SUPPLEMENTARY MATERIALS AND METHODS

Construction of CAR Lentiviral vectors

DNA sequences encoding for the anti-CD38 CAR (1G and 2G CD38 CAR) and the anti-CS1 CCR (CS1 CCR) were chemically synthesized (GeneArt ThermoFisher) and contain mouse derived scFv's. Linkage of 1G CD38 CAR to the P2A motif and the CS1 CCR was performed using In-Fusion® snap assembly (Takara Bio) according to the manufacturer's protocol. The pSFFV-Kana lentiviral vector was generated by replacing the ampicillin resistance gene of the pRRL–PGK–WPRE (a gift from Dr Hana Raslova, Villejuif, France) with the chemically synthesized Kanamycin resistance gene and by replacing the PGK promoter by the SFFV promoter. Insertion of the CAR and CCR coding sequences into the pSFFV-Kana vector was performed using In-Fusion® snap assembly.

Cell lines

The MM.1S, KMS11 and NCI-H929 MM cell lines have been previously described (Fayon et al., 2021). To generate Luciferase expressing cells, MM.1S, KMS11 or NCI-H929 were transduced with lentiviral particles produced from the pLenti CMV Puro LUC (Addgene #17477). MM.1S inactivated for the *CD38* and/or *SLAMF7* (CS1) genes have been previously described (Fayon et al., 2021). All MM cell lines were cultured in complete RPMI medium: RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (all from ThermoFisher) at 37°C + 5% CO_2.

NIH-3T3 cells were transduced with retroviral particles to obtain NIH-3T3 feeder cells expressing CD38, CS1 or both targets. NIH-3T3 cells were cultured in DMEM medium: DMEM (Gibco) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine at 37°C + 5% CO_2. HEK-293T cells were cultured under the same conditions as NIH-3T3 cells.

Lentiviral production

HEK293T cells were co-transfected with lentiviral CAR vector and packaging plasmids (pMD2G and psPAX2 all from Addgene) using calcium phosphate precipitation method (ThermoFisher) following the manufacturer's protocol. Lentiviral supernatants were collected at 72-hours post-transfection and concentrated using high-speed ultracentrifugation. To generate the lentiviral stocks, the resulting concentrated lentivirus batches were suspended in PBS and stored at -80°C.

CAR-T production and Genome editing

Primary peripheral blood mononuclear cells (PBMCs) were obtained from the "Etablissement Français du Sang". In all cases, informed consent of volunteers was obtained in accordance with the Declaration of Helsinki and with approval of the Saint-Louis Hospital Internal Review Board. PBMCs were isolated from cytapheretic residues by centrifugation on a Pancoll (Human; PAN™ Biotech) gradient (2/1 ratio of diluted blood to Pancoll), using Leucosep tubes (ThermoFisher) according to the manufacturer's protocol, and frozen at -80°C in FBS containing 10% DMSO.

PBMCs were activated with CD3/CD28 activation beads (at 0.4/1 bead cell ratio, ThermoFisher) in CTS™ OpTmizer™ T Cell Expansion SFM (Gibco) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, IL-2 (10 ng/ml prior to electroporation, 5 ng/ml after, BioLegend) and hIL-7 (1 ng/ml, BioLegend) (henceforth referred to as OpTmizerC), at 37°C + 5% CO_2 for 24 hours, followed by genome editing by CRISPR/Cas9. In brief, ribonucleoprotein (RNP) complexes were generated by incubating single guide RNA (sgRNA) targeting *CD38* (100 µM, Sigma-Aldrich) with the Cas9nuclease (ThermoFisher) (1:2 molecular ratio, respectively), for 20 minutes at room-temperature. The RNP complex was then electroporated into $5x10^6$ activated T cells in 100 µL of 2M electroporation buffer (Chicaybam et al., 2013) in a 2B-Nucleofector Unit (Lonza). Transfected T cells were maintained in culture, using CD3/CD28 dynabeads (at 0.8/1 bead cell ratio) in OpTmizerC at 37°C + 5% CO_2, for another 24 hours prior to transduction with lentiviral particles. Lentiviral transduction was performed in culture plates coated with

RetroNectin (15 µg/ml, Takara Bio), T cells were combined with lentiviral particles (Multiplicity of Infection ≥10) at 37°C + 5% CO_2. After 24 hours the medium was changed, and cells were kept in culture under standard conditions.

Flow cytometry staining

For the detection of CAR expression at the surface of T cells, cells were stained in PBS 1x + 0.5% Bovine Serum Albumin (BSA) with an anti-murine scFv-APC (JacksonImmunoResearch, #115-136-072, 1/200) or a biotinylated-CD38 recombinant protein (Acro biosystems, #CD8-H82E7, 1/50) and a fluorescein isothiocyanate (FITC) conjugated-CS1 recombinant protein (Acro biosystems, #SL7-HF2H7, 1/50) recognizing the respective scFv's. Following 1 hour incubation at 4°C, cells were washed and followed with second incubation with an APC-(BioLegend, #405207, 1/100) or BV421-(BioLegend, #405225, 1/50) streptavidin conjugated antibody for 30 min at 4°C. Knock down efficiency was determined by staining cells with APC-Cy7-CD4 (BioLegend, #300518, 1/200), BUV395-CD8 (BD, #563795, 1/200) and BV510-CD38 (BD, #563251, 1/200) in FACS buffer (PBS 1x, 2.5% FBS and 0.1% sodium azide) for 20 min at 4°C. Cells were fixed in a 5% formalin solution (Sigma-Aldrich) in PBS prior to analysis. To determine reconstitution in the humanized mice, cells were treated with Fc block (BD, #564220) for 10 min at RT, followed by staining with Zombie UV™ (BioLegend, # 423107, 1/1000) according to the manufacturers protocol. Cells were next stained for 20 min at 4°C in FACS buffer with PerCP-CD45 (BioLegend, #304026, 1/100), BV421-CD3 (BioLegend, #317344, 1/200), SparkNir685-CD19 (BioLegend, #302270, 1/80), BUV737-CD56 (BD, #612766, 1/80), SparkBlue550-CD14 (BioLegend, #367148, 1/80), BUV805-CD15 (BD, # 742057, 1/200), Alexa Flour 647-Cd1c (BioLegend, #331510, 1/200). Samples were collected using either the Fortessa (BD), Attune NxT (Invitrogen) or Cytek® Aurora (Cytek) flow cytometers and analysed using FlowJo™ V10 (FlowJo BD).

Cytotoxicity assay

CAR-T cytotoxicity was determined by assessing tumour cell viability following a co-culture with CAR-T. Sixteen hours prior to the cytotoxicity assay, anti-CD3/CD28 beads were magnetically removed from the cultures and CAR-T were mixed with luciferase expressing target cells at different effector to target (E/T) ratios. After a 4-hour or 24-hour co-culture, the luciferase activity was determined using Bright-Glo™ Luciferase Assay System (Promega) and bioluminescence was measured with a CLARIOstar plate reader (BMG Labtech). Cell viability was determined by subtracting baseline killing by non-transduced (NT) T-cells using the following formula: % of viable cells = RLU[E/T CAR]/RLU[E/T NT]*100% (RLU: relative light units)

Long term proliferation test

NIH-3T3 feeder cells were treated with Mitomycin C (4µg/ml, Sigma-Aldrich) for 1 hour at 37°C, then washed with culture medium and plated in 24 well plates. CAR-T were co-cultured in feeder cells at an initial ratio of 1/4 ($2.5x10^4$:$1x10^5$) CAR-T to feeder ratio. Every 2-3 days the CAR-T were transferred to fresh feeder cells. Cell proliferation was determined by counting the viable cells, with dead cells excluded by trypan blue (Invitrogen) staining, using the Countess II (Invitrogen). Cell numbers were quantified using ImageJ (ImageJ) software.

Cytokine production

Cytokine secretion was determined by collecting the supernatant of the long-term proliferation assay. The supernatant was collected at day 4 and stored at -20°C. Cytokine secretion was quantified using a bead-based immunoassay (LEGENDplex™, BioLegend) according to the manufacturer's protocol. The data was acquired using the Attune NxT and analysed using the LEGENDplex software provided by the manufacturer.

Intravenous MM.1Sluc xenograft model

All the following *in vivo* experiments were performed with the approval of the appropriate ethical committee (agreement # APAFIS#19755-2019031316181939 v2) according to the conditions established by the European Union. Six to 12-week-old female NOD/SCID/$\gamma c^{-/-}$ mice were inoculated with $3x10^6$ MM.1Sluc cells by tail vein injection (i.v) at day 0, followed, 14 days later, by i.v infusion of $1x10^6$ CAR-T. Bioluminescence was measured with the IVIS Imaging System (PerkinElmer) every 7 days after tumour injection. The study contained four to eight randomized mice in each group which were injected with CAR-T generated from three to five different healthy donors. Data was further analysed using Aura imaging software (Spectral Instruments Imaging)

Subcutaneous MM.1Sluc xenograft model

MM.1Sluc cells ($0.5x10^6$ cells per mouse) were injected subcutaneously in the back of NSG mice. $MM.1S^{wt}$ cells were injected in the left flank and $MM.1S^{CS1ko}$ cells were injected in the right flank of the mice. CAR-T ($1x10^6$ cells per mouse) were administered i.v in the tail vein 4 days post tumour injection. Tumour progression was followed by luminescent imaging every 7 days using the IVIS Imaging System. The study contained four mice in each group which were injected with CAR-T generated from two different healthy donors. Data was further analysed using Aura imaging software (Spectral Instruments Imaging).

Humanized mice model

Haematopoietic progenitors were obtained from human cord blood samples by centrifugation on a Pancoll gradient (2/1 ratio of diluted blood to Pancoll) using Leucosep tubes according to the manufacturers protocol. CD34+ cells were isolated by magnetic cell sorting using a CD34 MicroBead Kit (Miltenyi Biotec) according to the manufacturers protocol and frozen at -80°C, in FBS medium containing 10% DMSO, or cultured immediately. Fresh or frozen CD34+ cells were cultured in IMDM medium: IMDM (Gibco) completed with 20%BIT (Stemcell^{TM} technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 50 ng/ml SCF (Miltenyi Biotec), 50 ng/ml FLT3 (Miltenyi Biotec) 20 ng/ml TPO

(Miltenyi Biotec), 10 ng/ml IL-3 (Miltenyi Biotec) for 16 hours at 37°C + 5% CO_2. The CD34+ cells were pooled from 4 different donors and $0.35x10^6$ cells per mouse were administered i.v in the tail vein in sub lethally irradiated NSG mice. Five and a half-weeks post injection of $CD34^+$ cells, mice were injected with $1x10^6$ CAR-T per mouse (i.v in the tail vein). The mock condition corresponds to T cells transduced with a pSFFV lentiviral vector driving expression of a CAR lacking the scFv domain and the GFP. Blood was collected from submandibular vein of the mice to monitor the reconstitution prior to CAR-T injection. Blood, serum and bone marrow were collected post CAR-T treatment for further analysis by flow cytometry. Prior to analysis, blood and organ samples were passed on a Pancoll gradient (2/1 ratio of diluted blood to Pancoll) to obtain the cells. The study contained 11 mice that were randomized into 3 groups which were injected CAR-T produced from 2 different healthy donors.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: Surface expression of CAR constructs

FACS analysis of CAR surface expression on T cells transduced with CD38 CAR constructs determined by staining with an anti-murine scFv. The histogram (left) shows the scFv expression levels on non-transduced T cells (NT), or T cells transduced with first generation (1G) CD38 CAR, second generation (2G) CD38-28, 2G-BB or DCAR. Histograms show the percentages of scFv positive cells (left) and the Mean Intensity of fluorescence (MIF, right). The histograms show the mean ±SEM from 3 healthy donors. Significance was calculated using a one-way ANOVA with Tukey's multiple comparison test. *$p \leq 0.05$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

Supplementary Figure 2: Surface expression of CD38 in edited CAR-T

Expression of CD38 in CRISPR/Cas9 edited T cells or T cells transduced with first generation (1G) CD38 CAR $1G^{ko}$, second generation (2G) CD38-28^{ko}, 2G-BB^{ko} or $DCAR^{ko}$, compared to non-transduced cells edited (NT^{ko}) or not (NT^{wt}). The histogram shows a representative healthy donor. The bar graphs show the mean ±SEM of three separate healthy donors. Significance was calculated using a one-way ANOVA with Tukey's multiple comparison test. ****$p \leq 0.0001$

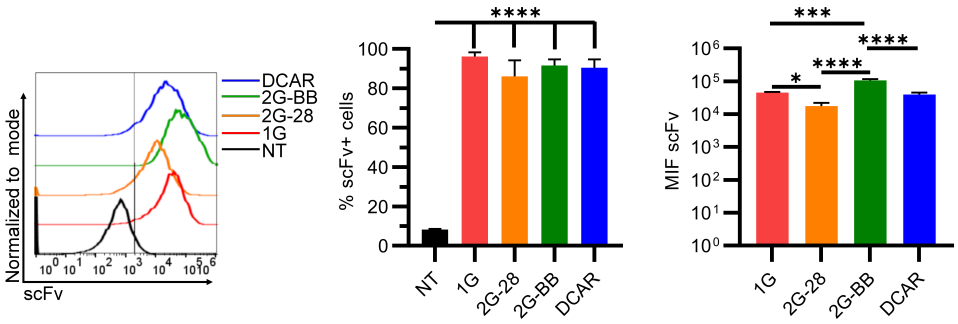
Supplementary Figure 3: Cytotoxicity of DCAR-T against different multiple myeloma cell lines.

CAR-T cytotoxicity was determined by co-culturing MM.1Sluc^{wt}, KMS-11luc^{wt} and NCI-H929luc^{wt} cells for 24 hours in the presence DCAR-T at different effector/target (E/T) ratios. Cell viability was assessed by luciferase activity and normalized to non-transduced T cells. Each E/T ratio shows the mean ±SEM from three independent experiments.

Supplementary Figure 4: Surface expression of CD38 and CS1 in humanized mice

Expression of CD38 (a) and CS1 (b) in humanized mice. The histograms show a representative mouse. The left bar graphs show the percentage of positive cells for the total haematological population (CD45+, red) and B cells (CD19+, blue), while the right bar graphs show the mean intensity of fluorescence (MIF) for the total haematological population (CD45+, red) and B cells (CD19+, blue). The bar graphs show the mean ±SEM of 11 mice. Significance was calculated using a Mann-Whitney test. ***$p \leq 0.001$ ****$p \leq 0.0001$.

Supplementary figure 1



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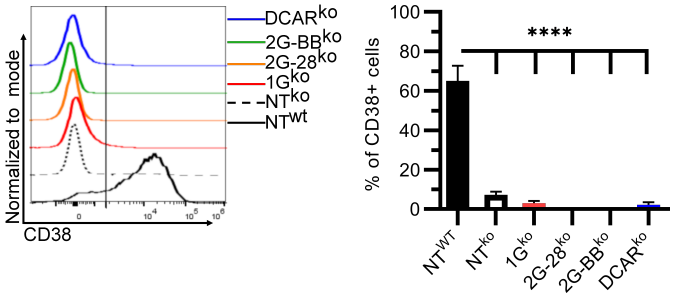

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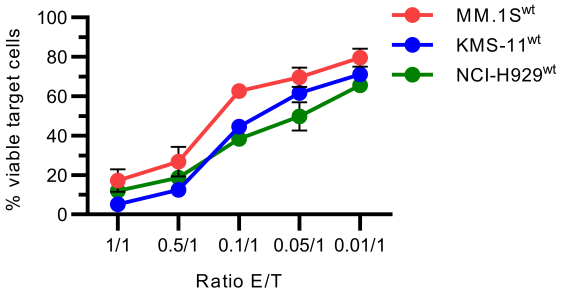

Supplementary figure 4