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

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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 with which we transduced T cells from healthy donors and multiple myeloma patients. We then evaluated the pre-clinical efficacy and safety of the transduced T cells. 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 patients with acute myeloid leukemia and multi-drug resistant multiple myeloma 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 of CD34+ hematopoietic progenitor cells, monocytes, natural killer cells, and to a lesser extent T and B cells but did not inhibit the outgrowth of progenitor cells into various myeloid lineages and, furthermore, were 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 efforts to diminish the undesired effects of this immunotherapy using appropriate strategies.

Introduction

Multiple myeloma (MM), a 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 that MM could be successfully treated through (cellular) immunotherapy.[3,4] The curative potential of cellular

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immunotherapy in MM is illustrated by the induction of long-term sustained remissions after allogeneic stem cell transplantation or donor lymphocyte infusions 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 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 retroviral or lentiviral transduction of cytotoxic T cells, CAR expressed on the cell surface redirect the cytotoxic T cells toward the original target of the antibody in a non-HLA-restricted manner,[7,8] making it possible to apply the therapy regardless of the patient's HLA type. 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 the malignant plasma cells from patients with MM. 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 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 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-CAR. We transduced T cells from healthy individuals and MM patients with the CD38-CAR 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 introducing a caspase-9-based suicide gene.

Methods

Bone marrow mononuclear cells from patients with multiple myeloma or acute myeloid leukemia

Bone marrow mononuclear cells containing 5-20% malignant plasma cells or ~50% acute myeloid leukemia (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.

Peripheral blood mononuclear cells from healthy individuals

Peripheral blood mononuclear cells were isolated from the 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] (*Online Supplementary Table S1*) were kindly provided by Genmab. Cloning methods are described in the *Online Supplementary Methods.*

Retroviral chimeric antigen receptor transduction into T cells

Transduction methods are described in the *Online Supplementary Methods*.

Flow cytometry-based cell lysis assays

To detect the lysis of various cell subsets by CART cells in mononuclear cells from whole bone marrow or peripheral blood, serial dilutions of CART cells were incubated with CFSE-labeled bone marrow mononuclear cells or peripheral blood mononuclear cells for 24 h. 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%.

Bioluminescence imaging-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 h with a SpectraMax luminometer (Molecular Devices) within 15 min after the addition of 125 μg/mL beetle luciferin (Promega).[11] The percent lysis was then calculated as in the flow-based cytotoxicity assay above.

Experimental animals

RAG2$^{-/-}\gamma c^{-/-}$ 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-chimeric antigen receptor-transduced T cells against multiple myeloma 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 in the *Online Supplementary Methods*.

Results

Generation of CD38-chimeric antigen receptor-transduced T cells

We used the variable heavy and light chain sequences of three different CD38 antibodies with CD38 binding affinities comparable to that of daratumumab (*Online*

Supplementary Table S1), which is now being tested in clinical trials. T cells from healthy peripheral blood mononuclear cells 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 CAR 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 phycoerythrin-conjugated streptavidin revealed the expression of all three CAR on >95% of the T cells, whereas T cells transduced with an empty vector (mock-transduced T cells) displayed only background staining (Figure 1B, left panel). The CAR-transduced cells contained variable levels of both CD4+ and CD8+ cells (Figure 1B, right panel).

CD38-dependent proliferation and cytokine secretion of CD38-chimeric antigen receptor-transduced T cells

To analyze their proliferative and functional properties, neomycin-selected, highly purified CD38-CART cells were expanded using irradiated feeder cells in the presence of phytohemagglutinin and interleukin-2. While the mock T cells initially expanded better than the CD38-CART cells (Figure 1C, left panel), the growth disadvantage of CD38-CART cells disappeared in the second round of expansion (Figure 1C, right panel), indicating that transduction of the 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 the irradiated CD38+ MM cell line UM9 and used the CD38- MM cell line U266 as a control (Figure 1D, left panel). CD38-CART cells, but not mock T cells, specifically proliferated and produced interferon-γ, tumor necrosis factor-α and interleukin-2 (Figure 1D, right panel), but not interleukin-4, -5 or -10 (*data not shown*) upon stimulation with UM9 cells. These results indicate 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 multiple myeloma cell lines by CD38-chimeric antigen receptor-transduced T 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 bioluminescence imaging-based cytotoxicity assays.[11,14] As expected, there was no CD38-CAR-specific lysis of the CD38- U266 cell line (Figure 2A). In contrast, all three types of CD38-CART cells, but not mock T cells, effectively lysed the CD38+ MM cell line UM9 in a cell-dose dependent manner (Figure 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-CAR (028, 056, 026), we continued our investigation with one type of CD38-CART cell (CAR056). Flow cytometry and bioluminescence imaging-based cytotoxicity assays, performed using other malignant cell lines expressing various levels of CD38 (*Online Supplementary Figure S1*) as target cells, revealed a good correlation between CD38 expression and CD38-CART cell-mediated lysis (Figure 2C). 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 (Figure 2C).

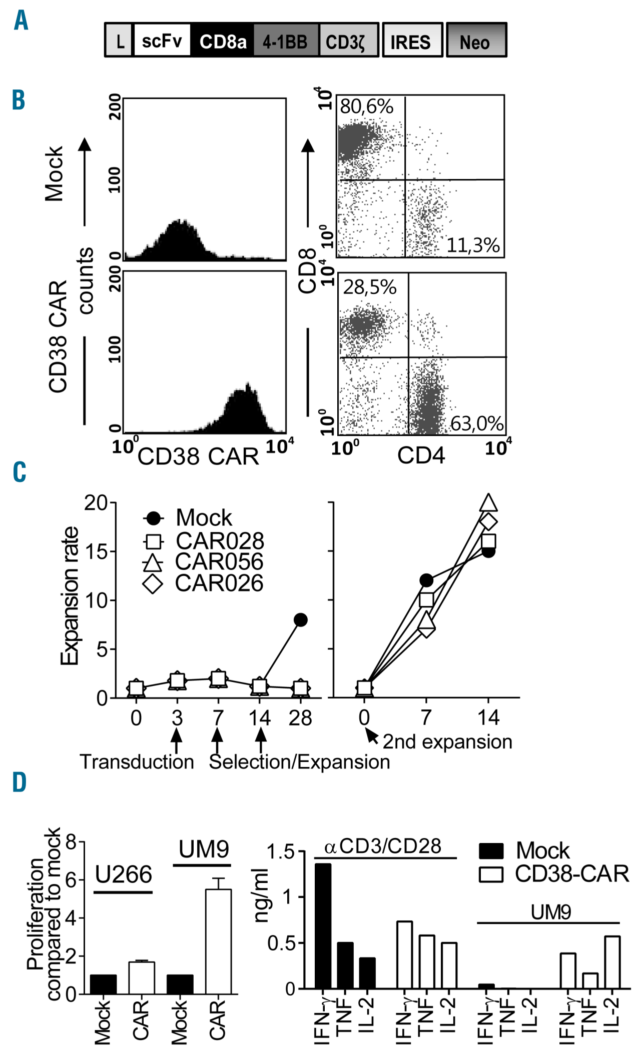

Figure 1. CD38-CAR construct and CD38-CART-cell phenotype. (A) Schematic overview of the CD38-CAR construct. The CD38-scFv sequence is based on three different antibody sequences (028, 056 and 026, see also *Online Supplementary Table S1*), with CD8a as a transmembrane domain and 4-1BB and CD3ζ as intracellular 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 phycoerythrin-labeled streptavidin. The results for CD38-CART cells generated with CAR056, representative of all three CAR, are shown. The expression of surface markers CD4 and CD8 (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 mock and CD38-CART cells stimulated with αCD3/CD28 beads or the MM cell line UM9. The cytokine secretion was measured with a flow cytometry-based CBA kit (BD) in the cell-free supernatants after 24 h of stimulation. The graph shows the secretion of interferon (IFN)-γ, tumor necrosis factor (TNF) and interleukin (IL)-2. Secretion of IL-4, -5 and -10 was below the detection limits. These data are not therefore shown in this figure. Similar results were obtained in two independent assays.

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Lysis of primary multiple myeloma and acute myeloid leukemia cells by CD38-chimeric antigen receptor-transduced T 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 bone marrow mononuclear cells without isolating them from other cells.[15] As depicted in Figure 3A, primary CD138+CD38+ MM cells from 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 bone marrow mononuclear cells of two AML patients' malignant cells, which were identified as CD13+ CD45+ cells and expressed either low/intermediate (patient 1) or high (patient 2) levels of CD38, were effectively lysed by CD38-CART cells (Figure 3A). Finally, CD38-CART cells that were generated (Figure 3B) from a MM patient were effective towards autologous malignant MM cells in bone marrow mononuclear cells, indicating the feasibility of generating effective CD38-CART cells also from MM patients.

Fully functional CD38-chimeric antigen receptor-transduced T cells are negative for CD38

While CD38-CART cells had no apparent functional deficiencies, a phenotyping assay revealed that, despite a mixed effector/central memory phenotype, they lost the expression of CD38 (Figure 4A). Interestingly, when we co-cultured CD38-CART cells with an autologous CD19-CART cell population, these CD19-CART 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 (*Online Supplementary Figure S2*), indicating that the loss of CD38 was not associated with detectable T-cell dysfunction. Nonetheless, since the CD38 molecule could also play a role in migration, we evaluated whether CD38- CD38-CART cells could migrate properly through endothelial layers in a transwell migration assay (Figure 4B). These assays revealed no differences between the mock-transduced, CD38+ and CD38-CAR-transduced CD38- T cells, ruling out an apparent migratory dysfunction of CD38-CART cells.

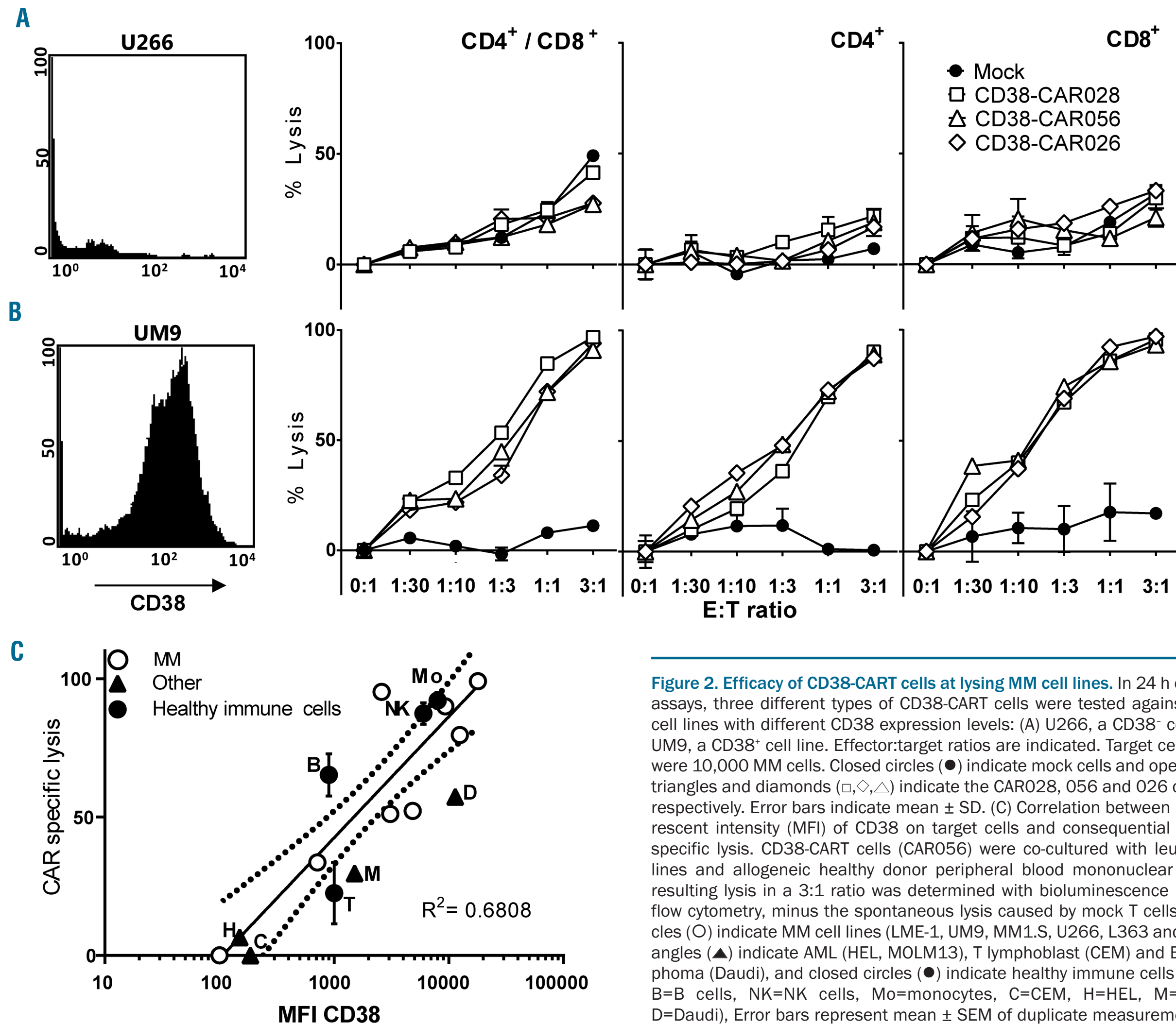

Figure 2. Efficacy of CD38-CART cells at lysing MM cell lines. In 24 h cytotoxicity assays, three different types of CD38-CART cells were tested against two MM cell lines with different CD38 expression levels: (A) U266, a CD38- cell line, (B) UM9, a CD38+ cell line. Effector:target ratios are indicated. Target cells per well were 10,000 MM cells. Closed circles (●) indicate mock cells and open squares, triangles and diamonds (□,◇,△) indicate the CAR028, 056 and 026 constructs, respectively. 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 peripheral blood mononuclear cells. The resulting lysis in a 3:1 ratio was determined with bioluminescence imaging 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), and 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.

In vivo efficacy of CD38-chimeric antigen receptor-transduced T cells against multiple myeloma tumors growing in a humanized microenvironment

To substantiate the *in vitro* results, we questioned whether the CD38- CART cells could mediate anti-MM effects *in vivo* after systemic injection in our recently developed model in Rag2$^{-/-}\gamma c^{-/-}$ mice, in which a humanized bone marrow-like niche for MM cells is generated by subcutaneous implantation of ceramic scaffolds coated with human bone marrow stromal cells[11,12] (Figure 4). Thus, we implanted such scaffolds seeded with luciferase-transduced UM9 MM cells in the back of the mice (6 scaffolds per mouse). Upon detection of the luciferase signal by bioluminescence imaging, we treated the mice with intravenous 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 fast progression. Although not curative, treatment of the tumor-bearing mice with CD38-CART cells induced a significant anti-tumor effect (Figure 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 (Figure 4D), thus ruling out tumor escape due to "antigen loss" variants.

Impact of CD38-chimeric antigen receptor-transduced T cells on CD38+ normal hematopoietic cells and hematopoietic progenitor cells

Besides the high levels expressed 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, natural killer cells, B cells and monocytes. We, therefore, evaluat-

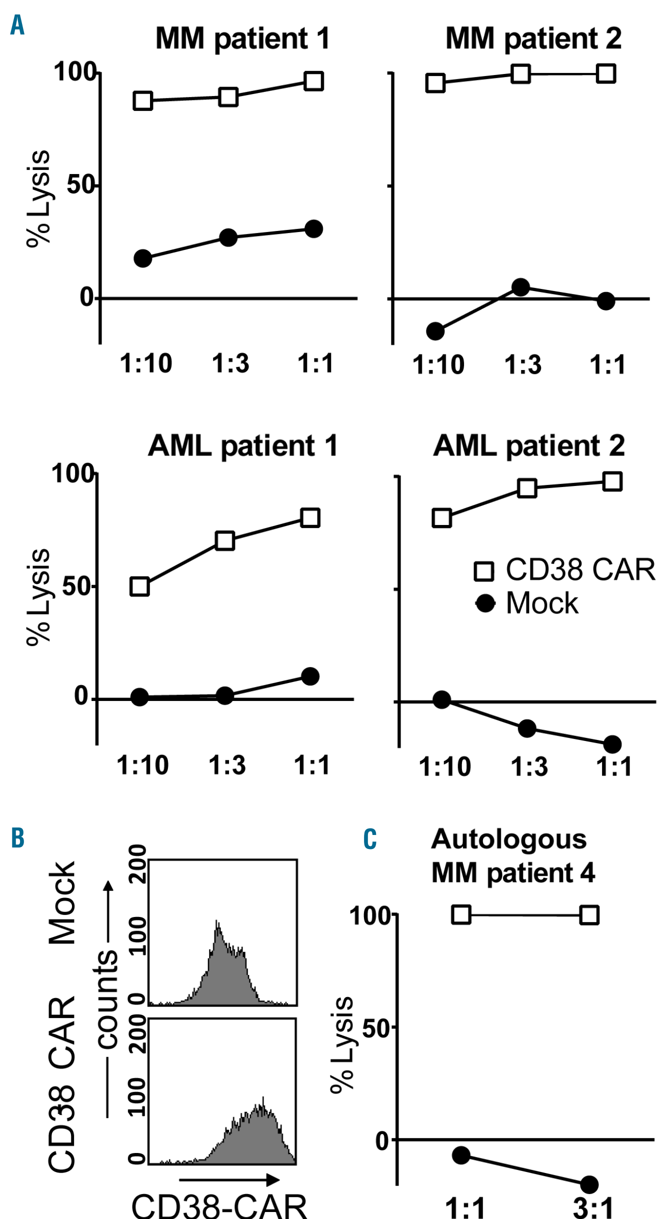

Figure 3. Efficacy of CD38-CART cells generated from healthy individuals at lysing primary MM cells. (A) Bone marrow-derived mononuclear cells from three MM patients, all three refractory to lenalidomide and bortezomib, and bone marrow mononuclear cells from two AML patients were co-incubated with no,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

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ed the possible negative impact of CART cells on these cell subsets by co-incubating unsorted bone marrow mononuclear cells with CD38-CART cells. CD38-CART cells appeared to eliminate the CD38+ fractions of mature T, B, natural killer and monocyte cell subsets (Figure 5A) and the CD38+ fraction of CD34+ cells (Figure 5B) in a 4 h assay. The lysis of CD34+CD38+ cells did not, however, have any influence on the development of colony-forming units of monocytes or of granulocytes in a 14-day hematopoietic precursor cell colony-forming assay[18,19] (Figure 5C,D).

Specific elimination of CD38-chimeric antigen receptor-transduced T cells using a suicide gene (iCasp9)

Although CD38-CART cells did not lyse the CD38- fractions of mature hematopoietic cells and did not inhibit the outgrowth of these cell populations, a cautious approach toward the clinical application of this construct is still required. As a first step towards safer application of CD38-CART cells, we tested the possibility of controlling 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

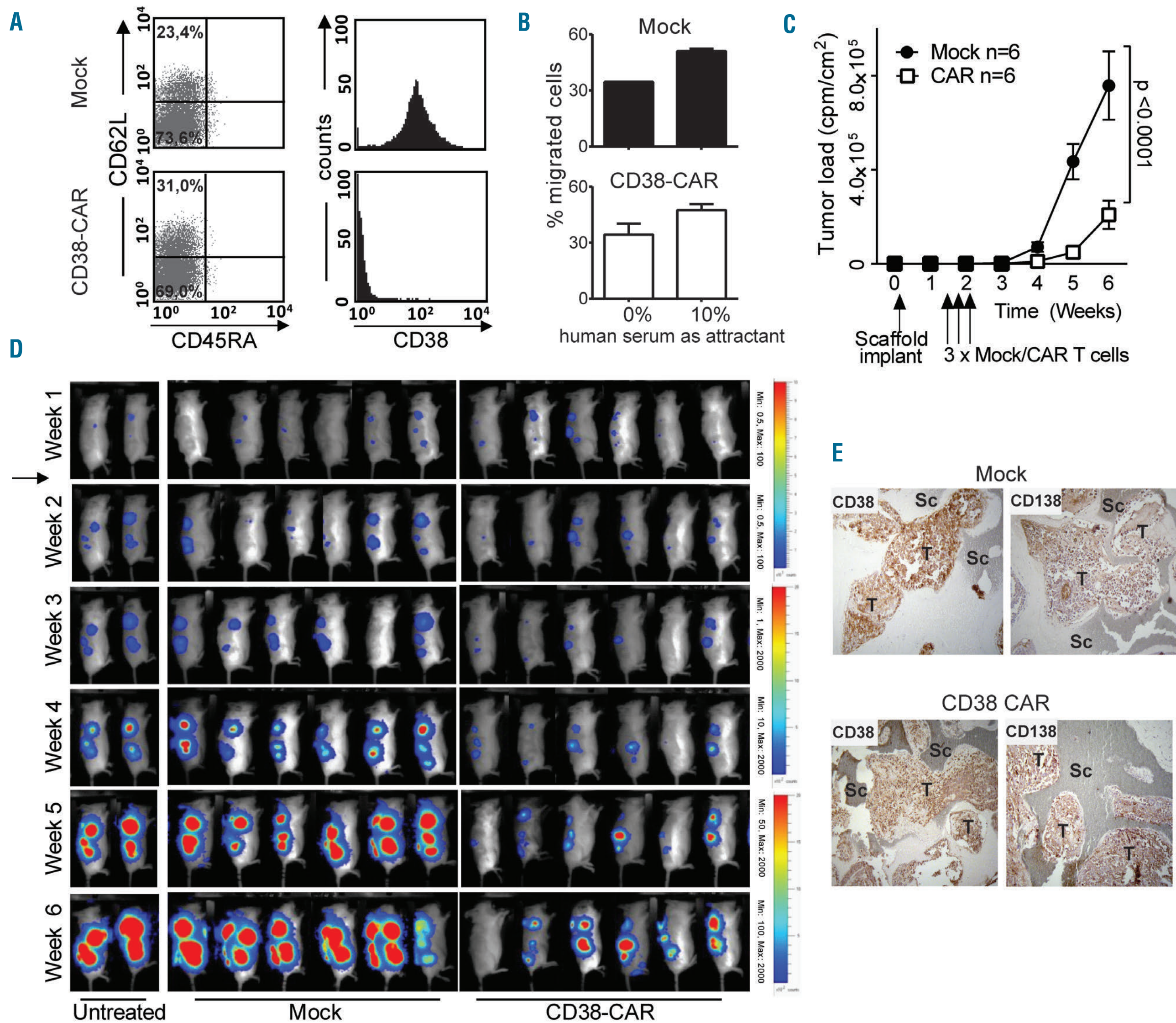

Figure 4. Tumor growth in mock- and CD38-CART-cell-treated mice. (A) Analysis of CD38-CART cells after 2 weeks of *in vitro* culture, with fluorescence-labeled monoclonal antibodies for CD45RA and CD62L and CD38. (B) Leukocyte transmigration assay, in which mock and CART cells were cultured in a transwell system in the inserts with human umbilical vein endothelial cells, which were activated with tumor necrosis factor (TNF)-α. Spontaneous TNFα-induced transmigration was compared to active migration induced by 10% 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 bioluminescent imaging measurements. Each group contained six mice, each harboring six scaffolds. Results are mean tumor load (cpm/cm^2) of six mice per group. Closed circles (●) indicate mock cells and open squares (□) indicate CAR056 cells. The error bars represent mean + SEM, n=6. The differences between groups were analyzed after week 6 using an unpaired Student T test, P<0.0001 (D) Bioluminescent imaging of mice on the right side; mice were implanted with fully humanized bone marrow stromal cell scaffolds each coated with 1×10^6 UM9-GFP-Luc tumor cells. At 7, 9 and 13 days after implantation, mice were injected intravenously with 20×10^6 mock or CD38-CART cells. (E) Representative immunohistochemistry figure: remaining tumors were stained with CD38 and CD138 antibody, T = tumor, sc = scaffold.

vector containing a green fluorescent protein (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 (Figure 6A, lower panel). As expected, the dimerizer treatment also resulted in a proportional decrease in the lysis of the MM cell line UM9. (Figure 6B). There was still some remaining lysis due to the surviving iCasp9-negative CD38-CART cells, indicating that triggering the suicide gene did not induce bystander damage to the cells in the close vicinity. When tested after sorting for GFP+ cells (Figure 6C,D), almost all GFP+ cells died after treatment with the dimerizer (Figure 6C) and there was no CD38-specific lysis left (Figure 6D), confirming the results obtained in previous studies,[20,21] and suggesting the possibility of controlling CD38-CART cells using the iCasp9 suicide gene without undesired consequences.

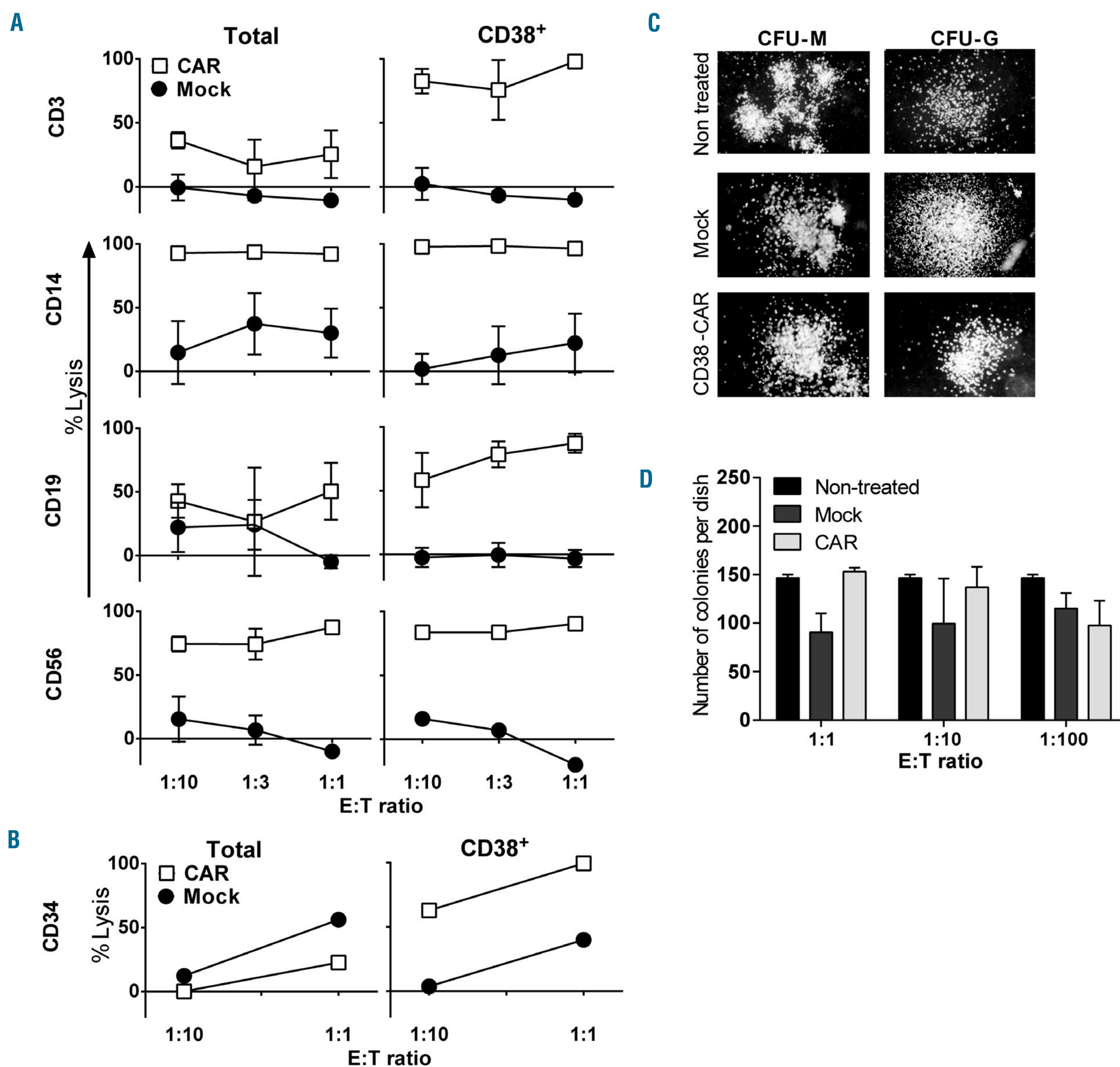

Figure 5. The impact of CD38-CART cells on non-malignant hematopoietic cells in bone marrow and outgrowth of hematopoietic cell lineages. (A) Bone marrow mononuclear cells from three MM patients were co-incubated with none, mock- or CD38-CART cells for 16 h. The graphs depict the resulting lysis of the total or the CD38+ fractions of CD3+ (T cells), CD56+ (mainly natural killer cells), CD14+ (monocytes) and CD19+ (B cells) subsets at three effector:target rations, determined with flow cytometry and calculated as described in the Methods section. Results are from three individual experiments combined. Closed circles (●) indicate mock cells and open squares (□) indicate CAR056 cells. Error bars represent mean ± SEM, n=3. (B) CD34+ fraction of bone marrow mononuclear cells from healthy donors was co-incubated with none, mock- or CD38-CART cells for 4 h at different target:effector cell ratios before being transferred into the semisolid hematopoietic progenitor cell culture medium. After 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 cells and open squares (□) indicate CAR056 cells. (C) After 14 days of culture in plastic dishes, colony-forming unit-monocytes (CFU-M), and CFU-granulocytes (CFU-G) were visible. (D) The numbers of CFU-M and CFU-G colonies were determined microscopically. Results of a representative experiment are shown as mean ± SD.

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Discussion

While cellular immunotherapy of hematologic malignancies has been applied for many decades in the most non-specific form as allogeneic stem cell transplantation or donor lymphocyte infusions, 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 T-cell receptor-gene transferred T cells, tumor infiltrating T cells and more recently with cytotoxic T cells endowed with tumor-reactive CAR. Among these strategies, CAR-based therapies are perhaps the most appealing, as CART cells recognize their target antigens in an MHC-independent manner. Setting out to develop a CAR-based strategy for MM, we have been encouraged by the highly promising clinical results of therapy with daratumumab, which targets CD38, a type II transmembrane glycoprotein, expressed at high and uniform levels in most, if not all, MM cells in all stages of the disease.[10] Daratumumab has recently been administered to several patients at moderate to high doses and for prolonged periods with little or no toxicity, despite the fact that the 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-CAR, we started the study using three distinct human CD38 antibodies, which showed similar binding affinities to CD38 as that of daratumumab (*Online Supplementary Table S1*). Based on successful usage of 4-1BB-containing CAR in recent studies[22–24] we constructed CAR containing 4-1BB (CD137) co-stimulatory and CD3ζ activating domains. Our results demonstrate the successful generation of CD38-CAR and CD38-CART cells regardless of the antibody sequences. T cells transduced with these CD38-CAR 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 from patients who had become resistant to various chemotherapies. This suggests that CD38-CAR therapy could be a viable choice for patients with few 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⁻, but long-term cultured

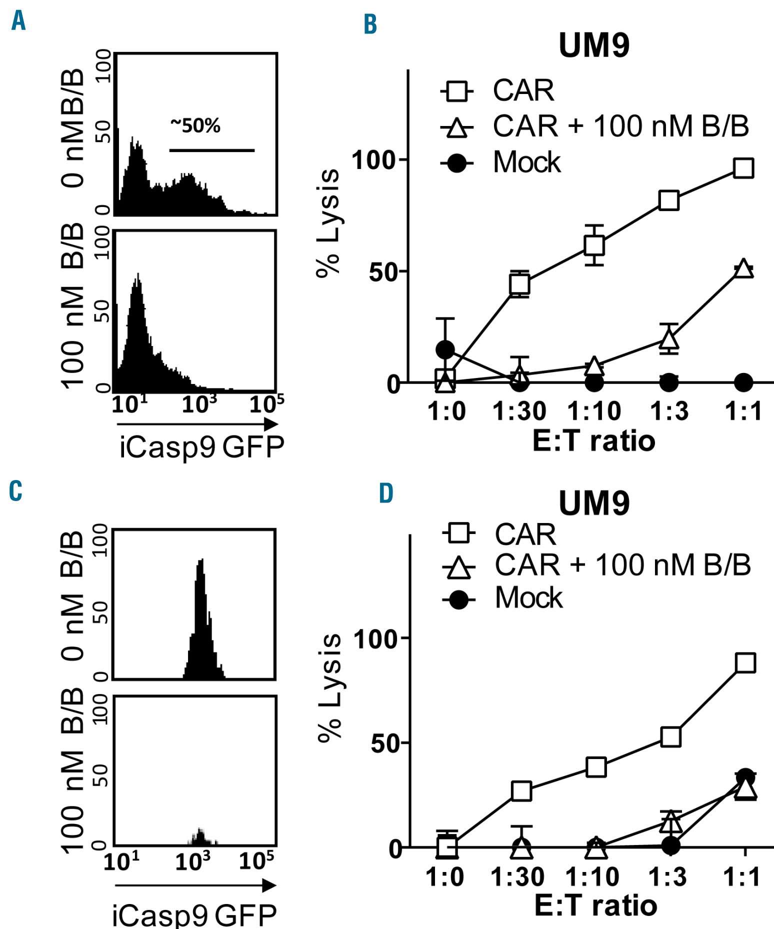

Figure 6. Dimerizer AP20187-mediated elimination of the iCasp9 suicide gene-transduced CD38-CAR T cells. (A) Flow cytometry histogram plots: CD38-CAR T cells that were additionally transduced with the iCasp9-GFP construct. The upper panel shows the untreated cells: 50% GFP⁺; the lower panel shows the cells treated with 100 nM dimerizer AP20187 (B/B). (B) Lysis of the 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 gene-transduced cells (50% of all CAR⁺ cells in (A). The residual cytolysis is thus caused by the CAR⁺ cells that were not transduced with iCasp9 n=2, mean ± SD. (C) CD38-CART iCasp9-GFP^{high} sorted cells. The upper panel shows the untreated cells 100% GFP⁺; the lower panel shows the cells treated with 100 nM dimerizer B/B. (D) Lysis of the UM9 cell line by iCasp9^{high}-CD38-CART cells that were untreated or treated with the dimerizer. Closed circles (●) indicate mock cells and open diamonds (◇) and triangles (△) indicate CAR056 without and with B/B, respectively. n=2, mean ± SD.

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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 importantly, in our model, unlike all previously reported CAR studies, the human MM tumors were grown to larger masses in a fully humanized bone marrow 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 the efficacy of CART-cell treatment could be improved if the therapy were to 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 induced by the 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 to identifying the potential drawbacks and risks of 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$^-$ fractions of important immune cells, such as B and T cells, were also unaffected. These results suggest that the therapy will spare sufficient numbers of T and B cells for these 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 determine whether an intact immune response would be possible in the absence of CD38. A partial solution to this issue came from the analyses of CD38-CART cells: remarkably, we discovered that the CD38-CART cells, regardless of which single chain variant fragment 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$^-$ CD38-CART cells, however, had no growth disadvantage, had a highly activated status, displayed CD38-dependent proliferation, cytokine production, and cytotoxic activities and showed no other detectable functional aberrancies. This was also the case for CD19-CART cells which became CD38$^-$ after co-culture with CD38-CART cells (*Online Supplementary Figure S2*). Furthermore CD38-CART cells did not show any defects in transmigration assays and they also mediated significant anti-MM effects *in vivo*, thus indicating their capacity to migrate properly 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$^+$, and (ii) CD38 expression is not essential for T cells to fulfill their functions. This latter conclusion is also supported by the fact that there is still no evidence, even from CD38 knockout mice,[32] that CD38-deficient effector T cells are functionally defective.

On the other hand, the relatively broad expression of the target antigen of CD38-CART cells increases the risk of the so-called "cytokine release syndrome" due to massive activation of CART cells, as has been observed in previous trials with ERBB2- and CD19-CART cells.[35–37] Although the interleukin-6 receptor antagonist tocilizumab appears to reduce cytokine release syndrome[38] it would still be desirable to minimize the occurrence of such severe side effects. Furthermore, since we cannot rule out toxicities occurring due 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 of CD38-CART cells, and probably also in the case of other CART-cell approaches targeting the 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 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 iCasp9 gene[20,46] can effectively control CART cells. These results, which are in agreement with those of other studies,[20,45,47] provide positive prospects for 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.

Considering all the data 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. These results warrant further studies aimed at diminishing the undesired effects of CD38-CART cells against normal CD38$^+$ cells through optimizing the formers' CD38 affinity and improving *in vivo* controllability.

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