Title: Generation and optimization of off-the-shelf immunotherapeutics targeting TCR-V β 2+ T cell malignancy

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Supplementary Fig. 1: mCAR-Vβ2 T cell development.



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Supplementary Fig. 1: mCAR-V β 2 T cell development.

a, Representative flow cytometry of total T cells from a CTCL patient with a V β 2+ malignancy stained with a panel of anti-V β antibodies showing the distribution of V β family usage and predominance of V β 2. **b**, Key components of CAR-V β 2 construction in a lentiviral vector. **c**, Key components of TCR- β chain TRBV20-1 containing construction in a lentiviral vector. **d**, GFP and surface CAR-V β 2 expression in mCAR-V β 2 T cells determined by flow cytometry. **e**, mcherry and surface TRBV20-1 expression in Jurkat-TRBV20-1 cells determined by flow cytometry. **f**, Live Jurkat-TRBV20-1 cell counts after overnight in vitro killing by allogeneic CAR-CD19 T cells (black) or mCAR-V β 2 T cells (red) at 1:1 of E:T ratio, determined by flow cytometry. **g**, Lentiviral transduction efficiency of CAR-CD19 or mCAR-V β 2 in CD8 T cells from a V β 2+ CTCL patient, determined by flow cytometry detection of GFP. **h**, Live CTCL counts of three V β 2+ CTCL patients after overnight in vitro killing by purified autologous CAR-CD19 T cells (black) or mCAR-V β 2 T cells (red, blue and green) at different E:T ratios, determined by flow cytometry. Source data are provided as a Source Data file.



Supplementary Fig. 2: Allogeneic mCAR-V β 2 T cell generation.

Supplementary Fig. 2: Allogeneic mCAR-V β 2 T cell generation.

a, Flow cytometric histograms showing CD3 expression 2 days post TRAC KO in mCAR-V β 2 T cells before (left) or after (right) residual CD3+ cell depletion. **b**, HLA-A/B/C and **c**, HLA-DR/DP/DQ expression on mCAR-V β 2 T cells with (red) or without (normal control (NC), black) triple (TRAC/B2M/CIITA) KO, determined by flow cytometry. **d**, Lentiviral transduction efficiency of mCAR-V β 2 in CD8 and CD4 T cells from a healthy donor following triple KO, determined by flow cytometry detection of GFP. **e**, Activation marker CD137, CD25 and CD69 expression on triple KO mCAR-V β 2 T cells after overnight in vitro culture alone or mixed with CTCL cells from three different V β 2+ patients and one V β 13.2+ patient, determined by flow cytometry. **f**, V β 2+ PTCL cell count, **g**, V β 2-normal CD4+ T cells from a V β 2 + PTCL patient and treated with (red) or without (NC, black) triple KO mCAR-V β 2 T cells. **e**, n=3 replicates of each group. *p<0.05 and ****p<0.001 by one-way ANOVA. **f-h**, n=3 mice in each group (**f**, p=0.032, **h**, p=0.0026).



Supplementary Fig. 3: CAR expression levels in humanized CAR-Vβ2 T cells.

Supplementary Fig. 3: CAR expression levels in humanized CAR-V β 2 T cells.

a-b, Live counts of CTCL cells from a V β 2+ patient after overnight culture with a set of CAR-V β 2 T cells humanized via two different in silico strategies provided by **a**, the BioPhi algorithm or **b**, a third-party contractor (mAbvice), as determined by flow cytometry. c, GFP+% and d, average GFP expression intensity of the GFP+ population of CAR-V β 2 T cells after humanization using the BioPhi in silico algorithm, as determined by flow cytometry. e, GFP+% and f, average GFP expression intensity of the GFP+ population of CAR-V β 2 T cells after humanization by mAbvice, as determined by flow cytometry. Source data are provided as a Source Data file.

2									
a	hCAR-Vβ2-								
	(V1-	(V7-4-							
	46*01_2_V	46*01_2_V	46*01_2_V	46*01_2_V	46*01_3_V	46*01_3_V	46*01_3_V	46*01_3_V	1*02_1_V4-
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	hCAR-Vβ2-								
	(V7-4-								
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	1*01_3)	39*01_1)	39*01_2)	1*01_2)	1*01_3)	39*01_1)	39*01_2)		
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	46*01_1_V	46*01_1_V	46*01_1_V	46*01_1_V	1*01_V7-	1*01_V7-	1*01_V4-	1*01_V3D-	2*06_V7-
	7-3*01_1)	7-3*01_2)	4-1*01_1)	3D-20*02)	3*01_1)	3*01_2)	1*01_1)	20*02)	3*01_1)
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	(V1-	CAR-CD19	mCAR-Vβ2						
	2*06_V7-	2*06_V4-	2*06_V3D-	3*01_V7-	3*01_V7-	3*01_V4-	3*01_V3D-		
	3*01_2)	1*01_1)	20*02)	3*01_1)	3*01_2)	1*01_1)	20*02)		

Supplementary Fig. 4: Immune reactivity of candidate humanized CAR-Vβ2.

h					HC 1				
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	GFP	GFP	GFP	, ", ", ", ", ", ", ", ", ", ", ", ", ",	GEP	, ",",",",","," CFP	GFP	GFP	GFP
	2 1/20 1.38	u ¹ 2 u ¹	2 1.22 1.23	2 1/3 0.62	109 0.84	1	1.34 1.34 1.34	N	100 1.00
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	108 077 1 07 101 52.4 44.1	u ¹ 08 07 45.6 51.1	u ⁴ 08 09 09 07 38.7 57.2	Q8 8 07 42.5 53.4	08 07 42.4 55.6	08 Q7 40.3 56.3	08 07 30.2 66.5	08 8 9 9 9 07 31.4 65.7	08 0 07 30.0 67.4
	GFP	GEP 05 Q6	GFP	GFP GFP	GFP	GFP 05 06	GFP	CFP 05 06	GFP Q6
	u ¹ 1.25 1.68	u ⁴ 1.60 1.74	u ⁴ .141 1.75	u ⁴ 1.10 1.64	u ⁴ 1.36 1.84	u ⁵ 0.98 2.08	u ¹ 115 1.41	u ¹ 0.93	u ¹ 1.89 0.92
	Q8 0 0 07 26.2 70.9	08 Q7 26.5 70.2	Q8 Q7 26.8 70.1	08 07 28.9 68.3	08 00 07 028.9 67.9	08 0 07 25.7 71.3	08 07 24.3 73.2	08 07 07 44.2 54.1	Q8 8 Q7 44.4 52.8
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	¹⁰ Q5 Q6 11 ⁴ 2.75 6.44	25 Q6 4.08 7.65	^{10²} Q5 Q6 ^{10⁴} 3.87 6.01	^{10²} Q5 Q6 ^{10⁴} 4.94 7.57	US Q6 US 6.74	4.15 7.15	25 Q6 3.46 7.00	¹² Q5 Q6 12 4.41	05 Q6 6.23 4.96
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Supplementary Fig. 4: Immune reactivity of candidate humanized CAR-V β 2.

a, The sample layout used to test for the presence of pre-existing IgM antibody directed against the panel of allogeneic humanized CAR-V β 2 T cells in sera from **b**, three healthy controls or **c**, three CTCL patients, as determined by flow cytometry. **d**, MLR assay to detect killing by CD8 T cells from two donors mixed with TRAC-KO (black) or TRAC/B2M/CIITA (triple)-KO hCAR-V β 2 T cells generated from two additional donors. n=3 replicates of each group. ****p<0.0001 by two-way ANOVA. Source data are provided as a Source Data file.



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Supplementary Fig. 5: CRISPR-AAV system for CAR-Vβ2 T cell generation.

Supplementary Fig. 5: CRISPR-AAV system for CAR-V β 2 T cell generation.

a, Representative flow cytometry showing CD4 and CD8 population percentages and expression of GFP as CAR reporter on AAV-dependent allogeneic mCAR-V β 2 T cells. **b**, Expression of GFP as CAR reporter on mCAR-V β 2 T cells two days and ten days post triple KO and AAV-mCAR-V β 2 transduction, determined by flow cytometry. **c**, Patient derived V β 2+ CTCL cell counts after overnight in vitro co-culture with allogeneic triple KO mCAR-V β 2 pan-T cells generated via CRISPR/AAV system (red) compared to allogeneic triple KO CAR-CD19 pan-T cells generated via lentivirus transduction (black), at different effector to target (E:T) ratios. **d**, Representative flow cytometry showing CD3- purity, CD4 and CD8 population percentages and expression of GFP as CAR reporter on lenti-CAR-CD19, lenti-hCAR-V β 2 and AAV-hCAR-V β 2 T cells. **c**, n=3 replicates in each group. *p<0.05 by two-way ANOVA. Source data are provided as a Source Data file.



Supplementary Fig. 6: hCAR-Vβ2 T cell recovery in cytokine optimization.

Supplementary Fig. 6: hCAR-V β 2 T cell recovery in cytokine optimization.

a, CD8+ % in 7-day and 12-day cytokine expanded CD3- hCAR-V β 2 T cells, determined by flow cytometry. n=3 replicates in each group. **b**, CD45RA+CD45RO+ % of 7-day and 12-day cytokine expanded resting CD8+CD3- hCAR-V β 2 T cells, determined by flow cytometry. **c**, Live total T cell counts during cytokine expansion in vitro, determined by trypan blue cell counting. **d**, Percentage of viable total T cells following 12-day cytokine expansion, determined by trypan blue cell counting. **e**, IFN γ + % of 12-day cytokine expanded resting CD8+CD3- hCAR-V β 2 T cells, determined by Flow cytokine expanded resting CD8+CD3- hCAR-V β 2 T cells, determined by flow cytokine expanded resting CD8+CD3- hCAR-V β 2 T cells, determined by flow cytokine expanded resting CD8+C3- hCAR-V β 2 T cells, determined by flow cytometry. Source data are provided as a Source Data file.



Supplementary Fig. 7: Humanized anti-V β 2 therapeutic antibody with enhanced ADCC.

Supplementary Fig. 7: Humanized anti-V β 2 therapeutic antibody with enhanced ADCC.

a, Lens Culinaris Agglutinin (LCA) expression on the surface of parental expiCHO cells, presorted expiCHO cells post Fut8 KO or post-sorted expiCHO cells post Fut8 KO, determined by flow cytometry. **b**, SDS-PAGE of purified humanized anti-V β 2 antibody sample and denatured protein sample from protein G beads post antibody elution. **c**, Live Jurkat-TRBV20-1 cell counts, after overnight co-culture with NK cells from a healthy donor as effector cells at different E:T ratios without antibody addition (black) or mixed with 100ng/ml mouse anti-V β 2 antibody (blue) or humanized anti-V β 2 antibody (red), determined by flow cytometry. n=3 replicates in each group. **p<0.01 and ****p<0.0001 by two-way ANOVA. Source data are provided as a Source Data file.

Supplementary Fig. 8: Flow cytometry gating strategies.



Supplementary Fig. 8: Flow cytometry gating strategies.

a, Gating strategy for Fig. 1 c-f, Fig. 4 c-d and Fig. 6 c-h. b, Gating strategy for Fig. 2 b-l and Fig.

3. c, Gating strategy for Fig. 4 e-k. d, Gating strategy for Fig. 5 c-f.

Supplementary Table 1.

No.	cytokine combination
1	IL2+IL7+IL15
2	IL7+IL15
3	IL15
4	IL7+IL15+IL21
5	IL15+IL21
6	IL2+IL12
7	IL2+IL18
8	IL2+IL12+IL18
9	IL12+IL15
10	IL15+IL18
11	IL12+IL15+IL18
12	IL7+IL12+IL15
13	IL7+IL15+IL18
14	IL7+IL12+IL15+IL18
15	IL12+IL15+IL21
16	IL15+IL18+IL21
17	IL12+IL15+IL18+II21
18	IL7+IL12+IL15+IL21
19	IL7+IL15+IL18+IL21
20	IL7+IL12+IL15+IL18+IL21

Supplementary Table 2.

Gene	sgRNA sequence		
TRAC	TCAGGGTTCTGGATATCTGT		
B2M	AAGTCAACTTCAATGTCGGA		
CIITA	GTGGCACACTGTGAGCTGCC		
TRBV12-3	GCAAAGGGACACACAGCAGA		
Fut8	AGTTGAAACTCTGAAAATGC		