooled results from two independent experiments using three separate PBMC donors. Data points indicate mean $\pm$ 1 SEM. (B) PBMCs treated as described (A) at the 10 nM rapamycin concentration were analyzed by flow cytometry to determine the absolute CD4 and CD8 CAR T-cell counts.  $n=6$  per group per time point, pooled results of two independent experiments using three PBMC donors. Axis is split to allow separation of CD4 and CD8 T cells to be seen on the same scale. Symbols (\*) indicate statistical significance for two-way ANOVA, main column analysis of effect of rapamycin treatment on cell expansion or enrichment over time series. Data points indicate mean $\pm$ 1 SEM; some error bars not visible due to eclipse by data symbol. PBMCs were transduced with MOI=5 with UB-VV100 and expanded for 18 days with rapamycin (study day 21 after transduction). T cells were assessed by flow cytometry for (C) representative flow staining of surface FMC63 detection and (D) memory phenotype. Representative plots show a single donor. CAR+ T cells are overlaid in red over total T-cell population. Memory phenotype was quantified for CD8+ T cells for day 7 no rapamycin, day 21 no rapamycin, day 21+10 nM rapamycin, in both the CAR+ and CAR-negative fractions. Naïve (CCR7+CD45RA+). Symbol (#) indicates  $p$  value of  $< 0.05$  for two-way ANOVA, Tukey's multiple comparison's test for comparing the 'naïve' population of the CAR+ fraction to the naïve population of the CAR-negative fraction on study day 21+10 nM rapamycin.  $n=6$  from replicate measurements of three unique PBMC donors per condition, results from one experiment. ANOVA, analysis of variance; CAR, chimeric antigen receptor; CM, central memory (CCR7+CD45RA-); EM, effector memory (CCR7-CD45RA+); EMRA, effector memory re-expressing CD45RA (CCR7-CD45RA+); MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell.

### Activation, transduction, and expansion in B-ALL & DLBCL patient samples



**Figure 4** UB-VV100 transduces functional CAR T cells from patient samples. In two separate experiments, PBMCs from patients with B-ALL and DLBCL were transduced with UB-VV100 at MOI of 5. (A) On day 3, the frequencies of T cells expressing CD25 were assessed in B-ALL and DLBCL samples. On day 7 post UB-VV100 transduction, cells were further cultured in the absence or presence of 10 nM rapamycin. \* $P < 0.05$ , Student's t-test. Bars indicate mean. (B) The frequencies of CAR T cells on days 7, 14, and 20 were determined by flow cytometry. On day 20, rapamycin-expanded CAR T cells from B-ALL and DLBCL patient samples were cocultured with Nalm-6 or CD19KO Nalm-6 tumor cells. (C) After 24 hours of coculture, the frequency of non-viable Nalm-6 tumor targets was determined by viability dye staining via flow cytometry. \*\*\*\* $P < 0.001$ , two-way ANOVA, main effect analysis for Nalm-6 identity. (D) The concentration of IFN- $\gamma$  in the coculture supernatant was also measured in both B-ALL and DLBCL samples. Data points indicate mean  $\pm$  1 SEM. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , two-way ANOVA, main effect analysis for Nalm-6 identity. ANOVA, analysis of variance; CAR, chimeric antigen receptor; IFN- $\gamma$ , interferon gamma; MOI, multiplicity of infection.

due to disease condition and prior cancer therapies. This can lead to increased heterogeneity in the overall cellular composition and the quality of patient T cells (eg, CD4:CD8 ratio, differentiation state, and T-cell subtypes), which could impact T-cell permissiveness to transduction, potency, and persistence properties of CAR T cells.<sup>18–20</sup> To evaluate the function of UB-VV100 and transduced T cells with patient material, PBMCs from B-ALL (n=3) and R/R DLBCL (n=5) patients were transduced with UB-VV100 (day 0). In both B-ALL and R/R DLBCL patient samples, UB-VV100 mediated T-cell activation and generated CAR T cells (figure 4A). To determine if rapamycin treatment can enrich patient-derived CAR T cells, cells were further cultured in the presence or absence of 10 nM rapamycin starting on day 7. Treatment with rapamycin led to a higher frequency of CAR T cells by day 20 in all patient samples compared with the no rapamycin control group (figure 4B). Rapamycin-enriched patient CAR T cells were functional as demonstrated by in vitro cytokine secretion and killing of Nalm-6 cells (figure 4C,D). Taken together, these data demonstrate that UB-VV100 can generate functional CAR T cells from patient PBMCs from relevant disease indications.

#### UB-VV100 biodistribution in humanized mice following intraperitoneal administration indicates selective transduction of immune cells

We evaluated the activity, biodistribution, and safety profile of UB-VV100 in a CD34+ hematopoietic stem cell humanized mouse model, using endogenous B-cell depletion as a surrogate indicator of UB-VV100-transduced CAR T-cell activity.<sup>21</sup> For delivery of UB-VV100 in the clinic, an intranodal injection could potentially maximize UB-VV100 exposure in a tissue enriched with T cells while minimizing systemic distribution. While NSG mice lack developed lymph nodes, injection through the intraperitoneal route results in drainage through abdominal lymphatics<sup>22</sup>; hence, this route was chosen for murine efficacy and safety studies. CD34-humanized mice were treated with doses of 0.4E+06, 2E+06, and 10E+06 transducing units (TUs). Animals developed a UB-VV100 dose-dependent reduction in B cells, with animals treated with 10E+06 TU reaching levels consistent with B-cell aplasia. In contrast, animals treated with vehicle or 10E+06 TU of a cocal-pseudotyped vector displaying an anti-CD3 scFv but encoding a CAR of irrelevant specificity exhibited B-cell levels comparable to the untreated

control animals, demonstrating antigen-specific activity (figure 5A and online supplemental table S3). Vehicle-treated animals exhibited a slight reduction in B cells over the study period, consistent with past reports that circulating B-cell levels gradually reduce over time in CD34-humanized mice.<sup>23</sup> Circulating CAR T cells were detected in a UB-VV100 dose-dependent manner but not in mice treated with the control CAR encoding vector, suggesting antigen-dependent expansion of CAR T cells. The detection of B-cell depletion (day 11) prior to the detection of circulating CAR T cells in the peripheral blood (day 18) may be due to B-cell killing in sites other than the peripheral blood or may reflect occluded or reduced detection of surface CAR after engagement with cognate antigen.

We evaluated biodistribution and acute toxicity of UB-VV100 in CD34-humanized mice injected intraperitoneal with 36E+06 or 360E+06 TU of UB-VV100. Animals were treated with 1 mg/kg of rapamycin via intraperitoneal injection every other day beginning study day 5. There was a UB-VV100 dose-dependent reduction in B cells as highlighted earlier (online supplemental figure 3). Animals tolerated both dose levels of UB-VV100 without acute adverse events. On final study termination and necropsy, organs from all study animals were evaluated for histopathology. There were no UB-VV100-related clinical signs or effects on body weights, food consumption or local injection site irritation, and no postmortem macroscopic, microscopic, or organ weight differences that could be attributed to UB-VV100 treated as compared with vehicle control animals. On study day 28, organs were evaluated for presence of transduced cells by qPCR. Integration events were detected in a dose-dependent manner; the highest level of integrated vector genomes was detected in the spleen and liver, followed by lower levels in the bone marrow, injection site, and lung (figure 5B). Organs from animals treated with UB-VV100 were further evaluated by ISH against the UB-VV100 transgene to evaluate the identity of transduced cells. Semiquantitative colocalization scoring of integration events in spleen and liver characterized transduced cells as either human T cells (0–10% human CD3+ of transgene+cells) or murine macrophages (91%–100% murine CD68+ of transgene+cells) (figure 5C, full tissue section quantified in online supplemental table S7 and S8).

#### **Biodistribution in canines following intranodal or intraperitoneal administration indicates that UB-VV100 primarily transduces immune cells/tissues**

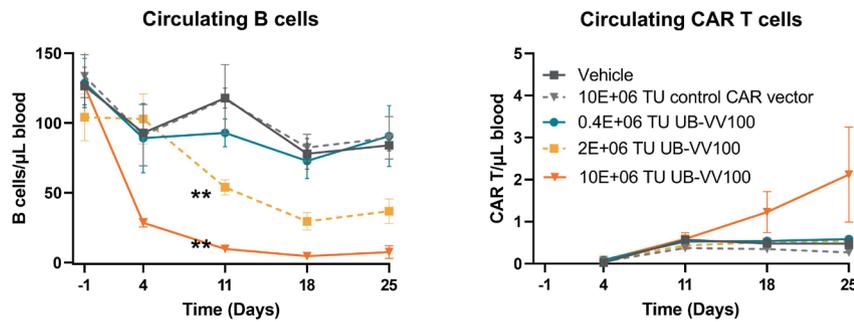
As lymph node injections are not feasible in immunocompromised NSG/NCG mouse models, biodistribution was also evaluated in canines, which can adequately assess coccal-mediated transduction,<sup>9,10</sup> though no targeted activation of T cells is expected due to a lack of cross-reactivity with the antihuman CD3 scFv surface engineering. UB-VV100 surface engineered viral particles containing an *EGFP* payload (CD3-cocal-GFP) were administered to healthy beagles by bilateral, ultrasound-guided inguinal lymph node injection of 4E+08 TU CD3-cocal-GFP or

intraperitoneal injection of 40E+08 TU CD3-cocal-GFP (figure 6A). CD3-cocal-GFP was well tolerated. Intranodal injection resulted in detection of integrated vector genomes primarily in the injected inguinal lymph nodes, with a 10-fold lower frequency of transduced cells detected in downstream draining medial iliac and lumbar lymph nodes. Intraperitoneal injection resulted in detection of integrated vector genomes primarily in the blood and medial iliac and lumbar lymph nodes through which interstitial fluid from the intraperitoneal cavity drains (figure 6B). There was no quantifiable detection of integrated vector genomes in any non-lymphoid tissues with either route of administration (ROA). ISH analysis identified the large majority of transduction events from both ROAs (ranging from >70% to >99% across lymph node samples) as immune cells (transgene+ and canine CD45+), including macrophages (transgene+ and canine CD68+) and T cells (transgene+ and canine CD3+). Despite a lack of canine T-cell targeting with particle surface engineering, transduction of canine T cells was observed in lymphoid tissues from intranodal injected animals, indicating the intranodal ROA is effective at delivering lentivirus to the target T-cell population (figure 6C, quantified in online supplemental table S9).

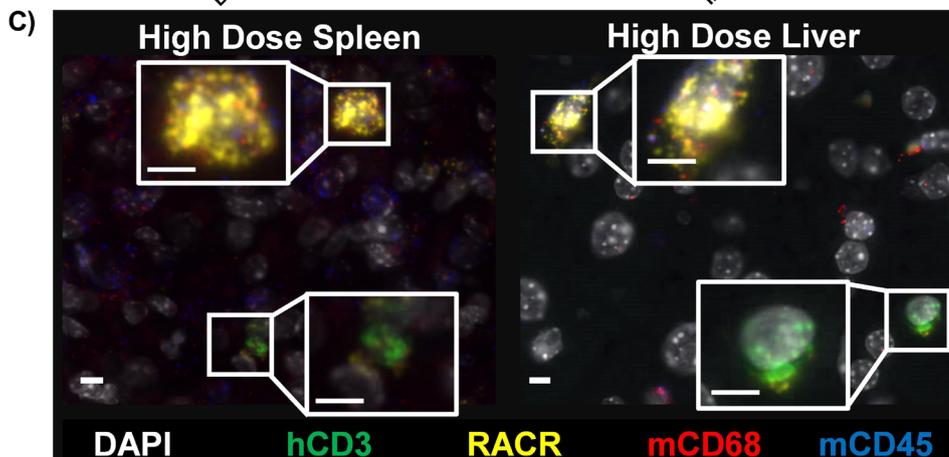
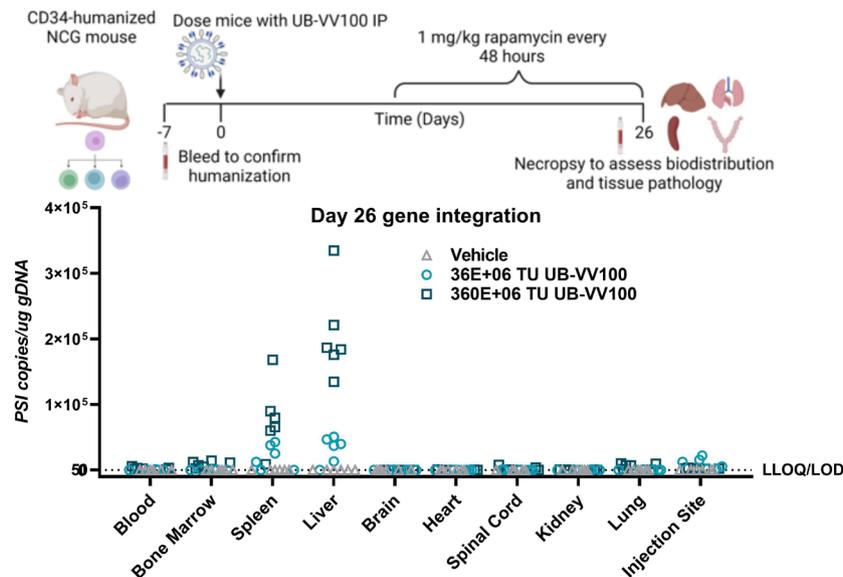
#### **UB-VV100 controls systemic leukemia in a Nalm-6 xenograft mouse model**

We evaluated the efficacy of in vivo generated CAR T cells in an aggressive tumor model. To do this, we developed a systemic Nalm-6 tumor model in PBMC-humanized NSG mice in which the mouse MHC class I and II genes have been knocked out to reduce graft-versus-host disease, and minimized transduction caused by activation driven by xenoreactivity.<sup>24</sup> Mice were implanted with Nalm-6 cells via tail vein injection day -4, humanized with PBMCs via intraperitoneal injection day -1, and treated with vector intraperitoneal day 0 (figure 7A). Administration of 27, 80, and 270 million TU of UB-VV100 resulted in a dose-dependent activation of human T cells as measured by CD71 expression 4 days after administration (figure 7B). By day 11, circulating CAR T cells were detectable in all UB-VV100-treated mice in a dose-dependent manner (figure 7C). By study day 12, animals treated with UB-VV100 displayed significant tumor reduction compared with vehicle treated animals, and animals treated with the highest dose showed complete tumor elimination (figure 7D). Animals treated with all doses of UB-VV100 tolerated the treatment without signs of toxicity. All doses of UB-VV100 significantly improved animal survival as compared with vehicle-treated animals (figure 7E). Transduction of circulating murine CD45+ cells was not detected at any time point, and transduction of Nalm-6 tumor cells could not be evaluated due to absence of detectable tumor cells by flow cytometry in UB-VV100 treated mice during serial bleeds or at terminal euthanasia in the represented study (online supplemental figure 4). In an independent replicate study, Nalm-6 tumor cells were detectable in a few mice

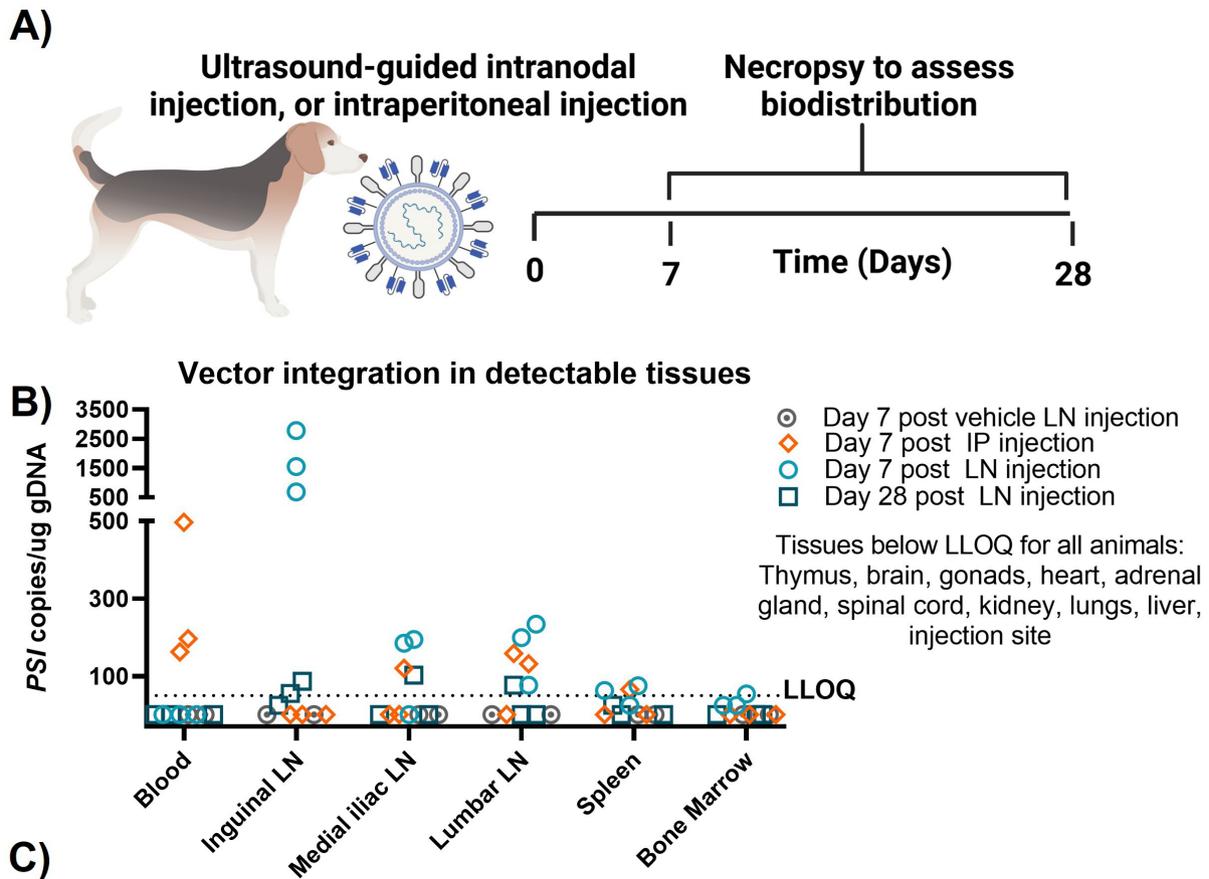
### A) Depletion of endogenous B cells in CD34-humanized mice



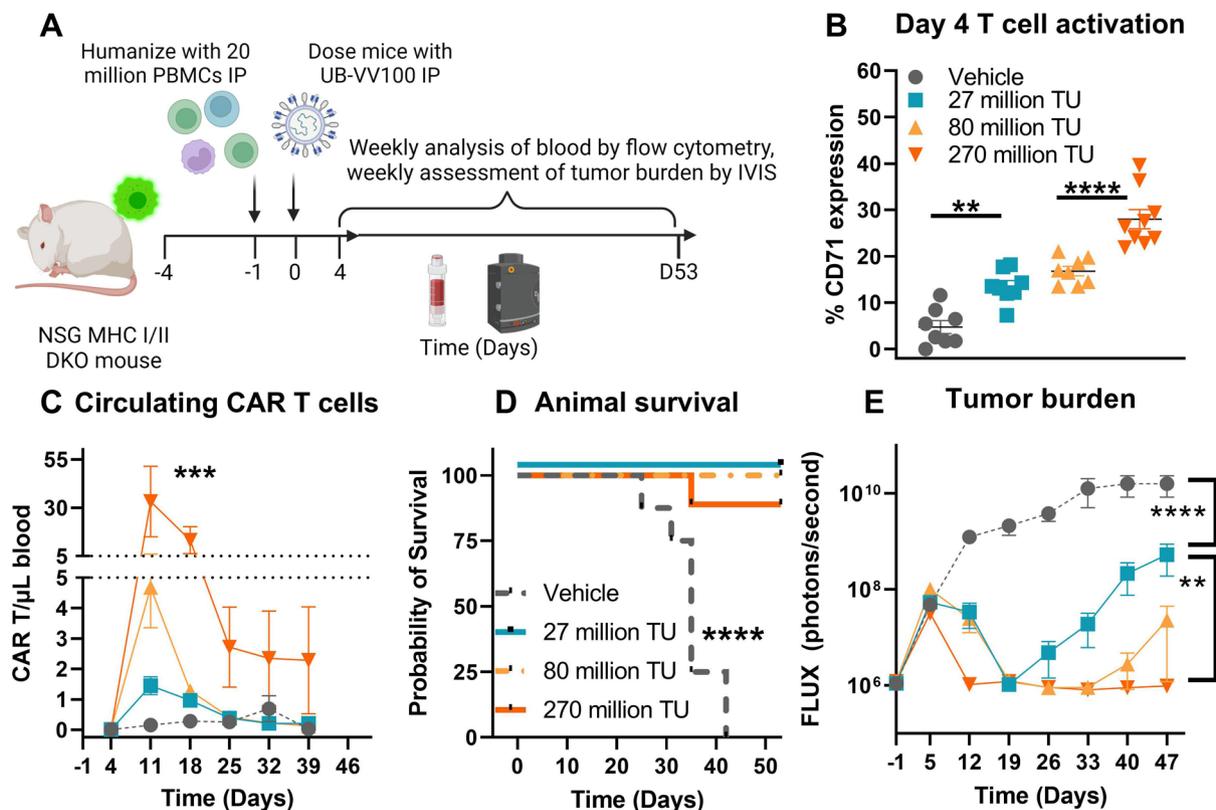
### B) Biodistribution of UB-VV100 in rapamycin treated CD34-humanized mice



**Figure 5** UB-VV100 mediates B-cell depletion in CD34-humanized mice with a favorable biodistribution profile. (A) CD34-humanized NSG mice were injected with 0.4, 2.0, or 10.0 E+06 TUs of UB-VV100. Control animals were injected with vehicle only or 10E+06 TU of coc pseudotyped vector displaying an  $\alpha$ CD3 scFv and encoding an irrelevant (control) CAR. Circulating B cells and circulating T cells were enumerated by flow cytometry once a week for 25 days. **\*\*** $P < 0.01$ , two-way ANOVA, Tukey's multiple comparison's test for main effect of vector dose. (B) CD34-humanized NSG mice were treated with either 36E+06 or 360E+06 TU UB-VV100 and were administered 1 mg/kg rapamycin every 48 hours beginning on day 5. Biodistribution of transduced cells was evaluated on day 28 by detection of the viral element *PSI* in genomic DNA tissue samples using qPCR. (C) Liver and spleen of mice treated with 360E+06 TU UB-VV100 were analyzed by RNA ISH to characterize the identity of cells expressing UB-VV100 RNA transcripts using colocalization analysis. Representative images depict human T cells (CD3+) and mouse macrophages (CD68+) transduced by UB-VV100. White indicates DAPI nuclear stain; green denotes human CD3; yellow denotes RACR sequence of transduced cells; red denotes murine CD68; blue denotes murine CD45. ANOVA, analysis of variance CAR, chimeric antigen receptor; ISH, in situ hybridization; RACR, rapamycin-activated cytokine receptor; scFv, single-chain variable fragment; TU, transducing unit.



**Figure 6** Biodistribution of surface-engineered lentiviral vectors in a canine. (A) Canines were treated with either  $4\text{E}+08$  TU CD3-cocal-GFP via ultrasound-guided bilateral inguinal lymph node injection or  $4\text{E}+9$  TU CD3-cocal-GFP via intraperitoneal injection. Necropsy was performed after either 1 or 4 weeks to assess lentiviral integration biodistribution profiles. (B) Biodistribution of transduced cells was evaluated by detection of the viral element *PSI* in genomic DNA blood and tissue samples using qPCR. Only organs with transduction events detected over the LLOQ are shown. (C) RNA ISH was performed to characterize the identity of cells expressing *EGFP* RNA transcripts using colocalization analysis. Representative images depict canine T cells transduced by CD3-cocal-GFP in the inguinal lymph node, medial iliac lymph node, and spleen 1 week after intranodal injection. Original magnification  $\times 40$ ; scale bars indicate  $5\ \mu\text{m}$ . White denotes DAPI nuclear stain; green denotes eGFP of transduced cells; yellow denotes canine CD3; red denotes canine CD68; blue denotes canine CD45. eGFP, enhanced green fluorescent protein; ISH, in situ hybridization; IP, intraperitoneal; LLOQ, lower limit of quantification; LN, lymph node; TU, transducing unit.



**Figure 7** UB-VV100 treatment results in in vivo transduction of CAR T cells and clearance of Nalm-6 tumor. (A) Animals were engrafted with Nalm-6 tumor on study day  $-4$  via tail vein injection, humanized with  $20 \times 10^6$  PBMCs on day  $-1$  via intraperitoneal injection, and treated with 27 million, 80 million or 270 million TU of UB-VV100 on study day 0 via intraperitoneal injection. (B) T-cell activation was assessed on day 4 by flow cytometry.  $**P < 0.01$ ,  $****P < 0.0001$ , one-way ANOVA, Tukey's multiple comparison test between the indicated groups. (C) Circulating CAR T cells were enumerated by flow cytometry surface staining against FMC63.  $***P < 0.001$ , one-way ANOVA for day 11.  $n = 7-9$  per group per time point. (D) Animal survival was evaluated during the study. (E) Average tumor burden as assessed by bioluminescent imaging. (F) Heatmap of bioluminescent imaging data overlaid with mouse images. The last observed in-life photon flux data were reported for deceased animals at the indicated time points.  $**P < 0.01$ ,  $****P < 0.0001$ , two-way ANOVA, multiple comparisons between the indicated UB-VV100 dose level across the entire observation period.  $n = 7-9$  per group,  $****P < 0.0001$ , Mantel-Cox test. ANOVA, analysis of variance; CAR, chimeric antigen receptor; IVIS, In Vivo Imaging System; PBMC, peripheral blood mononuclear cell; TU, transducing unit.

ethanized 42 and 85 days after UB-VV100 treatment. The recovered Nalm-6 tumor cells exhibited bright CD19 expression, an absence of FMC63 expression, and an absence of intracellular P2A expression as compared with Nalm-6 tumor recovered from vehicle treated animals (online supplemental figure 5). While the possibility of Nalm-6 transduction cannot be definitively disproven, we did not observe this phenomenon in this study series.

During model development, we observed that treatment of PBMC-humanized mice with rapamycin inhibited humanization, both in the context of healthy animals and Nalm-6 tumor bearing animals (online supplemental figure 6A,B). Based on literature on the use of rapamycin in humanized mouse models, this is likely due to inhibition of allogeneic interactions between human PBMCs and Nalm-6 tumor cells or xenogeneic interactions with mouse antigens which are required for sustained survival of human T cells in the mouse environment.<sup>25-28</sup> To decouple the need for PBMC humanization from the mechanism of action of the RACR system, we employed ex vivo manufactured CAR T cells in a Raji tumor-bearing

NSG model. We found that administration of 0.05 or 0.5 mg/kg of rapamycin 5 $\times$  a week promoted dose-dependent relative enrichment and total expansion of ex vivo manufactured CAR T cells (containing the same payload construct as UB-VV100) infused at a dose of 1 million CAR<sup>+</sup> cells per mouse. Treatment with 0.5 mg/kg rapamycin, but not lower doses of rapamycin, was associated with complete tumor clearance (online supplemental figure 7 and online supplemental table S11). We also evaluated in our in vivo disease model the use of a rapamycin analog in which mTOR binding is greatly reduced (rapalog, AP21967). This rapalog can still engage the RACR heterodimeric cytokine receptor to provide a positive IL-2/IL-15 signal, but with substantially reduced immunosuppressive effects on all immune cells. Using 1, 5, or 10 mg/kg rapalog in combination with UB-VV100 in our systemic Nalm-6 tumor model, we observed rapalog dose-dependent enhancement of CAR T-cell expansion and improved survival (online supplemental figure 8 and online supplemental table S12).

## DISCUSSION

We found that surface engineering of lentiviral particles with the cocal glycoprotein and anti-CD3 scFv enabled engagement, activation, and transduction of CD3+T cells to express an anti-CD19 CAR and the RACR system. The resulting CAR T cells exhibited antigen-specific tumor engagement and preferential expansion compared with non-transduced cells *in vitro* in the presence of rapamycin. *In vivo*, treatment with UB-VV100 resulted in T cell activation, CAR T-cell transduction, and elimination of endogenous and malignant B cell targets. UB-VV100 biodistribution in humanized mice and canines was limited primarily to immune cells and was not associated with tissue pathology.

Unstimulated T cells exist in the G<sub>0</sub> phase of the cell cycle where they are not readily transduced by LVVs; they require external stimulation to enter the G<sub>2</sub>/M phase and permit gene transfer.<sup>6 29–31</sup> Buchholz and colleagues have published several studies on their efforts to develop LVVs to overcome this.<sup>6–8</sup> Pseudotyping LVVs with a modified paramyxovirus envelope to target CD3 has demonstrated *in vivo* transduction of CAR T cells and depletion of malignant and endogenous B-cell targets, particularly in combination with exogenous human IL-7 and bead activation.<sup>6 7</sup> *In vivo* CAR T-cell manufacturing has also been accomplished by repeated doses of T cell-targeted nanoparticles bearing a CAR-encoding DNA or mRNA payload.<sup>32 33</sup> In our hands, administration of a single dose of UB-VV100 without any stimulants resulted in robust *in vivo* T-cell activation, producing sufficient CAR T cells to eradicate a systemic B-cell malignancy. Additional vector surface engineering could further refine the quality of T-cell engagement and tissue tropism to enhance *in vivo* transduction.

*In vivo* administration of LVVs carries the risk of off-target transduction, and VivoVec particles could exhibit wide tissue tropism via cocal binding its cognate receptors (including the LDL-R family).<sup>6 34</sup> Although we observed widespread anatomical distribution of VivoVec-transduced cells by qPCR, analysis by ISH revealed transduced cells were predominantly of immune origin (T cells and macrophages, which traffic to nearly all tissues), even in the context of high-volume intraperitoneal delivery, and even in the absence of anti-CD3 scFv targeting. Our observations represent an important extension of previous work with VSV-G pseudotyped LVVs, which reported biodistribution to the liver, spleen, and bone marrow, but did not identify transduced cell types.<sup>35 36</sup> Although transduction of macrophages is inefficient *in vitro* due to their quiescent state, other groups have reported uptake of LVVs by murine Kupffer cells (a type of macrophage found in the liver), resulting in sequestration of particles from surrounding hepatocytes and transduction of the Kupffer cells.<sup>37 38</sup> Macrophage transduction in our models may therefore be a result of macrophage scavenging behavior, followed by pH-dependent viral entry into the cytosol mediated by cocal.<sup>39</sup> It is not clear if human macrophages, which exhibit

robust postentry restriction of lentiviral transduction via SAMHD1, would exhibit the same susceptibility to transduction *in vivo*.<sup>40</sup> Nonetheless, generation of CAR+ macrophages in the clinical setting could promote clearance of CAR-targeted cells and contribute to the holistic drug mechanism of action.<sup>41</sup>

The use of UB-VV100 in the clinical setting poses efficacy and safety challenges that may not replicate in preclinical models using healthy donor derived materials. Despite potential differences in the quality of T cells expected in human patient populations, UB-VV100 generated functional CAR T cells from B-ALL and R/R DLBCL-derived PBMCs, suggesting *in vivo* manufacturing is still effective in this patient population. An added risk with the *in vivo* approach is the potential transduction of CD19+ malignant B cells with a CD19-binding CAR leading to epitope masking and subsequent antigen escape.<sup>14</sup> UB-VV100 was intentionally designed to encode a CD19 CAR comprising an FMC63 scFv positioned on a short hinge in order to minimize cis engagement with the CD19 target epitope, which is located in a membrane distal position.<sup>15 16</sup> Indeed, Nalm-6 cells transduced with UB-VV100 retained sensitivity to CAR T-cell killing, and no antigen escape via this mechanism was observed in murine systemic tumor models.

Due to the high complexity and sensitive timeline of the systemic Nalm-6 tumor model in PBMC humanized mice, we have to date been unable to develop a study protocol in which rapamycin administration does not interfere with either humanization or tumor growth. Data from *in vitro* studies, *in vivo* studies using *ex vivo* manufactured CAR T cells, and *in vivo* studies using rapalog support that the rapamycin–RACR axis may be of clinical benefit to patients. The holistic approach of *in vivo* transduction paired with RACR-mediated expansion has the potential to mitigate efficacy and safety concerns of existing CAR T-cell therapies. On the efficacy side, CAR T cells that expand in their natural context may have a more favorable phenotype and enhanced activity compared with those expanded in prolonged *ex vivo* culture, which has been associated with undesirable differentiation and exhaustion.<sup>42–45</sup> On the safety side, the kinetics of *in vivo* CAR T generation may reduce the severity of cytokine release syndrome (CRS). In hematological malignancies, CRS has been correlated with lymphodepletion regimens, the initial infused dose of CAR T cells, and tumor burden at the time of treatment.<sup>46 47</sup> *In vivo* CAR T-cell generation and subsequent expansion is anticipated to result in more gradual kinetics of CAR T cells engaging tumor, possibly blunting CRS.

The removal of lymphodepletion has many potential benefits for patients. Lymphodepletion is currently required to promote full CAR T-cell efficacy but also increases the risk of serious infection in a vulnerable patient population.<sup>48</sup> UB-VV100 is designed to be used with rapamycin in the absence of lymphodepletion, whereby rapamycin provides protection against immune-mediated deletion of nascent CAR T cells via mTOR

inhibition of non-transduced cells while simultaneously supporting CAR T-cell proliferation via RACR.<sup>49–51</sup> Rapamycin may also dampen some forms of immune toxicity without interfering with CAR T-cell function.<sup>52 53</sup>

Safe and effective engineering of CAR T cells in vivo has the potential to expand access to this class of life-saving therapies in both solid and liquid tumors by reducing the cost for therapy, reducing the wait time for treatment and avoiding the need for lymphodepleting chemotherapy, as well as providing potential safety and efficacy advantages over ex vivo manufactured CAR T cells.

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## 1 **Supplementary methods**

2 All animal studies were conducted under an approved IACUC. Female NSG mice, 6-12 weeks of age,  
3 lacking expression of MHC I and II (NSG DKO, stock number 025216) were purchased from the  
4 Jackson Laboratories. Female CD34-humanized NCG mice were purchased from Charles River.

5 B cell depletion in CD34-humanized NCG mice: Circulating human B cells were evaluated by flow  
6 cytometry from all mice on day -7 before beginning treatment. Animals were treated with vehicle,  
7 36E+06 TU, or 360E+06 TU, UB-VV100 via intraperitoneal injection day 0. Circulating B cells were  
8 evaluated by flow cytometry day 28.

9 Rapamycin-mediated inhibition of T cell expansion in PBMC-humanized mice: Female MHC I/II DKO  
10 NSG mice were engrafted 20 million PBMCs via intraperitoneal injection study day -1. Mice were  
11 treated with 1 mg/kg rapamycin beginning study day 5. Circulating total human T cells were evaluated  
12 by flow cytometry.

13 RACR-driven expansion of ex vivo manufactured CAR T cells: Raji tumor cells (ATCC CCL-86)  
14 engineered to express green fluorescent protein and firefly luciferase (*GFP::ffluc*) were acquired from  
15 Seattle Children Therapeutics. Cryopreserved negative-selected pan T cells were purchased from  
16 Bloodworks Northwest (Seattle, WA). CAR T cells were manufactured by overnight bead activation  
17 with 1:1 ratio CD3/CD28 dyna beads: T cells, transduction of MOI = 5 with UB-VV100 for 72 hours.  
18 Cells were expanded in complete RPMI and 50 IU/mL human IL-2 as previously described for two  
19 weeks and evaluated for transduction efficiency by surface staining against FMC63, and cryopreserved.  
20 Animals were injected with 0.5E+06 tumor cells via intravenous injection study day -4. Animals were  
21 infused with 1 million CAR+ T cells, or an equivalent dose of total donor-matched T cells, by

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22 intraperitoneal injection day 0 Animals were dosed with 0, 0.005, 0.05, or 0.5 mg/kg rapamycin 5x  
23 weekly by intraperitoneal injection starting study day 0.

24 Rapalog mediated expansion of in vivo transduced CAR T cells: Female NSG MHC I/II DKO mice  
25 were engrafted with 0.5E+06 Nalm-6 tumor cells study day -4, humanized with 20E+6 PBMCs day -1,  
26 and treated with 50 million TU UB-VV100 via intraperitoneal injection day 0. Animals were treated  
27 with 1, 5, or 10 mg/kg rapalog (AP21967, Takara Bio, Shingha, Japan) 3x weekly beginning study day 5.  
28 Circulating CAR T cells were evaluated by flow cytometry as previously described.

29

30 **Supplementary figures**31 **Table SI. List of antibody clones used in manuscript.**

Figure	Conjugate	Clone	Vendor and catalog number
Figure 2A, B; Figure 3A, B	hCD3 PE	UCHT1	Biologend 300408
Figure 2A, B; Figure 3A, B	hCD4 APC	A161A1	Biologend 357408
Figure 2A, B; Figure 3A, B	hCD8 BV421	SK1	Biologend 344748
Figure 2A, B; Figure 3A, B	hCD25 CD25	M-A251	Biologend 356108
Figure 2A, B; Figure 3A, B	FMC63 CAR idiotype FITC	Y45	ACRO Biosystems FM3-FY45
Figure 2A, B; Figure 3A, B	Live/Dead Zombie NIR	N/A	Biologend 423106
Figure 2C	Live/Dead Zombie Violet	N/A	Biologend 423114
Figure 2C	hCD3 PerCPCy5.5	UCHT1	Biologend 300430
Figure 2C	hCD8 AF700	SK1	Biologend 344724
Figure 2C	hCD4 BV510	SK3	Biologend 344634
Figure 2C	P2A A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 2C	Nalm6/Nalm6KO mCherry	N/A	N/A
Figure 2E	hCD3 PerCPCy5.5	UCHT1	Biologend 300430

Figure 2E	hCD8 AF700	SK1	Biologend 344724
Figure 2E	hCD4 BV510	SK3	Biologend 344634
Figure 2E	P2A A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 2E	hCD107a PE	H4A3	Biologend 328608
Figure 2E	Live/Dead Zombie NIR	N/A	Biologend 423106
Figure 2E	Nalm6/Nalm6KO GFP::ffluc FITC	N/A	N/A
Figure 2F	hCD8 PerCpCy5.5	SK1	Biologend 344710
Figure 2F	hCD4 BV650	RPA-T4	Biologend 300536
Figure 2F	hCD3 A700	UCHT1	Biologend 300424
Figure 2F	hCD25 BV421	BC96	Biologend 312218
Figure 2F	hCD19 PECy7	HIB19	Biologend 302216
Figure 2F	hCD19 FITC	EPR5906	Abcam ab196468
Figure 2F	Live/Dead Zombie NIR	N/A	Biologend 423106
Figure 3 C,D	hCD3 PerCpCy5.5	UCHT1	Biologend 300430
Figure 3 C,D	hCD8 AF700	SK1	Biologend 344724
Figure 3 C,D	hCD4 BV510	SK3	Biologend 344634
Figure 3 C,D	FMC63 CAR idiotype PE	R19M	CytoArt 200106
Figure 3 C,D	P2A A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 3 C,D	hCD45RA BV605	HI100	Biologend 304134
Figure 3 C,D	hCCR7 BV421	G043H7	Biologend 353208
Figure 3 C,D	Live/dead Zombie NIR	N/A	Biologend 423106
Figure 4A,B	Live/dead Zombie NIR	N/A	Biologend 423106
Figure 4A,B	hCD3 R718	UCHT1	BD Biosciences 566953
Figure 4A,B	hCD8 or hCD4 BV510	SK1 or SK3	Biologend 344732
Figure 4A,B	hCD20 BV650	2H7	Biologend 302336
Figure 4A,B	FMC63 CAR idiotype PE	R19M	Cytoart 200106
Figure 4A,B	hCD19 PECy7	HIB19	Biologend 302216

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Figure 4A,B	hCD25 BV421	BC96	Biologend 302630
Figure 4A,B	hCD19 (intracellular) A488	EPR5906	Abcam ab196468
Figure 4A,B	hCD79a (intracellular) PerCpCy5.5	HM47	Biologend 333508
Figure 4A,B	P2A (intracellular) A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 4C	hCD8 PerCpCy5.5	SK1	Biologend 344710
Figure 4C	hCD4 A700	SK3	Biologend 344622
Figure 4C	hCD19 PECy7	HIB19	Biologend 302216
Figure 4C	hCD3 BV650	UCHT1	Biologend 300468
Figure 4C	Nalm-6 or Nalm-6 KO GFP	N/A	N/A
Figure 5A, S7	Live/dead LIVE/DEAD Fixable Violet	N/A	Invitrogen L34964
Figure 5A, S7	hCD45 PERCP5.5	2D1	Biologend 368504
Figure 5A, S7	hCD3 AF700	UCHT1	Biologend 300424
Figure 5A, S7	hCD20 PE	2H7	Biologend 302306
Figure 5A, S7	hCD4 BV650	RPA-T4	Biologend 300535
Figure 5A, S7	hCD8 BV605	SK1	Biologend 344741
Figure 5A, S7	hCD25 PECY7	M-A251	Biologend 356122
Figure 5A, S7	hCD71 APCCY7	CY1G4	Biologend 334110
Figure 5A, S7	FMC63 CAR idotype PE	Y45	ACRO Biosystems FM3-HPY53
Figure 5A, S7	FITC dextran	N/A	Millipore 46944-100MG-F
Figure 7B, C, S4, S8	Live/dead LIVE/DEAD Fixable Violet	N/A	Invitrogen L34964
Figure 7B, C, S4, S8	hCD45 PERCP5.5	2D1	Biologend 368504
Figure 7B, C, S4, S8	hCD3 AF700	UCHT1	Biologend 300424
Figure 7B, C, S4, S8	hCD4 BV650	RPA-T4	Biologend 300535
Figure 7B, C, S4, S8	hCD8 BV605	SK1	Biologend 344741
Figure 7B, C, S4, S8	hCD25 PECY7	M-A251	Biologend 356122
Figure 7B, C, S4, S8	hCD19 APCCY7	6D5	Biologend 115530
Figure 7B, C, S4, S8	FMC63 CAR idotype PE	Y45	ACRO Biosystems FM3-HPY53
Figure 7B, C, S4, S8	mCD45 BV510	30-F11	Biologend 103137
Figure 7B, C, S4, S8	Nalm6 GFP::ffluc	N/A	N/A

All flow panels	Human TruStain Fc blocker blocker	N/A	Biologend 422302
All flow panels	Mouse TruStain Fc blocker blocker	93	Biologend 101320

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34 **Table SII. Human patient characteristics.**

Age (years)	Disease Status	Organs involved	Treatment	Date of diagnosis	Date of PBMC
50-60	Refractory	Lymph nodes, spleen	6 courses <sup>1</sup> R-CHOP	3/30/2020	11/18/2020
70-80	Refractory	Small intestine, lymph nodes	3 courses R-CHOP	7/01/2020	12/08/2020
60-70	Relapse	Bone marrow, Liver, Spleen	2015 – 6 courses R-CHOP 2018 – 4 courses <sup>2</sup> RDHAP Autologous bone marrow transplant	1/01/2015	9/21/2020
50-60	Relapse	Lymph nodes	6 courses R-CHOP	2/01/2020	11/20/2020
80-90	Relapse	Skin	5 courses R-CHOP	10/16/2019	12/22/2020

35 <sup>1</sup>Rituximab, Cyclophosphamide, hydroxydaunorubicin, Oncovin, Prednisone36 <sup>2</sup>Rituximab, Dexamethasone, Cytarabine, Cisplatin

37

38 **Table SIII: B cell depletion in CD34-humanized mice (Figure 5A)**

Purpose	<ul style="list-style-type: none"> <li>To evaluate dose-dependent activity of UB-VV100 by measuring depletion of endogenous B cells in CD34-humanized mice</li> <li>To evaluate antigen-specificity of B cell depletion</li> </ul>
Species/Strain	Female CD34-humanized Nod.Cg-Prkdc <sup>scid</sup> IL2rg <sup>tm1Wjl</sup> /SzJ (NSG, Jackson stock 005557), acquired from Jackson, 14 weeks post humanization

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Age/Weight	17 weeks of age at study start, 17-25 g in weight			
Randomization and enrollment criteria	Mice were evaluated for >30% humanization upon receipt from the vendor. Animals were assigned study arms by creating blocks of animals arranged by B cell abundance. Animals were assigned within blocks using Microsoft Excel random number generator. Resulting groups were evaluated for average and standard deviation B cell abundance and humanization before final acceptance. No additional confounders were used to account for study assignment			
Study site	Study was approved under IACUC protocol PROTO202000003. Animals were housed in social housing units at Fred Hutchinson Cancer research center under ABSL2 barrier containment on a 12/12 hour light/dark cycle. Animals had nestlet enrichment and ad libitum access to food and water.			
Study Design TU = Transducing units	Group	Vector dose	Vector identity	Sample size
	1	0E+06 TU	Vehicle	4
	2	10E+06 TU	VivoVec vector encoding control CAR	4
	3	0.4E+06 TU	UB-VV100	4
	4	2E+06 TU	UB-VV100	4
	5	10E+06 TU	UB-VV100	4
Study blinding	No study blinding was implemented in this design. Animal behavior, health, and humane endpoint criteria were recorded by a technician external to the sponsor.			
Dosing route and frequency	Vector test article (UB-VV100 or VivoVec vector encoding a control CAR) or vehicle control (PBS) was administered via single intraperitoneal injection on study day 0.			
Sample collection and frequency	Blood was collected via retroorbital sinus on study days -1, 4, 11, 18, and 25			
Outcomes	<ul style="list-style-type: none"> <li>Health: Grooming, posture, activity level, bodyweight</li> <li>UB-VV100 efficacy: Circulating B cells as enumerated by flow cytometry from serial blood draws</li> <li>UB-VV100 efficacy: Circulating CAR T cells as enumerated by flow cytometry from serial blood draws</li> </ul>			

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40 **Table SIV: UB-VV100 biodistribution in CD34-humanized mice (Figure 5B-C and Figure S2)**

Purpose	<ul style="list-style-type: none"> <li>To evaluate biodistribution and tissue tropism of UB-VV100</li> <li>To evaluate tolerability of UB-VV100 test article in humanized mice</li> </ul>
Species/Strain	Female CD34-humanized NOD- <i>Prkdc</i> <sup>em26Cd52</sup> <i>Il2rg</i> <sup>em26Cd22</sup> /NjuCtrl (NCG) mice from Charles River Laboratories
Age/Weight	28 weeks of age at study start, 20-26 g in weight

Randomization and enrollment criteria	Animals were assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. Animals were randomized separately. Animals in poor health or at extremes of body weight range were not be assigned to groups. Animals were distributed to groups to account for equal distribution by humanization and human donor (2 total donors were used).		
Study site	Study was approved under Charles River Laboratories IACUC protocol Umoja 2021-3225. Animals were housed in microisolator units under ABSL2 barrier containment on a 12/12 hour light/dark cycle. Animals had ad libitum access to food and water.		
Study Design TU = Transducing units	Group	Vector dose	Sample size
	1	0E+06 TU	6
	2	36E+06 TU	6
	3	360E+06 TU	6
Study blinding	No study blinding was implemented in this design. All data and reports were prepared by a contract research organization external to the sponsor.		
Dosing route and frequency	UB-VV100 test article was administered by intraperitoneal injection on day 0. Rapamycin was administered via intraperitoneal (IP) injection at a dose of 1 mg/kg in PBS and 1% DMSO every 48 hours beginning study day 4 until study termination.		
Sample collection and frequency	Blood was collected via retroorbital sinus on day -7 and on day 26 via cardiac puncture upon study termination.		
Outcomes	<ul style="list-style-type: none"> <li>• Health: dermal irritation scoring, clinical observations for health and appearance, bodyweight, and daily food consumption (no changes related to test article noted)</li> <li>• Enumeration of circulating B cells by flow cytometry (results reported in Figure S2)</li> <li>• Evaluation of macroscopic pathology and organ weights upon necropsy (no abnormalities related to vector test article noted)</li> <li>• Evaluation of microscopic pathology (no abnormalities related to vector test article noted)</li> <li>• Biodistribution of UB-VV100 transduced cells by ddPCR targeted against the viral PSI integration element</li> <li>• Identification of UB-VV100 transduced cells by ISH against the UB-VV100 transgene</li> </ul>		
Organs evaluated for histopathology, ddPCR, and flow	Organ	Analysis	
	Aorta, bone marrow, brain, esophagus, eye, gallbladder, adrenal glands, heart, kidney, large and small intestine, liver, lungs, skeletal muscle, optic nerve, sciatic nerve, pancreas, injection site ovary, spinal cord, spleen, stomach, thymus, tongue, trachea, bladder, uterus, vagina	Macroscopic and microscopic pathology	
	Bone marrow, brain, adrenal gland, heart, kidney, liver, lung, ovary, injection site, spinal cord, spleen	qPCR against vector PSI integration element	
	Spleen, liver	ISH against UB-VV100 transgene	

## Preclinical pharmacology of a lentiviral vector designed to generate CAR T cells in vivo

42 **Table SV: Biodistribution of VivoVec particles in Beagle dogs (Figure 6A-C)**

Purpose	<ul style="list-style-type: none"> <li>To evaluate biodistribution and tissue tropism of anti-human CD3scFv coated coccal pseudotyped (VivoVec) lentiviral vectors in Beagle dogs</li> <li>To evaluate biodistribution and tissue tropism of VivoVec vectors when administered via lymph node injection</li> </ul>				
Species/Strain	Male and female purpose-bred laboratory Beagle dogs sourced from Marshall Bioresources				
Age/Weight	7 to 9 months in age, 7 to 11 kg in weight				
Randomization and enrollment criteria	Animals were randomly assigned to groups upon arrival and evaluated for health before acceptance into the study.				
Study site	Animals were housed at Charles River Laboratories under IACUC approval for protocol Umoja 2021-3232. Animals were given ad libitum access to water and access to weight-appropriate rationed food for 4-6 hours per day. Animals were acclimated for a minimum of 9 days upon receipt to the facility in social housing, and were singly housed for the study period.				
Study Design TU = Transducing units	Group	Sample size (males/females)	Vector dose	Route of administration	Necropsy day
	1	1M/1F	Vehicle only	Bilateral inguinal LN	7
	2	1M/2F	4E+08 TU	Bilateral inguinal LN	7
	3	1M/2F	40E+08 TU	Intraperitoneal injection	7
	4	1M/2F	4E+08 TU	Bilateral inguinal LN	28
Study blinding	No study blinding was implemented in this design. All data and reports were prepared by a contract research organization external to the sponsor.				
Dosing route and frequency	Test article is a coccal-pseudotyped lentiviral vector particle engineered to express anti-human CD3-scFv, and encodes an enhanced green fluorescent protein (eGFP) payload. Canines were treated by bilateral ultrasound guided inguinal lymph node injection in a volume of 0.6 mL, or via intraperitoneal injection in a volume of 6 mL, once on study day 0.				
Outcomes	<ul style="list-style-type: none"> <li>Weekly bodyweights and clinical observations (no adverse events noted)</li> <li>Mortality (no unscheduled deaths noted)</li> <li>Clinical pathology performed once pre-dose and once prior to necropsy for all groups : Clinical chemistry, hematology, coagulation, urinalysis</li> <li>Macroscopic and microscopic histopathology (no pathology related to vector test article noted)</li> <li>Biodistribution analysis by qPCR</li> <li>Identification of transduced cells via ISH</li> </ul>				

Organs evaluated for histopathology, ddPCR, and flow	Organ	Analysis
	Aorta, bone marrow, brain, esophagus, eye, gallbladder, adrenal glands, heart, kidney, large and small intestine, liver, lungs, skeletal muscle, optic nerve, sciatic nerve, pancreas, injection site ovary, spinal cord, spleen, stomach, thymus, tongue, trachea, bladder, uterus, vagina	Macroscopic and microscopic pathology
	Blood, brain, gonads, heart, liver, spinal cord, adrenal gland, kidney, lungs, bone marrow, spleen, injection site, thymus, superficial inguinal lymph node, medial iliac lymph node, lumbar lymph node	qPCR against vector PSI integration element
	Inguinal lymph node, medial iliac lymph node, spleen	ISH against eGFP transgene

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44

45 **Table SVI. In situ hybridization probes (Advanced Cell Diagnostics)**

Target	Gene ID(s)	Animal	Sequence or catalog #
eGFP	Viral payload	Canine	#400288
Canine panCD3	<i>Cd3d, Cd3e, Cdef</i>	Canine	#1124138-C4, 1124128-C4, 406278-C4
Canine CD45	<i>Ptprc</i>	Canine	# 1123128-C3
Canine CD68	<i>Cd68</i>	Canine	#577868-C4
UB-VV100 RACR	Viral payload	Humanized mouse	# 1048558-C1
Human panCD3	<i>Cd3d, Cd3e, Cdef</i>	Humanized mouse	# 426628-C2
Murine CD45	<i>Ptprc</i>	Humanized mouse	#318658-C3
Murine CD68	<i>Cd68</i>	Humanized mouse	# 316618-C4

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47

48 **Table SVII: Semi-quantitative scoring of RACR+ cells by multiplex RNA ISH in liver sections**  
49 **from humanized mouse biodistribution study**

Tissue	Cell Phenotype	Proportion of Events (%) <sup>a</sup>					
		PBS (IP)		36E+06 TU UB-VV100 (IP)		360E+06 TU UB-VV100 (IP)	
		1501	2502	2504	2506	3501	3503

9

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Liver	All RACR+ Cells (DAPI+:RACR+)	0	1-10	1-10	1-10	11-20	11-20	1-10
	RACR+: CD45+ (Leukocyte)	--	91-100	0	91-100	91-100	91-100	91-100
	RACR+: CD3+ Pool (T Cells)	--	0	0	0	0	1-10	1-10
	RACR+: CD68+ (Macrophages)	--	91-100	91-100	91-100	91-100	91-100	91-100
	RACR+: CD45-, CD3-, CD68- (Non-immune cells)	--	0	0	0	0	0	0

50 <sup>a</sup> Proportion of events are reported from semi-quantitative scoring of RACR expression and cell type marker co-localization.  
 51 Scoring was performed by a blinded pathologist from Advanced Cell Diagnostics, Inc. Each column indicates one individual  
 52 with an animal ID. N=1 representative animals per control group and N=3 representative animals per UB-VV100 treated  
 53 group were analyzed by RNA ISH.  
 54 NA = sample not analyzed; IP = intraperitoneal; c = cell.

55

56

57 **Table SVIII: Semi-quantitative scoring of RACR+ cells by multiplex RNA ISH in spleen sections**  
 58 **from humanized mouse biodistribution study**

Tissue	Cell Phenotype	Proportion of Events (%) <sup>a</sup>						
		PBS (IP)	Low Dose (IP)			High Dose (IP)		
		1501	2502	2506	2601	3501	3503	3602
Spleen	All RACR+ Cells (DAPI+:RACR+)	0	1-10	1-10	1-10	21-30	21-30	21-30
	RACR+: CD45+ (Leukocyte)	--	91-100	91-100	91-100	91-100	91-100	91-100
	RACR+: CD3+ Pool (T Cells)	--	0	0	0	1-10	0	1-10
	RACR+: CD68+ (Macrophages)	--	91-100	91-100	91-100	91-100	91-100	91-100
	RACR+: CD45-, CD3-, CD68- (Non-immune cells)	--	0	0	0	0	0	0

59 <sup>a</sup> Proportion of events are reported from semi-quantitative scoring of RACR expression and cell type marker co-localization.  
 60 Scoring was performed by a blinded pathologist from Advanced Cell Diagnostics, Inc. Each column indicates one individual  
 61 with an animal ID. N=1 representative animals per control group and N=3 representative animals per UB-VV100 treated  
 62 group were analyzed by RNA ISH.

63 NA = sample not analyzed; IP = intraperitoneal; c = cell.

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69 **Table SIX: Semi-quantitative scoring of EGFP+ cells by multiplex RNA ISH in canine**  
 70 **biodistribution study**

Tissue	Cell Phenotype	Proportion of Events (%) <sup>a</sup>								
		IN Delivery (1 wk)			IN Delivery (4 wks)			IP Delivery (1 wk)		
		2001 <sup>b</sup>	2501	2502	4001	4502 <sup>d</sup>	4601	3001	3501	3502
Inguinal Lymph Node (injected LN)	All EGFP+ Cells (DAPI+:EGFP+)	NA	1-10	< 1	0	NA	< 1	0	<< 1	<< 1
	EGFP+: CD45+ (Leukocyte) <sup>c</sup>	NA	81-90	21-30	--	NA	91-100	--	81-90	91-100
	EGFP+: CD3+ Pool (T Cells)	NA	11-20	51-60	--	NA	1-10	--	51-60	91-100
	EGFP+: CD68+ (Macrophages)	NA	51-60	1-10	--	NA	1-10	--	61-70	91-100
	EGFP+: CD45-, CD3-, CD68- (Non-immune cells)	NA	< 1	21-30	--	NA	1-10	--	1-10	0
Medial Iliac Lymph Node	All EGFP+ Cells (DAPI+:EGFP+)	< 1	< 1	0	0	<< 1	<< 1	<< 1	1-10	<< 1
	EGFP+: CD45+ (Leukocyte) <sup>c</sup>	91-100	81-90	--	--	51-60	91-100	81-90	71-80	91-100
	EGFP+: CD3+ Pool (T Cells)	1-10	11-20	--	--	41-50	91-100	11-20	71-80	91-100
	EGFP+: CD68+ (Macrophages)	1-10	1-10	--	--	31-40	71-80	41-50	61-70	91-100
	EGFP+: CD45-, CD3-, CD68- (Non-immune cells)	< 1	1-10	--	--	21-30	1-10	1-10	11-20	0
Spleen <sup>e</sup>	All EGFP+ Cells (DAPI+:EGFP+)	<< 1 (10 cells)	<< 1 (5 cells)	NA	NA	NA	NA	NA	NA	<< 1 (6 cells)
	EGFP+: CD45+ (Leukocyte) <sup>c</sup>	<i>51-60</i>	<i>0</i>	NA	NA	NA	NA	NA	NA	<i>41-50</i>
	EGFP+: CD3+ Pool (T Cells)	<i>21-30</i>	<i>51-60</i>	NA	NA	NA	NA	NA	NA	<i>41-50</i>
	EGFP+: CD68+ (Macrophages)	<i>71-80</i>	<i>51-60</i>	NA	NA	NA	NA	NA	NA	<i>81-90</i>
	EGFP+: CD45-, CD3-, CD68- (Non-immune cells)	<i>1-10</i>	<i>41-50</i>	NA	NA	NA	NA	NA	NA	<i>11-20</i>

71

72 <sup>a</sup> Proportion of events are reported from semi-quantitative scoring of EGFP expression and cell type marker co-localization.  
 73 Scoring was performed by a blinded pathologist from Advanced Cell Diagnostics, Inc. If less than 10 EGFP+ cells per tissue  
 74 section were identified, the cell type populations are italicized and the total number of EGFP+ cells is noted in parenthesis  
 75 Each column indicates one individual with an animal ID.

76 <sup>b</sup> Inguinal lymph node tissue from animal 2001 not available was for analysis.

77 <sup>c</sup> Canine CD45 ISH probe set covers 7 of 8 *Ptprc* transcripts encoding or CD45

78 <sup>d</sup> Medial iliac lymph node tissue from animal 4502 was not available for analysis.

79 <sup>e</sup> Only spleen samples with quantifiable qPCR levels (>50 vg/ug DNA) were analyzed by multiplex RNA ISH

80 NA = sample not analyzed; IP = intraperitoneal; wk = week; c = cell.

81

82

## Preclinical pharmacology of a lentiviral vector designed to generate CAR T cells in vivo

83 **Table SX: UB-VV100 controls systemic Nalm-6 (Figure 7A-E, Supplemental figure SX)**

Purpose	<ul style="list-style-type: none"> <li>To evaluate the dose-dependent activity of UB-VV100 in humanized, immune-compromised mice engrafted with systemic Nalm-6 tumor</li> <li>To evaluate the role of anti-CD3 scFv in CAR T cell transduction in vivo</li> <li>To evaluate the tolerability of UB-VV100 when administered to humanized mice bearing systemic tumor</li> </ul>															
Species/Strain	Female NSG mice lacking endogenous MHC I/II (NOD.Cg-Prkdcscid H2-K1tm1Bpe H2-Ab1em1Mvw H2-D1tm1Bpe Il2rgtm1Wjl/SzJ).															
Age/Weight	8-12 weeks of age at study start, 17-25g in weight															
Randomization and enrollment criteria	Animals were randomized in blocks to account for tumor burden on day -1, animal age, and animal weight. 34 animals were enrolled initially. 1 animal was retrospectively rejected from the study due to failed humanization as measured by flow cytometry.															
Study site	Studies were conducted at Seattle Children's Hospital under IACUC protocol ACUC00654. Animals were housed in ABSL2 containment barrier conditions in social housing with ad libitum access to food and water. Upon study start, high-calorie gel was provided ad libitum on the cage floor to encourage feeding. Animals were provided with nestlets and plastic hut enrichment.															
Study Design TU = Transducing units	<table border="1"> <thead> <tr> <th>Group</th> <th>Vector dose (TU/Animal)</th> <th>Number of Animals</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>Vehicle (0)</td> <td>8</td> </tr> <tr> <td>3</td> <td>UB-VV100 (<math>2.7 \times 10^7</math>) TU</td> <td>8</td> </tr> <tr> <td>4</td> <td>UB-VV100 (<math>8.0 \times 10^7</math>) TU</td> <td>8</td> </tr> <tr> <td>5</td> <td>UB-VV100 (<math>2.7 \times 10^8</math>) TU</td> <td>9</td> </tr> </tbody> </table>	Group	Vector dose (TU/Animal)	Number of Animals	1	Vehicle (0)	8	3	UB-VV100 ( $2.7 \times 10^7$ ) TU	8	4	UB-VV100 ( $8.0 \times 10^7$ ) TU	8	5	UB-VV100 ( $2.7 \times 10^8$ ) TU	9
Group	Vector dose (TU/Animal)	Number of Animals														
1	Vehicle (0)	8														
3	UB-VV100 ( $2.7 \times 10^7$ ) TU	8														
4	UB-VV100 ( $8.0 \times 10^7$ ) TU	8														
5	UB-VV100 ( $2.7 \times 10^8$ ) TU	9														
Study blinding	The technician who recorded clinical scores, bodyweight data, tumor progression data, and evaluated humane endpoint criteria was blinded to the study arms. All other personnel had study keys.															
Dosing route and frequency	<b>Tumor:</b> $2.5 \times 10^5$ Nalm-6 tumor cells were administered via tail vein injection once on Study Day -4. <b>Humanization:</b> $2.0 \times 10^7$ PBMCs were administered via IP injection once on Study Day -1. <b>Drug:</b> UB-VV100 or vector vehicle (10 mM Tris, 10% sucrose, 0.1% poloxamer 188 (v/v), pH 7.1) was administered to the indicated study arms via IP injection once on Study Day 0.															
Sample collection and frequency	Blood was collected by serial retroorbital bleeds on days 4, 11, 18, 25, 32, and 39. Tumor burden was evaluated by non-invasive bioluminescent imaging days -1, 5, 12, 19, 26, 33, 40, and 47.															
Outcomes	<ul style="list-style-type: none"> <li>Animals were evaluated for bodyweight and semi-quantitative clinical scoring 2x weekly (all adverse health events were related to tumor progression, with clinical scores improving at increasing doses of vector)</li> <li>Circulating CAR T cells were evaluated weekly by flow cytometry</li> <li>Nalm-6 tumor burden was evaluated weekly by bioluminescent imaging</li> </ul>															

85 **Table SXI: Rapamycin mediates expansion of ex vivo manufactured CAR T cells in vivo (Figure**  
 86 **S4)**

Purpose	To assess the ability of the rapamycin/RACR axis to drive CAR T cell enrichment, expansion, and tumor clearance in vivo			
Species/Strain	Female NSG mice lacking endogenous MHC I/II (NOD.Cg-Prkdcscid H2-K1tm1Bpe H2-Ab1em1Mvw H2-D1tm1Bpe Il2rgtm1Wjl/SzJ).			
Age/Weight	7 weeks of age at study start, 17-25g in weight			
Randomization and enrollment criteria	Animals were randomized in blocks to account for tumor burden on day -1, animal age, and animal weight.			
Study site	Study was approved under IACUC protocol PROTO202000003. Animals were housed in social housing units at Fred Hutchinson Cancer research center under ABSL2 barrier containment on a 12/12 hour light/dark cycle. Animals had nestlet enrichment and ad libitum access to food and water.			
Study Design TU = Transducing units	Group	CAR T cell infusion	Rapamycin (mg/kg), 3x weekly	Number of Animals
	1	$3 \times 10^6$ Mock transduced T cells	0	5
	2	$3 \times 10^6$ Mock transduced T cells	0.005	7
	3	$3 \times 10^6$ Mock transduced T cells	0.05	5
	4	$3 \times 10^6$ Mock transduced T cells	0.5	5
	5	$3 \times 10^6$ transduced T cells (30% CAR+)	0	5
	6	$3 \times 10^6$ transduced T cells (30% CAR+)	0.005	6
	7	$3 \times 10^6$ transduced T cells (30% CAR+)	0.05	6
	8	$3 \times 10^6$ transduced T cells (30% CAR+)	0.5	6
Study blinding	No study blinding was implemented in this design			
Dosing route and frequency	$5 \times 10^5$ Raji tumor cells were implanted via retroorbital injection on Study Day -4. $3 \times 10^6$ transduced T cells (30% FMC63 CAR+) or $3 \times 10^6$ donor-matched mock transduced T cells (0% FMC63 CAR+) were infused into mice via IP injection once on Study Day 0. The indicated dose of rapamycin was administered to mice 5x weekly from Study Days 0-46 via IP injection in 1% DMSO diluted in PBS.			
Sample collection and frequency	Blood was collected by serial retroorbital bleeds on days 4, 18, 25, 32, 39, and 46.			
Outcomes	<ul style="list-style-type: none"> <li>Animals were evaluated for bodyweight 2x weekly</li> <li>Circulating CAR T cells were evaluated weekly by flow cytometry</li> <li>Raji tumor burden was evaluated weekly by bioluminescent imaging</li> </ul>			

87

## Preclinical pharmacology of a lentiviral vector designed to generate CAR T cells in vivo

88 **Table SXII: Rapalog mediated expansion of CAR T cells in vivo**

Purpose	To assess the ability of rapalog to drive expansion of in vivo transduced CAR T cells			
Species/Strain	Female NSG mice lacking endogenous MHC I/II (NOD.Cg-Prkdcscid H2-K1tm1Bpe H2-Ab1em1Mvw H2-D1tm1Bpe Il2rgtm1Wjl/SzJ).			
Age/Weight	10-14 weeks of age at study start, 17-25g in weight			
Randomization and enrollment criteria	Animals were randomized in blocks to account for tumor burden on day -1, animal age, and animal weight.			
Study site	Studies were conducted at Seattle Children's Hospital under IACUC protocol ACUC00654. Animals were housed in ABSL2 containment barrier conditions in social housing with ad libitum access to food and water. Upon study start, high-calorie gel was provided ad libitum on the café floor to encourage feeding. Animals were provided with nestlets and plastic hut enrichment.			
Study Design TU = Transducing units	Group	Vector dose	Rapalog dose (mg/kg)	Number of Animals
	1	0 (Vehicle)	0 mg/kg	5
	2*	0 (Vehicle)	10 mg/kg	5
	3	50E+06 TU UB-VV100	0 mg/kg	9
	4	50E+06 TU UB-VV100	1 mg/kg	9
	5	50E+06 TU UB-VV100	5 mg/kg	9
	6	50E+06 TU UB-VV100	10 mg/kg	8
	*10 mg/kg of rapalog was found to have no impact on tumor progression, and was removed from the final figure for visual clarity			
Study blinding	No study blinding was performed for this study.			
Dosing route and frequency	<b>Tumor:</b> 0.5E+06 Nalm-6 tumor cells were administered via tail vein injection once on Study Day -4. <b>Humanization:</b> $2.0 \times 10^7$ PBMCs were administered via IP injection once on Study Day -1. <b>Drug:</b> UB-VV100, Cocal, or vector vehicle (10 mM Tris, 10% sucrose, 0.1% polaxamer 188 (v/v), pH 7.1) only were administered to the indicated study arms via IP injection once on Study Day 0. <b>Rapalog</b> (AP21967) was formulated in 10% DMSO and administered to mice via intraperitoneal injection 3x weekly beginning study day 5.			
Sample collection and frequency	Blood was collected from the retroorbital sinus on study days 5, 12, 19, and 26			
Outcomes	<ul style="list-style-type: none"> <li>Animals were evaluated for bodyweight 2x weekly</li> <li>Circulating CAR T cells were evaluated weekly by flow cytometry</li> <li>Tumor burden was evaluated weekly by bioluminescent imaging</li> </ul>			

89

90 Supplementary figure 1. PBMCs were transduced with UB-VV100 at MOI 5 in the presence of IL2 and no

91 additional stimulation. Surface FMC63 CAR expression was measured 7 days after addition of UB-VV100. A)

92 Representative flow cytometry gating tree for analysis of CAR expression on CD3+ and CD3- cells. B) FMC63  
93 CAR expression on CD3- cells after transduction with a “No payload” empty vector control or UB-VV100 in 3  
94 different healthy donors. The empty vector control is an  $\alpha$ CD3 scFv engineered vector bearing no transgene.  
95 PBMCs transduced with the empty vector control serve as a flow cytometry gating control for CAR expression as  
96 these cells do not express the CAR.

97 Supplementary figure 2: Co-transduction of PBMCs and Nalm-6 tumor cells with UB-VV100 results in Nalm-6  
98 tumor elimination.  $5 \times 10^5$  Nalm-6 cells and  $5 \times 10^5$  PBMCs were mixed and transduced with either UB-VV100, or a  
99 CD3-Cocal engineered vector encoding a CAR of irrelevant specificity (control CAR). Wells were analyzed for  
100 A) survival of Nalm-6 tumor targets and B) expansion of CAR+ T cells. Data shown are mean  $\pm$  standard error  
101 from 8 unique PBMC donors

102

103 Supplementary figure 3: Circulating B cells in CD34 humanized NCG mice after treatment with UB-VV100. B  
104 cell were enumerated by flow cytometry 7 days before dosing, and 28 days after administration with vehicle or  
105 UB-VV100. \* indicates  $p < 0.05$ , one-way ANOVA Dunn's test for multiple comparisons.

106

107 Supplementary figure 4. Representative gating strategy for identification of circulating CAR T cells and tumor  
108 cells. PBMC humanized mice bearing systemic Nalm-6 tumor were treated with vehicle or  $270 \times 10^6$  TU UB-  
109 VV100 on day 0. Nalm-6 tumor cells, human T cells, and murine CD45+ cells were identified by flow cytometry  
110 and assessed for expression of surface FMC63. Representative plots are shown for study day 11 A) vehicle treated  
111 mice (N = 8) UB-VV100 treated mice (N=9), and for study day 32 C) vehicle treated mice (N=6) and D) UB-  
112 VV100 treated mice (N=8).

113 Supplementary figure 5. Representative gating strategy to assess Nalm-6 transduction in UB-VV100 treated mice.  
114 PBMC humanized mice bearing systemic Nalm-6 tumor were treated with vehicle or  $100 \times 10^6$  TU UB-VV100 on  
115 day 0. Bone marrow was harvested from animals upon humane endpoint and evaluated for CD19, FMC63, and

### Preclinical pharmacology of a lentiviral vector designed to generate CAR T cells in vivo

116 P2A expression in Nalm-6 tumor cells. Tumor was successfully recovered from all animals treated with A)  
117 Vehicle (2 representative plots from N = 5 total animals). Tumor was detected by flow cytometry in a total of 3  
118 animals ( N = 8 total animals) upon humane endpoint on B) day 42 and C) Day 85. Nalm-6 tumor was not  
119 detected on upon euthanasia in the 5 remaining UB-VV100 treated mice.

120 Supplementary figure 6. Rapamycin-mediated inhibition of PBMC humanization in mice. Circulating total  
121 human T cell populations were evaluated by flow cytometry in A) healthy mice (N=3-8 mice per group per time  
122 point) and B) Nalm-6 tumor bearing mice (N=3-5 per group per time point). Bars indicate +/- 1 SEM. \*\*, \*\*\*  
123 indicate p values of < 0.01, <0.001 respectively, two-way ANOVA, Tukey's multiple comparisons test.

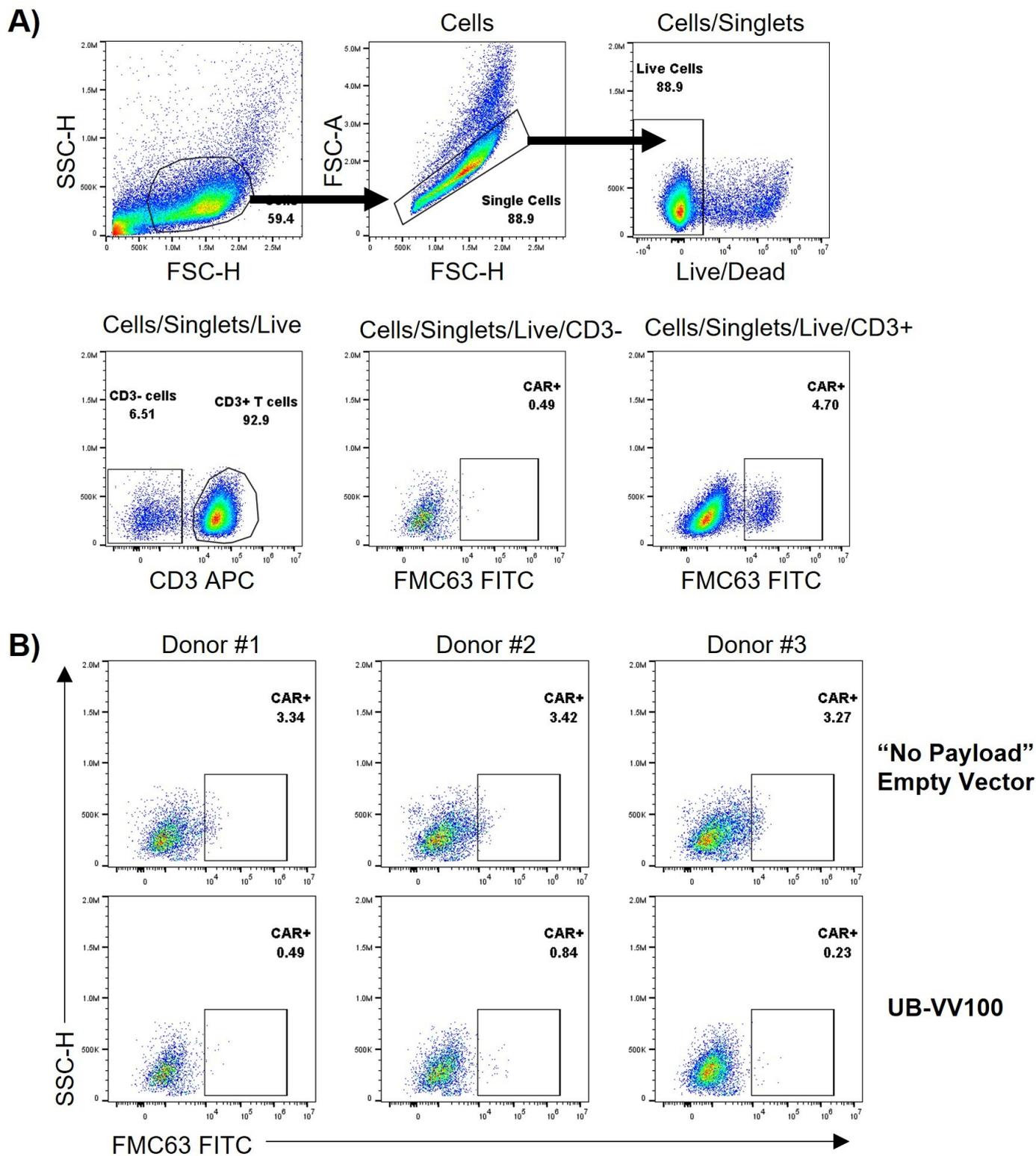
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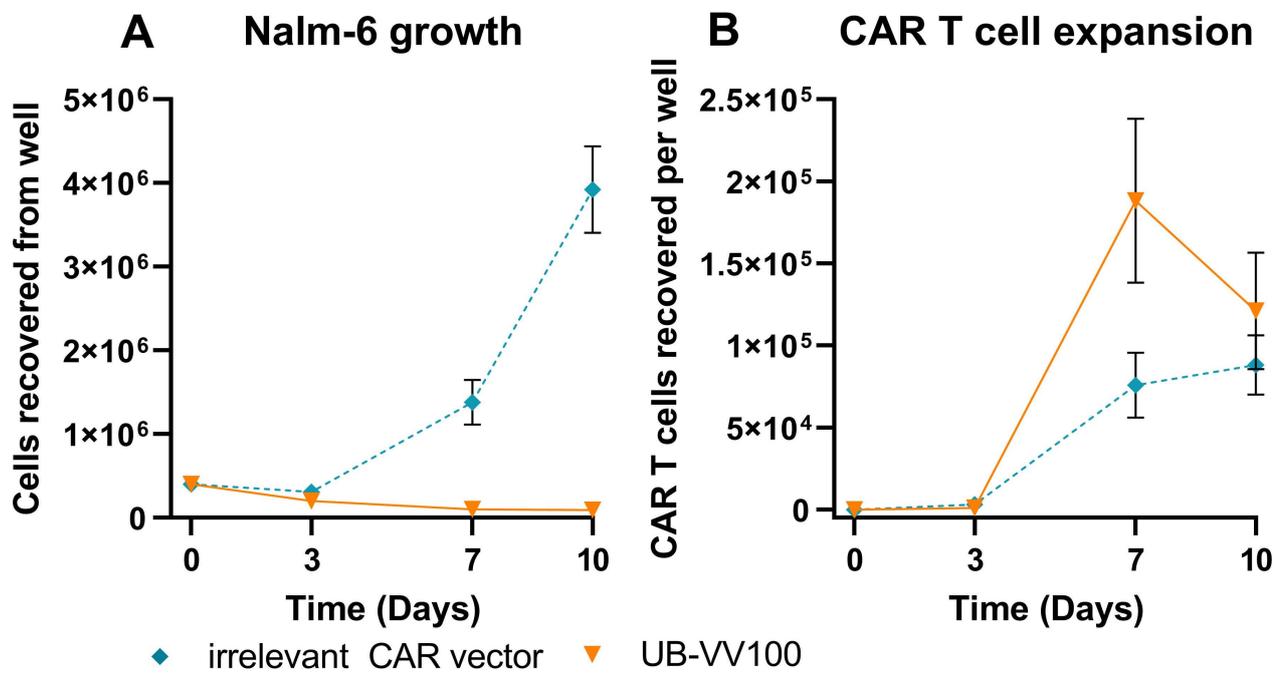
125 Supplementary figure 7. T cells from a single donor were activated with CD3/CD28 beads and then transduced  
126 with UB-VV100 (CAR T cells) or not transduced (mock transduced T cells) and then infused into NSG MHC I/II  
127 knockout mice 5 days after implantation of 5E+05 Raji tumor cells. Animals were treated 5x weekly with 0,  
128 0.005, 0.05, or 0.5 mg/kg rapamycin (N=5-6 per group). A) Animals treated with mock T cells (3 million T cells)  
129 or CAR T cells (3 million total T cells, 30% of which were CAR+) were monitored for survival. Data from a  
130 single experiment are shown as 2 survival graphs for visual clarity. B) Circulating CAR T cells were monitored  
131 in the blood by flow cytometry and quantified by absolute circulating number using counting beads and by  
132 fraction of CD3+ T cells with surface expression of FMC63. Data points indicate mean +/- 1 SEM. Error bars are  
133 not visible at some data points due to eclipse by the data symbol.

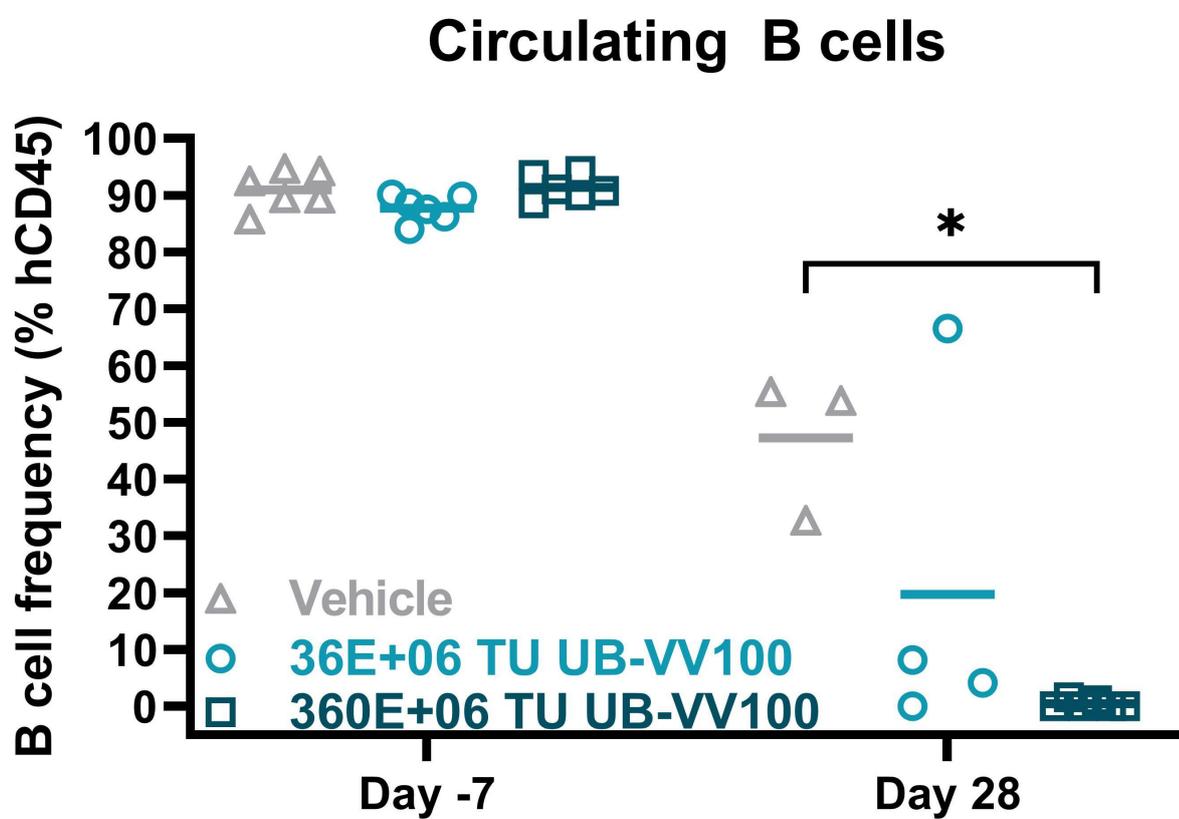
134

135 Supplementary figure 8. Rapalog mediated expansion of in vivo transduced CAR T cells. Nalm-6 tumor bearing  
136 animals were treated with 50 million TU UB-VV100 on day 0, and then treated with 0, 1, 5, or 10 mg/kg rapalog  
137 3x weekly via intraperitoneal injection beginning day 5. A) Animals were evaluated for survival. \*\* indicates p <  
138 0.01, Mantel-Cox test, relative to 0 mg/kg rapalog treatment arm. B) Animals were evaluated for circulating CAR

- 139 T cell expansion by flow cytometry. Bars indicate median. \* indicates  $p < 0.05$ , one-way ANOVA, Tukey  
140 multiple comparison's test.

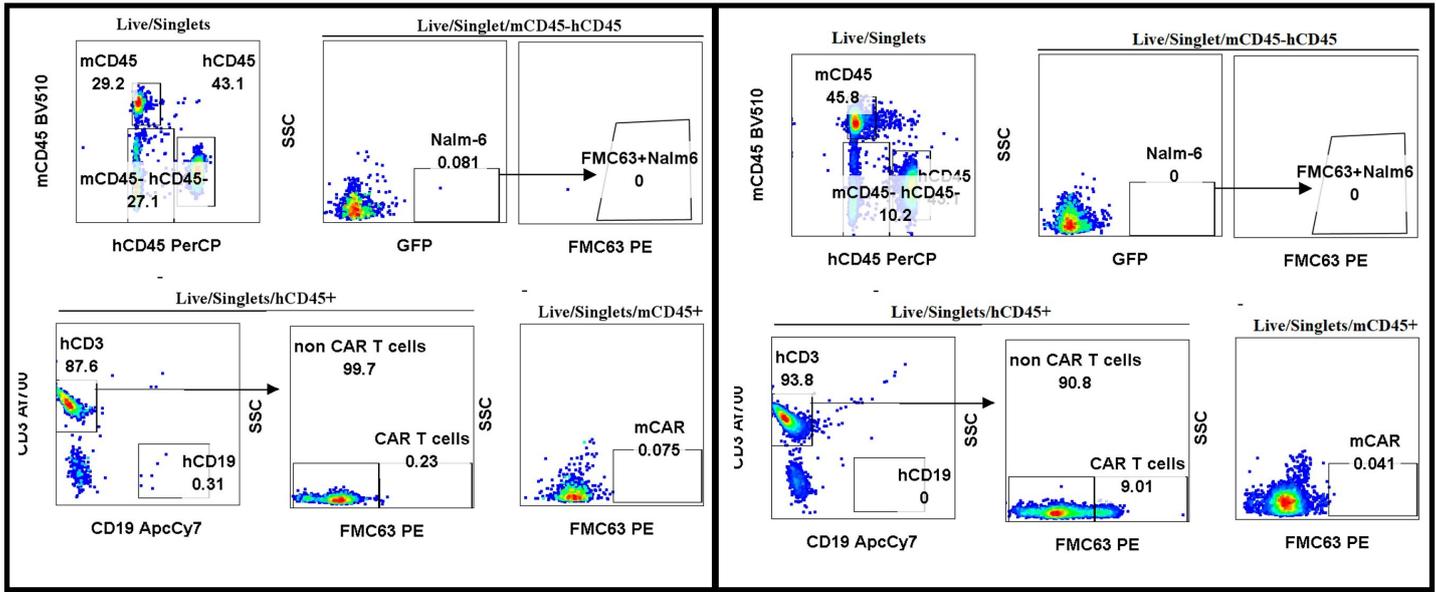






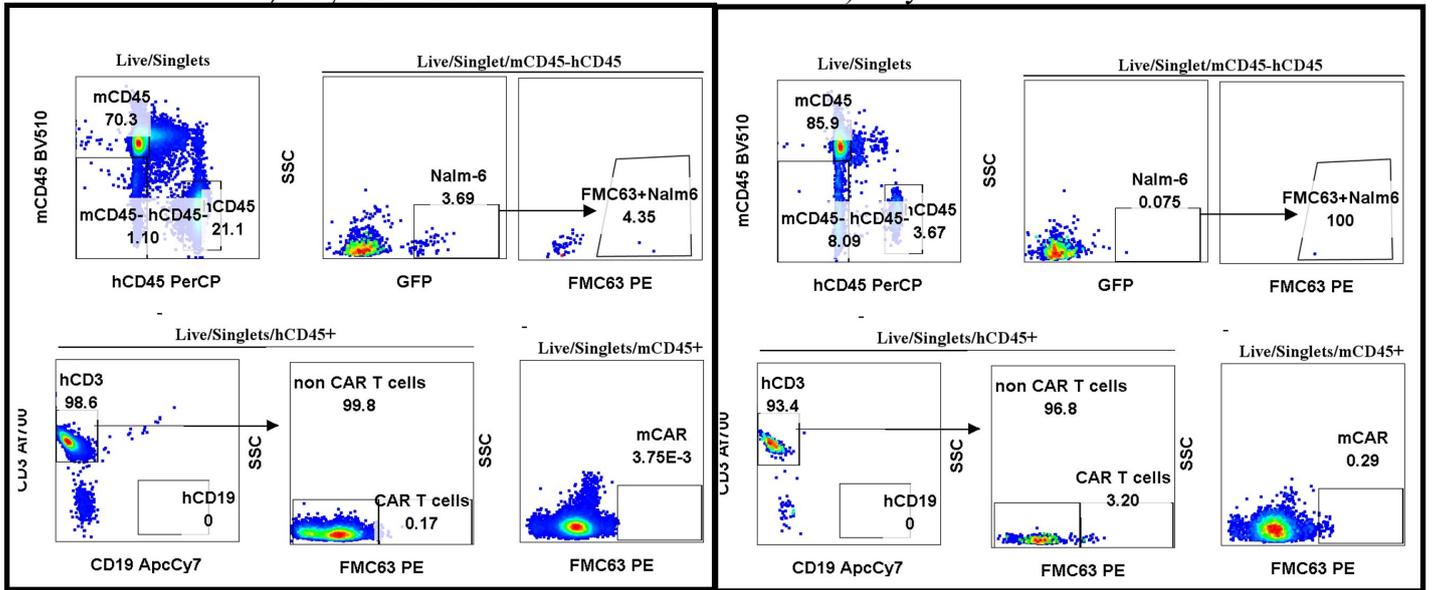
A) Day 11 Vehicle

B) Day 11 270 E+06 TU UB-VV100

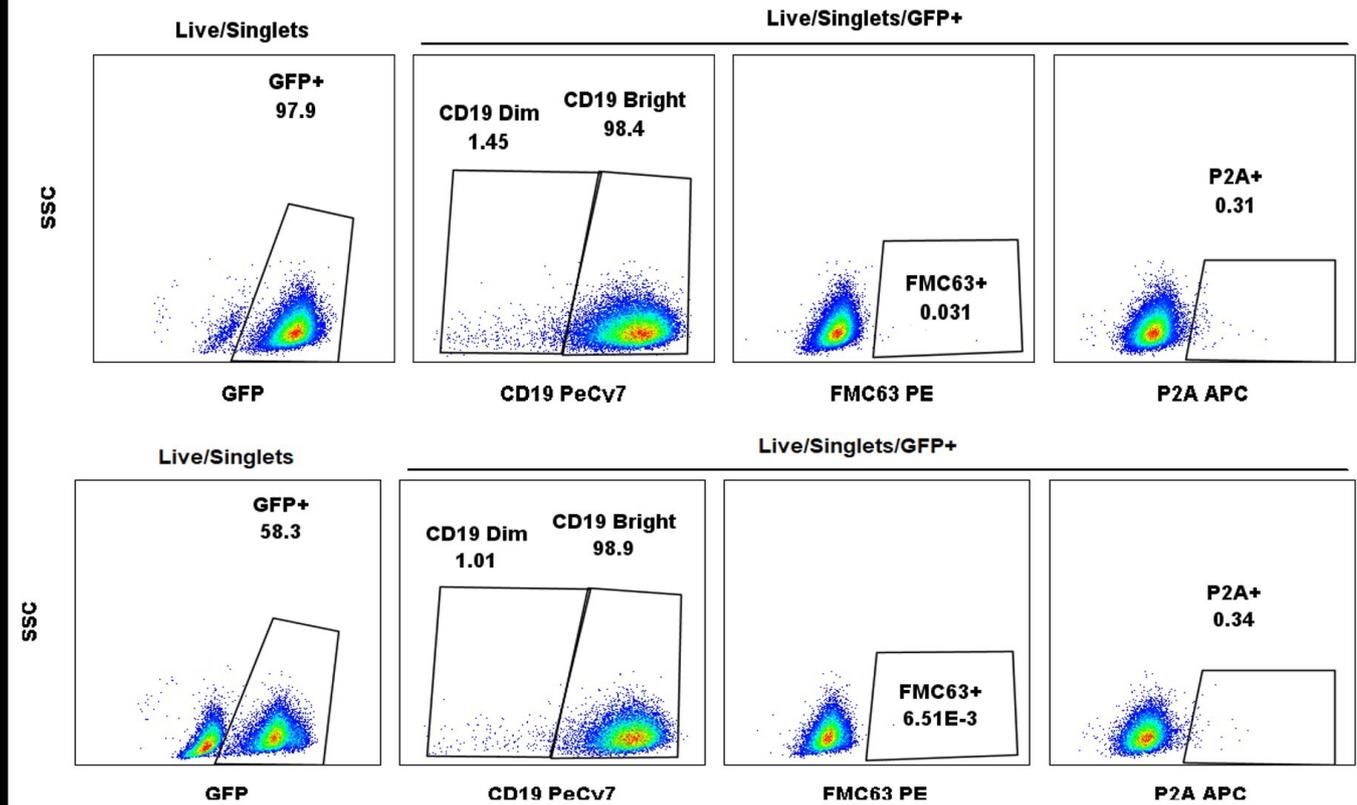


C) Day 32 Vehicle

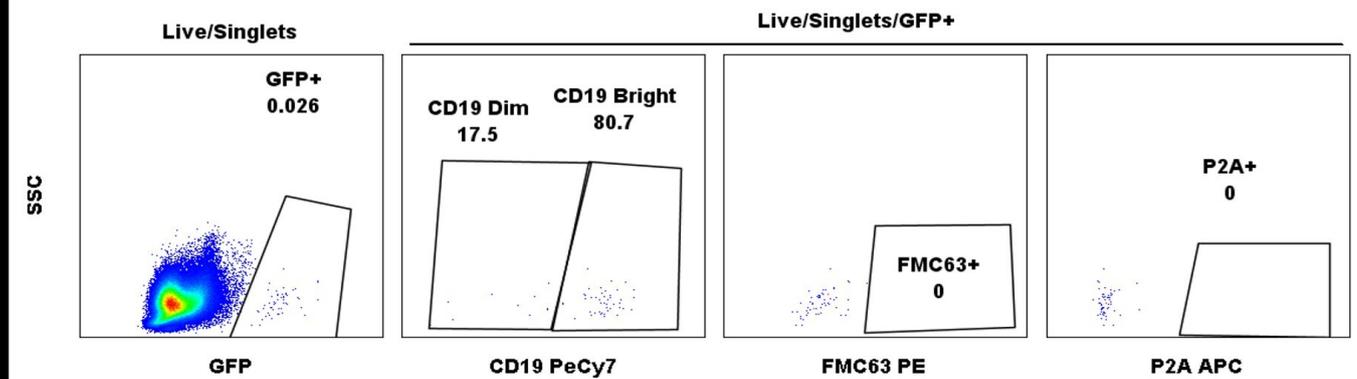
D) Day 32 270 E+06 TU UB-VV100



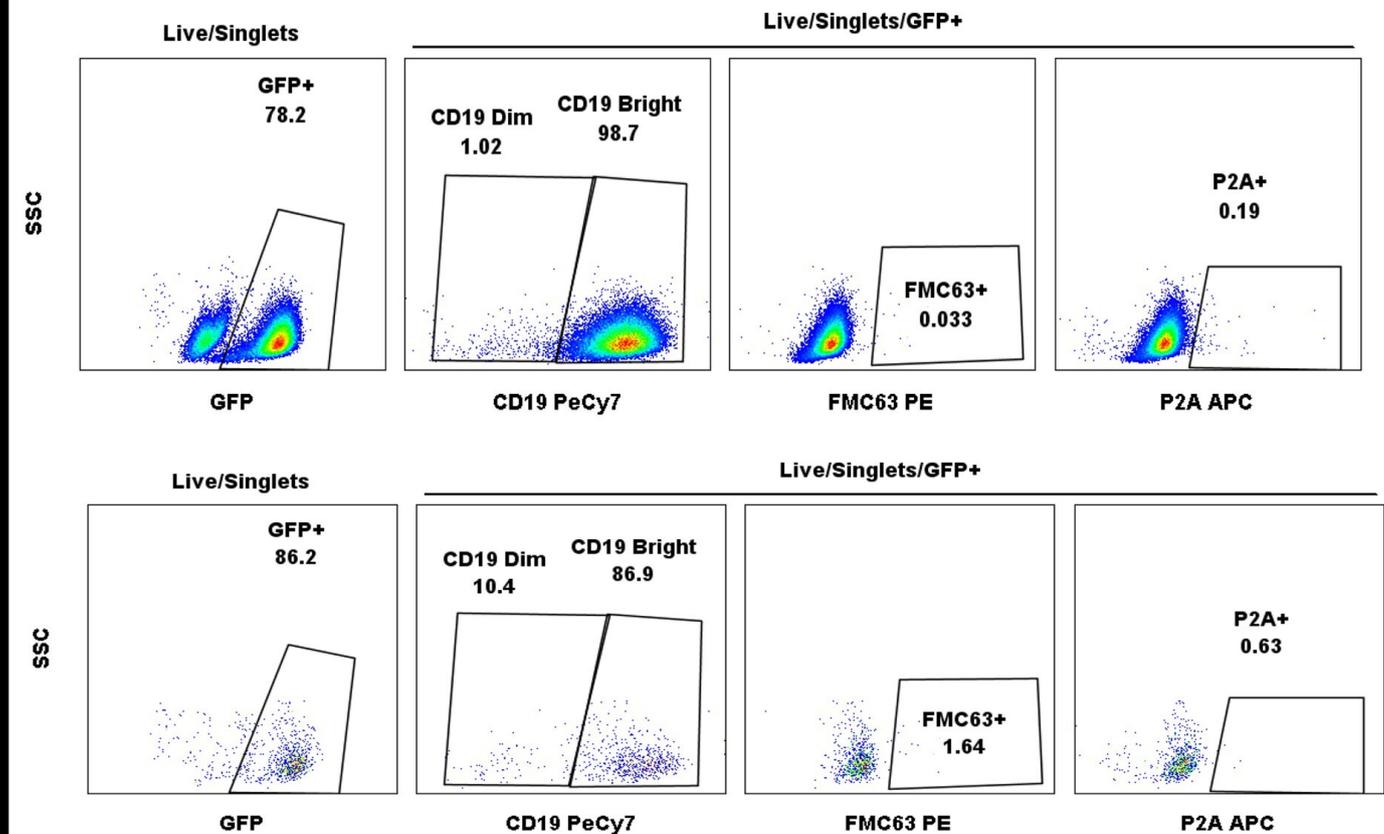
### A) Bone marrow Vehicle Day 26

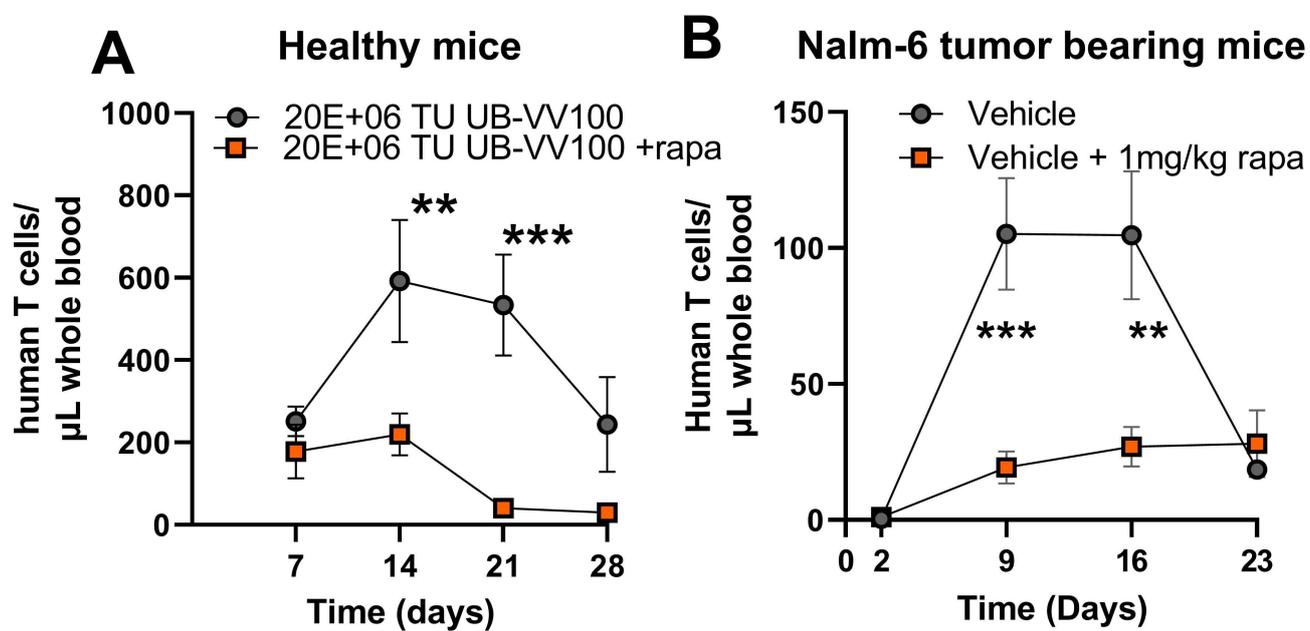


### B) Bone marrow 100E+06 TU UB-VV100 Day 42

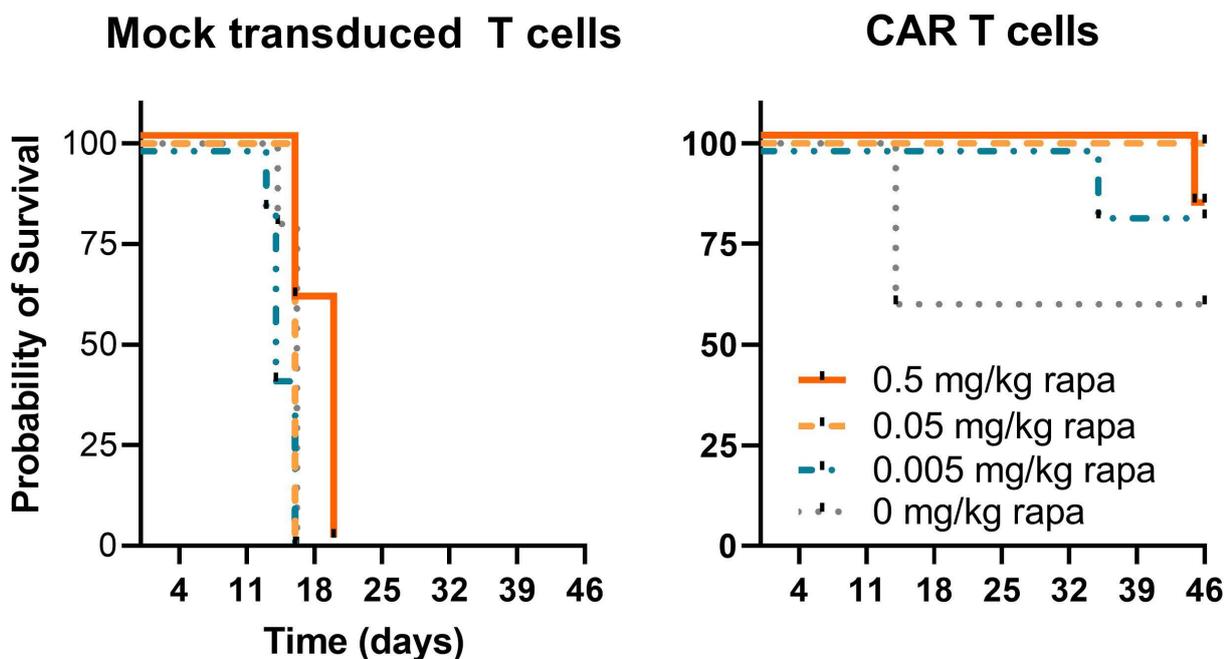


### C) Bone marrow 100E+06 TU UB-VV100 Day 85





## A) Survival in Raji tumor-bearing mice



## B) CAR T cell enrichment and expansion in vivo

