Pre-clinical evaluation of CD38 chimeric antigen receptor engineered T cellsfor the treatment of multiple myeloma

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Supplementary figures and methods

Figure S1

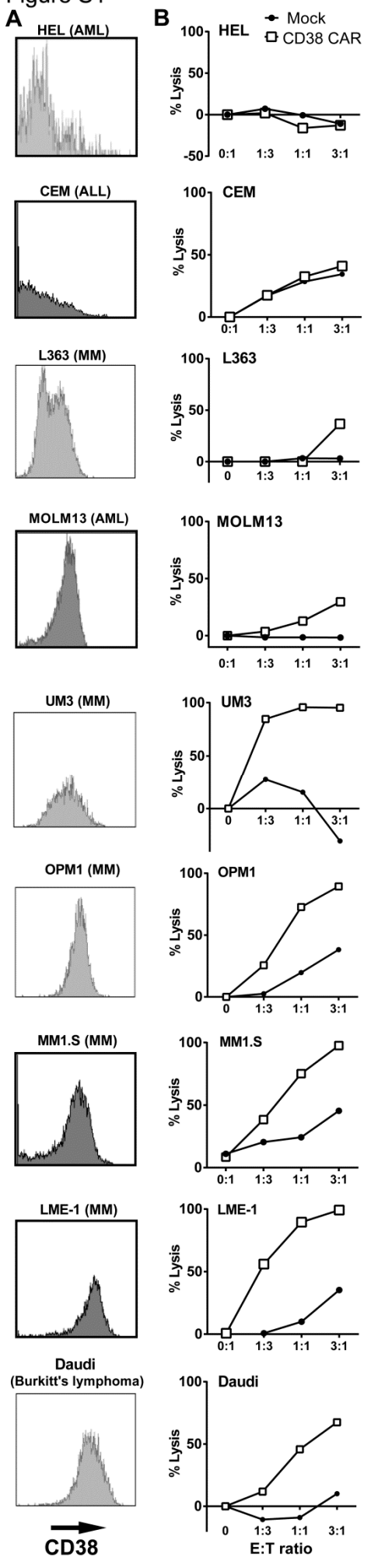

Figure S1 Efficacy of CD38-CAR T cells to lyse different cell line. In 24 h, cytotoxicity assays, CD38-CAR T cells (CAR056) were tested against two AML, one ALL, one Burkit's Lymphoma and 5 MM cell lines. (A) CD38 expression histogram of cell lines, determined by flow cytometry (B)Either BLI or flow cytometry measured cytotoxicity of CD38 CAR T cells in different effector:target ratios as indicated.

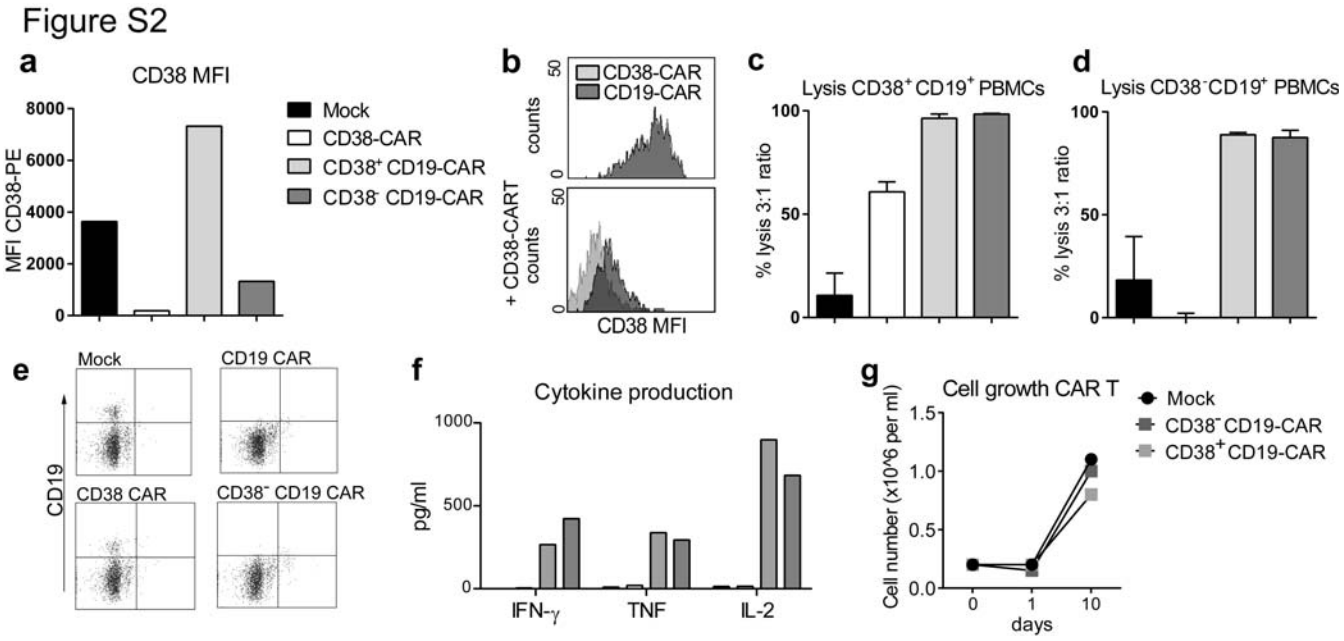

Figure S2. Function analysis of CD38$^+$ or CD38$^-$ CD19-CAR T cells. We treated the CD19-CAR T cells (with NGFR expression) with irradiated CD38-CAR T cells in a 1:1 ratio and analyzed the CD38 expression on CD38-CART cells and treated (CD38$^-$) and untreated (CD38$^+$) CD19 CAR T cells after 24 hours as measured with flow cytometry calculated MFI (Fig S2 A) and depicted as a histogram (Fig. S2 B; upper panel: CD19-CART alone, lower panel: CD19-CART treated with CD38-CART). The CD38 expression dropped significantly and we analyzed their capacity to eliminate CD19$^+$ PBMCs. When incubated overnight with PBMCs, we saw a nearly 100% lysis of CD19$^+$ PBMCs in cultures containing CD19CART cells (Fig S2 C). The CD38-CART cells lysed ~50% of the CD19$^+$ PBMCs, this is because roughly half of the CD19$^+$ PBMCs were also CD38$^+$. When solely looking at the CD19$^+$ CD38$^-$

PBMCs, only the CD19-CAR T cells eliminated these $CD19^+$ cells (Fig S2 D and target PBMCs plots in E). When analyzing the cytokine release upon exposure to $CD19^+$ cell line, the CD19-CAR T cells mainly produced IFN-γ, IL-2 and TNF. This cytokine production was not significantly different between $CD38^+$ or $CD38^-$ CD19-CAR T cells (Fig S2 F). When co-incubated with previously described feeder cells (containing irradiated allogeneic PBMCs and EBV LCL cells) $CD38^+$ or $CD38^-$ CD19-CAR T cells both expanded, similar to mock (Fig S2 G).

Table S1. Apparent affinity of selected CD38 antibodies

CD38 antibody	Affinity to CHO-CD38 cells (µg/mL)	Affinity to Daudi-luc cells (µg/mL)
3003-026	0.63	0.25
3003-028	0.32	0.11
3003-056	0.51	0.13
daratumumab	0.17	0.16

Table S1. Apparent affinity of selected CD38 antibodies. These CD38 antibodies were generated at Genmab BV. Indicated values are the apparent affinities (µg/ml) determined by flow cytometry binding to target cell lines CHO-CD38 and Daudi.

Name	AA sequence
028	VH 028 QVQLVQSGAEVKKPGSSVKVSCK**AF**GGTF**S**SYAISWVRQAPG QGLEWMGRII**R**FLG**IA**NYAQ**K**FQGRVTL**I**ADKST**N**TAYMELSS LRSEDTAVYYC**A**GEPGERDPDAVDIWGQGTMVTVSS
	VL 028 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYN**S**YPLTFGGGTKVEIK
056	VH 056 QVQLVQSGAEVKKPGSSVKVSCK**PS**GGTF**R**SYAISWVRQAPG QGLEWMGRII**V**FLG**KV**NYAQ**R**FQGRVTL**T**ADKST**T**TAYMELS SLRSEDTAVYYC**T**GEPGARDPDA**F**DIWGQGTMVTVSS
	VL 056 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYN**N**YPLTFGGGTKVEIK
026	VH 026 QVQLVQSGAEVKKPGSSVKVSCKAFGGTFSSYAISWVRQAPG QGLEWMGRIIRFLG**KT**N**H**AQKFQGRVTL**T**ADKSTNTAYMELS SLRSEDTAVYYCAGEPG**D**RDPDAVDIWGQGTMVTVSS
	VL 026 DIQMTQSPSSLSASVGDRVTITCRASQGIRSWLAWYQQKPEK APKSLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC QQYN**S**YPLTFGGGTKVEIK

Table S2. Sequences of the selected CD38 antibodies. The sequences of the variable heavy chain (VH) and variable light chain (VL) of the CD38 antibodies. Sequences listed were used to generated CARs with corresponding names.

Supplementary Methods

Cell lines

Unmodified or luciferase (Luc-GFP)-transduced human MM cell lines, U266, UM9, LME-1, UM9, MM1.S, U266, L363 and UM3, human leukemic cell lines HEL, MOLM13, CEM and Daudi were cultured in RPMI-1640 (Invitrogen) + 10% FBS (Integro BV) + antibiotics (penicillin;10.000 U/ml, streptomycin; 10,000 µg/ml) as described[1]. HUVECs were cultured in EGM2 medium (Lonza). All cell lines were authenticated by surface expression of appropriate receptors maximal six months before the most recent experiment.

Retroviral constructs

CD38-CAR Constructs The sequences of the variable light and heavy chain of three different human CD38 antibodies, which are distinct than the recently documented daratumumab[2] were kindly provided by Genmab. The sequences were synthetically produced (GeneArt Invitrogen) in a scFv (single chain variable fragment) format with a signaling sequence and a G_4S linker. The CAR design is depicted in figure 1A. A G_4S linker separates the heavy and light chain sequences. The CD8a leader sequence placed in front of the scFv sequence facilitates signaling to the plasma membrane. To complete the CAR construct, the scFv was cloned in frame with a CD8a transmembrane domain, a 4-1BB and a CD3ζ sequence, identical to the second generation CAR design that has been used by Porter *et al*.[3]. Downstream of the CAR, an IRES induces the simultaneous transcription of the neomycine resistance gene. The generated CAR constructs were cloned into a pBullet retroviral vector under a CMV promoter and sequence was confirmed with Big dye v3.1 terminator and ABI sequencer (Biosystems).

Caspase-9 based suicide gene construct. The inducible caspase-9 retroviral pMSCV plasmid was kindly provided by David Spencer (Addgene, plasmid 15567)[4,5].

Generation of retroviral particles

The CAR constructs, gag-pol (pHIT60), and envelop (pCOLT-GALV) vectors (Roche) were transfected using Fugene-6 transfection reagent (Roche) into Phoenix Ampho packaging cells, cultured in DMEM + 10%FCS + antibiotics (penicillin;10.000 U/ml, streptomycin; 10.000µg/ml) Three days after transfection, cell free supernatants containing retroviral particles were collected. The virus particles were concentrated using the RetroX kit following the instructions of the manufacturer (ClonTech) and were frozen at -80°C until use.

Retroviral CAR Transduction into T cells

PBMCs from healthy donors or MM patients were stimulated with αCD3/CD28 Dynabeads™ (1×10^6 beads/10^6 cells) (Invitrogen) in RPMI-1640 + 10% FBS. After 48 hours, a total of 1×10^6 cells were retrovirally transduced by incubation of 50 µl of concentrated CAR retrovirus per 1×10^6 cells and 8 µg/ml Polybrene. After immediate centrifugation for 30 minutes at 32°C, the cells were cultured at 37°C and received a second hit after 24 hours. To obtain a pure population of CD38-CAR$^+$ T cells, transduced T cells were selected and expanded using culture medium (RPMI-1640, 10% human serum, antibiotics) supplemented with 800 µg/ml neomycin (Gibco, Karlsruhe, Germany) and 50 IU/ml rhIL-2 (Proleukin®, Novartis). After one week a fraction of expanding CAR-transduced T cells were sorted for CD4$^+$ and CD8$^+$ cells using MACS (Miltenyi Biotec). Unsorted and sorted T cells were expanded in culture medium containing IL2 (50 IU/ml) and were restimulated for several rounds (14 days/round) using a feeder cell/cytokine mixture consisting of irradiated EBV cell lines (5 Gy) and allogeneic PBMCs (2.5 Gy),100 U/ml IL-2 and 1 ng/ml PHA.

Retroviral transduction of CAR positive T cells with inducible caspase-9

Selected and expanded CAR-positive T cells were transduced with pMSCV-F-del Casp9.IRES.GFP, after stimulation with αCD3/CD28 Dynabeads™ (1×10^6 beads/10^6 cells)

as described above. GFP-positive transduced cells were sorted with FACS-Aria II sorter and were subsequently treated with increasing concentrations of B/B homodimerizer (Clontech, identical to AP20187 ARIAD Pharmaceuticals, Cambridge, MA)[4] to test the functional expression of the inducible caspase-9. After dimerization cells were analyzed for their GFP, AnnexinV and Topro3 expression with flow cytometry.

Tritium-based proliferations assays

To determine (CD38-dependent) proliferative capacity, Mock and CAR T cells were seeded in triplicates in 96-well plates in 3:1 ratio with 50 Gy irradiated UM9 cells. [^{3}H]Thymidine (^{3}H -TdR) (1 µCi per well; Amersham Biosciences) was added after 72 h and the cells were cultured for another 16 h before harvesting. ^{3}H-TdR incorporation was determined by liquid scintillation[6].

Cytokine measurements

To determine the broad array of cytokines produced by CAR T cells, we used the Cytokine Bead Array (CBA) Human Th1/Th2 cytokine kit (BD) according to manufacturer protocol. In brief, a mixture of capture beads (IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ), PE-detection reagent and cell supernatant were incubated for 3 hours. Beads were washed and analyzed by a standardized flow cytometry assay.

Flow cytometry

Flow cytometry assays were performed on FACS Calibur, CantoII or LSR Fortessa (BD). Different cell subsets in PBMC or in BM-MNC in various flow cytometry-based assays were determined using fluorescein conjugated antibodies specific for human CD3, CD4, CD7 CD8, CD13, CD14, CD16, CD19, CD33, CD38, CD45, CD56 and CD133, CD138 (BD Bioscience). CAR surface expression by flow cytometry was done with Biotinylated protein L (GeneScript;

Piscataway, NJ) and phycoerythrin (PE)-conjugated streptavidin (BD Bioscience) as described[7]. Cytotoxicity assays using flow cytometry: percentage cell lysis in a treated sample was calculated as follows and only if the analyzed target cell population contained >500 viable cells in the untreated samples. % lysis cells = 1 − (absolute number of surviving cells in treated wells / absolute number of surviving cells in untreated wells) × 100%.

Transmigration assay

To analyze the migration capacity of Mock and CAR T cells, the Cytoselect Leukocyte Transmigration Assay (Cell Biolabs, Inc) was used according to manufacturer's protocol. In brief, HUVECs were seeded on membranes in a 24 well plate, after 48 hours Mock and CAR T cells were seeded and migration over the membrane was detected with leukocyte tracker labeling.

Hematopoietic precursor cell (HPC) growth inhibition assays

HPC growth inhibition assays were performed as described previously[8]. Briefly, either 10, 100 or 1000 $CD34^+$ sorted BM cells were mixed with effector T cells at different T:BM cell ratios in 0.2 mL of RPMI culture medium. The cells cultured for 4 hours to create cell contact and then resuspended to a final volume of 1.2 mL with semisolid Methocult (Stem cell technologies, H4534). One milliliter of the semisolid suspension was then plated in 30-mm plastic dishes and incubated at 37°C in 5% CO_2. After 21 days, the number of colony-forming unit-granulocytes (CFU-G), and CFU-monocytes (CFU-M), were scored under a microscope.

***In vivo* efficacy of CD38-CAR T cells against MM tumors growing in a humanized microenvironment.**

To create a human bone marrow-like environment in mice, hybrid scaffolds consisting of three 2- to 3-mm^3 biphasic calcium phosphate particles were coated *in vitro* with human

mesenchymal stromal cells (MSC)(2×10^5 cells/scaffold). After a week of *in vitro* culture in a bone-formation promoting medium, humanized scaffolds were seeded with $CD38^+$ UM9 cells (1×10^6 cells/scaffold) to allow adherence to the hu-scaffolds and after 16 hours implanted subcutaneously into the mice, as described previously[1,9]. Seven days after implantation, when the tumors became detectable in the scaffolds by BLI, different groups of mice received tail i.v. injections of CD38-CAR T cells or with mock-transduced CAR T cells (20×10^6 cells/mice). The injections were repeated on day 9 and 13. Tumor growth was monitored by weekly BLI measurements as described previously[9]. All animal experiments were conducted after acquiring permission from the local ethical committee for animal experimentation and were in compliance with the Dutch Animal Experimentation Act.

Immunohistochemistry

Tumor were dissected from mice and fixed in formalin, subsequently decalcified with Cal-Rite™ (Thermo Scientific) and paraffin-embedded. Retrieved sections were blocked with 0.3% H_2O_2 in methanol. Sections were boiled for 10 minutes in a citrate buffer, pH 6, and then blocked with serum-free protein block. The slides were incubated overnight at 4°C with anti-38 (Monosan BLD2) or CD138 (IQP-153P). The sections were counterstained with hematoxylin, washed, and subsequently dehydrated through graded alcohol, cleared in xylene, and coverslipped.

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