All-trans retinoic acid works synergistically with the γ-secretase inhibitor crenigacestat to augment BCMA on multiple myeloma and the efficacy of BCMA-CAR T cells

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Abstract

B-cell maturation antigen (BCMA) is the lead antigen for chimeric antigen receptor (CAR) T-cell therapy in multiple myeloma (MM). A challenge is inter- and intra-patient heterogeneity in BCMA expression on MM cells and BCMA downmodulation under therapeutic pressure. Accordingly, there is a desire to augment and sustain BCMA expression on MM cells in patients that receive BCMA-CAR T-cell therapy. We used all-trans retinoic acid (ATRA) to augment BCMA expression on MM cells and to increase the efficacy of BCMA-CAR T cells in pre-clinical models. We show that ATRA treatment leads to an increase in *BCMA* transcripts by quantitative reverse transcription polymerase chain reaction and an increase in BCMA protein expression by flow cytometry in MM cell lines and primary MM cells. Analyses with super-resolution microscopy confirmed increased BCMA protein expression and revealed an even distribution of non-clustered BCMA molecules on the MM cell membrane after ATRA treatment. The enhanced BCMA expression on MM cells after ATRA treatment led to enhanced cytolysis, cytokine secretion and proliferation of BCMA-CAR T cells *in vitro*, and increased efficacy of BCMA-CAR T-cell therapy in a murine xenograft model of MM *in vivo* (NSG/MM.1S). Combination treatment of MM cells with ATRA and the γ-secretase inhibitor crenigacestat further enhanced BCMA expression and the efficacy of BCMA-CAR T-cell therapy *in vitro* and *in vivo*. Taken together, the data show that ATRA treatment leads to enhanced BCMA expression on MM cells and consecutively, enhanced reactivity of BCMA-CAR T cells. The data support the clinical evaluation of ATRA in combination with BCMA-CAR T-cell therapy and potentially, other BCMA-directed immunotherapies.

Introduction

Chimeric antigen receptor (CAR) T-cell therapy directed against B-cell maturation antigen (BCMA) is under clinical investigation for treating multiple myeloma (MM). Several trials have reported efficacy of BCMA-CAR T-cell therapy[1–4] and as of June 2022, two BCMA-CAR T-cell products have received clinical approval.[5] In the KarMMa study, Idecabtagene vicleucel has achieved an overall response rate (ORR) of 73%, and the mean progression-free survival (PFS) was 8.8 months.[6] In the Cartitude-1 study, Ciltacabtagene autoleucel has achieved an ORR of 97%, and the mean PFS had not been reached at 24 months.[7] Even though the ORR, in particular for Ciltacabtagene autoleucel, is very high and the PFS exceeds by far the benchmarks that have been set in heavily pretreated MM patients by other treatment modalities, many patients ultimately relapse. There is an ongoing correlative research effort to delineate the impact of BCMA antigen density and BCMA downmodulation on MM cells, as well as BCMA-CAR T-cell engraftment and persistence on outcome. Based on the available data with CAR T cells from preclinical and clinical development, the prevailing paradigm is that higher antigen density and higher CAR T-cell frequency is expected to correlate with favorable clinical outcome.[8–10]

BCMA is a tumor necrosis family receptor, that is physio-

logically expressed on plasma cells, their precursors and polyclonal plasmablasts, and that is absent on hematopoietic stem cells and non-hematopoietic cells and tissues.[11,12] BCMA expression has been demonstrated at the gene and protein level on malignant plasma cells from treatment-naïve and previously treated MM patients.[11–13] There is considerable inter- and intra-patient variation in BCMA expression, making BCMA hardly detectable by conventional analyses such as flow cytometry and immunohistochemistry in some patients.[1,15,16] The range in BCMA antigen density (i.e., molecules per MM cell) that has been reported in several studies has raised concerns that BCMA expression on at least a subset of MM cells may be insufficient for appropriate BCMA-CAR T-cell recognition and stimulation.[17] Indeed, antigen density on target cells has been identified as a key parameter that determines CAR T-cell function and therapeutic efficacy.[18–20] Staggered thresholds in antigen density are required for inducing cytolytic activity, cytokine secretion and proliferation.[21–23] These preclinical assessments are supported by clinical studies showing that low antigen density correlates with inferior clinical outcome after CAR T-cell therapy.[8–10]

The principle strategies for modulating antigen expression on MM cells are regulation at the gene level and at the protein level. At the protein level, γ-secretase inhibitors (GSI) have been shown to increase the level of BCMA expression on myeloma cell lines and on primary myeloma cells.[17] In order to accomplish regulation at the gene level, we focused on all-trans retinoic acid (ATRA). ATRA is approved for the treatment of acute promyelocytic leukemia. Retinoic acids can influence gene expression and protein production in mammalian cells.[24] ATRA induces changes in post-translational modifications such as histone acetylation in tumor cells.[25–28] These epigenetic changes have been shown to augment CD38 expression on MM cells and subsequently, enhanced efficacy of the CD38-targeting antibody daratumumab.[25,29,30]

Here, we analyzed the effect of ATRA treatment on BCMA expression on MM cell lines and primary MM cells. We measured BCMA expression on MM cells after ATRA treatment by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and flow cytometry and applied super-resolution microscopy for antigen quantification and distribution analyses on the MM cell surface. We further examined the recognition of MM cells by BCMA-CAR T-cells after ATRA treatment *in vitro* and in a murine xenograft model of MM *in vivo*.

Methods

Human subjects

Peripheral blood and bone marrow samples were obtained from healthy donors and patients after written informed consent to participate in research protocols approved by the Institutional Review Boards of the University of Würzburg.

Cell lines

The OPM-2, NCI-H929 and MM.1S cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The OPM-2 and MM.1S cell lines were modified with firefly-luciferase_GFP by lentiviral transduction.

All-trans retinoic acid and crenigacestat treatment

Myeloma cells were cultured in RPMI-1640 (Gibco, Darmstadt, Germany) supplemented with 10% fetal bovine serum at $1x10^6$ cells/mL, 1% glutamin and 1% penicillin and streptomycin (all from Gibco). ATRA (Sigma Aldrich, Darmstadt, Germany) and crenigacestat (LY3039478, MedChemExpress, New Jersey, USA) were reconstituted in dimethyl sulfoxide and added to the medium to a final concentration of 25, 50 or 100 nM (ATRA) and 10 nM (crenigacestat), respectively.

CAR T-cell preparation

A codon-optimized single chain variable fragment (scFv) comprising the variable heavy (VH) and variable light (VL) chain of anti-BCMA mAb BCMA50,[31] separated by a (G4S)3 linker, was synthesized (GeneArt, Regensburg, Germany) and fused to a spacer and transmembrane domain, a 4-1BB_CD3ζ signaling module, a T2A element and a truncated epidermal growth factor receptor (EGFRt) in an epHIV7 lentiviral vector backbone.[32] Human $CD4^+$ and $CD8^+$ T cells were isolated, activated with CD3/CD28 Dynabeads (Gibco) and transduced. CAR-modified T cells were enriched using the EGFRt marker and expanded with irradiated $BCMA^+$ feeder cells.

Flow cytometry

Bone marrow mononuclear cells were stained with anti-CD38-BV421 and anti-CD138-FITC monoclonal antibodies (mAb) (Biolegend, Koblenz, Germany; Clone: HIT2 and DL-101, respectively) to identify malignant plasma cells and anti-BCMA-APC mAb (Biolegend; Clone: 19F2) or isotype control (Biolegend; mouse IgG2a,κ) according to the manufacturer's instructions. Cell viability was assessed by 7-AAD staining (BD, Heidelberg, Germany). T cells were stained with anti-EGFR-APC mAb (Biolegend, Clone: AY13) to detect the EGFRt transduction marker expressed in the CAR transgene cassette. Flow cytometry was done on a Canto II (BD) or a MACSquant-10 (Miltenyi Biotec, Bergisch-Gladbach, Germany) and data analyzed using FlowJo software (TreeStar, Ashland, OR).

Super-resolution microscopy

Myeloma cells were stained with anti-BCMA-AF647 or -

BV421, anti-CD138-FITC antibodies or isotype controls (Biolegend; Clones: 19F2 and MI15). *d*STORM (direct stochastic optical reconstruction microscopy) images were acquired on an Olympus IX-71 inverted microscope and reconstructed using the software rapidSTORM3.314. Quantification of BCMA was performed using a custom script written with Mathematica.[23,33]

In vitro studies with CAR T cells

Cytolytic activity was analyzed in a bioluminescence-based assay using firefly luciferase (ffluc)-transduced target cells and distinct effector to target cell (E:T) ratios. Proliferation of T cells was analyzed by CFSE dye dilution after a 72-hour co-culture with target cells (E:T ratio of 4:1). IFNγ and IL-2 secretion were measured by enzyme-linked immunosorbant assay (ELISA) (Biolegend) in supernatants obtained after a 20-hour co-culture of T cells with target cells (E:T ratio of 4:1).

In vivo studies with myeloma cells and CAR T cells

The University of Würzburg Institutional Animal Care and Use Committee approved all mouse experiments. Six- to 8-week old female NSG (NOD-scid IL2rγnull) mice were obtained from Charles River (Sulzfeld, Germany), inoculated by tail vein injection with $2x10^6$ MM.1S/ffluc_GFP and randomly allocated to the treatment groups. ATRA was formulated in PEG300, Tween80 and saline (8:1:9) and administered by intraperitoneal (i.p.) injection (30 mg/kg). Crenigacestat was formulated in PEG300 (Sigma Aldrich), Tween80 (Sigma Aldrich) and saline (8:1:9) and administered by i.p. injection (1 mg/kg). BCMA-CAR-modified and control non-CAR modified T cells were administered i.v. by tail vein injection ($1×10^6$ T cells, i.e., $0.5×10^6$ $CD4^+$ and $0.5×10^6$ $CD8^+$). Bioluminescence imaging (BLI) was performed on an IVIS Lumina (Perkin Elmer, Waltham, MA) following i.p injection of D-luciferin (0.3 mg/g body weight) (Biosynth, Staad, Switzerland), and data analyzed using Living Image software (Perkin Elmer).

Statistical analyses

Statistical analyses were performed using Prism software v6.07 (GraphPad, San Diego, California). Unpaired and paired *t*-tests were used to analyze data from *in vitro* experiments and from experiments on BCMA expression *in vivo*. *P* values<0.05 were considered statistically significant. For all other *in vivo* experiments, statistical analysis was performed with SPSS 28. The measurements per group were described by mean and standard deviation. Normality was checked by qq plots. As the qq plots showed no normal distributed data and due to the small sample size, non-parametric Mann Whitney U tests were used as significance test for comparing the measurements between groups. The CAR T-cell group was compared to CAR T cells + ATRA, and the CAR T cell + GSI group was compared to CAR T cells + GSI + ATRA. Exact *P* values were used due to the small sample size. For each time point, each pairwise comparison tested its own null hypothesis so that no adjustment of the α error was needed. *P* values <0.05 were considered statistically significant.

Results

All-trans retinoic acid treatment augments surface expression of BCMA protein on myeloma cell lines

We determined BCMA baseline expression on three commonly used myeloma cell lines by flow cytometry and found graded BCMA expression with MM.1S being $BCMA^{low}$ (ΔMFI: 5,349), OPM-2 being $BCMA^{intermediate}$ (ΔMFI: 8,037) and NCI-H929 being $BCMA^{high}$ (ΔMFI: 22,014) (Figure 1A). After treating each myeloma cell line with 50 nM ATRA for 72 hours, we found upregulation of BCMA expression on all three myeloma cell lines. However, the hierarchy in BCMA expression had remained unchanged: MM.1S (ΔMFI: 8,837) < OPM-2 (ΔMFI: 13,098) < NCI-H929 (ΔMFI: 32,201) (Figure 1A). We normalized the ΔMFI obtained at baseline to 1 and thus, the relative increase in BCMA expression after ATRA treatment was 1.8-fold in MM.1S, 1.7-fold in OPM 2, and 2.5-fold in NCI-H929 myeloma cells (Figure 1A). Upon discontinuation of ATRA treatment, BCMA expression returned to baseline within 72 hours in all three myeloma cell lines, but increased again with the same amplitude when ATRA treatment was reinstalled (Figure 1A). We did not detect an effect of ATRA treatment on the viability of these myeloma cell lines during the assay period (*Online Supplementary Figure S1*).

In order to derive insights into the density and distribution of BCMA molecules on the surface of myeloma cell lines, we further examined MM.1S cells with and without ATRA treatment by single-molecule sensitive *d*STORM super-resolution microscopy (Figure 1B and C).[33] The data showed variable BCMA expression between MM.1S cells however, there were no $BCMA^{negative}$ MM.1S cells detectable. Before ATRA treatment, the minimum and maximum density was 1.8 and 11.7 BCMA molecules per μm^2, respectively, with even BCMA distribution across the cell membrane. After treatment with 100 nM ATRA for 72 hours, there was a minimum of 3.1 BCMA molecules per μm^2 and a maximum of 13.6 BCMA molecules per μm^2, and BCMA still occurred as single molecules without cluster formation (Figure 1B).

All-trans retinoic acid and crenigacestat work synergistically in increasing BCMA expression on myeloma cell lines

We were interested in determining the combined effect of ATRA and the GSI crenigacestat, that in previous work has been shown to increase BCMA expression in myeloma cells at the protein level.[17] Therefore, we treated MM.1S and

OPM-2 myeloma cells with ATRA (100 nM) and with crenigacestat (10 nM) and analyzed BCMA expression by flow cytometry. We found that crenigacestat had a stronger effect on BCMA expression compared to ATRA and that the combined treatment with both compounds had the strongest effect. The relative increase in BCMA protein expression after treatment with crenigacestat alone and in combination with ATRA was 9.6-fold and 18.3-fold on MM.1S cells (Figure 1D) and 5.9-fold and 6.9-fold on OPM-2 cells (*Online Supplementary Figure S2*), respectively.

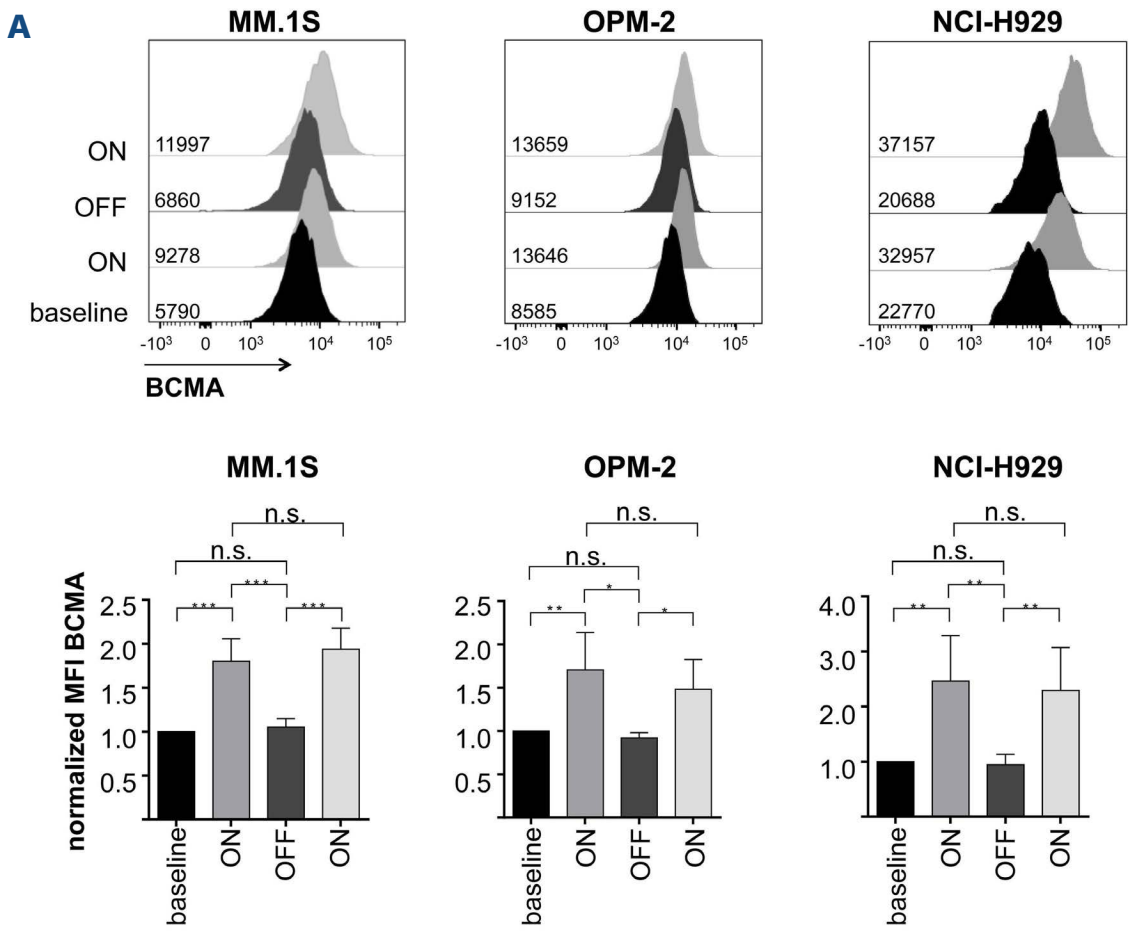
We hypothesized that ATRA induces epigenetic changes in myeloma cells that lead to increased *BCMA* gene expression and confirmed by RT-qPCR that this was indeed the case. In MM.1S and OPM-2 cells, the relative increase in *BCMA* transcripts after treatment with ATRA was 1.8-fold (Figure 1E) and 2.1-fold (*Online Supplementary Figure S3*), respectively.

Taken together, these data show that ATRA treatment leads to increased *BCMA* gene expression and increased expression of BCMA protein in myeloma cell lines. ATRA works synergistically with the GSI crenigacestat to induce a maximum increase in BCMA protein density on the surface of myeloma cells.

Primary myeloma cells show increased BCMA expression after all-trans retinoic acid treatment, alone and in combination with crenigacestat

In order to confirm these findings in primary myeloma cells, we obtained bone marrow from patients with newly diagnosed (ND, n=7) and relapsed/refractory (R/R, n=11) MM. Patients in the R/R cohort had previously received treatment with immunomodulatory drugs and/or proteasome inhibitors, none of the patients had received anti-BCMA therapy. We analyzed purified $CD38^{+}CD138^{+}$ malignant plasma cells by flow cytometry and found variable BCMA expression between patients as assessed by ΔMFI (range, 94-2,650). There was no significant difference in BCMA expression on myeloma cells from ND and R/R patients (Figure 2A). We submitted primary myeloma cells from n=5 patients (n=3 ND and n=2 R/R) that covered the spectrum of $BCMA^{low}$ to $BCMA^{high}$ expression at baseline to *in vitro* treatment with ATRA. In each of these patient samples, we detected an increase in BCMA expression by flow cytometry after treatment with ATRA for 72 hours (100 nM P=0.04; 50 nM P=0.01; 25 nM P=0.04) (Figure 2B; *Online Supplementary Figure S4*). The increase in ΔMFI for BCMA expression on primary myeloma cells after ATRA treatment was on average 1.6-fold (range, 1.2–2.2-fold). After 72 hours of ATRA treatment, there was no impact on the viability of primary myeloma cells (*Online Supplementary Figure S5*). BCMA expression on primary myeloma cells declined to baseline once exposure to ATRA was discontinued, and increased again upon re-exposure to the drug (Figure 2C).

Next, we evaluated the combination treatment of ATRA and GSI on primary myeloma cells from n=3 patients. Consistent with our observation in myeloma cell lines, we observed a stronger increase in BCMA expression with crenigacestat compared to ATRA treatment, and a maxi-



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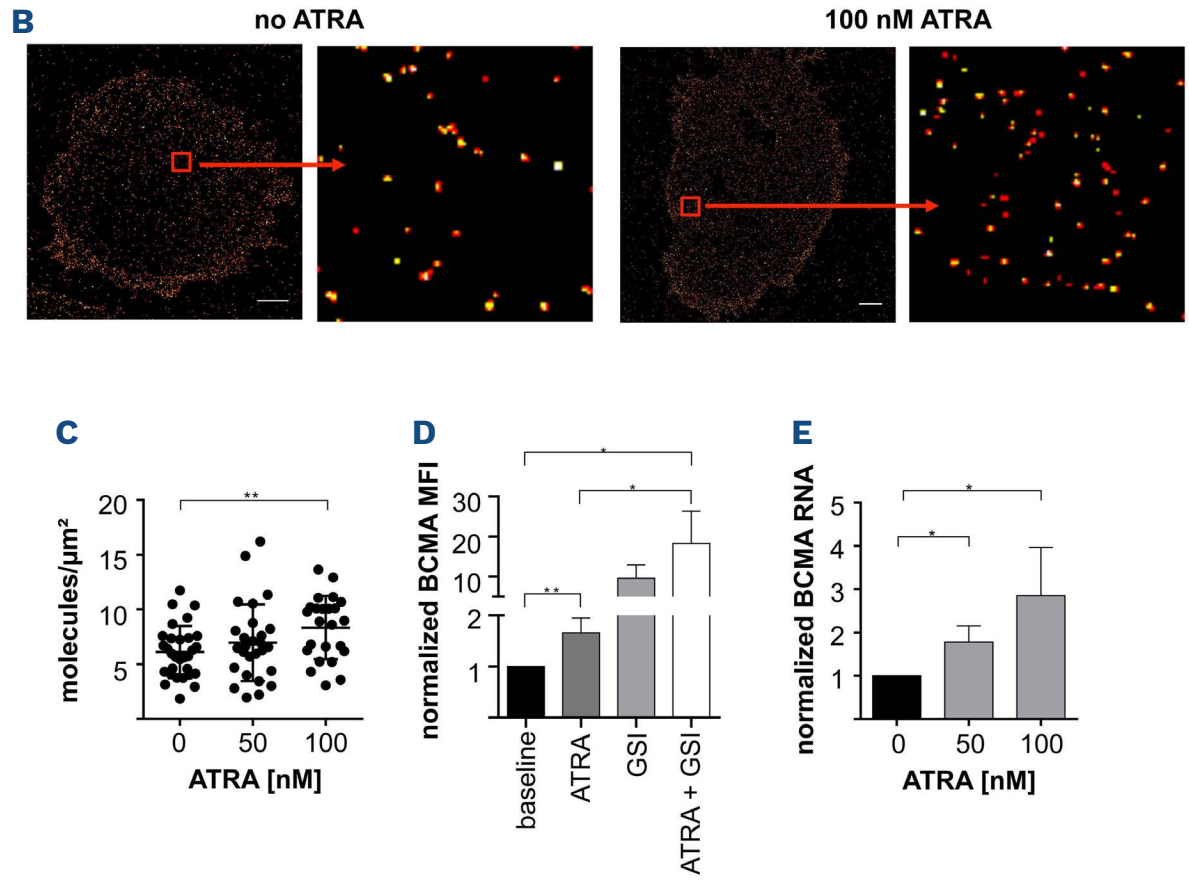

Figure 1. All-trans retinoic acid treatment enhances B-cell maturation antigen expression on myeloma cell lines. (A) B-cell maturation antigen (BCMA) expression on MM.1S, OPM-2 and NCI-H929 cell lines was analyzed by flow cytometry. Expression was measured on untreated myeloma cell lines, after 72 hours of all-trans retinoic acid (ATRA) treatment (50 nM), 24 hours after subsequent removal of the drug, and 72 hours after ATRA re-exposition. 7-AAD was used to exclude dead cells from analysis. Upper panel: representative overlay histograms. Lower panel: bar diagrams show relative increase normalized to completely untreated cells (n=5). (B) Representative photographs of BCMA molecule distribution on untreated and ATRA-treated MM.1S cells visualized by direct stochastic optical reconstruction microscopy (dSTORM). (C) Number of BCMA molecules on untreated and ATRA-treated MM.1S cells measured by dSTORM. (D) BCMA expression on MM.1S cells that had been treated with 100 nM ATRA and/or 10 nM γ-secretase inhibitors (GSI) crenigacestat for 72 hours was analyzed by flow cytometry (n=5). (E) *BCMA* RNA levels in MM.1S were analyzed by quantitative reverse transcription PCR (qRT-PCR) assay after incubation with increasing doses of ATRA for 48 hours (n=4). Bar diagrams show mean values + standard deviation. *P* values between indicated groups were calculated using unpaired *t*-test. n.s.: not significant. **P*<0.05, ***P*<0.01, ****P*<0.001.

mum increase in BCMA expression with the combination treatment. The relative increase in BCMA expression after crenigacestat treatment alone and in combination with ATRA was 6.4-fold and 7.7-fold, respectively (Figure 2D). Taken together, these data show that ATRA alone and in combination with crenigacestat augments BCMA surface expression on primary myeloma cells from patients with ND and R/R disease.

All-trans retinoic acid treatment does not impair BCMA-CAR T-cell viability and function

We sought to determine the effect of ATRA on T cells and confirmed that a 72-hour treatment of $CD4^+$ or $CD8^+$ BCMA-CAR T cells did neither diminish their viability nor expression of the BCMA-CAR as assessed by the percentage of live $CAR^{positive}$ T cells and the mean expression of the CAR_EGFRt transgene by flow cytometry (Figure 3A and B). We also performed functional testing with BCMA-CAR T cells after 1 week ATRA treatment and found similarly potent and specific cytolytic activity against BCMApositive target cells (Figure 3C).

The extracellular portion of membrane-bound BCMA can be shed from myeloma cells to release a shorter, soluble BCMA (sBCMA) protein isoform. Depending on the epitope recognized by the BCMA-CAR, sBCMA may interfere with BCMA recognition on myeloma cells. We confirmed that the reactivity of the BCMA-CAR used in this study that contains a scFv derived from the BCMA50 mAb, was not diminished in the presence of sBCMA (*Online Supplementary Figure S6*). Furthermore, we measured sBCMA in the supernatant of MM.1S, OPM-2 and NCI-H929 cells that had been treated with ATRA for up to 4 days and did not detect a higher concentration of sBCMA compared to non-ATRA-treated MM cells (Figure 3D; *Online Supplementary Figure S7*). Taken together, these data show that ATRA treatment does not affect the viability and function of BCMA-CAR T cells and does not lead to an increased release of sBCMA from MM cells.

Enhanced BCMA expression after all-trans retinoic acid treatment leads to enhanced reactivity of BCMA-CAR T cells against multiple myeloma cell lines *in vitro*

Next, we examined if the increase in BCMA expression after ATRA treatment leads to increased anti-myeloma re-

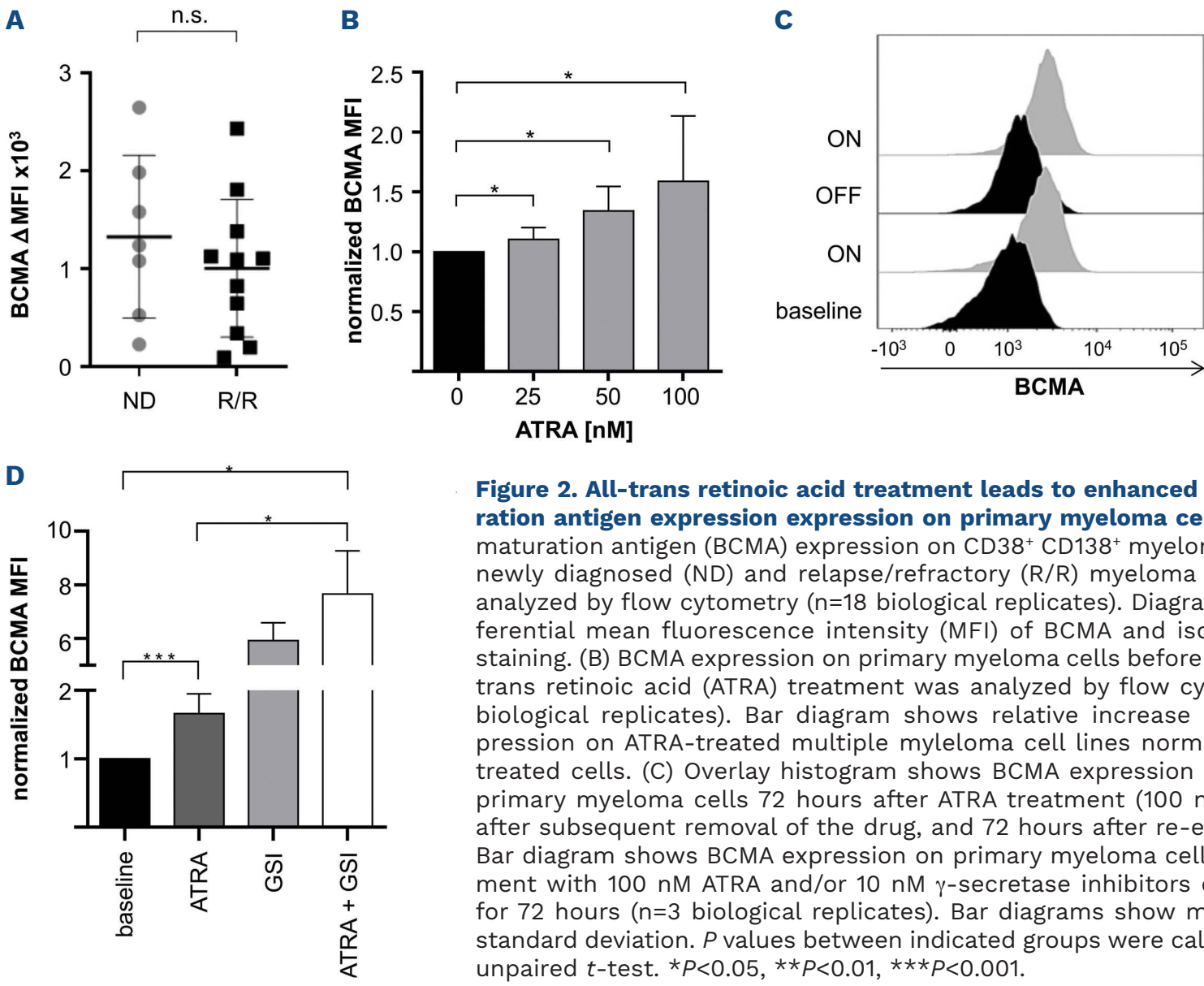

Figure 2. All-trans retinoic acid treatment leads to enhanced B-cell maturation antigen expression expression on primary myeloma cells. (A) B-cell maturation antigen (BCMA) expression on CD38⁺ CD138⁺ myeloma cells from newly diagnosed (ND) and relapse/refractory (R/R) myeloma patients was analyzed by flow cytometry (n=18 biological replicates). Diagram shows differential mean fluorescence intensity (MFI) of BCMA and isotype control staining. (B) BCMA expression on primary myeloma cells before and after all-trans retinoic acid (ATRA) treatment was analyzed by flow cytometry (n=5 biological replicates). Bar diagram shows relative increase of BCMA expression on ATRA-treated multiple myeloma cell lines normalized to untreated cells. (C) Overlay histogram shows BCMA expression on untreated primary myeloma cells 72 hours after ATRA treatment (100 nM), 24 hours after subsequent removal of the drug, and 72 hours after re-exposition. (D) Bar diagram shows BCMA expression on primary myeloma cells after treatment with 100 nM ATRA and/or 10 nM γ-secretase inhibitors crenigacestat for 72 hours (n=3 biological replicates). Bar diagrams show mean values + standard deviation. *P* values between indicated groups were calculated using unpaired *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001.

activity of BCMA-CAR T cells. We tested the cytolytic activity of CD8⁺ BCMA-CAR T cells from healthy donors and MM patients and found superior cytolysis of ATRA-treated compared to non-ATRA-treated myeloma cell lines (Figure 4A; *Online Supplementary Figure S8*). The increase in cytolysis of myeloma cells was consistent at several E:T ratios and – on example of MM.1S target cells – increased from 42.5% to 55.5% during the 4-hour assay period (E:T =5, *P*=0.036, Figure 4A). Consistent with our analysis of BCMA expression, we observed the strongest cytolysis when MM1.S cells had been treated with the ATRA plus crenigacestat combination. With ATRA plus crenigacestat-treated MM1.S target cells, the specific cytolysis by BCMA-CAR T cells increased to 74% (*P*=0,002; Figure 4A).

Our analyses also showed increased production of IFNγ and IL-2 from CD8⁺ and CD4⁺ BCMA-CAR T cells as assessed by ELISA after a 24-hour co-culture with ATRA-treated *versus* non-ATRA-treated myeloma target cells. In particular, there was a significant increase in IL-2 production from CD8⁺ BCMA-CAR T cells after co-culture with MM1.S cells that had been treated with ATRA and the ATRA plus crenigacestat combination (Figure 4B). By CFSE dye dilution, we detected a significant increase in proliferation of CD8⁺ and CD4⁺ BCMA-CAR T cells after stimulation with myeloma cells that had undergone treated with ATRA and the ATRA plus crenigacestat combination (Figure 4C). This proliferation was productive and led to an increase in the percentage of viable BCMA-CAR T cells and the absolute number of BCMA-CAR T cells at the end of the 72-hour assay period.

Collectively, these data show that the increased expression of BCMA on myeloma cells after ATRA treatment augments the anti-myeloma response of BCMA-CAR T cells. The increase in anti-myeloma function is strongest, when myeloma cells undergo concurrent treatment with ATRA and crenigacestat.

All-trans retinoic acid treatment enhances the efficacy of BCMA-CAR T-cell therapy in NSG/MM.1S mice

We sought to evaluate the effect of ATRA treatment on the anti-myeloma efficacy of BCMA-CAR T-cell therapy *in vivo*. In a first experiment, we inoculated NSG mice (n=6) with 2x10⁶ MM.1S cells by tail vein injection to establish systemic MM and then administered a 4-day treatment course with either ATRA (n=3 mice; 30 mg/kg i.p. qd) or solvent control (n=3 mice). After completion of ATRA treatment, we obtained bone marrow and determined BCMA expression on MM.1S cells by flow cytometry. We found significantly higher BCMA expression on MM.1S cells from mice in the ATRA treatment group compared to the con-

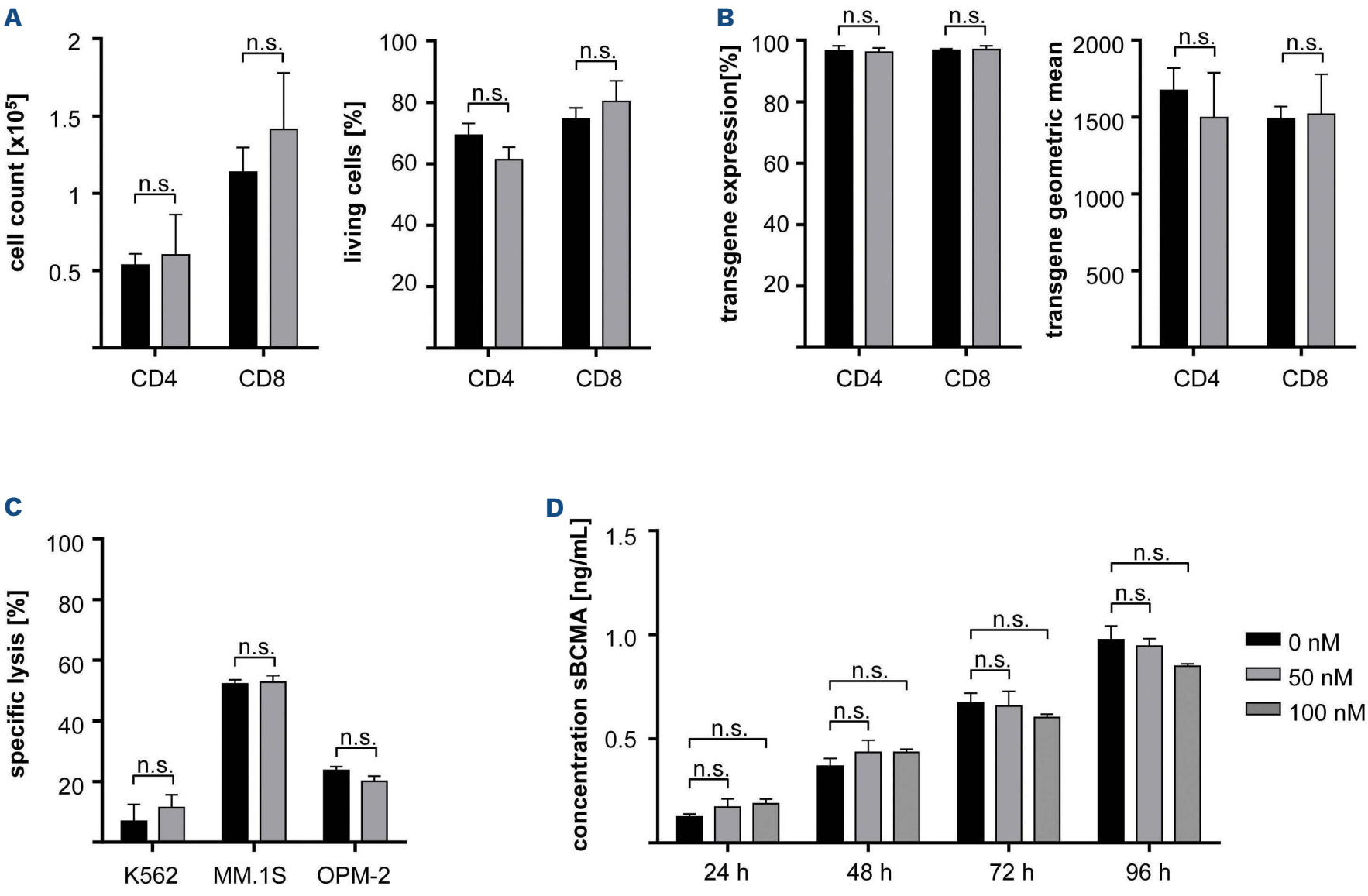

Figure 3. B-cell maturation antigen-CAR T cells are not impaired by all-trans retinoic acid treatment. (A) Cell count and viability of $CD4^+$ and $CD8^+$ B-cell maturation antigen chimeric antigen receptor (BCMA-CAR) T cells after incubation with 100 nM all-trans retinoic acid (ATRA) for 72 hours was analyzed by trypan blue staining (n=3 technical triplicates). Left bar diagram shows the total number of living cells, right bar diagram shows the percentage of viable cells. (B) Expression of the EGFRt-CAR transgene construct after incubation of $CD4^+$ and $CD8^+$ BCMA-CAR T cells with 100 nM ATRA for 72 hours was analyzed by flow cytometry (n=3 technical triplicates). Left bar shows percentage of CAR-positive cells, right bar shows geometric mean of transgene signal (C) Cytolytic activity of $CD8^+$ BCMA-CAR T cells was determined in a bioluminescence-based assay after 4 hours of co-incubation with target cells. T cells had been pretreated with 100 nM ATRA for 1 week. Assay was performed in triplicate wells with 5,000 target cells per well (n=3 biological replicates) (D) Soluble BCMA (sBCMA) concentration in the supernatant of MM.1S after incubation with increasing doses of ATRA was analyzed by enzyme-linked immosorbant assay. Bar diagrams show mean values + standard deviation. *P* values between indicated groups were calculated using (A to C) unpaired *t*-test or (D) two-way ANOVA. n.s.: not significant.

trol group (*P*=0.002; Figure 5A).

In a second experiment, we performed adoptive transfer of BCMA-CAR T-cells at an effective but non-curative dose ($1x10^6$ total CAR-T cells i.v., CD8:CD4 at 1:1 ratio). At this dose, the optimal anti-myeloma response is reached within 1 week after BCMA-CAR T-cell transfer, and MM relapse occurs rapidly within 2 weeks. Concurrent to BCMA-CAR T-cell therapy, we administered ATRA (30 mg/kg i.p.) and/or crenigacestat (1 mg/kg i.p.), or solvent control as per treatment schedule (Figure 5B). We obtained peripheral blood on day 2 after adoptive transfer and detected a higher percentage of BCMA-CAR T cells in mice that had received a concurrent treatment with either ATRA alone, crenigacestat alone or the ATRA plus crenigacestat combination (Figure 5C), consistent with superior engraftment and induction of *in vivo* proliferation.

Then, we analyzed myeloma burden and distribution by serial bioluminescence imaging (BLI). At 1 week after BCMA-CAR T-cell transfer, we observed an anti-myeloma effect in each of the treatment groups that had received BCMA-CAR T cells alone or with a concurrent treatment of ATRA, crenigacestat or the ATRA plus crenigacestat combination. We observed the deepest remissions in mice that had received concurrent therapy with the ATRA plus crenigacestat combination, followed by ATRA alone and crenigacestat alone (Figure 5D). Indeed, each of the concurrent treatment regimen that contained ATRA was superior to BCMA-CAR T-cell therapy alone. At 2 weeks after BCMA-CAR T-cell transfer, we observed rapid disease progression in each of the mice that had received BCMA-CAR T-cell therapy alone. We also observed disease progression in all but one of the mice that had received concur-

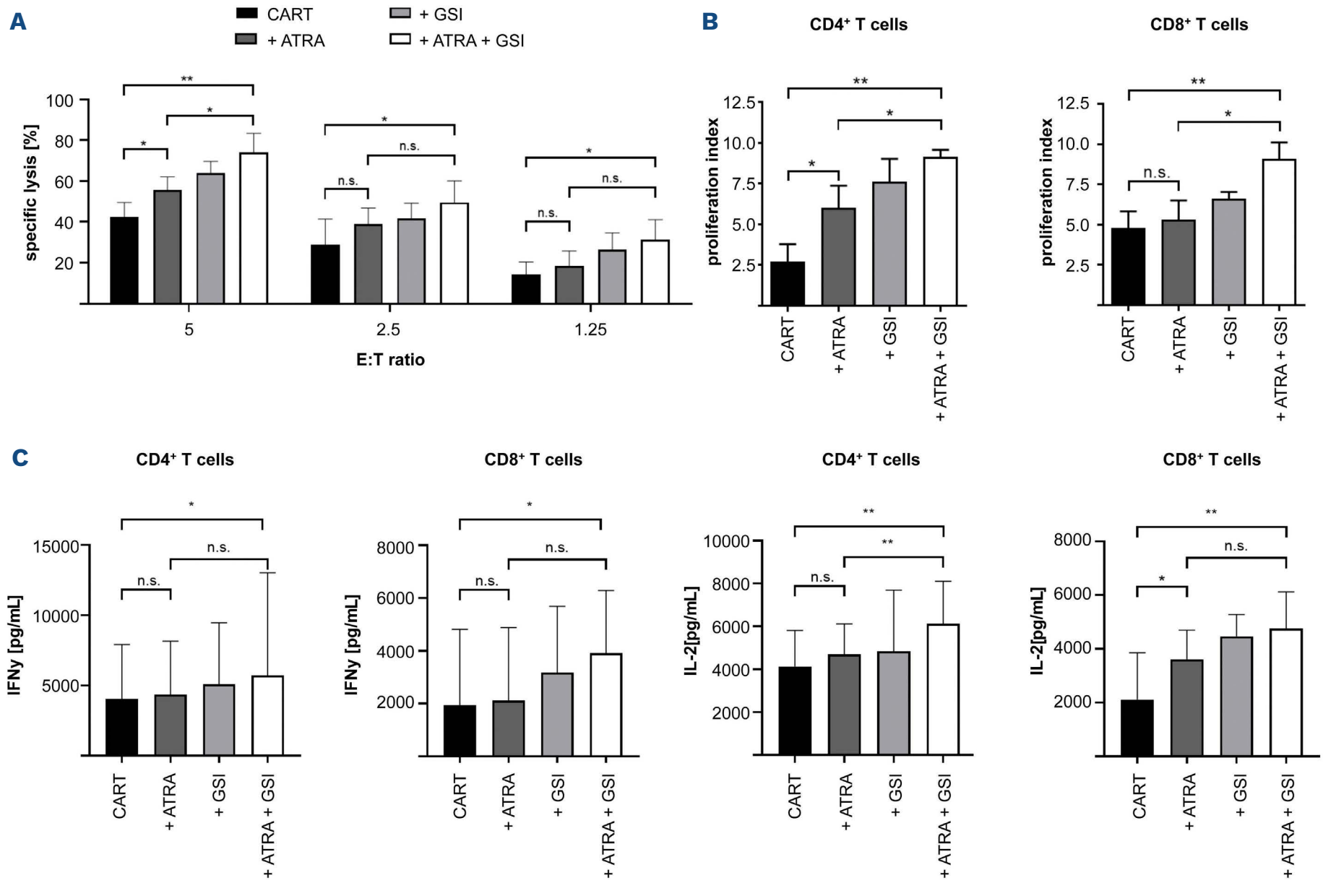

Figure 4. All-trans retinoic acid treatment enhances the anti-myeloma efficacy of B-cell maturation antigen-CAR T cells *in vitro*. (A to C) Prior to assay setup, MM.1S cells were incubated with 100 nM all-trans retinoic acid (ATRA) and/or 10 nM γ-secretase inhibitors (GSI) for 72 hours or were left untreated. ATRA was washed out before CAR T cells were added. (A) Cytolytic activity of CD8+ B-cell maturation antigen chimeric antigen receptor (BCMA-CAR) T cells was determined in a bioluminescence-based assay after 4 hours of co-incubation with MM.1S target cells. Assay was performed in triplicate wells with 5,000 target cells per well (n=4 biological replicates). (B) BCMA-CAR T cells were co-incubated with MM.1S cells for 24 hours. Cytokine release of BCMA-CAR T cells was determined in the supernatant by enzyme-linked immunosorbant assay. Assay was performed in triplicate wells (n=3 biological replicates). (C) CFSE-labeled BCMA-CAR T cells were co-incubated with MM.1S cells. Proliferative capacity of BCMA-CAR T cells was determined after 3 days by measuring the reduction of CFSE-signal (n=3 biological replicates). Bar diagrams show mean values + standard deviation, *P* values between indicated groups were calculated using unpaired *t*-test. n.s.: not significant. **P*<0.05, ***P*<0.01.

rent treatment with either ATRA alone or crenigacestat alone. In contrast, there was no disease progression in any of the mice that had received BCMA-CAR T cells and concurrent ATRA plus crenigacestat (Figure 5D). In these mice, the BLI signal remained at baseline (i.e., the level observed before MM.1S inoculation) and analysis of bone marrow confirmed the absence of MM.1S cells (Figure 5E). In particular, treatment with BCMA-CAR T cells and the ATRA plus crenigacestat combination conferred a superior anti-myeloma effect compared to the treatment with BCMA-CAR T cells and crenigacestat alone as assessed by BLI at day 21 and at day 28 (*P*=0.008 and *P*=0.036, respectively). In aggregate, these data show that ATRA treatment leads to increased BCMA expression on myeloma cells *in vivo* and augments the anti-myeloma efficacy of BCMA-CAR T cells. Combination treatment with ATRA and crenigacestat concurrent to BCMA-CAR T-cell therapy confers the strongest improvement of the anti-myeloma response both in terms of depth and duration of myeloma remission.

Discussion

There is a surging clinical and preclinical development of cellular and antibody-based immunotherapies directed against BCMA in MM, including BCMA-specific CAR T cells that have shown their potential to induce very high rates of complete remissions in patients that are resistant to conventional therapies.[1–4,34] However, a barrier to consist-

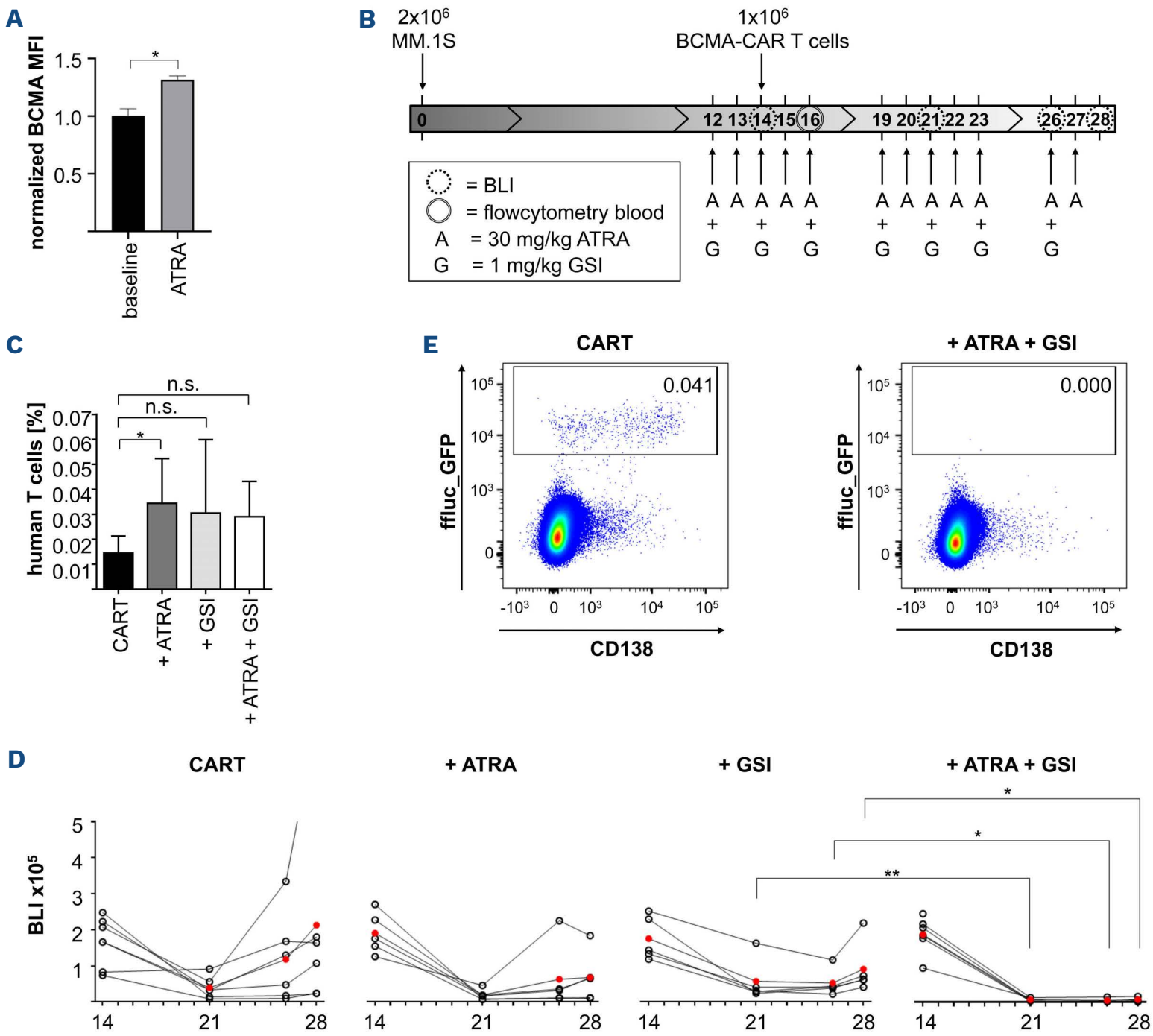

Figure 5. All-trans retinoic acid treatment enhances the anti-myeloma efficacy of B-cell maturation antigen-CAR T cells *in vivo*. (A) NSG mice were inoculated with 2x10^6 MM.1S cells. After 12 days, mice were intraperitoneally (i.p.) injected with 30 mg/kg all-trans retinoic acid (ATRA) for 4 days. BCMA expression on MM.1S cells obtained from bone marrow of untreated and ATRA-treated mice was analyzed by flow cytometry (n=3). (B) ATRA and crenigacestat treatment scheme for experiments in NSU+00a0\1D) NSG mice were inoculated with 2×10^6 MM.1S/ffluc_GFP cells. 14 days later, they were treated with 1×10^6 BCMA-CAR T-cells (CD4$^+$:CD8$^+$ ratio = 1:1). BCMA-CAR T cells were given alone or in combination with ATRA (30 mg/kg body weight as i.p. injection), γ -secretase inhibitors (GSI) crenigacestat (1 mg/kg body weight as i.p. injection) or both drugs (n=5-6 mice per group). 12 doses of ATRA and 7 doses of GSI were injected between day 12 and day 27. BLI was measured on day 14, 21, 26 and 28. (C) Percentage of human T cells (CD3$^+$ CD45$^+$) among living cells (7-AAD$^-$) in peripheral blood was measured two days after CAR T-cell injection (day 16) by flowcytometry. (D) The average radiance of MM.1S signal was analyzed to assess myeloma progression/regression in each treatment group. Bioluminescence (BLI) values were obtained as photon/sec/cm^2/sr in regions of interest encompassing the entire body of each mouse. Circles show the bioluminescence signals of single mice, red dots show the mean bioluminescene signals between day 14 (time point of CAR T-cell injection) and day 28 (end of experiment). (E) Percentage of MM.1S cells in bone marrow from mice treated with BCMA-CAR T cells alone or in combination with ATRA and GSI was analyzed by measuring CD138$^+$, ffluc_GFP$^+$, 7-AAD$^-$ cells by flow cytometry. (A and C) Bar diagrams show mean values + standard deviation, *P* values between indicated groups were calculated using unpaired *t*-test. **P*<0.05, ***P*<0.01. (D) *P* values between indicated groups were calculated using non-parametric Mann Whitney U tests for comparing CAR T *vs*. CAR T + ATRA and CAR T + GSI *vs*. CART + ATRA + GSI. **P*<0.05, ***P*<0.01.

ently inducing durable responses with BCMA-CAR T-cell therapy and eventually cure, is the heterogeneous and sometimes decreasing expression of BCMA on MM cells, which is presumed to be one driver of suboptimal and short-lived response in some MM patients.[1,15,16] We performed flow cytometric analysis on primary myeloma cells and confirmed a highly variable degree of BCMA expression both within the myeloma cell population in a given patient, and between patients. These findings are consistent with prior studies that analyzed BCMA expression on myeloma cells and reported inter- and intra-patient heterogeneity.[3,15,17]

We reasoned that *BCMA* gene expression could be increased with an epigenetic modifier such as ATRA and indeed, our data show a significant increase in *BCMA* transcripts by RT-qPCR and of BCMA protein expression by flow cytometry on several MM cell lines and on primary myeloma cells. We confirmed the presence of BCMA and the increase in BCMA expression after ATRA treatment on BCMA^{low} myeloma cells by super-resolution *d*STORM microscopy. We also showed an increase in BCMA expression after ATRA treatment on myeloma cells in a murine xenograft model *in vivo*. We confirmed that ATRA does not affect T-cell viability, CAR expression and the effector function of BCMA-CAR T cells. Furthermore, we analyzed whether ATRA treatment leads to increased shedding of sBCMA from myeloma cells. Despite enhanced expression of membrane-bound BCMA on myeloma cells, we did not detect an increase of sBCMA in the supernatant of several myeloma cell lines that we had submitted to ATRA treatment. These data suggest that the catalytic capacity of γ-secretase in myeloma cells gets saturated and that therefore, a further increase in BCMA protein expression does not result in increased release of sBCMA. sBCMA may interfere with the recognition of membrane-bound BCMA on myeloma cells and diminish anti-myeloma reactivity.[17,35] In our BCMA-CAR, we included a targeting domain that binds to a membrane-proximal BCMA epitope.[31] We did not observe reduced anti-myeloma reactivity from T cells expressing this BCMA-CAR in the presence of sBCMA.

Several prior studies showed a correlation between BCMA expression on target cells and ensuing anti-myeloma function of BCMA-CAR T cells.[17,36] Our data show that the increase in BCMA expression on myeloma cells that is induced by ATRA leads to a substantial increase in specific anti-myeloma reactivity of CD8$^+$ and CD4$^+$ BCMA-CAR T cells. BCMA-CAR T cells showed higher cytolytic activity, cytokine secretion and proliferation after stimulation with ATRA-treated *versus* non-ATRA-treated myeloma target cells. This effect was accentuated when myeloma cells were treated with ATRA in combination with the GSI crenigacestat. Of particular interest, we observed a strong increase in IL-2 production, *in vitro* proliferation and viability in CD8$^+$ and CD4$^+$ BCMA-CAR T cells, which are critical attributes to enable engraftment, proliferation and persistence of CAR T cells in a clinical setting in humans.[37] These data affirm the prior notion that antigen density on target cells is an important variable that determines the subsequent anti-tumor function of CAR T cells, even though *per se* a low number of antigen molecules can be sufficient for triggering cytolytic activity.[8,20,23] However, several prior studies have shown that diminished antigen expression limits CAR T-cell function in preclinical and clinical studies.[10,21]

A recent study has reported on the use of crenigacestat to increase BCMA expression on myeloma cells.[17] γ-secretase sheds BCMA from the cell surface, leading to the release of sBCMA. Crenigacestat and other GSI inhibit the multisubunit protease γ-secretase, thereby preventing the cleavage of BCMA from the cell surface and leading to increased reactivity of BCMA-CAR T cells.[17,38] We reasoned that ATRA as a regulator of BCMA expression at the gene level and crenigacestat as a regulator of BCMA expression at the protein level may have an additive and potentially, synergistic effect. Indeed, our data on BCMA expression and anti-myeloma function of BCMA-CAR T cells show that concurrent ATRA plus crenigacestat treatment confers a maximum effect. In particular, there was a near doubling of cytolytic activity against myeloma cell lines and a near doubling in the number of cell divisions that both CD8$^+$ and CD4$^+$ BCMA-CAR T-cells underwent *in vitro*, indicating that the combined ATRA plus crenigacestat treatment had brought BCMA expression to a sweet spot where the BCMA-CAR employed in this study conferred optimal stimulation for a productive T-cell response. We acknowledge that for other BCMA-CAR constructs that employ distinct targeting domains may have distinct requirements in antigen density. We also acknowledge that increased cytokine production from BCMA-CAR T cells may be associated with increased toxicity from cytokine release syndrome (CRS) but note that overall, BCMA-CAR T-cell therapy has been well tolerated.[6,7]

We assessed the effect of ATRA and crenigacestat treatment on the outcome of BCMA-CAR T-cell therapy in a murine xenograft model – NSG/MM.1S – that we have employed in previous work to determine the anti-myeloma efficacy of BCMA- and SLAMF7-specific CAR T cells. This model is characterized by rapid myeloma progression and – depending on the dose of CAR T cells that is administered – effective but non-curative and curative outcome of CAR T-cell therapy.[39] Our data show that anti-myeloma efficacy of BCMA-CAR T cells is increased by concurrent treatment with ATRA, and that a combination of ATRA and crenigacestat leads to significantly improved myeloma regression compared to mice that only received concurrent treatment with crenigacestat. The ability to perform long-term follow-up in this model is limited due to the rigors associated with repeated ATRA and crenigacestat i.p. injections and therefore, we focused our analyses on readouts that can be obtained with a short-term follow-up. The data suggest that the mechanism of action that leads to improved anti-myeloma efficacy is superior engraftment and proliferation of BCMA-CAR T cells *in vivo* and deeper remission due to superior elimination of myeloma cells.

ATRA is a clinically approved drug with favorable safety profile.[40,41] Crenigacestat is in clinical development to treat Alzheimer's disease and selected cancers and has been reported to possess an acceptable safety profile.[42–44] These data suggest that a combination therapy with ATRA

and crenigacestat, concurrent to BCMA-CAR T-cell therapy is feasible. Our data show that the effect of ATRA (alone and in combination with crenigacestat) on BCMA expression is rapidly reversible and therefore, ought to be administered concurrent to BCMA-CAR T-cell therapy. Our data suggest that the combination of ATRA with crenigacestat or another GSI will be most effective. In normal hematopoietic cells, the expression of BCMA is restricted to mature B cells and plasma cells. A prior study has shown that ATRA increases BCMA expression in normal B cells however, because the baseline BCMA expression on normal B cells is already sufficient for recognition by BCMA-CAR T cells, we do not anticipate a further increase in toxicity in a clinical setting.[45] The concurrent treatment of MM patients with ATRA and crenigacestat is one example of how BCMA expression can be increased and sustained to augment the efficacy of BCMA-CAR T cells. However, this requires that myeloma cells retain at least one allele of *BCMA*. In the event of a homozygeous *BCMA* loss, the use of ATRA or crenigacestat will be unable to rescue the efficacy of BCMA-CAR T cells.[14]

The modulation of BCMA expression at the gene or protein level is also relevant for other modalities of cellular and antibody-based immunotherapy directed against BCMA. The anti-tumor efficacy of T-cell-engaging bispecific antibodies and antibody-drug conjugates is also dependent on antigen density on target cells and in general, a higher antigen density compared to CAR T cells is required to unfold their therapeutic effect.[46,47] Recently, several studies have reported on dual- (or even triple-) antigen targeting with CAR T cells against MM, e.g., with BCMA in combination with GPRC5D or SLAMF7.[48,49] In preliminary work, we found that the effect of ATRA treatment on the expression of target antigens other than BCMA has to be determined empirically and may, with some antigens, result in diminished expression. In a dual-antigen targeting approach, the effect of ATRA treatment is anticipated to be strongest when expression of both target antigens is increased. However, in the event of antigen loss due to allele deletion or alternative splicing,[14,50] the reactivity of CAR T cells against the remaining antigen may be retained and even enhanced through the use of ATRA.

Disclosures

EGG, MH and SRP are co-inventors on a patent application on the use of BCMA-CAR T-cell therapy in combination with ATRA that has been filed by the University of Würzburg, Würzburg, Germany and licensed to T-CURX GmbH, Würzburg, Germany. MH is co-inventor on patent applications and granted patients related to CAR technologies and CAR T-cell therapy that have been filed by the Fred Hutchinson Cancer Research Center, Seattle, WA and the University of Würzburg, Würzburg, Germany that have been, in part, licensed to industry. MH is a co-founder and equity owner of T-CURX GmbH, Würzburg, Germany. MH further declares speaker honoraria from Novartis, Kite/Gilead, BMS/Celgene and Janssen. JAPS declares speaker or advisory honoraria and/or research funding from Jazz, Pfizer, Takeda, Janssen, BMS/Celgene, Gilead, Novartis. HE declares honoraria from BMS/Celgene, Janssen, Amgen, Takeda, Sanofi and GSK, research funding from BMS/Celgene, Janssen, Amgen, GSK and Sanofi, travel/accomodation expenses from BMS/Celgene, Janssen, Amgen, Takeda, Novartis and advisory/consulting from BMS/Celgene, Janssen, Amgen, Takeda, Sanofi, GSK. LGRL declares speaker honoraria and travel grants from Janssen and Amgen. SD has received speaker honoraria from BMS. The remaining authors declare that they have no conflicts of interest.

Contributions

EGG designed and performed experiments, analyzed data and wrote the manuscript. LGRL and LH designed and performed experiments and analyzed data. BSM, RG, SB, SF and MS performed experiments and analyzed data. SD, KMK and LR provided biologic material and analyzed data. JAPS and HE analyzed data and wrote the manuscript. MH designed experiments, analyzed data, wrote the manuscript and co-supervised the project. SRP designed and performed experiments, analyzed data, wrote the manuscript and supervised the project. All authors approved the final version of the manuscript.

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Data-sharing statement

Inquiries regarding sharing of de-identified data should be addressed to the corresponding author.

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