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Supplemental information

Scalable GMP-compliant gene correction of CD4+ T cells with IDLV template functionally validated *in vitro* and *in vivo*

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Supplemental methods

Donor templates (AAV6 and IDLV)

The AAV6 donor template was generated from a construct containing AAV2 inverted terminal repeats[1]; lab-grade AAV6 stocks were produced by InnovaVector (Pozzuoli (NA), IT), by transient triple-transfection of suspension HEK-293 cells, then purified by ultracentrifugation on a cesium chloride density gradient and characterized in terms of infectious titer (cell-based assay followed by qPCR). The AAV6 preparation utilized throughout this study contained $3.2x10^{12}$ infectious genomes/ml. The IDLV donor template was generated exploiting HIV-derived, third-generation self inactivating transfer construct[1]. Lab-grade IDLV stocks were prepared in SR-Tiget Vector Core by transient quadri-transfection of adherent HEK-293 T cells, concentrated by ultracentrifugation and titered as previously described[2]. Nine different stocks were used throughout this study (infectious titer: $7.78x10^{8}$-$5.8x10^{9}$ TU/ml; infectivity: $2.78x10^{3}$-$1.28x10^{4}$ TU/ng p24). Purified IDLV stocks were produced by the same transfection method, followed by chromatography steps, sterilizing filtration and QC testing as described[3]. The stock used for this study was characterized for: infectious titer by ddPCR ($1.83x10^{9}$ TU/ml); particle concentration with HIV-1 p24 antigen ELISA kit, PerkinElmer (infectivity = $4.94x10^{4}$ TU/ng p24) and by multiangle dynamic light scattering (MADLS) technology using Zetasizer Ultra, Malvern Panalytical (particle concentration = $7.72x10^{11}$ pp/ml and aggregates = 0.77%); endotoxin by the Endosafe PTS system, Charls River (2.94 EU/ml), for total proteins by DC protein assay, BioRad (8.67 mg/ml); host cell proteins by HEK293 HCP ELISA assay kit, Cygnus Technologies (0.15 µg/ml); total DNA by Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen (4.14 µg/ml); and for residual VSV.G plasmid measured by digital droplet PCR ($3.39x10^{8}$ copies/ml)[3].

DNA Extraction

Genomic DNA was extracted with QIAamp DNA Micro Kit (QIAGEN) according to manufacturers' instructions.

ddPCR assays

For digital droplet PCR (ddPCR) analyses, we analyzed 5–50 ng of gDNA per reaction with the QX200 Droplet Digital PCR System (Bio-Rad). TTC5 (Bio-Rad) was used for normalization. The sequences of non-commercial assays are reported in table S6. Thermal conditions for commercial ddPCR assays were 95°C x 10', (94°C x 30'', 55°C x 1', 72°C x 2') x 40 cycles, 98°C x 10'. Copy numbers and confidence intervals were calculated with QXManager version 1.2 or QuantaSoft Regulatory edition v 1.7 (Bio-Rad).

Flow-cytometry

50-500k cells or 50 µl of PB were incubated with antibodies (see antibodies list in table S7) for 10 minutes at 4°C and then washed with PBS (Corning) + 2% heat inactivated FBS (Euroclone). At least 20,000 events were acquired with BD FACSCanto II (Becton Dickinson) or Cytoflex (Beckman Coulter). PB samples were lysed with ACK solution for 10 min at RT before acquisition. We define TSCM: $CD62L^{+}CD45RA^{+}$ cells, CM: $CD62L^{+}CD45RA^{-}$ cells, EM: $CD62L^{-}CD45RA^{-}$ cells and TEMRA: $CD62L^{-}CD45RA^{+}$ cells. Assessment of T-cell receptor diversity was done with the IOTest® Beta Mark from Coulter (Beckman Coulter cat#IM3497), a multi-analysis tool designed for quantitative analysis of the TCR Vβ repertoire on whole blood samples. The kit is composed of 8 vials containing mixtures of conjugated TCR Vβ antibodies corresponding to 24 different specificities (about 70% coverage of normal human TCR Vβ repertoire). The staining protocol was adapted for use on purified $CD4^{+}$ T cells. Briefly $1x10^{5}$ cells are stained with directly FITC- and PE-coupled antibody mixes whereas anti CD3-V450 (clone UCHT1 BD Pharmingen cat#560365) is used to gate the specific population. The test is run on FACS Canto II (BD Pharmingen), equipped with DIVA Software and analyzed with FlowJo Software (FLOWJO, LLC). Control for fluorescence PMT and compensation settings are included in the kit. Data were analyzed with FCS Express 7 Research (De Novo Software) or FlowJo software (Tree Star). For in vivo experiments Flow Count Beads (FCB) were added to all samples before acquisition. This allows for absolute count and provides a precision analysis in rare population. Limit of quantification (LOQ, 3 cell/µL) represents the minimum acceptable concentration for each gate to consider reliable the percentage of the marker. Samples not satisfying this acceptance criteria are excluded from the analysis. Data were analyzed with CytExpert Reg. Edition 2.5 (Beckman Coulter). Gating strategies are reported in Figure S3A-D and S4A-B.

Functional assays

After thawing and overnight resting, CD4+ T-cells were activated using phorbol myristate acetate (PMA, 10 ng/mL Sigma) plus ionomycin (500 ng/mL, Sigma) for 5 hours and surface expression of CD40L and ability to bind soluble CD40 were followed over 2.5-24 hours. For AVV6 and IDLV comparison experiments (Fig. S1D), cells were stimulated with PMA/ionomycin for 2.5 hours and analyzed over 5.5-24 hours. T-cells were stained with a panel of antibodies including monoclonal mouse anti-human CD271, CD3, CD4, CD154 or CD40muIgG fusion protein (Vinci Biochem). CD40L fold expression or CD40 binding were calculated as follows: median fluorescence intensity (MFI) of the stimulated sample / MFI of the unstimulated sample.

The ability of CD40L expressing cells to trigger CD40 downstream signalling was evaluated by co-culturing cells with HEK-Blue™ CD40 cells (InvivoGen). CD4+ T-cells from both HDs and patients were stimulated with PMA/Ionomycin or left unstimulated. After 2.5 hours CD4+ T-cells were harvested, washed and co-cultured in 1:1 ratio with HEK-Blue™ CD40L cells for 24 hours in presence of anti hIL-1beta (InvivoGen) and anti-hTNF-alfa (InvivoGen). Recombinant human CD40L (InvivoGen) and unstimulated T-cells were used respectively as positive and negative control. After 24 hours, secreted SEAP was measured in the supernatant by QUANTI-Blue solution (InvivoGen). The absorbance was read by Omega reader (BMG Labtech) at 650 nm.

Residual Cas9 assay

To quantitate residual Cas9 protein during manufacturing process, whole cell extract from $8x10^5$ $CD4^+$ T cells was analyzed with Cas9 ELISA Assay (XpressBio cat#Cas9-1000) following technical data sheet indications.

Growth factor dependent growth assay

To study the growth factor dependency of CD4+ T cells, the effect of IL2, IL7, IL15 on proliferation was examined. Briefly, cryopreserved CD4+ T cells were thawed and plated in X-Vivo15, 5% Human Serum, 1% Pen/Strep with or without cytokines. The following cytokines were used at the indicated concentrations: 100 IU/ml IL7, 200 IU/ml IL15 and 50 IU/ml IL2. Every two/three day of incubation, the number of living cells was evaluated using trypan blue exclusion combined with automatic cell counting (Countess 3FL automated Cell, ThermoFisher).

p24 quantification

Concentration of p24 was measured with Ella automated immunoassay system (ProteinSimple) using the Simple Plex Human HIV-1 Gag p24 Cartridge (ProteinSimple) that is pre-loaded with a factory calibrated standard curve and allows triplicate analysis of each sample. 1 ml of supernatant was harvested at the indicated time points and stored at -80°C. A 0.5 ml QC aliquot of DP was dedicated to p24 analysis. Samples were thawed at room temperature for 30 minutes. After thawing the DP was centrifuged 5 min at 350rcf to remove cells. Thawed samples were diluted at least 2-fold with sample diluent SD30 and centrifuged to remove residual debris. According to the manufacturer instructions, the cartridge was loaded with 50 µL of diluted samples and 1ml of wash buffer and run on Ella instrument. Results were analyzed with Simple Plex Explorer software (Protein Simple).

Histopathology

Liver, spleen, lung, gut, brain, skin ears and back, were trimmed and embedded in wax blocks, sectioned and slides stained with haematoxylin and eosin. On selected sections immunohistochemical analysis was performed with Anti human CD3, marker T-cells (LN10 Leika). Liver, skin and lung were analysed for microscopic lesions (GvH reaction). The extent and severity was graded as GvH minimal (score 1), GvH mild (score 2), GvH moderate (score 3) and GvH marked (score 4) as described in detail in the tables of incidence for each organ analyzed. In the spleen, immune reconstitution of the white pulp characterized by infiltrates of medium-large sized mononuclear cell infiltrate was graded on a scale of 1 to 4 as minimal (1), mild (2), moderate (3), marked (4) as described in detail in the table of incidence.

The slides were independently peer reviewed by an experienced pathologist and a consensus reached on the findings.

Statistical analysis

Non-parametric statistical analyses were performed only on experimental data with at least 5 replicates, using Prism 9 (GraphPad Software), as reported in figure legends.

Supplemental data

Supplemental Figures

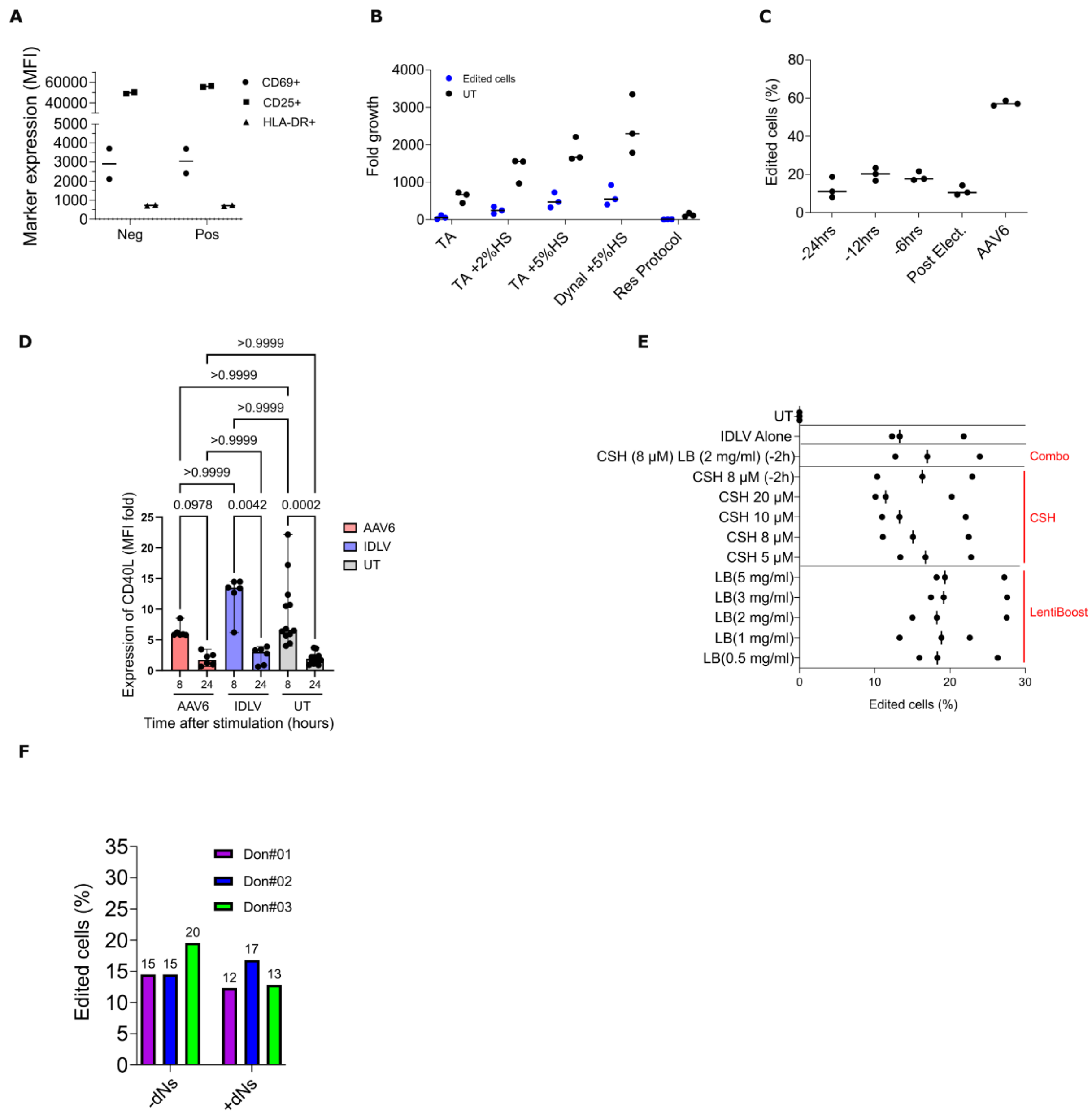
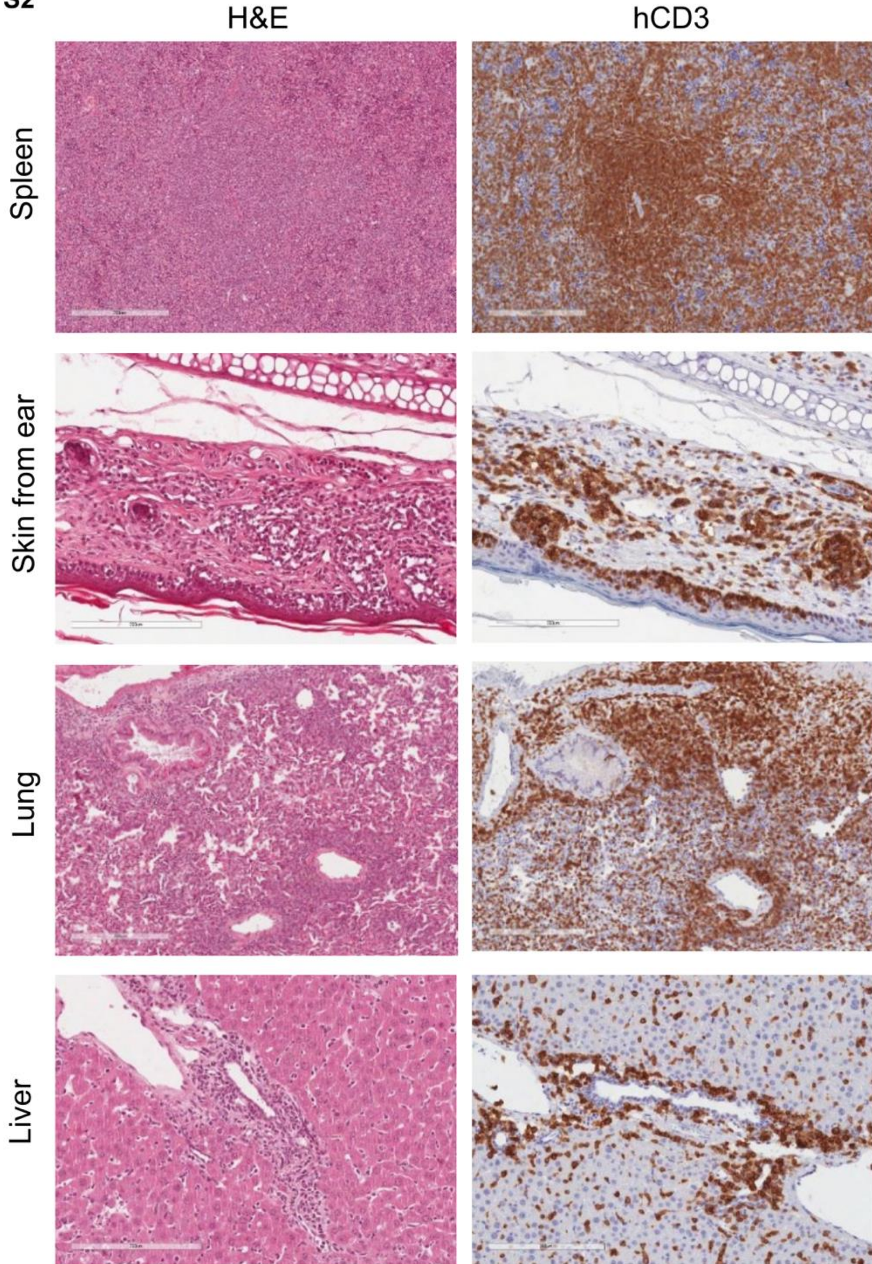


Figure S1: Optimization of a small and medium scale manufacturing process for gene editing of CD4+ T-cells A) Expression of activation markers (Mean Fluorescence Intensity, MFI) by flow cytometry at day 13 in cells edited after negative and positive selection for CD4+ cells. **B)** Cell culture and activation with TransAct (TA) or CD3/CD28 magnetic beads (Dynal) in presence or absence of Human Serum (HS). **C)** Percentage of edited cells with an IDLV donor template targeting AAVS1 locus to assess transduction timings in plastic plates. AAV6 added post electroporation. **D)** Relative

MFI of CD40L (median ± IQR) on UT (n = 12) or LNGFR+ HD derived CD4+ T cells, edited using AAV6 (n=6) or IDLV (n=6). To determine statistical differences between groups, data comparison was performed by Kruskal-Wallis test. **E)** Titration of transduction enhancers LentiBoost (LB) and CsH for IDLV transduction. **F)** Percentage of edited cells in presence of deoxynucleotides (dNTPs) during IDLV transduction.

Figure S2

A



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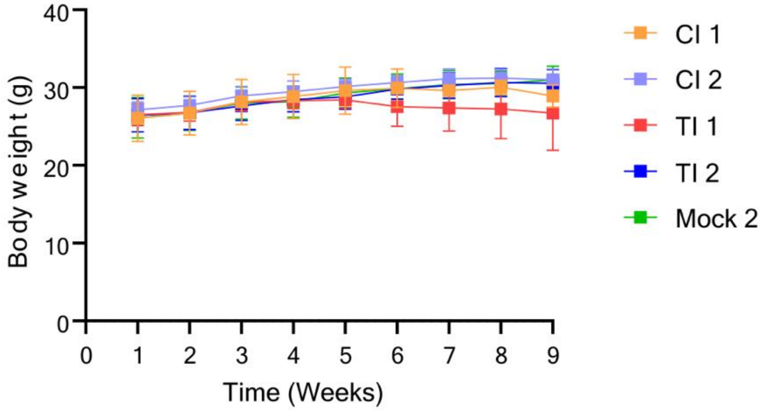
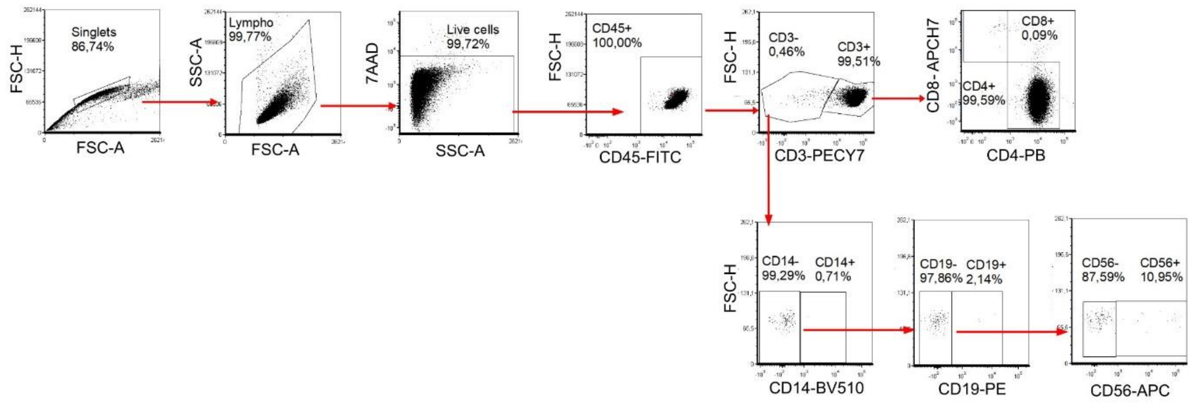


Figure S2: Engraftment of gene edited cells upon xenotransplantation A) GvHD histopathology. Representative images of H&E (left panels) and human CD3 (right panels) staining on spleen, skin from ear, lung and liver. Bars: 300 µm (spleen), 200 µm (skin from ear, lung and liver). **B)** Body weight of transplanted animals was recorded weekly until the end of study (Day 60).

Figure S3

A



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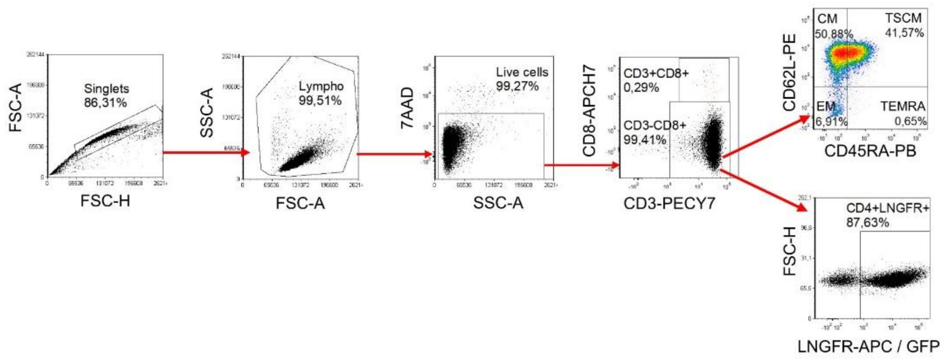

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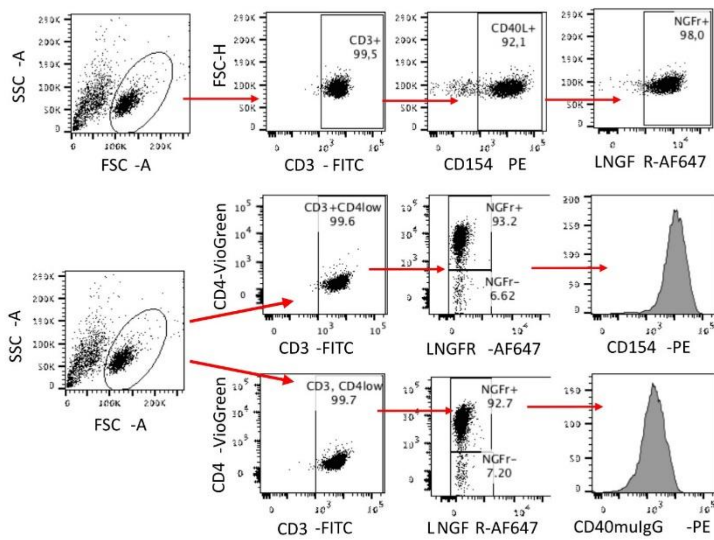

D

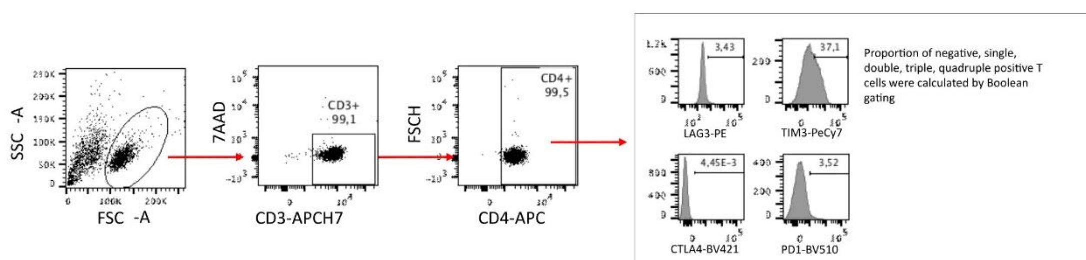

Figure S3: Flow cytometry gating strategies representative plots A) Purity of selected cells after CD4+ selection and of the cell product **B)** Immunophenotype of CD4+ cells and LNGFR+/GFP+ fraction **C)** Potency of edited cells by expression of LNGFR, CD40LG, and binding to CD40muIgG. **D)** Exhaustion panel

Figure S4

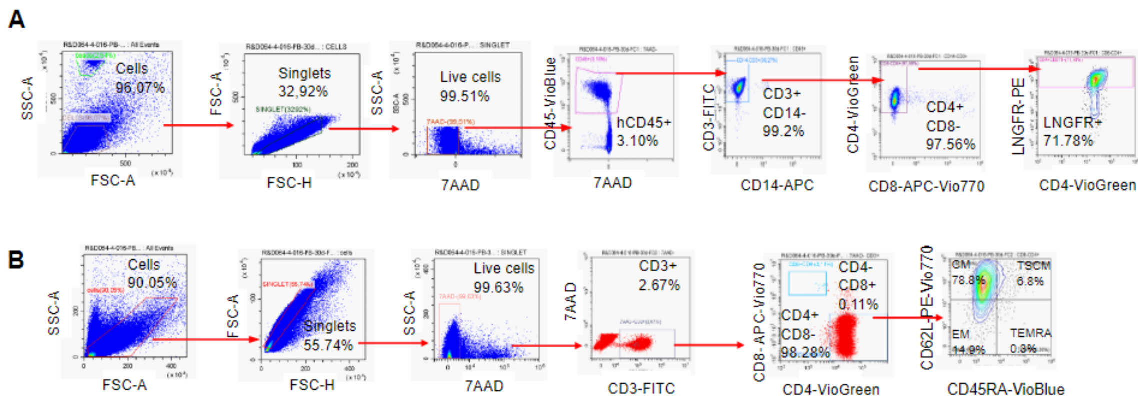

Figure S4: Flow cytometry gating strategies representative plots A) Fraction of corrected cells in PB of transplanted mice **B)** Immunophenotype of engrafted CD4+ cells. Same gating strategy was applied at end point analysis on BM and Spleen

Supplemental Tables

Table S1: Cell composition (WBC, CD3+, CD4+, CD8+ T cells) in patients' blood samples

**Sample #01 and #05 is the same patient at different timepoints*

Sample ID	Pt Age	WBC	CD3+	CD4+	CD8+
		Cells/µl	*%*	*%*	*%*
#01*	17 yo	5040	54,22	58,15	21,49
#02	15 yo	4780	54,89	40,94	48,63
#03	26 yo	2240	47,2	38,86	45,31
#04	46 yo	2500	16,22	76,43	19,61
#05*	18 yo	1600	n.a.	n.a.	n.a.

Table S2: Incidence table of spleen immune reconstitution in NSG mice xenotransplanted with human edited CD4+ T-cells *****cells did not engraft in one mouse. TI control item, CI test item.*

	CI 1	**TI 1**	**CI 2**	**TI 2**	**Mock 2**
	Donor 1 untreated $CD4^+$ T cells n=5	Donor 1 edited $CD4^+$ T cells n=5*	Donor 2 untreated $CD4^+$ T cells n=5	Donor 2 edited $CD4^+$ T cells n=5	Donor 2 electroporated not edited $CD4^+$ T cells n=5
Mononuclear cell (human) infiltration	5	4	5	5	5
Minimal	1	0	0	0	0
Mild	2	1	1	3	3
Moderate	2	1	4	2	2
Marked	0	2	0	0	0

Description and grading explanation

In the spleen, immune reconstitution of the white pulp characterized by infiltrates of medium-large sized mononuclear cell infiltrate (human CD3 positive T cell) was seen with different extent and graded on a scale of 1 to 4 as minimal (1), mild (2), moderate (3), marked (4); minimal referred of to the least extent discernible and marked the greatest extent possible.

Table S3: Incidence table of GvH damage in skin from back and ears of NSG mice xenotransplanted with human edited CD4+ T-cells *****cells did not engraft in one mouse. TI control item, CI test item.*

	CI 1	**TI 1**	**CI 2**	**TI 2**	**Mock 2**
	Donor 1 untreated $CD4^+$ T cells n=5	Donor 1 edited $CD4^+$ T cells n=5	Donor 2 untreated $CD4^+$ T cells n=5	Donor 2 edited $CD4^+$ T cells n=5	Donor 2 electroporated not edited $CD4^+$ T cells n=5
Skin from back					
GvH reaction	5	4	5	5	4
Minimal	1	1	3	3	1

Mild	2	2	1	1	1
Moderate	1	0	1	0	2
Marked	1	1	0	1	0
Skin from ears					
GvH reaction	5	4	5	5	5
Minimal	2	0	0	0	0
Mild	1	2	2	1	2
Moderate	0	2	2	2	2
Marked	2	0	1	2	1

Description and grading explanation

In the skin (back skin and ears) microscopic lesions (GvHD), characterized by medium-large size mononuclear cell infiltrate (human CD3+ T cell) admixed with small size mononuclear cells (murine F4/80 positive) and granulocytic/myeloid cells were seen with different extent and severity and graded as detailed below:

- GvH minimal (score 1): minimal lymphomonocytic infiltrate in the derma and adnexa, spongiosis and vacuolization of basal cells.
- GvH mild (score 2): mild lymphomonocytic infiltrate in the derma and adnexa, dermoepidermal junction and occasionally infiltrating the epidermis, spongiosis and vacuolization of basal cells.
- GvH moderate (score 3): moderate lymphomonocytic infiltrate in the derma and adnexa, dermoepidermal junction, infiltrating the epidermis, spongiosis, vacuolization of basal cells and necrotic keratinocytes.
- GvH marked (score 4): marked lymphomonocytic infiltrate in the derma and adnexa, dermoepidermal junction, infiltrating the epidermis, marked spongiosis, vacuolization of basal cells, necrotic keratinocytes. Necrosis of the epidermis with ulcer.

Table S4: Incidence table of GvH damage in lung of NSG mice xenotransplanted with human edited CD4+ T-cells ******cells did not engraft in one mouse. TI control item, CI test item.*

	CI 1	**TI 1**	**CI 2**	**TI 2**	**Mock 2**
	Donor 1 untreated $CD4^+$ T cells n=5	Donor 1 edited $CD4^+$ T cells n=5	Donor 2 untreated $CD4^+$ T cells n=5	Donor 2 edited $CD4^+$ T cells n=5	Donor 2 electroporated not edited $CD4^+$ T cells n=5
GvH reaction	5	4	5	5	5
Mild	1	0	0	1	1
Moderate	1	3	2	3	3
Marked	3	1	3	1	1

Description and grading explanation

In the lung, microscopic lesions (GvHD) characterized by infiltrates of medium-large sized mononuclear cell infiltrate (human CD3 positive T cell) admixed with small size mononuclear cells (murine F4/80 positive) and granulocytic/myeloid cells were seen with different extent and severity, and graded as detailed below:

- GvH mild (score 2): mild lymphomonocytic infiltrate peribronchial/perivascular with mild degeneration of epithelia of trachea and bronchioles
- GvH moderate (score 3): moderate lymphomonocytic infiltrate peribronchial/perivascular, minimal to mild infiltration into parenchyma and mild degeneration of epithelia of trachea and bronchioles
- GvH marked (score 4): marked lymphomonocytic infiltrate peribronchial/perivascular, mild up to severe infiltration into parenchyma and mild degeneration of epithelia of trachea and bronchioles

Table S5: Incidence table of GvH damage in liver of NSG mice xenotransplanted with human edited CD4+ T-cells ******cells did not engraft in one mouse. TI control item, CI test item.*

	CI 1	**TI 1**	**CI 2**	**TI 2**	**Mock 2**
	Donor 1 untreated $CD4^+$ T cells n=5	Donor 1 edited $CD4^+$ T cells n=5	Donor 2 untreated $CD4^+$ T cells n=5	Donor 2 edited $CD4^+$ T cells n=5	Donor 2 electroporated not edited $CD4^+$ T cells n=5
GvH reaction	5	4	5	5	5
Minimal	0	1	0	1	0
Mild	0	0	1	3	3
Moderate	5	1	3	1	2
Marked	0	2	1	0	0

Description and grading explanation

In the liver, microscopic lesions (GvHD) characterized by infiltrates of medium-large sized mononuclear cell infiltrate (human CD3 positive T cell) admixed with small size mononuclear cells (murine F4/80 positive) and granulocytic/myeloid cells were seen with different extent and severity and graded as detailed below:

- GvH minimal: minimal inflammatory lymphomonocytic infiltrate in few portal triads.
- GvH mild: mild inflammatory lymphomonocytic infiltrate in some of the portal triads.
- GvH moderate: moderate/marked inflammatory lymphomonocytic infiltrate of most or all of the portal triads, with spillover into the periportal hepatocytes. Minimal/mild perivenular inflammation. Occasional hepatocyte degeneration.
- GvH marked: moderate/marked inflammatory lymphomonocytic infiltrate of all of the portal triads, with spillover into the periportal hepatocytes. Moderate-to-severe perivenular inflammation that extends into the hepatic parenchyma forming bridging and associated with hepatocyte necrosis.

Table S6: ddPCR sequences

CD40LG	Probe 1	5'[FAM] TCA GTC TCC CTC TGA GAT GT[BHQ1] 3'
	Probe 2	5'[FAM] AGG CAA GAA GAG CGT CAA TTT GA [BHQ1] 3'
	Probe 3	5'[FAM] TCC ACT GAG GAG TAT AAT TGG CTG G [BHQ1] 3'
	Primer For	5'- ttaggagggggtctgataca-3'
	Primer Rev	5'- tcctcgatctgtgggaggaagagaa -3'

Table S7: List of antibodies

Target	Fluorochrome	Vendor	Clone	Cat. No.	Dilution
CD3	PECY7	Biolegend	HIT3a	300316	1:100
CD3	FITC	BD Pharmingen	SK7	345763	1:33
CD3	FITC	Miltenyi biotec	REA613	130-113-138	1:200
CD4	PB	BD Pharmingen	RPA-T4	558116	1:100
CD4	Viogreen	Miltenyi biotec	REA 623	130-113-230	1:100
CD8	APC-H7	BD Biosciences	SK1	641400	1:33
CD8	APC-Vio770	Miltenyi biotec	REA734	130-110-681	1:200
CD14	BV510	Biolegend	M5E2	301842	1:200
CD14	APC	Miltenyi biotec	REA599	130-110-520	1:400
CD19	PE	BD Biosciences	4G7	345777	1:50
CD45	FITC	BD Biosciences	2D1	345808	1:50
CD45	VioBlue	Miltenyi biotec	REA747	130-110-637	1:50
CD45RA	PB	Miltenyi biotec	T6D11	130-113-360	1:50
CD45RA	VioBlue	Miltenyi biotec	REA1047	130-117-743	1:50
CD56	APC	Miltenyi biotec	AF12-7H3	130-090-843	1:50
CD62L	PE	BD Biosciences	DREG-56	555544	1:50
CD62L	PE-Vio770	Miltenyi biotec	145/15	130-113-621	1:100
CD152 / CTLA4	PE	Miltenyi biotec	REA1003	130-116-810	1:50
LNGFR / CD271	APC	Miltenyi biotec	ME20.4-1.H4	130-113-418	1:50

LNGFR / CD271	AF647	BD Pharmingen	C40-1457	560326	1:25
LNGFR / CD271	PE	Miltenyi biotec	REA844	130-112-601	1:100
CD154	PE	Invitrogen	24-31	12-548-42	1:20
CD154	PE	Biolegend	24-31	310806	1:50
CD223 / LAG3	VioBlue	Miltenyi biotec	REA351	130-118-549	1:50
CD279 / PD1	PE-Vio770	Miltenyi biotec	REA1165	130-120-385	1:50
CD366 / TIM3	APC	Miltenyi biotec	REA635	130-119-781	1:50
7-AAD Viability Staining Solution	7-AAD	Biolegend	-	420404	1:100
CD40muIgG fusion protein	PE	Vinci Biochem	-	ANC-504-050	1:50

References

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