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Targeting B-cell maturation antigen increases sensitivity of multiple myeloma cells to MCL-1 inhibition

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To the Editor,

Interfering with the mechanisms by which malignant plasma cells develop drug resistance is critical for preventing relapse in multiple myeloma (MM). Downregulation of target antigen is one of the escape mechanisms limiting the success of promising therapeutic approaches such as B-cell maturation antigen (BCMA)-targeted immunotherapy. Despite high response rates and depth of responses after treatment with BCMA-targeting agents, patients eventually relapse. In most cases, the residual MM cells persisting after BCMA-based immunotherapy express reduced BCMA levels, suggesting that eliminating this BCMA MM cell pool could significantly delay or prevent relapse. An optimal therapeutic approach may therefore involve sequential treatment strategies targeting the critical anti-apoptotic pathways in refractory MM cells selected right after BCMA-based immunotherapy. However, ex vivo identification of potentially relevant sequential treatment strategies involving culture of primary MM cells for several days, has been limited due to the low viability of malignant plasma cells outside the bone marrow niche. Here, we describe a reproducible culture system for primary MM cells based on a synthetic hydrogel where viability of primary myeloma cells is preserved even in the absence of additional stromal subsets. Drug screening of MM samples in this 3D platform revealed a dynamic interplay between BCMA and MCL-1, showing that BCMA MM cells are highly sensitive to MCL-1 inhibitors (MCL-1i), and that pre-treatment with BCMA-blocking antibodies significantly increases MCL-1i efficacy in MM cells.

The BCMA (TNFRSF17) surface receptor is selectively present on plasma cells, and its expression is significantly higher in high- vs low-risk MM and in relapsed/refractory vs newly diagnosed patients. This progressive BCMA increase is due to loss of cells expressing a relatively lower level of this receptor, suggesting that malignant cells with highest BCMA expression may have a selective advantage over the
course of the disease. Multiple BCMA-targeting approaches are being tested in the clinic for the treatment of MM, including antibody-drug conjugates, bispecific BCMA×CD3 antibodies, and anti-BCMA chimeric antigen receptor (CAR) T cells. Signaling through the APRIL-BCMA axis promotes MM cell proliferation and survival, and induces the expression of the anti-apoptotic proteins BCL-2 and MCL-1 (ref. 5). MCL-1 is a key pro-survival factor for healthy and malignant plasma cells, and elevated MCL-1 expression in MM is associated with chemoresistance and shorter event-free survival. Several compounds targeting MCL-1 are currently tested in clinical trials, including the potent MCL-1 inhibitor (MCL-1i) S63845 (ref. 7). MM cell sensitivity to MCL-1i treatment is highly variable between patients, as is observed with BCMA-targeting immunotherapy, stressing the need for primary MM cell-based ex vivo studies on the factors conditioning the response to these treatment modalities.

Our culture approach for patient-derived MM cells is based on Puramatrix (PMX) hydrogel, which has previously been validated for MM cell co-culture with mesenchymal stromal cells (MSC) . A major advantage of PMX over other gels such as Matrigel or fibrin scaffolds is its chemically-defined composition (R-A-D-A repeats), therefore eliminating batch-to-batch variability issues. MM cells (patient information is listed in Table S1) were cultured in PMX supplemented with the pro-survival cytokines IL-6 and APRIL, which are found at high concentrations in the bone marrow and plasma of MM patients. The combination of both cytokines preserved MM cell viability significantly better than in unstimulated controls (Figure S1A). Primary MM cell survival in PMX was significantly higher than in 2D, as determined by cell viability percentages and by absolute cell number (Figure 1A-C). Compound diffusion in PMX was evaluated by exposing PMX-cultured MM cells to molecules added to the supernatant. In antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays with the anti-CD38 antibody Daratumumab, surface-bound
anti-CD38 was detected in all cells (Figure S1B), and MM cell line sensitivity to CD38 targeting in PMX was comparable to that in 2D (Figure S1C), showing that both small and large molecules diffuse efficiently in this hydrogel. Importantly, MM cells retained expression of plasma cell-associated markers when cultured in PMX. BCMA expression significantly increased over time, while CD38 levels remained mostly stable (Figure 1D and data not shown). Due to limited plasma cell viability in (2D) culture, previous reports testing ex vivo chemosensitivity of primary MM cells are based on their co-culture with either MSC or MS-5 cells. To relate to these previous studies, we compared MM drug responses in PMX supplemented with either cytokines or MSC. Specific apoptosis induced by the MCL-1 inhibitor (MCL-1i) S63845 in PMX + IL-6/APRIL was similar to that measured in PMX + MSC co-cultures (Figure 1E). Furthermore, it has been reported that primary MM cells with a t(11;14) translocation are highly sensitive to BCL-2 inhibition, and we confirmed this observation in PMX-based drug screenings (Figure S1D).

Over culture in the presence of IL-6 and APRIL, sensitivity of MM cells to MCL-1i decreased significantly (Figure 1F), evocative of drug sensitivity loss in the physiological niche. Both IL-6 and APRIL have been linked to acquisition of drug resistance in MM, while previous reports rely mostly on the study of cell lines.

Next, we assessed BCMA levels on MM cells that persisted after each treatment. Remarkably, BCMA expression on malignant cells that survived MCL-1 inhibition was significantly higher than on cells left untreated or exposed to other agents (Figure 2A). Distribution of BCMA expression in viable MM cells indicated that MCL-1 inhibition preferentially spares BCMA<sup>hi</sup> cells (Figure 2B). Taken together, these data reveal a connection between BCMA expression and dependence on MCL-1, and indicates that MCL-1i treatment eliminates BCMA<sup>low</sup> MM cells. This observation is especially relevant considering that residual MM cells remaining after BCMA CAR-T cell therapy show
significantly reduced BCMA levels, suggesting immune selection for BCMA-dim/negative clonal variants. Our data indicates that combining MCL-1 inhibitors with BCMA-targeting immunotherapies may increase their efficacy by depleting residual BCMA-low cells.

We next addressed the relation between BCMA blockade and MCL-1i-induced apoptosis by treating primary MM samples with a BCMA-targeting antibody (anti-BCMA). MM cell sensitivity to single-agent anti-BCMA was heterogeneous and did not correlate with BCMA expression in MM cells before treatment (Figure S2A and B). Importantly, there was a clear inverse correlation between MCL-1i and anti-BCMA sensitivity in MM samples: MCL-1i-resistant MM cells were highly sensitive to anti-BCMA, and vice versa (Figure 3A). We did not observe changes in MCL-1, BCL-2 or BCL-XL protein levels after anti-BCMA treatment, neither by FACS nor by western blot analysis (Figure 3B and Figure S2C-D). Considering that MCL-1i spares MM cells with highest BCMA expression, which may largely rely on BCMA signaling for survival, we next evaluated if co-treatment with MCL-1i and anti-BCMA has synergistic effects on MM cell apoptosis. In 5/7 primary MM samples tested, MCL-1i + anti-BCMA combination had a more than additive effect on MM cell killing (Figure 3C and D), but we did not observe consistent synergy when combining these drugs with primary MM samples. Next, we addressed whether increased MCL-1i efficacy in a context of BCMA blockade could be further enhanced by following a sequential treatment strategy. To this end, primary MM cells were cultured in the presence of anti-BCMA for 6 days before a 24h culture with MCL-1i. Strikingly, apoptosis induced by MCL-1 inhibition was significantly higher after a 6-day pre-treatment with anti-BCMA, as compared to treatment with an isotype control or a shorter (24h) BCMA blockade period (Figure 3E and F). Increased MM cell susceptibility towards sequential BCMA and MCL-1 targeting was also observed following co-culture with MSC in PMX (Figure S2E-H). Pre-treatment
with either Daratumumab (anti-CD38) or tocilizumab (anti-IL6R) did not sensitize MM cells to MCL-1 inhibition, suggesting that increased sensitivity to MCL-1i is specifically related to blockade of the APRIL-BCMA axis and not a general consequence of ADCC (Figure S2I and J). Limiting the potential toxicities associated with MCL1i therapy is important: MCL-1 is expressed on different healthy tissues, including cardiomyocytes, hematopoietic stem cells, oocytes, and lymphocytes. It is therefore relevant to find strategies to enhance the efficacy of MCL-1 inhibitors specifically in MM cells, which may lower the required doses for a persistent therapeutic effect. Our results suggest that blocking the APRIL-BCMA axis may render MM cells more dependent on pro-survival IL-6 signaling, forcing a scenario where cells are more sensitive to MCL-1 inhibition. Interestingly, patients with 1q21 amplification are highly sensitive to MCL-1 targeting, likely due to higher relative MCL1 expression resulting from amplification of 1q21 (ref. 8). The IL-6 receptor (IL-6R) locus is also located in this chromosomal region, suggesting that 1q21+ MM cells may largely rely on the IL-6 – MCL-1 axis for survival. Taken together, our data reveals the potential of MCL-1 inhibition as a strategy to eliminate BCMA<sub>low</sub> residual cells following BCMA-directed immunotherapy, and shows that anti-BCMA (pre-)treatment significantly enhances MCL-1i-induced apoptosis in MM cells.
References


Figure legends

Figure 1. Primary MM cell viability, phenotype, and drug sensitivity testing after culture in PMX hydrogel. (A) Representative flow cytometry plots of MM cells after culture. MM bone marrow mononuclear cells were seeded in PMX in the presence of IL-6 and APRIL (100 ng/mL each), for 7 days. All experiments involving primary MM cell culture were performed in this setting, unless otherwise stated. MM cells were gated as CD38+ CD138+ (left), and viable cells were identified as DiOC6+ TOPRO3-cells (right). (B) Frequency and (C) absolute cell number (expressed as fold-increase relative to 2D controls) of MM cells after 7 days in culture in 2D or PMX (n=18). Bars indicate mean + SEM. (D) Mean Fluorescence Intensity (MFI) of BCMA in MM cells was measured by flow cytometry after 2 or 7 days in PMX culture (n=12). (E) Simple linear regression analysis comparing specific apoptosis (%) induced by the MCL-1 inhibitor (MCL-1i) S63845, in n=12 MM samples cultured in PMX supplemented with either IL-6 + APRIL or mesenchymal stromal cells (MSC, 80,000/well 10). MCL-1i (1000 nM) was added on day 1, and MM viability was measured by flow cytometry 24h later. Specific apoptosis (%) was calculated by applying the following formula: [(%viable Nil - %viable treated)/ %viable Nil] x 100. (F) Specific apoptosis induced by MCL-1i in PMX-cultured MM cells (n=12). MCL-1i (100 nM) was added on day 1 or 6, and viability was measured 24h later (day 2 or 7, respectively). Each dot represents an individual sample. Statistical differences between 2 groups were analyzed using paired t-tests. *, p<0.05; **, p<0.01.

Figure 2. BCMA<sup>low</sup> MM cells are sensitive to MCL-1 inhibition. (A) Mean Fluorescence Intensity (MFI) of BCMA (expressed as x-fold of MFI in untreated controls) as measured by flow cytometry in alive MM cells after treatment with either 4 nM Bortezomib (Bor), 1 μM dexamethasone (Dex), 100 nM BCL-2 inhibitor (BCL-2i;
ABT-199), or 100 nM MCL-1i (S63845) for 24h (n=4-9). Each dot represents an individual sample. Statistical differences between groups were analyzed using a one-way ANOVA with Bonferroni’s multiple comparison test. **, p<0.01. (B) Representative histograms showing BCMA expression in MM cells from three different primary samples cultured in PMX. Histograms show BCMA in alive MM cells untreated (black) or after a 24h treatment with 100 nM MCL-1i (red).

**Figure 3. Pre-treatment with anti-BCMA increases MCL-1 inhibitor efficacy in MM cells.** (A) Simple linear regression analysis comparing specific apoptosis after 24h treatment with either 100 nM MCL-1i or 5 μg/mL anti-BCMA (α-BCMA; Vicky-1) (n=12). (B) Flow cytometry analysis of MCL-1 (left) and BCL-2 (right) expression in primary MM cells cultured for 2 or 7 days with IL-6 + APRIL (100 ng/mL each) in the presence of 5 mg/mL isotype control antibody (black) or anti-BCMA (grey). Mean + SEM (n=3). (C) Specific apoptosis induced by 100 nM MCL-1i (black), 5 mg/mL anti-BCMA (grey), or their combination (red) in seven primary MM samples cultured in PMX. Drugs were added on day 6, and MM cell viability was measured 24h later. (D) Plots comparing expected (EXP) to observed (OBS) specific apoptosis induced by combining anti-BCMA (5 μg/mL) and MCL-1i (100 nM) for 24h (n=7). Hypothetical expected (EXP) specific apoptosis assumes an additive effect of the two combined drugs, and was calculated by using the formula: \( [(\text{apoptosis drug A} + \text{apoptosis drug B}) - (\text{apoptosis drug A} \times \text{apoptosis drug B})/100]. \) In 5/7 samples, this combination showed a more than additive pro-apoptotic effect. (A,D) Each dot represents an individual sample. (E) Representative flow cytometry plots showing the proportion of alive MM cells (CD38+TOPRO3-) after the indicated treatments. MM samples were cultured for 6 days in the presence of either 5μg/mL isotype control antibody (black), anti-BCMA (red), or no antibody (blue). After this time, cells were treated with 100 nM MCL-1i (black, red) or
co-treated with MCL-1i + anti-BCMA (blue). MM viability was analyzed 24h later (i.e., on day 7) by FACS. (F) Cumulative plots showing apoptosis induced by MCL-1i in the conditions specified in (E). Mean + SEM (n=5). Statistical differences between 2 groups (D) were analyzed using paired t-tests. Statistical differences between 3 or more groups (F) were analyzed using a one-way ANOVA with Bonferroni’s multiple comparison test. *, p<0.05; **, p<0.01.
Supplementary Figure 1

A

Alive MM (x-fold of Nil)

B

CD38+ (%)

C

ADCC

Specific apoptosis (%)

Daratumumab (µg/mL)

D

CDC

Specific apoptosis (%)

Daratumumab (µg/mL)

BCL-2i

Specific apoptosis (%)

t(11;14)-

PMX

2D

*
Supplementary Figure 1. PMX hydrogel as a platform for *ex vivo* MM drug testing. (A) Primary MM samples (n=5) were cultured for 7 days in PMX hydrogel in the presence of 100 ng/mL IL-6, APRIL, or their combination. The graph shows the fold increase in the proportion (%) of alive MM cells relative to unstimulated controls. Statistical differences between groups were analyzed using a one-way ANOVA with Bonferroni’s multiple comparison test. *, p<0.05. (B) H929 cells were cultured in 2D or PMX before being treated with 1 µg/mL Daratumumab (Dara) for 24h. For flow cytometry analysis, cells were stained with a PE-labeled anti-CD38 antibody (clone HIT2) that binds to the same epitope as Dara. Thus, if Dara is bound to the cell surface, cells appear CD38(HIT2)-. The graph shows the proportion (%) of CD38(HIT2)+ cells measured in each setting. (C) Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) assays in PMX. L363 cells (MM cell line) were cultured for 24h in 2D or PMX before being treated with Dara at the indicated concentrations. 30 minutes after Dara addition, PBMCs (ratio PBMC:MM 10:1) or pooled human serum (25 µL/well in a final volume/well of 250 µL) were added to ADCC and CDC wells, respectively. Cell lysis was measured by flow cytometry 24h (ADCC) or 2h (CDC) after adding PBMC or serum. Error bars indicate Mean ± SD. Data were pooled from 2 independent experiments. (D) Specific apoptosis in PMX-cultured primary MM samples harboring or not the translocation t(11;14), after a 24h treatment with 100 nM BCL-2 inhibitor (BCL-2i; ABT-199). Each dot represents an individual sample. Statistical differences between both groups were analyzed using an unpaired t-test. *, p<0.05.
Supplementary Figure 2. Sensitivity of primary MM cells to BCMA targeting does not correlate with BCMA expression, and is maintained in the presence of MSC. (A) Specific apoptosis induced by 5 µg/mL anti-BCMA in primary MM samples (n=13) cultured in PMX. Anti-BCMA was added on day 1 or 6, and MM cell viability was measured 24h later (day 2 or 7, respectively). (B) Simple linear regression analysis relating BCMA mean fluorescence intensity (MFI) in untreated MM cells to specific apoptosis after 24h treatment with 5 µg/mL anti-BCMA at different time points in culture (day 2 and 7) (n=13). (C) Western blot analysis of MCL-1, BCL-2, and BCL-XL expression in H929 or OPM-2 MM cell lines after 24h treatment with APRIL (100 ng/mL), anti-BCMA (5 µg/mL), or their combination. (D) Quantification of protein expression as measured in (C), relative to expression in untreated cells. Bar graphs show averages of 2 independent experiments (+SEM). (E) Representative flow cytometry plots showing the proportion of alive MM cells (CD38+ TOPRO3-) after the indicated treatments. MM samples were co-cultured with mesenchymal stromal cells (MSC, 80.000/well) in PMX for 6 days in the presence of either 5 µg/mL isotype control antibody (black), anti-BCMA (red), or no antibody (blue). After this time, cells were treated with 100 nM MCL-1i (black, red) or co-treated with MCL-1i + anti-BCMA (blue). MM viability was analyzed 24h later (i.e., on day 7) by FACS. (F) Cumulative plots showing apoptosis induced by MCL-1i in the conditions specified in (E). Mean + SEM (n=3). (G) Plots comparing expected (EXP) to observed (OBS) specific apoptosis induced by combining anti-BCMA (5 µg/mL) and MCL-1i (100 nM) for 24h in the presence of MSC (n=3). (H) Mean Fluorescence Intensity (MFI) of BCMA in MM cells was measured by flow cytometry after 2 or 7 days in PMX + MSC co-culture (n=3). (I) MM cells were cultured in PMX and pre-treated with either daratumumab (0.1 µg/mL) or tocilizumab (5 µg/mL) before being exposed to MCL-1i (100 nM) for 24h. Bar graphs show specific apoptosis (Mean + SEM, n=3) induced by each individual agent and their combination. (J) Plots comparing expected (EXP) to observed (OBS) specific apoptosis induced by combining daratumumab or tocilizumab with MCL-1i in the settings described in (I). Statistical differences between 2 groups (G, H, J) were analyzed using paired t-tests. Statistical differences between 3 or more groups (D, F, I) were analyzed using a one-way ANOVA with Bonferroni’s multiple comparison test. (A, B, G, H, J) Each dot represents an individual sample.
## Supplementary Table 1

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**Supplementary Table 1. Clinical characteristics of MM patients (n=31).** ND, newly diagnosed; RR, relapsed/refractory; SMM, smoldering multiple myeloma; HD, hyperdiploidy.