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Sepantronium bromide (YM155) improves daratumumab-mediated cellular lysis of multiple myeloma cells by abrogation of bone marrow stromal cell-induced resistance.

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Contributions: ED performed the research, analyzed the data, and wrote the manuscript; RWJG, WAN, MT and ACMM assisted the design and the execution of the in vitro and in vivo experiments, analyzed the data and provided technical assistance; JK and ZS provided the retroviral construct and reviewed the manuscript; HY, JB provided the scaffolds for the in vivo experiments. JJLB, PWHIP provided the CD38 antibodies, assisted the design of the study, analyzed the data and reviewed the manuscript; . NWCJD, HML and TM designed and supervised the research and wrote the manuscript.

Preclinical evaluation of CD38 Chimeric Antigen Receptor engineered T cells for the treatment of Multiple Myeloma

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Running title: CD38-CART cells for Multiple Myeloma treatment

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Abstract

Adoptive transfer of Chimeric Antigen Receptor transduced T cells is a promising strategy for cancer immunotherapy. The CD38 molecule, with its high expression on Multiple Myeloma cells, appears a suitable target for antibody therapy. Prompted by this, we used three different CD38 antibody sequences to generate second generation retroviral CD38-Chimeric Antigen Receptor constructs; transduced T cells of healthy donors and Multiple Myeloma patients and evaluated their preclinical efficacy and safety. Irrespective of the donor and antibody sequence, CD38-Chimeric Antigen Receptor transduced T cells proliferated, produced inflammatory cytokines and effectively lysed malignant cell lines and primary malignant cells from acute myeloid leukemia and multi-drug resistant Multiple Myeloma patients in a cell-dose, and CD38-dependent manner, despite becoming CD38 negative during culture. CD38-Chimeric Antigen Receptor transduced T cells also displayed significant anti-tumor effects in a xenotransplant model, in which Multiple Myeloma tumors were grown in a human bone marrow-like microenvironment. CD38-Chimeric Antigen Receptor transduced T cells, also appeared to lyse the $CD38^+$ fractions $CD34^+$ hematopoietic progenitor cells, monocytes, natural killer cells, and to a lesser extent T and B cells but, did not inhibit the outgrowth progenitor cells into various myeloid lineages, and were furthermore effectively controllable with a caspase-9-based suicide gene. These results signify the potential importance of CD38-Chimeric Antigen Receptor transduced T cells as therapeutic tools for $CD38^+$ malignancies and warrant further diminishing their undesired effects using appropriate strategies.

Introduction

Multiple myeloma (MM), the malignant disorder of antibody producing clonal plasma cells is the second most common hematologic neoplasia worldwide.[1] Despite four decades of drug innovation, MM remains incurable with chemotherapy. Furthermore, the prognosis of MM patients, who become refractory to recently developed novel agents, is very poor.[2] On the other hand, clinical and experimental data collected over the past decades suggest the possibility to successfully treat MM through (cellular) immunotherapy.[3,4] The curative potential of cellular immunotherapy in MM is illustrated by the induction of long-term sustained remissions after allogeneic stem cell transplantation (SCT) or donor lymphocyte infusions (DLI) in a subset of patients.[5,6] A highly appealing and more specific immunotherapy strategy for cancer is the adoptive transfer of cytotoxic T cells (CTLs) that are genetically engineered to express Chimeric Antigen Receptors (CAR).[7,8] A CAR is an artificial hybrid receptor, in which the antigen recognizing domain of a tumor-reactive monoclonal antibody is fused with T-cell signaling domains. Upon retro- or lentiviral transduction of CTLs, CARs expressed on the cell surface redirect the CTLs toward the original target of the antibody in a non-HLA restricted manner[7,8], providing the possibility to apply the therapy regardless of the HLA type of the patient. Currently the most successful CAR-approaches are based on targeting the CD19 molecule, which is broadly expressed in several B cell malignancies but not on malignant plasma cells of MM patients. Among a few potential CAR candidates for MM[9], the CD38 molecule, with its high and uniform expression on malignant plasma cells, has long been suggested a suitable target for antibody therapy of MM. The utility of CD38 as a suitable target has indeed been supported by the results of recently initiated clinical trials in which MM patients were safely and effectively treated with the CD38-specific human monoclonal antibody daratumumab[10].

Encouraged by these clinical results, we now started to explore the feasibility of development of a CART cell therapy based on targeting the CD38 molecule. Using variable heavy and light chain sequences of three different human CD38 antibodies, we generated

three different CD38-CARs. We transduced T cells from healthy individuals and MM patients with CD38-CARs and evaluated them for essential functions such as antigen-specific proliferation and cytokine production, for *in vitro* and *in vivo* anti-tumor efficacy and for potential undesired effects such as targeting normal $CD38^+$ cell fractions in the peripheral blood and bone marrow. We also evaluated the feasibility of controlling CD38-CART cells by introduction of a caspase-9 based suicide gene.

Methods

Bone marrow Mononuclear cells (BM-MNC) from MM and AML patients

Bone marrow mononuclear cells containing 5-20% malignant plasma cells or ~50% AML blasts were isolated from bone marrow aspirates of MM/AML patients through Ficoll-Paque density centrifugation and cryopreserved in liquid nitrogen until use. All bone marrow and blood sampling from the patients was performed after informed consent and approved by the institutional medical ethical committee.

PBMC from healthy individuals

PBMCs were isolated from Buffy coats of healthy blood-bank donors by Ficoll-Paque density centrifugation after informed consent and approval by the institutional medical ethical committee.

Retroviral constructs

The sequences of three different human CD38 antibodies, which are distinct from, but display similar affinities to the recently documented daratumumab[10] (Table S1) were kindly provided by Genmab. Cloning methods are described in supplementary methods.

Retroviral CAR Transduction into T cells

Transduction methods are described in supplementary methods.

Flow cytometry-based cell lysis assays

To detect the lysis of various cell subsets by CART cells in whole BMNC or in PBMC, serial dilutions of CART cells were incubated with CFSE labeled BMMNC or PBMC for 24 hours. The cells were then harvested, stained for different CD markers and topro3 or LIVE/DEAD® Fixable Near-IR (Life Technologies L10119) and were quantitatively analyzed through volume-equalized measurements using a FACS Canto flow cytometer. For each cell subset identified with a CD marker, $CFSE^+$, $viable^+/Topro3^-$ cells were counted as surviving target cells. 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%.

BLI-based cell lysis assays

To determine the lysis of Luc-GFP-transduced human malignant cell lines by CD38-CART cells, serial dilutions of mock or CD38-CART cells were co-incubated with the malignant cell lines. The luciferase signal produced by surviving malignant cells was determined after 16-24 hours with a luminometer SpectraMax, Molecular Devices within 15 minutes after the addition of 125 µg/mL beetle luciferin (Promega).[11] The percent lysis was then calculated as in flow-based cytotoxicity assay above.

Experimental animals

RAG2$^{-/-}$γc$^{-/-}$ mice used in this study were originally obtained from the Amsterdam Medical Center (AMC, Amsterdam, the Netherlands). The mice were bred and maintained in filter top cages under specified pathogen-free conditions at the Central Animal Facility (GDL, Utrecht University, Utrecht, the Netherlands) and received sterile water and radiation-sterilized food pellets ad libitum.

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

To create a human bone marrow-like environment in mice, hybrid scaffolds were coated *in vitro* with human mesenchymal stromal cells. After a week of *in vitro* culture, humanized scaffolds were seeded with CD38^{+} UM9 cells and implanted subcutaneously into the mice, as described previously,[11,12] and described in supplementary methods.

Results

Generation of CD38-CART cells

We used the variable heavy and light chain sequences of three different CD38 antibodies with CD38 binding affinities comparable to daratumumab (Table S1), which is now being tested in clinical trials. T cells from healthy PBMC were transduced with the different CD38-CAR genes or with the empty vector (mock) separately. After selection of transduced cells to high purity by neomycin treatment, the surface expression of CARs was determined by incubating the T cells with biotinylated bacterial protein L, which specifically binds to the variable region of kappa light chains of antibodies.[13] Indirect staining with PE-conjugated streptavidin revealed the expression of all three CARs on >95% of the T cells, whereas T cells transduced with an empty vector (mock-transduced T cells) displayed only background staining (Fig. 1B left panel). The CAR-transduced cells contained variable levels of both $CD4^+$ and $CD8^+$ cells (Fig. 1B; right panel).

CD38-dependent proliferation and cytokine secretion of CD38-CART cells.

To analyze the proliferative and functional properties, the neomycin-selected, highly purified CD38-CART cells were expanded using irradiated feeder cells in the presence of PHA and IL-2. While the mock T cells initially expanded better than the CD38-CART cells (Fig. 1C; left panel), the growth disadvantage of CD38-CART cells disappeared in the second expansion round (Fig. 1C; right panel), indicating that transduction of CD38-CAR construct did not affect the proliferative capacity of T cells. We then tested whether CD38-CART cells can be activated by CD38-triggering. To this end, we co-cultured mock- and CD38-CAR-transduced T cells with irradiated $CD38^+$ MM cell line UM9 and used the $CD38^-$ MM cell line U266 as a control (Fig. 1D; left panel). CD38-CART cells, but not mock T cells, specifically proliferated and produced IFN-γ, TNF-α and IL-2 (Fig.1D; right panel), but not IL-4, -5 and -10 (data not shown) upon stimulation with UM9 cells. These results indicated that CD38 CART cells had no defects in cytokine production but displayed a typical Th1-like cytokine

response upon target recognition. Furthermore, the $CD38^-$ cell line U266 was unable to stimulate CD38-CART cells, demonstrating the proper antigen-specific function of CD38-CART cells.

CD38-dependent lysis of MM cell lines by CD38-CART cells

To determine the CD38-dependent lysis of malignant cells by CD38-CART cells, we first used luciferase-transduced MM cell lines with variable CD38 expression levels in BLI-based cytotoxicity assays.[11,14] As expected, there was no CD38-CAR-specific lysis of the $CD38^-$ U266 cell line (Fig. 2A). In contrast, all three CD38-CART cells, but not mock-T cells, effectively lysed $CD38^+$ MM cell line UM9 in a cell-dose dependent manner (Fig. 2B), showing the feasibility of generating effective CART cells with any of the CD38 antibody sequences we used. Since there was no functional difference between the three different CD38-CARs (028, 056, 026), we continued our investigation with one type of CD38-CART cell (CAR056). Flow cytometry and BLI-based cytotoxicity assays, executed using other malignant cell lines expressing various levels of CD38 (Supplementary Fig.S1) as target cells revealed a good correlation between the CD38 expression and CD38-CART cell-mediated lysis (Fig. 2C). Though one AML cell line, the Burkitt lymphoma-derived cell line Daudi as well as normal T cells appeared less sensitive to CD38-CART cell mediated lysis as compared to MM cell lines with similar levels of CD38 expression (Fig. 2C).

Lysis of primary MM and AML cells by CD38-CART cells.

To test the efficacy of CD38-CART cells against primary MM and AML cells, we used a previously described flow cytometry-based *ex vivo* cytotoxicity assay, in which the lysis of malignant cells is tested, directly in the BM-MNCs without isolating them from other cells.[15] As depicted in Figure 3A, primary $CD138^+CD38^+$ MM cells of three different MM patients, who were refractory to treatment with lenalidomide, and bortezomib (left panel), were effectively lysed by CD38-CART cells, but not by mock-transduced T cells. Similarly in the BM-MNCs of two Acute Myeloid Leukemia (AML) patients malignant cells, which were

identified as $CD13^+$ $CD45^+$ cells and expressed either low/intermediate (patient 1) or high CD38 (patient 2) were effectively lysed by CD38-CART cells (Fig 3A). Finally, CD38-CART cells that were generated (Fig 3B) from a MM patient were effective towards autologous malignant MM cells in BM-MNCs indicating the feasibility of generating effective CD38CART cells also from MM patients.

Fully-functional CD38 CART cells are negative for CD38

While CD38-CART cells had no apparent functional deficiencies, a phenotyping assay revealed that they, despite a mixed effector/central memory phenotype, lost the expression of CD38 (Fig. 4A). Interestingly, when we co-cultured CD38-CART cells with an autologous CD19 CART cell population, these CD19CART cells also became largely negative for CD38 expression but fully maintained their capacities to proliferate, secrete cytokines and kill the relevant target cells in a CD19 dependent fashion (supplementary Figure S2), indicating that the loss of CD38 was not associated with detectable T cell dysfunction. Nonetheless, since CD38 molecule could also play a role in migration, we also evaluated whether CD38 negative CD38-CART cells would properly migrate through endothelial layers in a transwell migration assay (fig 4B). These assays revealed no differences between the mock-transduced, CD38-positive and CD38-CAR-transduced CD38-negative T cells, ruling out an apparent migratory dysfunction of CD38-CART cells.

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

To substantiate the in vitro results, we questioned whether the CD38 negative CART cells could mediate *in vivo* anti-MM effects after systemic injection in our recently developed model in $Rag2^{-/-}\gamma c^{-/-}$ mice, in which a humanized BM like-niche for MM cells are generated by s.c. implantation of ceramic scaffolds coated with human bone marrow stromal cells (huBMSCs)[11,12] (Fig4). Thus, we implanted huBMSC-coated scaffolds seeded with luciferase-transduced UM9 MM cells in the back of the mice (6 scaffolds per mouse). Upon detection

of luciferase signal by BLI, we treated the mice by i.v. injections of CD38-CART cells using a previously established treatment scheme.[16] Mock-transduced T cells were used as controls. As illustrated in Figure 4B, in the control group treated with mock T cells, tumors showed a fast progression. Although not curative, treatment of the tumor-bearing mice with CD38-CART cells induced a significant anti-tumor effect (Fig. 4B,C) underscoring the potential of CD38-CART cells to properly infiltrate and lyse MM tumors growing in their natural, protective niche. Post mortem analyses revealed that the remaining $CD138^+$ tumors were still positive for CD38 (Fig. 4D), thus ruling out tumor escape due to "antigen loss" variants.

Impact of CD38-CART cells on $CD38^+$ normal hematopoietic cells and hematopoietic progenitor cells.

Besides the high expression levels in MM cells, the CD38 molecule is expressed at intermediate levels on a subset of hematopoietic progenitor cells[17] and on a fraction of normal hematopoietic cells including activated T cells, NK cells, B cells and monocytes. We therefore evaluated the possible negative impact of CAR-T cells on these cell subsets by co-incubating unsorted BM-MNCs with CD38-CART cells. CD38-CART cells appeared to eliminate the $CD38^+$ fractions of mature T, B, NK and monocyte cell subsets (Fig. 5A) and the $CD38^+$ fraction of $CD34^+$ cells (Fig. 5B) in a 4 hour assay. The lysis of $CD34^+CD38^+$ cells had however no influence on the development of colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) in a 14-day hematopoietic precursor cell (HPC) colony forming assay[18,19] (Fig. 5C and D).

Specific elimination of CD38-CART cells using a suicide gene (iCasp9)

Although CD38-CART cells did not lyse the CD38 negative fractions of mature hematopoietic cells and did not inhibit the outgrowth of these cell populations, still a cautious approach toward the clinical application is required. Therefore, as a first step towards a more safe application of CD38-CART cells, we tested the possibility to control

them with a suicide gene based on the inducible caspase-9 (iCasp9) gene that is activated with a small dimerizer molecule AP20187 (B/B).[20] Thus, we inserted an iCasp9 vector containing a GFP marker gene into the CD38-CART cells by retroviral transduction. Around 50% of the CD38-CART cells were transduced as detected by GFP expression (figure 6A, upper panel) When tested without sorting the iCasp9 transduced (GFP+) cells, all iCasp9 transduced, GFP+ but none of the iCasp9 non-transduced, GFP-CD38-CART cells were eliminated upon incubation with the dimerizer AP20187 (Fig. 6A lower panel). As expected, the dimerizer treatment also resulted in a proportional decrease in the lysis of the MM cell line UM9. (Fig. 6B). There was still a remaining lysis due to the surviving iCasp9 negative CD38-CART cells, indicating that the triggering of suicide gene induced no bystander damage to the cells in the close vicinity. When tested after sorting of GFP+ cells (Fig 6C and 6D), almost all GFP+ cells died after treatment with the dimerizer (Fig. 6C) and there was no CD38 specific lysis left (Fig 6D), confirming the results obtained by previous studies[20,21], and suggesting the possibility to control CD38-CART cells using the iCasp9 suicide gene without undesired consequences.

Discussion

While cellular immunotherapy of hematological malignancies has been applied for many decades in the most non-specific form as allo-SCT or DLI, it has recently entered a more specific level of innovation with several encouraging strategies, including vaccination with antigen-loaded dendritic cells or adoptive immunotherapy with TCR-gene transferred T cells, tumor infiltrating T cells and more recently with CTLs endowed with tumor-reactive CARs. Among these strategies, CAR-based therapies are perhaps the most appealing, as CART cells recognize their target antigens in an MHC-independent manner. Set out to develop a CAR-based strategy for MM, we have been encouraged by the highly promising clinical results of the therapy with daratumumab, which targets CD38, a type II transmembrane glycoprotein, expressed with high and uniform levels in most, if not all, MM cells in all stages of the disease[10]. Recently daratumumab has been applied to several patients at moderate to high doses and for prolonged periods with little or no toxicity. This, despite the fact that CD38 molecule is also expressed, albeit at lower levels, on a fraction of hematopoietic cells, cerebellar purkinje cells, liver and lung smooth muscle cells, and insulin-secreting β cells of pancreas.[17] Our study was therefore designed to test the feasibility, potential efficacy and pitfalls of a CD38-based CART cell approach for MM. To investigate the feasibility of generating CD38-CARs, we started the investigation using three distinct human CD38 antibodies, which displayed similar binding affinities to CD38 as daratumumab (Supplementary Table S1). Based on successful usage of 4-1BB-containing CARs in recent studies[22–24] we have constructed CARs containing 4-1BB (CD137) co-stimulatory and CD3ζ activating domains. Our results demonstrate the successful generation of CD38-CARs and CD38-CART cells regardless of the antibody sequences. T cells transduced with these CD38-CARs are highly proliferative, produce inflammatory Th1 like cytokines and, most importantly, are effective in killing malignant cells and normal hematopoietic cells in a CD38-dependent fashion, with some subtle differences between cell lines or hematopoietic cell types.

More importantly, CD38-CART cells appeared capable of eliminating primary $CD38^+$ MM cells of patients who became resistant to various chemotherapies. This suggests that CD38-CAR therapy could be a viable option for patients with little or no further chemotherapy options. These *in vitro* data were substantiated by the results obtained in our *in vivo* model. Although we did not observe the complete eradication of MM cells in our *in vivo* assays, we need to note that, since our CD38-CART cells appeared to lose their CD38 expression upon culture, we primarily designed our *in vivo* assays to determine the anti-tumor efficacy of these CD38 negative, but long-term cultured CD38-CART cells. This may have negatively influenced the anti-tumor efficacy, since it is known that long-term cultured T cells rapidly lose their in vivo persistence capacities.[25,26] In addition, and perhaps even more important, in our model, unlike all previously reported CAR studies, the human MM tumors are grown to larger masses in a fully humanized BM microenvironment. The MM microenvironment is known to provide essential signals for survival, growth and more importantly immune resistance of MM cells.[11,12,27,28] Since our model includes some of the microenvironment related aspects, our results suggest that it the efficacy of CART cell treatment could be improved if the therapy would be combined with immune checkpoint inhibitors and or with survivin and/or MCL-1 inhibitors which are effective modifiers of cell adhesion mediated immune resistance (CAM-IR) induced by tumor microenvironment.[11]

Unlike a number of earlier reports, which mainly focused on the anti-tumor efficacy of CD38-CART cells,[29–31] we devoted a considerable part of our investigation on identifying the potential drawbacks and risks of the CD38-CART cell therapy. Although CD38-CART cells eliminated the $CD38^+$ fractions of immune cell subsets as well as the $CD38^+$ fraction of hematopoietic progenitor cells, we observed no inhibition of the outgrowth of hematopoietic lineages from $CD34^+CD38^-$ progenitor cells. Furthermore, CD38-CART cells did not induce complete depletion of mature hematopoietic cells in the periphery. The CD38 negative fractions of important immune cells, such as B and T cells, were unaffected as well. These results suggest that the therapy will spare sufficient numbers of T and B cells to maintain

their functions. However, since CD38 is a well-known T cell activation molecule, and has also been implicated in chemotaxis,[32] T cell development[33], dendritic cell trafficking and humoral immune responses[34], it would be relevant to answer the question whether an intact immune response would be possible in the absence of CD38. A partial answer to this issue came from the analyses of CD38-CART cells: we remarkably discovered that the CD38-CART cells, regardless of which scFv was used, became completely devoid of CD38 expression on their surface in various independently generated batches of cells. The loss of CD38 was thus unlikely to be caused by a genetic defect, but was most probably due to the "self lysis" of the $CD38^+$ fractions, which was also described in another CD38-CAR study[29]. Our CD38 negative CD38-CART cells, however, had no growth disadvantage, displayed highly activated status, CD38-dependent proliferation, cytokine production, cytotoxic activities and showed no other detectable functional aberrancies. This was also the case for CD19CART cells which became CD38 negative after co-culture with CD38-CART cells (Figure S2). Furthermore CD38-CART cells did not show any defects in a transmigration assays and they also mediated significant anti-MM effects *in vivo*, thus indicating their capacity to properly migrate and infiltrate into the MM niches and to kill them. Thus, it seems likely that i) not all activated T cells have to be CD38 positive and ii) CD38 expression is not essential for T cells to fulfill their functions. This conclusion is also supported by the fact that there is yet no evidence, even in CD38 KO mice,[32] that CD38-deficient effector T cells are defective in function.

On the other hand, the relative broad expression of the target antigen of CD38-CART cells increases the risk of the so called "cytokine release syndrome (CRS)" due to massive activation of CAR-T cells, as have been observed in the previous trials with ERBB2- and CD19-CART cells.[35–37] Although the IL-6R antagonist tocilizumab appears to successfully reduce CRS[38] it would still be desirable to minimize the occurrence of such severe side effects. Furthermore, since we cannot rule out toxicities occurring die to the possible attack of non-hematopoietic $CD38^+$ cells, development of an optimal CD38-CART cell therapy

would require the improvement of the target-specificity as well as the *in vivo* control on CD38-CART cells, and probably also in the case of other CART cell approaches targeting kappa light chain[39], CD138[40], Lewis Y antigen[41], BCMA[42], CS1[43,44], and CD44v6.[45] One future option to improve the target-specificity could be the optimization of the target cell affinity of CART cells. In addition, suicide genes may enable the *in vivo* control of adoptively transferred CART cells. Indeed, in our first attempt to improve the safety profile of CD38-CART cells we observed, that the inducible caspase 9 (iCasp9) gene[20,46] can effectively control CART cells. These results, which are in agreement with other studies[20,45,47] provide positive prospects for the future clinical trials. The safety profile of CART cells could also be improved by the generation of inducible CAR constructs or using the recently developed dual CAR technologies.

Taken together, we conclude that CD38-CART cells are powerful immunotherapeutic tools and can be beneficial especially for MM patients who have no other chemotherapy options. Therefore these results warrants further studies towards diminishing their undesired effects against normal $CD38^+$ cells through optimizing their CD38 affinity and improving *in vivo* controllability.

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Figure Legends

Figure 1 CD38-CAR construct and CD38-CART cell phenotype

(A) Schematic overview of the CD38-CAR construct. CD38-scFv sequence is based on three different antibody sequences (028, 056 and 26, see also supplementary Table S1), with CD8a as transmembrane domain and 4-1BB and CD3ζ as intracellular domains. (B) CAR expression on the cell surface of healthy donor T cells was determined by binding of biotinylated protein L to the scFv domain (left panel), stained with PE labeled streptavidin. Depicted are the results for CD38-CART cells generated with CAR056, representative for all three CARs. The expression of surface markers CD4 and CD8 on (right panel) was determined by fluorescence-labeled monoclonal antibodies. (C) The expansion of mock and CD38-CART cells after transduction (left panel) and after the second round of stimulation (right panel; new stimulation set at "0"). (D) The relative ^{3}H-thymidine uptake (left panel) of mock and CD38-CART cells after 72 h stimulation with the CD38^{+} MM cell line UM9: responder ratio of 3:1. Error bars represent mean + SEM, n=3. The results are expressed as relative stimulation index, compared to mock and considered significant if the stimulation index is >=3. The cytokine secretion (right panel) from of mock and CD38-CART cells stimulated with αCD3/CD28 beads or CD38-specific with the MM cell line UM9. The cytokine secretion was measured with the flow cytometry-based CBA kit (BD) in the cell free supernatants after 24 hours of stimulation. Graph shows the secretion of IFN- γ, TNF and IL-2. Secretion of IL-4, -5 and -10 were below the detection limits. These data are therefore not shown in this figure. Similar results were obtained in two independent assays.

Figure 2 Efficacy of CD38-CART cells to lyse MM cell lines. In 24 h cytotoxicity assays, three different CD38-CART cells were tested against two MM cell lines with different CD38 expression levels (A) U266, a CD38-negative cell line, (B) UM9, a CD38^{+} cell line. Effector:Target ratios are indicated. Target cells per well were 10,000 MM cells. Closed

circles (●) indicate mock and open squares, triangle and diamond (□,◇,△) indicate CAR028, 056 and 026. Error bars indicate mean + SD. (C) Correlation between mean fluorescent intensity (MFI) of CD38 on target cells and consequential CD38-CAR specific lysis. CD38-CART cells (CAR056) were co-cultured with leukemic cell lines and allogeneic healthy donor PBMCs. The resulting lysis in a 3:1 ratio was determined with BLI or flow cytometry, minus the spontaneous lysis caused by mock T cells. Open circles (⭘) indicate MM cell lines (LME-1, UM9, MM1.S, U266, L363 and UM3), triangles (▲) indicate AML (HEL, MOLM13), T lymphoblast (CEM) and Burkitt Lymphoma (Daudi), closed circles (●) indicate healthy immune cells (T= T cells, B=B cells, NK= NK cells, Mo= monocytes, C=CEM, H=HEL, M=MOLM13, D=Daudi), Error bars represent mean + SEM of duplicate measurements..

Figure 3. Efficacy of CD38-CART cells generated from healthy individuals to lyse primary MM cells. (A) Bone marrow derived mononuclear cells (BM-MNCs) of three MM patients, all three refractory to lenalidomide, and bortezomib and BM-MNCs of two AML patients were co-incubated with no, mock- or CD38-CART cells generated from healthy PBMC for 16 h. Closed circles (●) indicate mock and open squares (□) indicate CAR056T cells (representative for all CARs). The graphs depict the resulting lysis of $CD138^+/CD38^+$ cells (MM) or $CD13^+/CD7^+/CD45dim/CD38^+$ (AML1, moderate CD38 expression) and $CD33^+/CD133^+/CD45dim/CD38^+$ (AML2, high CD38 expression) in three E:T ratios. The % lysis in these flow cytometry assays was calculated as described in the methods section. (B) Efficacy of CD38CART cells generated from a MM patient: CAR expression on the cell surface of patient's T cells was determined by flow cytometry with protein L staining (see also figure 1). (C) Bone marrow derived mononuclear cells (BM-MNCs) of the MM patient were co-incubated with autologous mock- or CD38-CART cells for 16 h. The graph depicts resulting lysis of $CD138^+/CD38^+$ cells in two ratios, determined in flow cytometry based assays. .

Figure 4. Tumor growth in Mock- and CD38-CART cell treated mice. (A) Analysis of CD38-CART cells after two weeks of *in vitro* culture, with fluorescence labeled monoclonal antibodies for CD45RA and CD62L and CD38. (B) Leukocyte transmigration assay, where mock and CART cells were cultured in a transwell system in the inserts with endothelial (HUVEC) cells, which were activated with TNF-α. Spontaneous TNFα-induced transmigration was compared to active migration induced by 10% of human serum in the lower compartment. % migrated cells = (Relative Fluorescence Units (RFU) of cells in lower compartment / RFU of total cells in both compartments) * 100%. (C) Analysis of tumor load in mice by quantification of BLI measurements. Each group contained six mice, each harboring 6 scaffolds. Results are mean tumor load (cpm/cm^2) of 6 mice per group. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. The error bars represent mean + SEM, n=6. The differences between groups were analyzed after week 6 in, an unpaired student's T test, $p < 0.0001$ (D) Bioluminescent imaging of mice on right side, mice were implanted with fully humanized BMSC scaffolds each coated with 1×10^6 UM9-GFP-Luc tumor cells. 7, 9 and 13 days after implantation, mice were i.v. injected with 20×10^6 Mock or CD38-CART cells. (E) Representative immunohistochemistry figure, remaining tumor were stained with CD38 and CD138 antibody, T = tumor, sc = scaffold.

Figure 5. The impact of CD38-CART cells on non-malignant hematopoietic cells in BM and outgrowth of Hematopoietic cell lineages. (A) BM-MNCs of 3 MM patients were co-incubated with none, mock- or CD38-CART cells for 16 h. The graphs depict the resulting lysis of the total or the CD38$^+$ fractions of CD3$^+$ (T cells), CD56$^+$, (mainly NK cells), CD14$^+$ (monocytes) and CD19$^+$ (B cells) cell subsets in three ratios, determined with flow cytometry and calculated as described in the methods section. Results are 3 individual experiments combined. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. Error bars represent mean + SEM, n=3. (B) CD34$^+$ fraction of BM-MNCs from healthy donors were co-incubated with none, mock- or CD38-CART cells for 4 h at different T:BM cell ratios

before transferring into the semisolid HPC culture medium. After the incubation, cells were analyzed by flow cytometry for surviving $CD34^+$ cells with CD38 expression. The graphs depict the resulting lysis of the total or the $CD38^+$ fraction of $CD34^+$ cells. Closed circles (●) indicate Mock and open squares (□) indicate CAR056. (C) After 14 days of culture in plastic dishes, colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) were visible, (D) the number of CFU-M, and CFU-G colonies were determined microscopically. Results of a representative experiment are shown mean + SD.

Figure 6. Dimerizer AP20187-mediated elimination of the iCasp9 suicide gene transduced CD38-CAR T cells. (A) Flow cytometry histogram plots, CD38-CAR T cells that were additionally transduced with the iCasp9-GFP construct. Upper panel shows the untreated cells 50% GFP^+; lower panel the cells treated with 100nM dimerizer AP20187 (B/B). (B) The lysis of UM9 cell line by iCasp9-transduced CD38-CAR T cells that were untreated or treated with the dimerizer. The significant reduction of GFP^+ cells (A) is a consequence of cell death activated by the dimerizer B/B. Note (in B) the decrease in cytolysis is proportional to the elimination of the suicide gen transduced cells (50% of all CAR-positive cells in A. The residual cytolysis is thus caused by the CAR-positive cells that were not transduced with iCasp9 n=2, mean + SD. (C) CD38-CART iCasp9-GFP-high sorted cells. Upper panel shows the untreated cells 100% GFP^+; lower panel the cells treated with 100nM dimerizer B/B. (D) The lysis of UM9 cell line by iCasp9-High-CD38-CART cells that were untreated or treated with the dimerizer. Closed circles (●) indicate Mock and open diamond (◇) and triangle (△) indicate CAR056 – and + B/B. n=2, mean + SD.

Figure 1

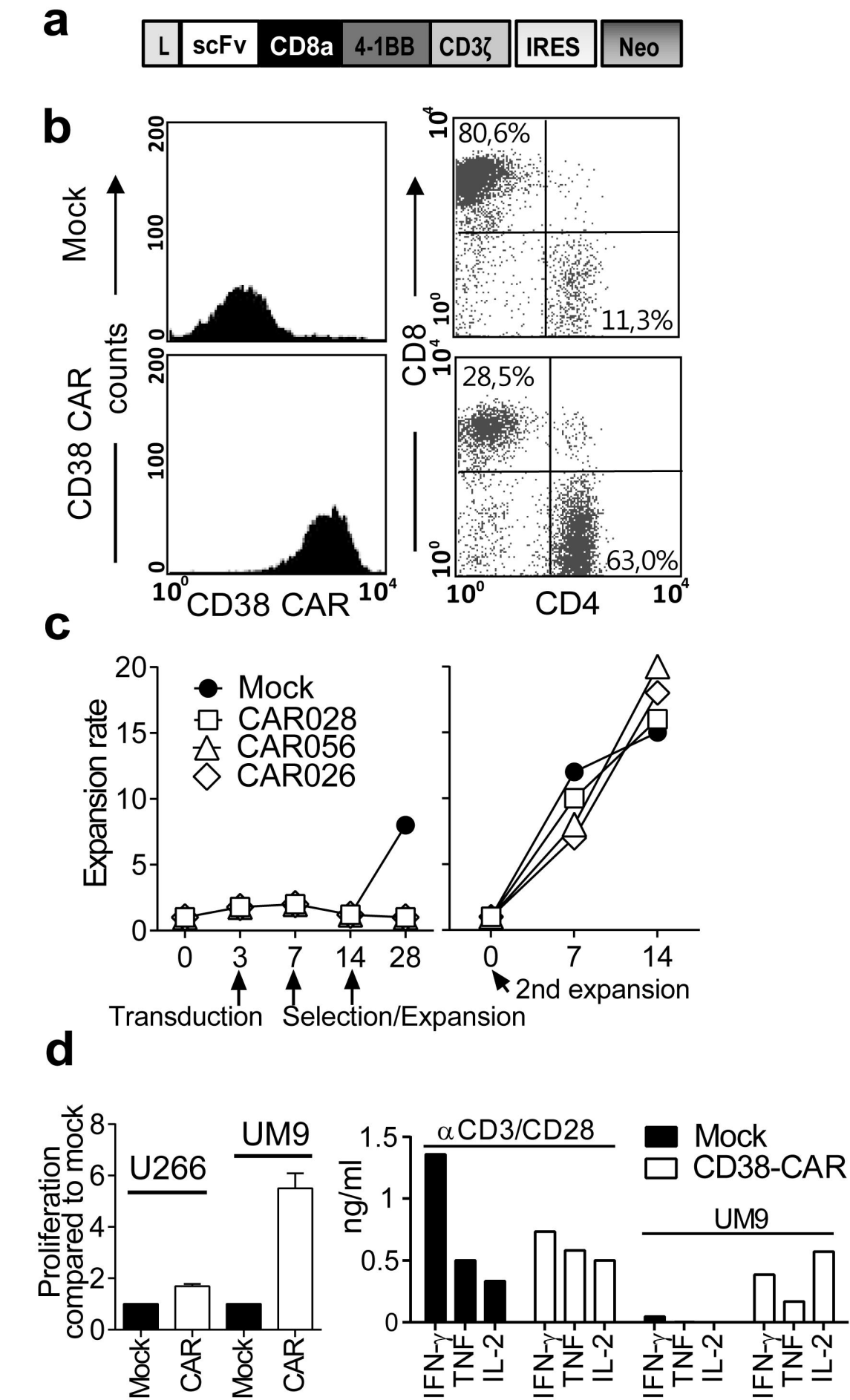

a
L
scFv
CD8a
4-1BB
CD3ζ
IRES
Neo
b
Mock
CD38 CAR
counts
200
100
0
10^0
CD38 CAR
10^4
CD8
80,6%
11,3%
28,5%
63,0%
CD4
c
Expansion rate
Mock
CAR028
CAR056
CAR026
0
5
10
15
20
0
3
7
14
28
Transduction
Selection/Expansion
2nd expansion
d
Proliferation compared to mock
U266
UM9
Mock
CAR
ng/ml
αCD3/CD28
CD38-CAR
IFN-γ
TNF
IL-2

Figure 2

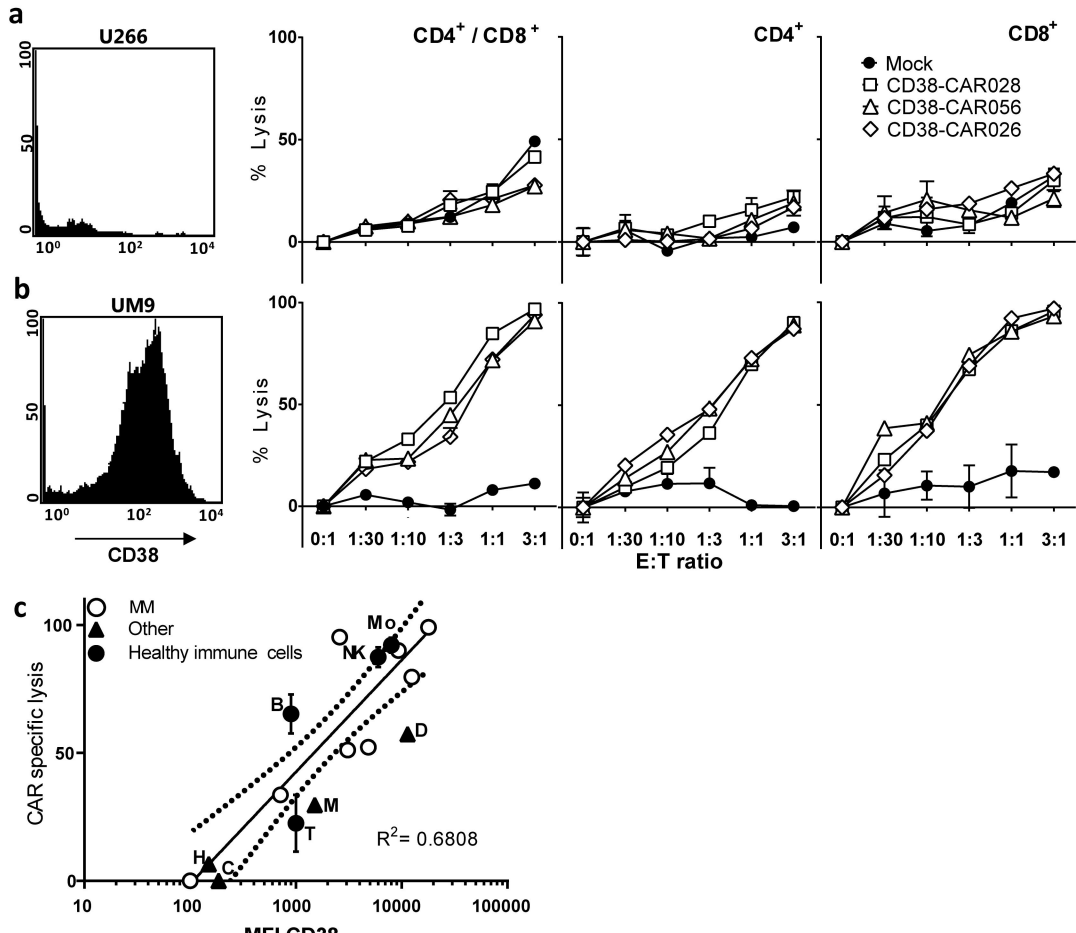

a
U266
b
UM9
CD38
CD4+ / CD8+
CD4+
CD8+
Mock
CD38-CAR028
CD38-CAR056
CD38-CAR026
% Lysis
0:1 1:30 1:10 1:3 1:1 3:1
E:T ratio
c
MM
Other
Healthy immune cells
CAR specific lysis
MFI CD38
R2= 0.6808
Mo
NK
B
D
M
T
H
C

Figure 3

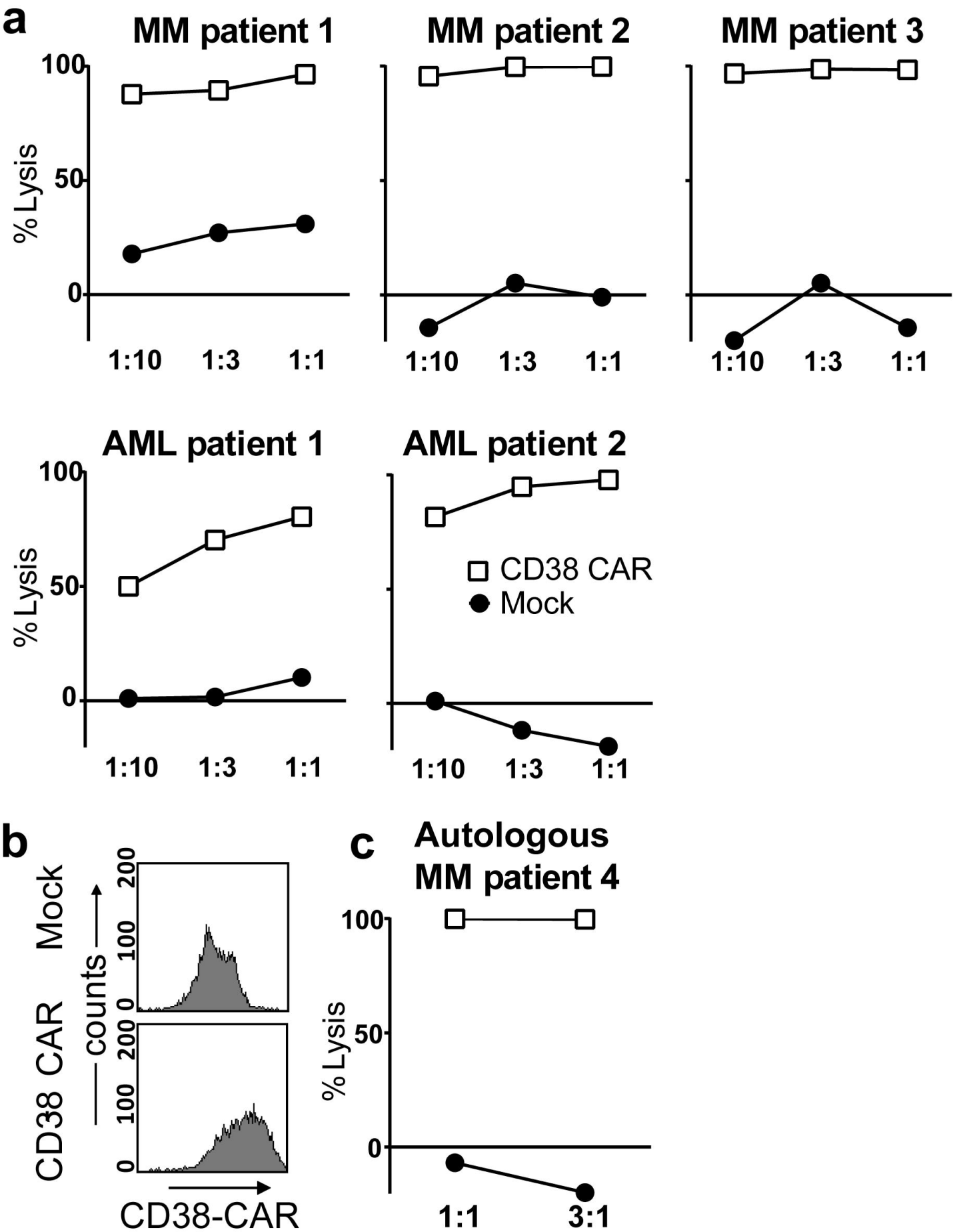

a
MM patient 1
MM patient 2
MM patient 3
AML patient 1
AML patient 2
% Lysis
100
50
0
1:10
1:3
1:1
CD38 CAR
Mock
b
CD38 CAR
Mock
counts
200
100
0
CD38-CAR
c
Autologous
MM patient 4
% Lysis
100
50
0
1:1
3:1

Figure 4

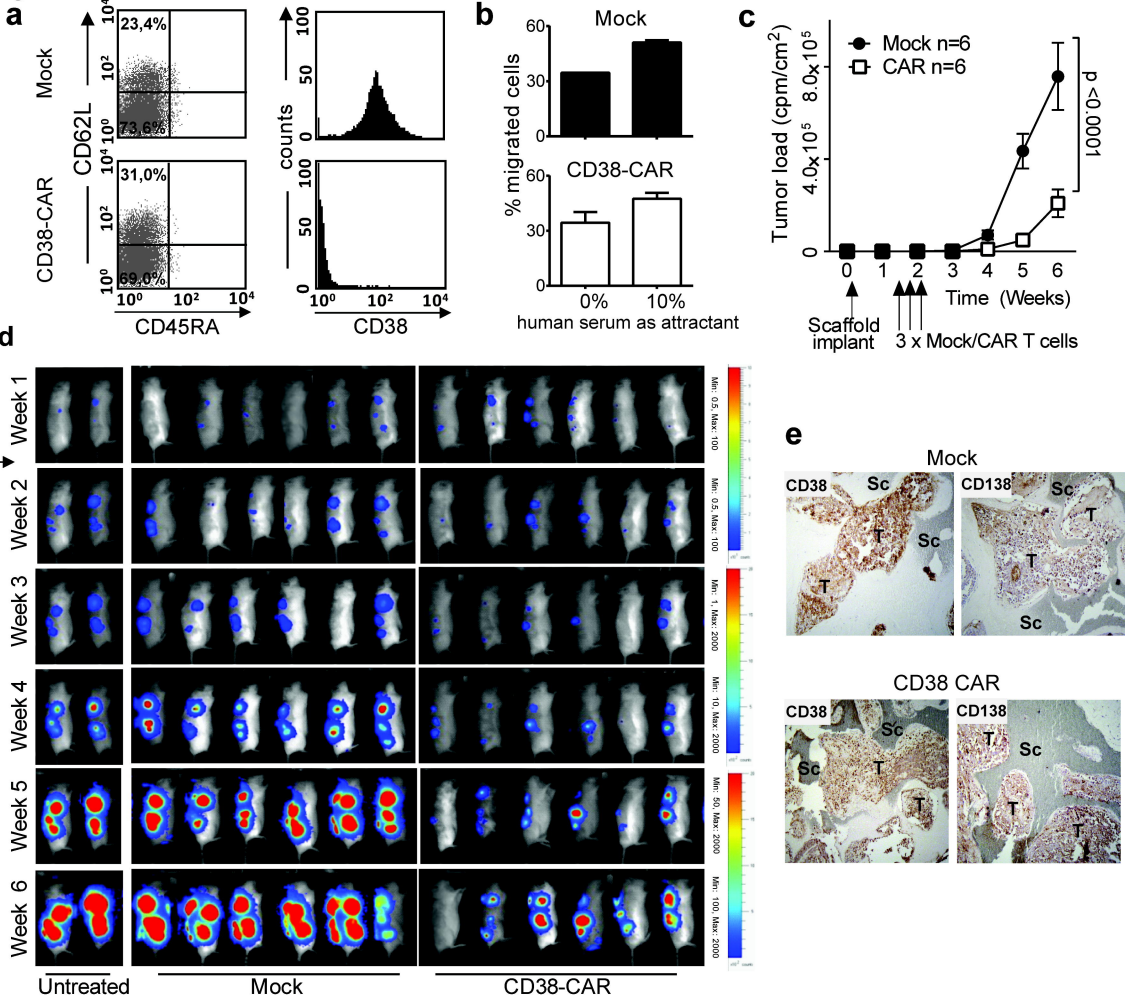

a
Mock
CD38-CAR
CD62L
CD45RA
23,4%
73,6%
31,0%
69,0%
counts
CD38
b
Mock
CD38-CAR
% migrated cells
0%
10%
human serum as attractant
c
Mock n=6
CAR n=6
p <0.0001
Tumor load (cpm/cm²)
Time (Weeks)
Scaffold implant
3 x Mock/CAR T cells
d
Week 1
Week 2
Week 3
Week 4
Week 5
Week 6
Untreated
Mock
CD38-CAR
e
Mock
CD38 CAR
CD38
CD138
Sc
T

Figure 5

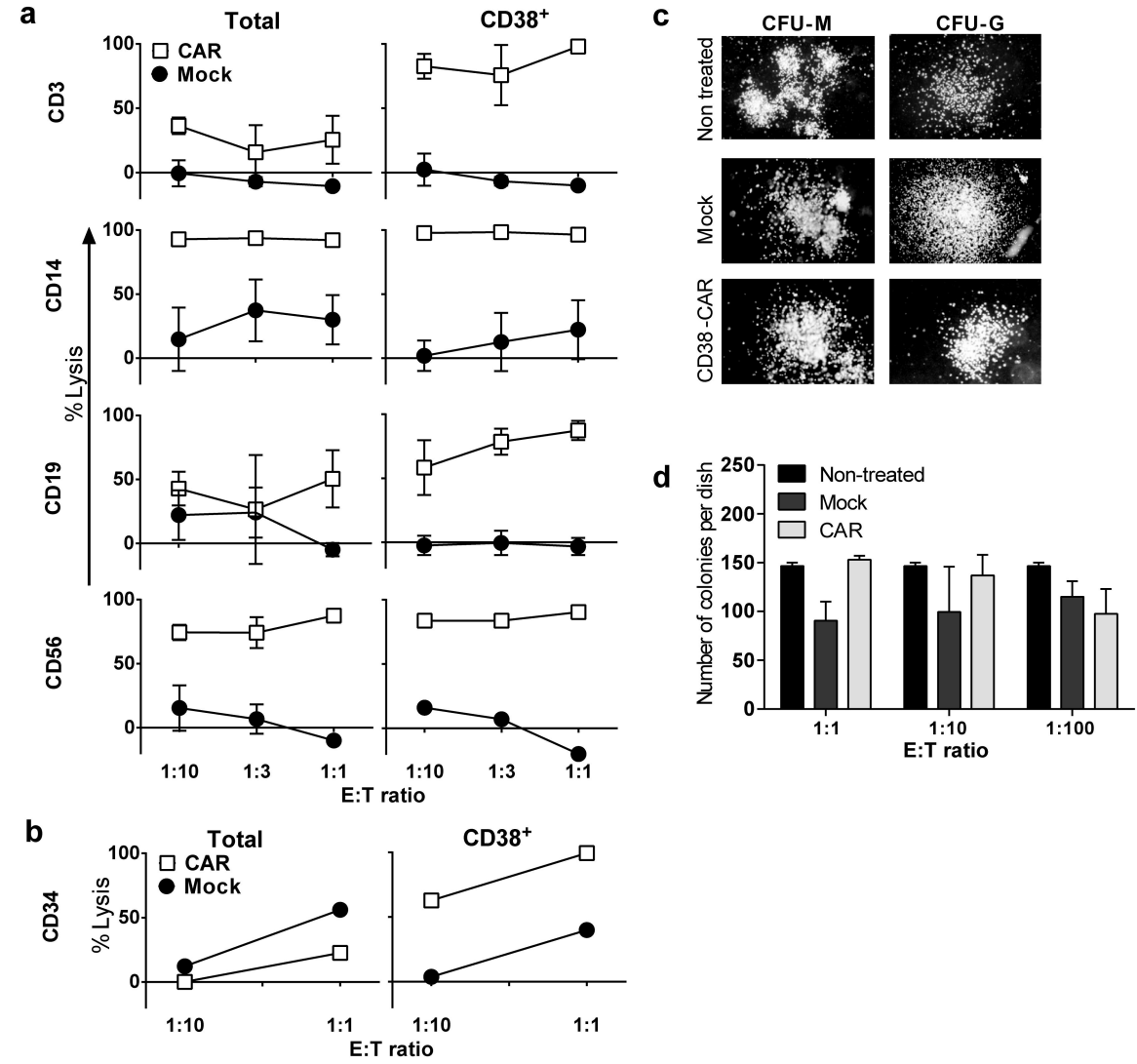
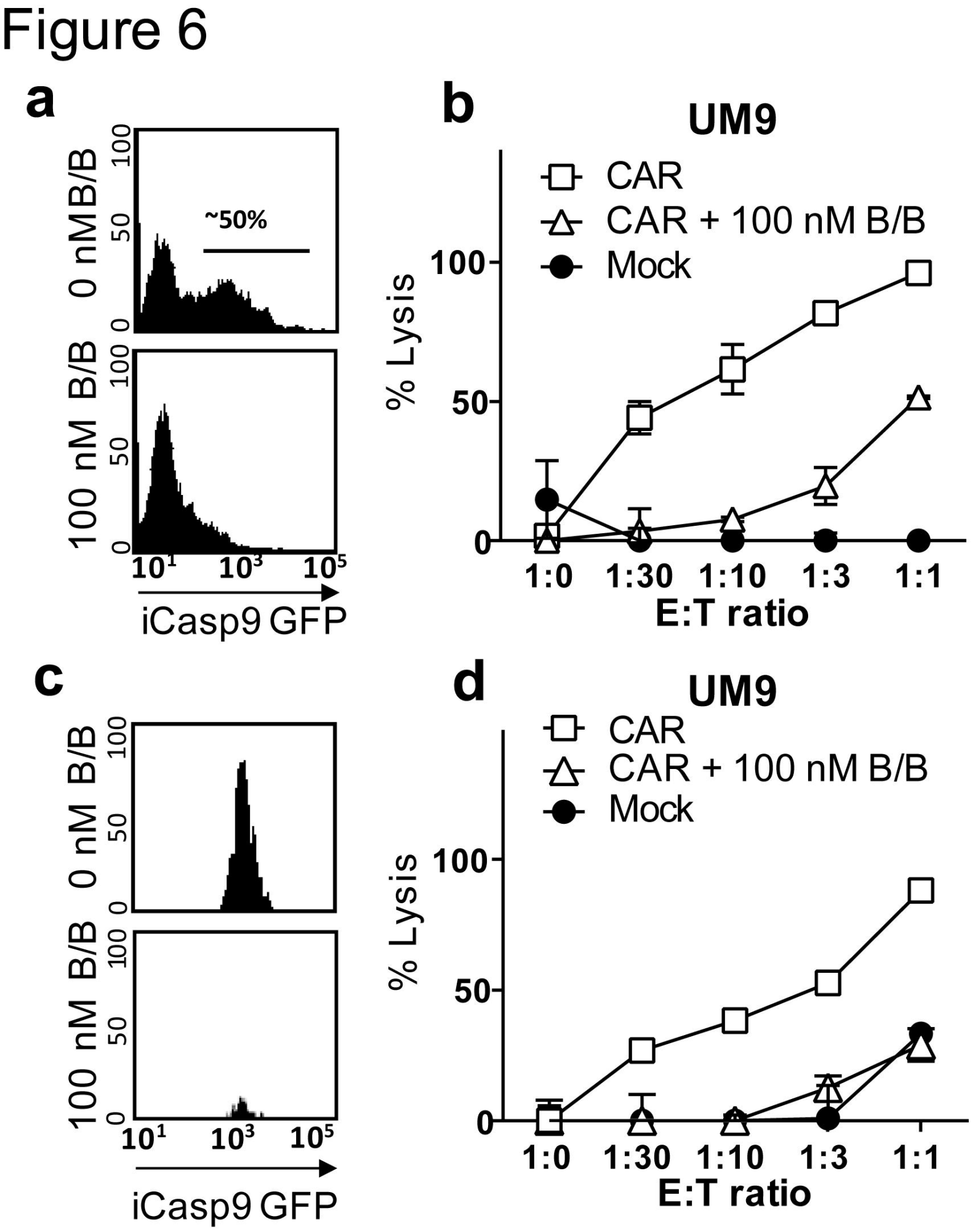

a
Total
CD38+
CAR
Mock
CD3
CD14
CD19
CD56
% Lysis
100
50
0
1:10
1:3
1:1
E:T ratio
b
CD34
c
CFU-M
CFU-G
Non treated
Mock
CD38 -CAR
d
Number of colonies per dish
250
200
150
Non-treated
Mock
CAR
1:100

Figure 6


a
0 nM B/B
100 nM B/B
~50%
100
50
0
10^1
10^3
10^5
iCasp9 GFP
b
UM9
CAR
CAR + 100 nM B/B
Mock
% Lysis
100
50
0
1:0
1:30
1:10
1:3
1:1
E:T ratio
c
0 nM B/B
100 nM B/B
100
50
0
10^1
10^3
10^5
iCasp9 GFP
d
UM9
CAR
CAR + 100 nM B/B
Mock
% Lysis
100
50
0
1:0
1:30
1:10
1:3
1:1
E:T ratio