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-humaniced 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

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	Biolgend 300408
Figure 2A, B; Figure 3A, B	hCD4 APC	A161A1	Biolegend 357408
Figure 2A, B; Figure 3A, B	hCD8 BV421	SK1	Biolegend 344748
Figure 2A, B; Figure 3A, B	hCD25 CD25	M-A251	Biolegend 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	Biolegend 423106
Figure 2C	Live/Dead Zombie Violet	N/A	Biolegend 423114
Figure 2C	hCD3 PerCPCy5.5	UCHT1	Biolegend 300430
Figure 2C	hCD8 AF700	SK1	Biolegend 344724
Figure 2C	hCD4 BV510	SK3	Biolegend 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	Biolegend 300430

Figure 2E	hCD8 AF700	SK1	Biolegend 344724
Figure 2E	hCD4 BV510	SK3	Biolegend 344634
Figure 2E	P2A A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 2E	hCD107a PE	H4A3	Biolegend 328608
Figure 2E	Live/Dead Zombie NIR	N/A	Biolegend 423106
Figure 2E	Nalm6/Nalm6KO GFP::ffluc FITC	N/A	N/A
Figure 2F	hCD8 PerCpCy5.5	SK1	Biolgend 344710
Figure 2F	hCD4 BV650	RPA-T4	Biolegend 300536
Figure 2F	hCD3 A700	UCHT1	Biolegend 300424
Figure 2F	hCD25 BV421	BC96	Biolegend 312218
Figure 2F	hCD19 PECy7	HIB19	Biolegend 302216
Figure 2F	hCD19 FITC	EPR5906	Abcam ab196468
Figure 2F	Live/Dead Zombie NIR	N/A	Biolegend 423106
Figure 3 C,D	hCD3 PerCPCy5.5	UCHT1	Biolegend 300430
Figure 3 C,D	hCD8 AF700	SK1	Biolegend 344724
Figure 3 C,D	hCD4 BV510	SK3	Biolegend 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	Biolegend 304134
Figure 3 C,D	hCCR7 BV421	G043H7	Biolegend 353208
Figure 3 C,D	Live/dead Zombie NIR	N/A	Biolegend 423106
Figure 4A,B	Live/dead Zombie NIR	N/A	Biolegend 423106
Figure 4A,B	hCD3 R718	UCHT1	BD Biosciences 566953
Figure 4A,B	hCD8 or hCD4 BV510	SK1 or SK3	Biolegend 344732
Figure 4A,B	hCD20 BV650	2H7	Biolegend 302336
Figure 4A,B	FMC63 CAR idiotype PE	R19M	Cytoart 200106
Figure 4A,B	hCD19 PECy7	HIB19	Biolegend 302216

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

Figure 4A,B	hCD25 BV421	BC96	Biolegend 302630
Figure 4A,B	hCD19 (intracellular) A488	EPR5906	Abcam ab196468
Figure 4A,B	hCD79a (intracellular) PerCpCy5.5	HM47	Biolegend 333508
Figure 4A,B	P2A (intracellular) A647	3H4	Novus Biolgics NBP2-59627AF647
Figure 4C	hCD8 PerCpCy5.5	SK1	Biolegend 344710
Figure 4C	hCD4 A700	SK3	Biolegend 344622
Figure 4C	hCD19 PECy7	HIB19	Biolegend 302216
Figure 4C	hCD3 BV650	UCHT1	Biolegend 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	Biolegend 368504
Figure 5A, S7	hCD3 AF700	UCHT1	Biolegend 300424
Figure 5A, S7	hCD20 PE	2H7	Biolegend 302306
Figure 5A, S7	hCD4 BV650	RPA-T4	Biolegend 300535
Figure 5A, S7	hCD8 BV605	SK1	Biolegend 344741
Figure 5A, S7	hCD25 PECY7	M-A251	Biolegend 356122
Figure 5A, S7	hCD71 APCCY7	CY1G4	Biolegend 334110
Figure 5A, S7	FMC63 CAR idiotype 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	Biolegend 368504
Figure 7B, C, S4, S8	hCD3 AF700	UCHT1	Biolegend 300424
Figure 7B, C, S4, S8	hCD4 BV650	RPA-T4	Biolegend 300535
Figure 7B, C, S4, S8	hCD8 BV605	SK1	Biolegend 344741
Figure 7B, C, S4, S8	hCD25 PECY7	M-A251	Biolegend 356122
Figure 7B, C, S4, S8	hCD19 APCCY7	6D5	Biolegend 115530
Figure 7B, C, S4, S8	FMC63 CAR idiotype PE	Y45	ACRO Biosystems FM3-HPY53
Figure 7B, C, S4, S8	mCD45 BV510	30-F11	Biolegend 103137
Figure 7B, C, S4, S8	Nalm6 GFP::ffluc	N/A	N/A

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

32

33

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 [1]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 [2]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 [1]Rituximab, Cyclophosphamide, hydroxydaunorubicin, Oncovin, Prednisone

36 [2]Rituximab, Dexamethasone, Cytarabine, Cisplatin

37

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

Purpose	• To evaluate dose-dependent activity of UB-VV100 by measuring depletion of endogenous B cells in CD34-humanized mice • To evaluate antigen-specificity of B cell depletion
Species/Strain	Female CD34-humanized Nod.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/SzJ (NSG, Jackson stock 005557), acquired from Jackson, 14 weeks post humanization

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

<table>
<tr><td>Age/Weight</td><td>17 weeks of age at study start, 17-25 g in weight</td></tr>
<tr><td>Randomization and enrollment criteria</td><td>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</td></tr>
<tr><td>Study site</td><td>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.</td></tr>
<tr><td>Study Design
TU = Transducing units</td><td>
<table>
<tr><th>Group</th><th>Vector dose</th><th>Vector identity</th><th>Sample size</th></tr>
<tr><td>1</td><td>0E+06 TU</td><td>Vehicle</td><td>4</td></tr>
<tr><td>2</td><td>10E+06 TU</td><td>VivoVec vector encoding control CAR</td><td>4</td></tr>
<tr><td>3</td><td>0.4E+06 TU</td><td>UB-VV100</td><td>4</td></tr>
<tr><td>4</td><td>2E+06 TU</td><td>UB-VV100</td><td>4</td></tr>
<tr><td>5</td><td>10E+06 TU</td><td>UB-VV100</td><td>4</td></tr>
</table>
</td></tr>
<tr><td>Study blinding</td><td>No study blinding was implemented in this design. Animal behavior, health, and humane endpoint criteria were recorded by a technician external to the sponsor.</td></tr>
<tr><td>Dosing route and frequency</td><td>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.</td></tr>
<tr><td>Sample collection and frequency</td><td>Blood was collected via retroorbital sinus on study days -1, 4, 11, 18, and 25</td></tr>
<tr><td>Outcomes</td><td>• Health: Grooming, posture, activity level, bodyweight
• UB-VV100 efficacy: Circulating B cells as enumerated by flow cytometry from serial blood draws
• UB-VV100 efficacy: Circulating CAR T cells as enumerated by flow cytometry from serial blood draws</td></tr>
</table>

39

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

<table>
<tr><td>Purpose</td><td>• To evaluate biodistribution and tissue tropism of UB-VV100
• To evaluate tolerability of UB-VV100 test article in humanized mice</td></tr>
<tr><td>Species/Strain</td><td>Female CD34-humanized NOD-$Prkdc^{em26Cd52}$ $Il2rg^{em26Cd22}$ /NjuCrl (NCG) mice from Charles River Laboratories</td></tr>
<tr><td>Age/Weight</td><td>28 weeks of age at study start, 20-26 g in weight</td></tr>
</table>

<table>
<tr><td>Randomization and enrollment criteria</td><td>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. Aanimals were distributed to groups to account for equal distribution by humanization and human donor (2 total donors were used).</td></tr>
<tr><td>Study site</td><td>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.</td></tr>
<tr><td>Study Design
TU = Transducing units</td><td>
<table>
<tr><th>Group</th><th>Vector dose</th><th>Sample size</th></tr>
<tr><td>1</td><td>0E+06 TU</td><td>6</td></tr>
<tr><td>2</td><td>36E+06 TU</td><td>6</td></tr>
<tr><td>3</td><td>360E+06 TU</td><td>6</td></tr>
</table>
</td></tr>
<tr><td>Study blinding</td><td>No study blinding was implemented in this design. All data and reports were prepared by a contract research organization external to the sponsor.</td></tr>
<tr><td>Dosing route and frequency</td><td>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.</td></tr>
<tr><td>Sample collection and frequency</td><td>Blood was collected via retroorbital sinus on day -7 and on day 26 via cardiac puncture upon study termination.</td></tr>
<tr><td>Outcomes</td><td>

Health: dermal irritation scoring, clinical observations for health and appearance, bodyweight, and daily food consumption (no changes related to test article noted)
Enumeration of circulating B cells by flow cytometry (results reported in Figure S2)
Evaluation of macroscopic pathology and organ weights upon necropsy (no abnormalities related to vector test article noted)
Evaluation of microscopic pathology (no abnormalities related to vector test article noted)
Biodistribution of UB-VV100 transduced cells by ddPCR targeted against the viral PSI integration element
Identification of UB-VV100 transduced cells by ISH against the UB-VV100 transgene

</td></tr>
<tr><td>Organs evaluated for histopathology, ddPCR, and flow</td><td>
<table>
<tr><th>Organ</th><th>Analysis</th></tr>
<tr><td>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</td><td>Macroscopic and microscopic pathology</td></tr>
<tr><td>Bone marrow, brain, adrenal gland, heart, kidney, liver, lung, ovary, injection site, spinal cord, spleen</td><td>qPCR against vector PSI integration element</td></tr>
<tr><td>Spleen, liver</td><td>ISH against UB-VV100 transgene</td></tr>
</table>
</td></tr>
</table>

41

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	• To evaluate biodistribution and tissue tropism of anti-human CD3scFv coated cocal pseudotyped (VivoVec) lentiviral vectors in Beagle dogs • To evaluate biodistribution and tissue tropism of VivoVec vectors when administered via lymph node injection
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	(see nested table below)
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 cocal-pseudotyped lentiviral vector particle engineered to express anti-human CD3-scFv, and encodes a 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	• Weekly bodyweights and clinical observations (no adverse events noted) • Mortality (no unscheduled deaths noted) • Clinical pathology performed once pre-dose and once prior to necropsy for all groups : Clinical chemistry, hematology, coagulation, urinalysis • Macroscopic and microscopic histopathology (no pathology related to vector test article noted) • Biodistribution analysis by qPCR • Identification of transduced cells via ISH

Study Design:

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

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

43

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	*Cd3d*, *Cd3e*, *Cdef*	Canine	#1124138-C4, 1124128-C4, 406278-C4
Canine CD45	*Ptprc*	Canine	# 1123128-C3
Canine CD68	*Cd68*	Canine	#577868-C4
UB-VV100 RACR	Viral payload	Humanized mouse	# 1048558-C1
Human panCD3	*Cd3d*, *Cd3e*, *Cdef*	Humanized mouse	# 426628-C2
Murine CD45	*Ptprc*	Humanized mouse	#318658-C3
Murine CD68	*Cd68*	Humanized mouse	# 316618-C4

46

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 (%)[a]**						
		PBS (IP)	**36E+06 TU UB-VV100 (IP)**			**360E+06 TU UB-VV100 (IP)**		
		1501	**2502**	**2504**	**2506**	**3501**	**3503**	**3504**

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 [a] 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 (%)[a]**						
		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 [a] 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.
64

65

66

67

68

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

Tissue	Cell Phenotype	Proportion of Events (%)[a]								
		IN Delivery (1 wk)			IN Delivery (4 wks)			IP Delivery (1 wk)		
		2001[b]	2501	2502	4001	4502[d]	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)[c]	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)[c]	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[e]	All EGFP+ Cells (DAPI+:EGFP+)	<< 1 (10 cells)	<< 1 (5 cells)	NA	NA	NA	NA	NA	NA	<< 1 (6 cells)
	EGFP+: CD45+ (Leukocyte)[c]	*51-60*	*0*	NA	NA	NA	NA	NA	NA	*41-50*
	EGFP+: CD3+ Pool (T Cells)	*21-30*	*51-60*	NA	NA	NA	NA	NA	NA	*41-50*
	EGFP+: CD68+ (Macrophages)	*71-80*	*51-60*	NA	NA	NA	NA	NA	NA	*81-90*
	EGFP+: CD45-, CD3-, CD68- (Non-immune cells)	*1-10*	*41-50*	NA	NA	NA	NA	NA	NA	*11-20*

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

[b] Inguinal lymph node tissue from animal 2001 not available was for analysis.

[c] Canine CD45 ISH probe set covers 7 of 8 *Ptprc* transcripts encoding or CD45

[d] Medial iliac lymph node tissue from animal 4502 was not available for analysis.

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

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

Michels KR, *et al. J Immunother Cancer* 2023; 11:e006292. doi: 10.1136/jitc-2022-006292

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	• To evaluate the dose-dependent activity of UB-VV100 in humanized, immune-compromised mice engrafted with systemic Nalm-6 tumor • To evaluate the role of anti-CD3 scFv in CAR T cell transduction in vivo • To evaluated the tolerability of UB-VV100 when administered to humanized mice bearing systemic tumor
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	(see table below)
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	**Tumor:** 2.5×10^5 Nalm-6 tumor cells were administered via tail vein injection once on Study Day -4. **Humanization:** 2.0×10^7 PBMCs were administered via IP injection once on Study Day -1. **Drug:** 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	• 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) • Circulating CAR T cells were evaluated weekly by flow cytometry • Nalm-6 tumor burden was evaluated weekly by bioluminescent imaging

Study Design:

Group	Vector dose (TU/Animal)	Number of Animals
1	Vehicle (0)	8
3	UB-VV100 (2.7×10^7) TU	8
4	UB-VV100 (8.0×10^7) TU	8
5	UB-VV100 (2.7×10^8) TU	9

84

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

<table>
<tr><td>Purpose</td><td>To assess the ability of the rapamycin/RACR axis to drive CAR T cell enrichment, expansion, and tumor clearance in vivo</td></tr>
<tr><td>Species/Strain</td><td>Female NSG mice lacking endogenous MHC I/II (NOD.Cg-Prkdcscid H2-K1tm1Bpe H2-Ab1em1Mvw H2-D1tm1Bpe Il2rgtm1Wjl/SzJ).</td></tr>
<tr><td>Age/Weight</td><td>7 weeks of age at study start, 17-25g in weight</td></tr>
<tr><td>Randomization and enrollment criteria</td><td>Animals were randomized in blocks to account for tumor burden on day -1, animal age, and animal weight.</td></tr>
<tr><td>Study site</td><td>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.</td></tr>
<tr><td>Study Design
TU = Transducing units</td><td>
<table>
<tr><th>Group</th><th>CAR T cell infusion</th><th>Rapamycin (mg/kg), 3x weekly</th><th>Number of Animals</th></tr>
<tr><td>1</td><td>3×10^6 Mock transduced T cells</td><td>0</td><td>5</td></tr>
<tr><td>2</td><td>3×10^6 Mock transduced T cells</td><td>0.005</td><td>7</td></tr>
<tr><td>3</td><td>3×10^6 Mock transduced T cells</td><td>0.05</td><td>5</td></tr>
<tr><td>4</td><td>3×10^6 Mock transduced T cells</td><td>0.5</td><td>5</td></tr>
<tr><td>5</td><td>3×10^6 transduced T cells (30% CAR+)</td><td>0</td><td>5</td></tr>
<tr><td>6</td><td>3×10^6 transduced T cells (30% CAR+)</td><td>0.005</td><td>6</td></tr>
<tr><td>7</td><td>3×10^6 transduced T cells (30% CAR+)</td><td>0.05</td><td>6</td></tr>
<tr><td>8</td><td>3×10^6 transduced T cells (30% CAR+)</td><td>0.5</td><td>6</td></tr>
</table>
</td></tr>
<tr><td>Study blinding</td><td>No study blinding was implemented in this design</td></tr>
<tr><td>Dosing route and frequency</td><td>5×10^5 Raji tumor cells were implanted via retroorbital injection on Study Day -4. 3×10^6 transduced T cells (30% FMC63 CAR+) or 3×10^6 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.</td></tr>
<tr><td>Sample collection and frequency</td><td>Blood was collected by serial retroorbital bleeds on days 4, 18, 25, 32, 39, and 46.</td></tr>
<tr><td>Outcomes</td><td>• Animals were evaluated for bodyweight 2x weekly
• Circulating CAR T cells were evaluated weekly by flow cytometry
• Raji tumor burden was evaluated weekly by bioluminescent imaging</td></tr>
</table>

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	(see design table below) *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 preformed for this study.
Dosing route and frequency	**Tumor:** 0.5E+06 Nalm-6 tumor cells were administered via tail vein injection once on Study Day -4. **Humanization:** 2.0×10^7 PBMCs were administered via IP injection once on Study Day -1. **Drug:** 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. **Rapalog** (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	• Animals were evaluated for bodyweight 2x weekly • Circulating CAR T cells were evaluated weekly by flow cytometry • Tumor burden was evaluated weekly by bioluminescent imaging

Study Design:

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

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 αCD3 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. $5x10^5$ Nalm-6 cells and $5x10^5$ 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 ± 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 270E+06 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 100E+06 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.

124

125 Supplemental 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.