mTOR inhibitors sensitize multiple myeloma cells to venetoclax via IKZF3- and Blimp-1-mediated BCL-2 upregulation

Physical interference with anti-apoptotic function of BCL-2 family proteins provides a novel therapeutic paradigm for hematological malignancies, the survival of which is often dependent on BCL-2 or MCL-1.[1] Among several agents targeting BCL-2 family proteins, venetoclax (ABT-199) was the first agent with selectivity for BCL-2 to enter the clinic.[2] Based on randomized phase III studies, venetoclax was approved for the treatment of patients with chronic lymphocytic leukemia and, more recently, for treatment-naive acute myeloid leukemia (AML) patients older than 75 who are unfit for intensive induction therapy.[3] Promising signals from phase I trials in multiple myeloma (MM) led to a placebo-controlled phase III trial of venetoclax in combination with bortezomib and dexamethasone.[4] In the BELLINI trial, the venetoclax group was superior to the placebo group in terms of progression-free survival, but failed to achieve prolongation of overall survival due to higher incidence of treatment-related deaths.[5] The results thus indicate that venetoclax can contribute activity to a standard-of-care regimen but might be combined more effectively with other, more-targeted therapeutic agents for the treatment of MM.

Previous studies have revealed that clinical efficacy of venetoclax is enriched in patients whose tumors carry the t(11;14) chromosomal translocation,[4] and could be predicted by the ratio of *BCL2/MCL1* mRNA expression in MM cells.[6] We confirmed the correlation between the *BCL2/MCL1* ratio and the sensitivity to venetoclax in MM cell lines used in this study (Figure 1A). In order to identify optimal combination partners for venetoclax, we performed chemical library screening for compounds that increase the *BCL2/MCL1* ratio in MM.1S cells, in which t(11;14) was absent and baseline *BCL2* expression was relatively low (Figure 1A). We found that the *BCL2/MCL1* ratio was most strikingly increased by mTOR inhibitors (mTORC1-specific inhibitors everolimus and temsirolimus, and a dual mTORC1/2 inhibitor torkinib) among 66 compounds in the library (Figure 1B; *Online Supplementary Table S1*). Next, we confirmed the increase in the *BCL2/MCL1* ratio in other MM cell lines. Everolimus and torkinib significantly upregulated *BCL2* expression at mRNA and protein levels in t(11;14)-positive KMS12-BM and KMS-21 cells in a time- and dose-dependent manner (Figure 1C; *Online Supplementary Figure S1A*). mTOR inhibitors only marginally affected the expression of *MCL1* mRNA, and therefore the net effect was to increase the *BCL2/MCL1* ratio. The increase was much more robust in t(11;14)-negative MM.1S than t(11;14)-positive MM cells because of the lower baseline expression of *BCL2* in the former (Figure 1A; note that pretreatment expression levels were adjusted in Figure 1C to visualize the changes clearly). These results suggest that mTOR inhibitors are strong candidates in combination with venetoclax for the treatment of MM regardless of the presence of t(11;14).

Next, we examined the mechanisms by which mTOR inhibitors upregulated *BCL2* mRNA expression in MM cells. To this end, we comprehensively analyzed the binding of known transcription factors in the vicinity of the transcription start sites of the *BCL2* gene using the ChIP-Atlas platform.[7] Among B-cell transcription factors, IKZF3 and Blimp-1, but not IKZF1 or FOXO1, were highly accumulated at acetylated H3K27-enriched promoter/enhancer regions of *BCL2*: GRCh37/hg37: 60,985,600-60,987,400, including P1 and P2 promoters,[8] in MM cells (Figure 2A; *Online Supplementary Figure S1B*). This is compatible with the results of biochemical studies, in which IKZF3 is a pivotal transcriptional activator of *BCL2* in T lymphocytes[9] and co-operates with Blimp-1 to maintain the survival of MM cells.[10] The expression levels of Blimp-1 and IKZF3, but not IKZF1, were positively correlated with *BCL2* expression in primary MM cells, implying their active involvement in *BCL2* transcription (*Online Supplementary Figure S1C*). Consistent with this view, short hairpin RNA (shRNA)-mediated knockdown of IKZF3 or Blimp-1 significantly decreased the abundance of *BCL2* mRNA in KMS12-BM cells (Figure 2B, upper panel). Dual inhibition of IKZF3 and Blimp-1 additively suppressed *BCL2* mRNA expression despite the fact that sh-IKZF3 negatively affected the expression of Blimp-1 and *vice versa* due to mutual transcriptional regulation of the two genes in MM cells (*Online Supplementary Figure S1D*).[10] Our attempts to obtain more prominent consequences of IKZF3 and/or Blimp-1 downregulation using other shRNA sequences failed due to induction of massive cell death because the two molecules are indispensable for the survival of MM cells (*data not shown*). The mTOR inhibitor everolimus markedly increased the abundance of IKZF3 and Blimp-1 in MM cells, both at the mRNA and protein level (*Online Supplementary Figure S2A*). This increase resulted in >10-fold accumulation of IKZF3 and Blimp-1 on the P1 promoter region of *BCL2*, which was proportional to the level of *BCL2* transactivation in MM.1S cells treated with everolimus (Figure 2B, lower panel). As anticipated, *BCL2* transactivation resulted in an increased abundance of BCL-2 protein with a reciprocal decrease in BCL-XL expression (*Online Supplementary Figure S2B*). Everolimus-mediated upregulation of BCL-2 and its regulatory proteins was retained even if everolimus was combined with venetoclax (*Online Supplementary Figure S2B*). Mechanistically, we found that everolimus activated AKT kinase via phosphorylation at serine-473, which in turn phosphorylates and inactivates EZH2 to de-repress *BCL2* transcription via erasure of a repressive histone mark, H3K27 trimethylation, on the *BCL2* promoter in MM cells (*Online Supplementary Figure S2C*). This is in line with our previous observation[11] and suggests that inhibition of the mTORC1 complex is sufficient for *BCL2* upregulation because mTOR inhibitor-mediated AKT activation is stronger when mTORC2 activity is spared.[12]

Having demonstrated that mTOR inhibitors upregulated BCL-2 expression, we next examined whether everolimus and torkinib could enhance the sensitivity of MM cells to venetoclax-mediated killing. Isobologram analyses of drug interactions revealed that mTOR inhibitors exerted a strong synergy with venetoclax in MM cells under both non-adherent and adherent conditions (Figure 2C). The synergistic effect was confirmed in primary MM cells derived from patients without t(11;14) (Figure 2D, right panel) and even in those from t(11;14)-positive patients, which showed higher baseline sensitivity to venetoclax (Figure 2D, left panel). It is well chronicled that MM cells acquire drug resistance through their interaction with stromal cells and/or extracellular matrix proteins.[11] The synergistic effect of mTOR inhibitors and venetoclax in the presence of fibronectin (adherent conditions) strongly suggests that this combination could be effective *in vivo* and potentially overcomes cell adhesion-mediated drug resistance. In order to test this notion, we attempted to reproduce the combined effect of everolimus and venetoclax in a murine xenograft model

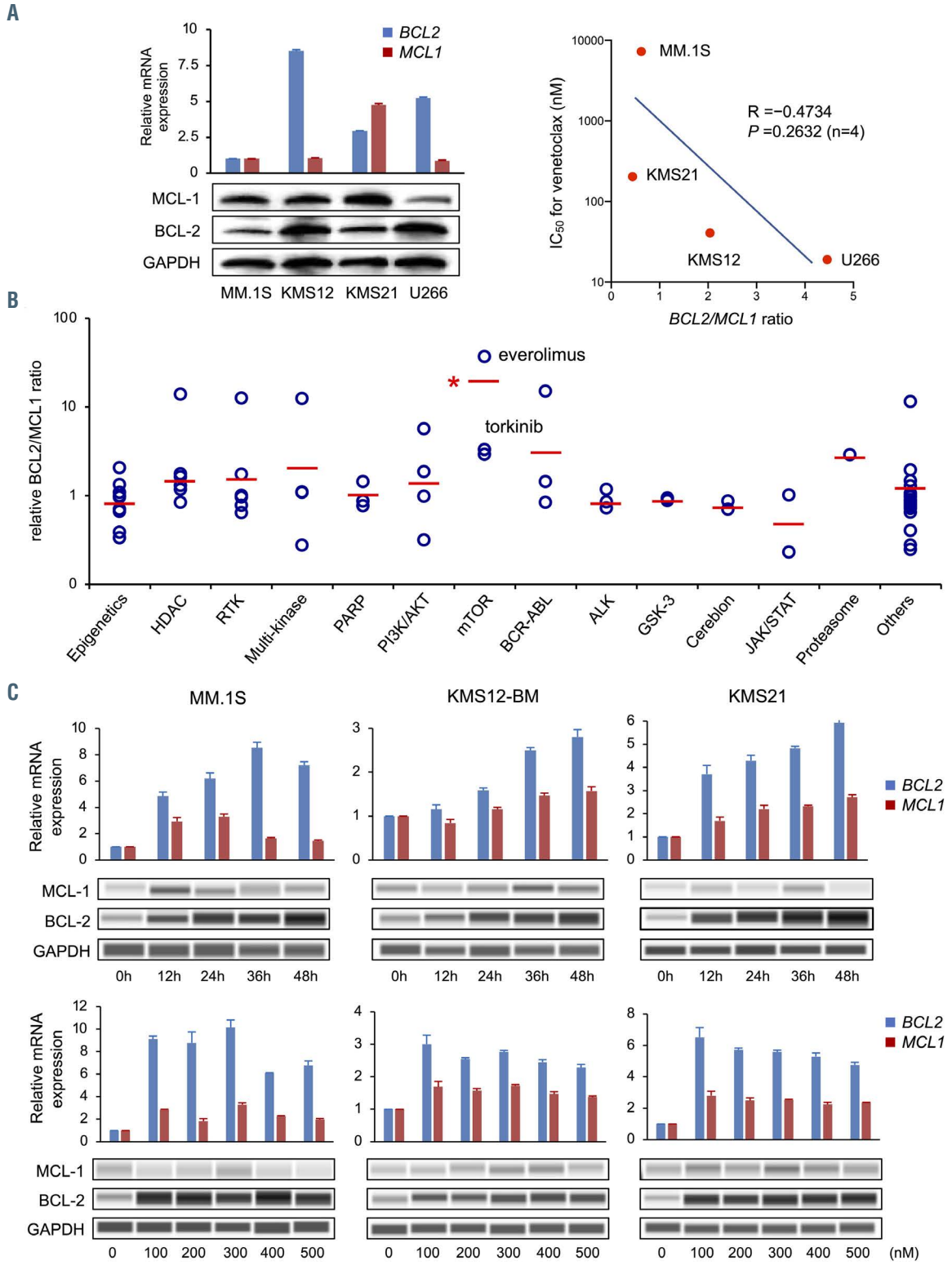

Figure 1. mTOR inhibitors increase the *BCL2/MCL1* mRNA expression ratio in multiple myeloma cells. (A) Left panel: we prepared total RNA and whole cell lysates from the indicated cell lines to evaluated the expression of BCL-2, MCL-1 and GAPDH (loading control). The expression levels of *BCL2* and *MCL1* mRNA were determined by real-time quantitative polymerase chain reaction (qRT-PCR), normalized to that of GAPDH, and quantified by the 2-$\Delta\Delta$*Ct* method with the values of MM.1S cells set at 1.0. Right panel: we determined the half maximal inhibitory concentration (IC_{50}) of 4 MM cell lines for venetoclax and calculated the Pearson's correlation coefficient with the *BCL2/MCL1* ratio. (B) MM.1S cells were cultured for 24 hours in the absence or presence of 66 small molecule inhibitors at IC_{50} (*Online Supplementary Table S1*). The categories of target molecules are shown on the x-axis. The relative ratio of *BCL2/MCL1* expression is shown on the y-axis. Bars indicate the means of each group of inhibitors. **P*<0.05 by one-way ANOVA with the Student-Newman-Keuls multiple comparison test. (C) The indicated MM cell lines were cultured in the presence of 200 nM everolimus for the indicated periods (upper panels) or various concentrations of everolimus for 24 hours (lower panels). The expression level of *BCL2* and *MCL1* was determined by qRT-PCR and shown as relative mRNA expression with pre-treatment values set at 1.0. Whole cell lysates were simultaneously prepared and subjected to immunoblotting for BCL-2, MCL-1 and GAPDH proteins.

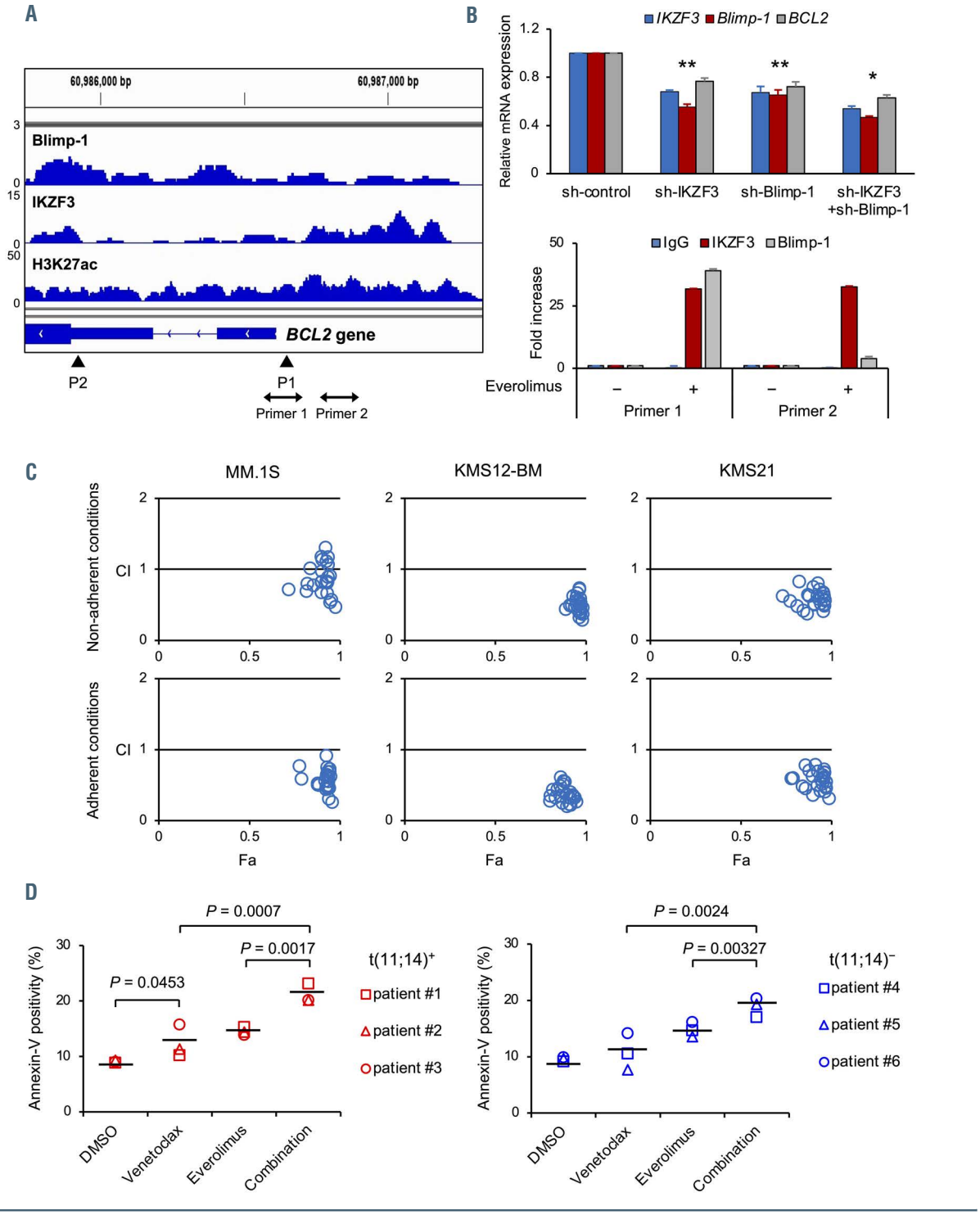

Figure 2. mTOR inhibitors enhance the binding of IKZF3 and Blimp-1 to the *BCL2* promoter. (A) The chromatin landscape of *BCL2* promoter/enhancer regions on a peak browser of ChIP-Atlas (*http://chip-atlas.org*).[7] Data were assembled from the database of human multiple myeloma (MM) cells (Blimp-1 and histone H3 acetylated at lysine-27) and human pre-B cells (IKZF3). Arrowheads indicate the location of P1 and P2 promoters.[8] (B) Upper panel: KMS12-BM cells were transduced with short hairpin RNA (shRNA)-expression vectors containing a scrambled sequence (sh-Control), shRNA against IKZF3 (sh-IKZF3), shRNA against Blimp-1 (sh-Blimp-1), or both sh-IKZF3 and sh-Blimp-1 (the nucleotide sequences are available on request). The expression level of *IKZF3, Blimp-1* and *BCL2* was determined by real-time quantitative polymerase chain reaction (qRT-PCR), normalized to that of *GAPDH*, quantified by the $2^{-\Delta\Delta Ct}$ method, and shown as fold-increases against the values obtained with sh-Control. **P*<0.01 and ***P*<0.05 by one-way ANOVA with the Student-Newman-Keuls multiple comparison test. Lower panel: chromatin suspensions were prepared from KMS12-BM cells cultured with (+) or without (−) 200 nM everolimus for 24 hours and immunoprecipitated with anti-IKZF3 antibody, anti-Blimp-1 antibody or isotype-matched immunoglobulin (Ig) G. The resulting precipitants were subjected to qRT-PCR with primer 1 (forward: 5'-GTCCGGTATTCGCAGAAGTC-3' [−108 to −88] and reverse: 5'-CTCCTTCATCGTCCCCTCTC-3' [−239 to −219]), which covers the P1 promoter region of the BCL2 gene, and primer 2 (forward: 5'-GTGCCGAGCGCTAGAAGC-3' [−361 to −343] and reverse: 5'-GGGAGAACTTCGTAGCAGTCAT-3' [−467 to −445]), which covers the upstream enhancer region, as indicated in the left panel. The data were normalized to the values of input, quantified by the $2^{-\Delta\Delta Ct}$ method, and

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shown as fold-increases against the values obtained with control IgG immunoprecipitants from untreated KMS12-BM cells. (C) The indicated MM cell lines were treated with venetoclax in combination with everolimus in 96-well plates coated with (adherent conditions) or without (non-adherent conditions) fibronectin for 72 hours. Dose-response curves of each combination were generated to make non-constant normalized isobolograms at half maximal inhibitory concentration (IC_{50}) using CompuSyn software. The isobolograms shown are representative of at least three independent experiments. A combination index (CI) <1.0 indicates the synergism of the two drugs.[11] (D) Left panel: CD138-positive cells were isolated from the bone marrow of MM patients carrying t(11;14) and treated with vehicle alone (dimethyl sulfoxide [DMSO]), 50 ng/mL venetoclax, 50 nM everolimus, and the combination of venetoclax and everolimus for 24 hours. Right panel: the same experiments using CD138-positive cells from MM patients who were negative for t(11;14). The y-axis shows the percentage of annexin-V-positive cells assessed by flow cytometry. Bars indicate the means of three samples. *P*-value was determined by one-way ANOVA with Tukey's multiple comparison test. No brackets mean *P*>0.05. Informed consent was obtained in accordance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of Jichi Medical University.

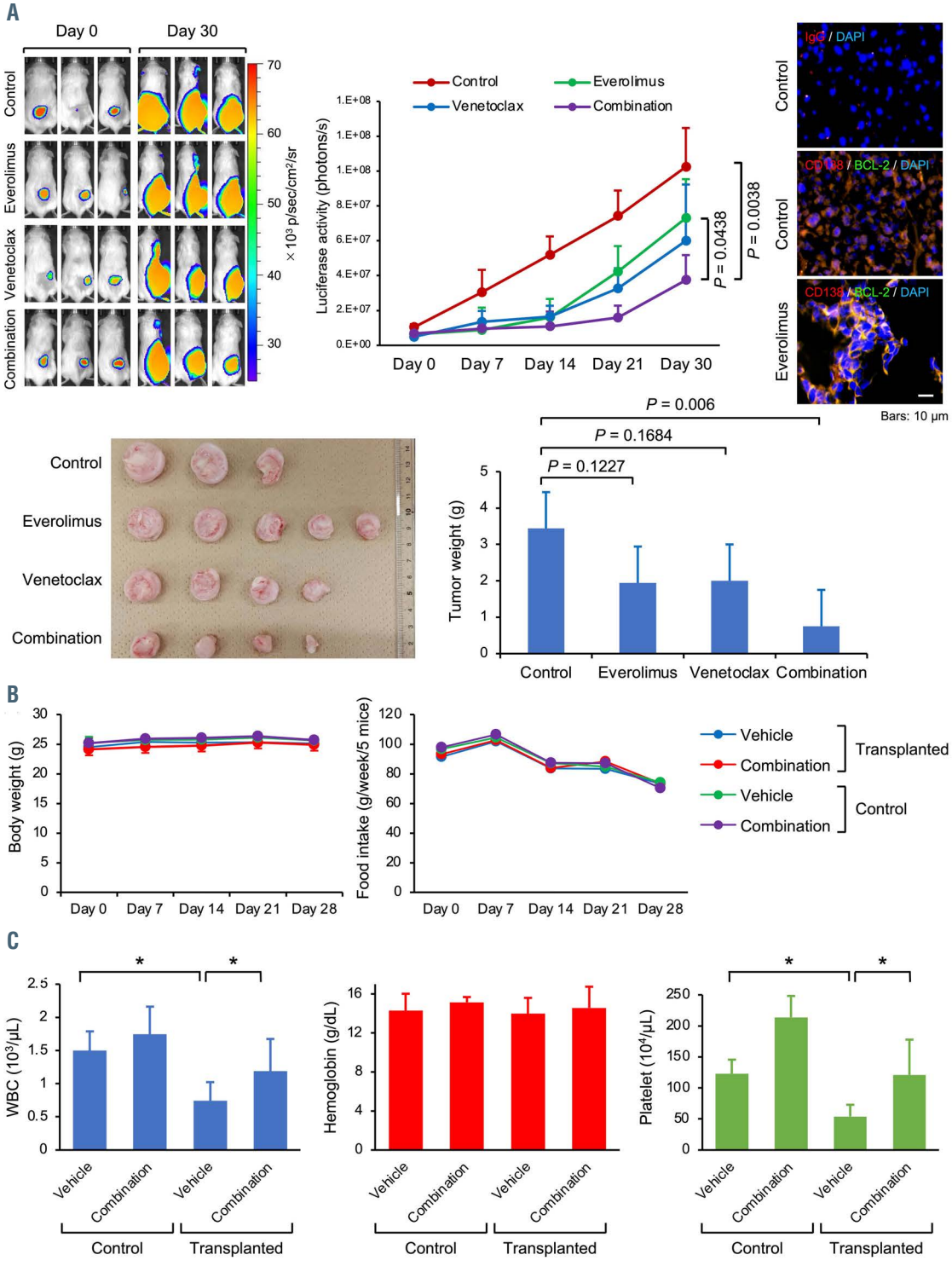

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Figure 3. The mTOR inhibitor everolimus exhibits synergistic effects with venetoclax in a murine multiple myeloma model. (A) We inoculated 5x10^5 luciferase-expressing KMS12-BM cells subcutaneously in the right thigh of male NOD/SCID mice (Charles River Laboratories, Wilmington, MA) and randomized them into four treatment groups when measurable tumors developed (day 0). Each group was treated with the vehicle alone (0.9% NaCl, orally, 5 times a week), everolimus alone (4 mg/kg, orally, twice a week), venetoclax alone (40 mg/kg, orally, 5 times a week), and the combination of everolimus and venetoclax (n=5 each) for 3 weeks. Upper left panel: tumor-derived luciferase activity was measured *ex vivo* by the IVIS Imaging System after D-luciferin injection. Representative photographs of NOD/SCID mice on day 0 and day 30 are shown (original magnification: x2). Upper middle panel: quantitative data of *in vivo* bioluminescence imaging shown in the left panel. The signal intensity is shown as photon units (photons/s). *P*-value was determined by one-way ANOVA with Tukey's multiple comparison test. Upper right panel: tumor sections were prepared from vehicle-treated (control) and everolimus-treated mice at day 7 and stained with anti-BCL-2 rabbit polyclonal antibody or isotype-matched immunoglobulin G (IgG), followed by FITC-conjugated anti-rabbit IgG antibody (green), and PE-conjugated anti-human CD138 antibody (red). Nuclei were counterstained with DAPI (blue). Only merged images are shown. Data shown are representative of multiple independent experiments. Lower left panel: representative photographs of tumors resected on day 30 (original magnification: x2). Lower right panel: the means ± standard deviation (S.D.) (bars) of the weights of resected tumors shown in the left panel. *P*-value was determined by one-way ANOVA with Tukey's multiple comparison test. (B) We inoculated 5x10^5 luciferase-expressing KMS12-BM cells (transplanted) or culture medium alone (control) subcutaneously in the right thigh of male NOD/SCID mice and randomized them into two treatment groups when measurable tumors developed in transplanted mice (day 0). Each group was treated with the vehicle alone (0.9% NaCl, orally, 5 times a week) or the combination of everolimus (4 mg/kg, orally, twice a week) and venetoclax (40 mg/kg, orally, 5 times a week) for 4 weeks. Left panel: we measured body weights of mice on the indicated days. The means ± S.D. (bars) are shown (n=5). No significant difference was noted between the groups by one-way ANOVA with Tukey's multiple comparison test. Right panel: we estimated food intake of each group of mice (n=5 in a same cage) by calculating the consumption of supplied diets during a week (the day 0 value means food consumption between day -7 and day 0). (C) We measured the counts of white blood cells (WBC), hemoglobin, and platelets in peripheral blood of recipient mice on day 21 of treatment. The means ± S.D. (bars) are shown (n=5). ***P*<0.05 by Student's *t*-test. All animal studies were approved by the Institutional Animal Ethics Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals formulated by the National Academy of Sciences.

of MM. First, we determined that the maximal tolerated doses of everolimus and venetoclax were 4 mg/kg, twice a week and 40 mg/kg, fives times a week, respectively, for NOD/SCID mice in a pilot experiment (*data not shown*). We inoculated luciferase-expressing KMS12-BM cells subcutaneously in the right thigh of NOD/SCID mice and, when measurable tumors developed, started the treatment with vehicle alone (0.9% NaCl), everolimus alone, venetoclax alone or the combination of everolimus and venetoclax for randomly assigned groups of mice (n=5 each). The combined treatment with everolimus and venetoclax significantly retarded the growth of inoculated tumors as evidenced by luciferase activity traced *ex vivo* (Figure 3A, upper panels) and the size of tumors resected on day 30 (Figure 3A, lower panels), whereas either everolimus or venetoclax alone showed only moderate effects at the doses and schedules used. A histopathological examination of resected tumors confirmed the growth-inhibitory effect of the combination of the two drugs and mTOR inhibition-mediated BCL-2 up-regulation *in vivo* (Figure 3A, upper right panel). Everolimus has already been approved for the treatment of breast cancer, renal cell carcinoma and neuroendocrine tumors by the Food and Drug Adminstration, and is known to cause gastrointestinal toxicity such as mucositis, diarrhea, nausea and vomiting.[13] Neither decreased food intake nor weight loss was observed in everolimus- or everolimus/venetoclax-treated mice, although food intake slightly declined during experiments in all groups (Figure 3B). Moreover, we measured complete blood counts on day 21 to check for neutropenia, the most common side effect of venetoclax in MM patients,[4,5] and other hematological toxicity that might be exacerbated in combination with everolimus. As shown in Figure 3C and the *Online Supplementary Figure S2D*, tumor implantation caused a significant decrease in leukocytes and platelets in NOD/SCID mice probably due to remote effects of MM cells on hematopoiesis.[14] Notably, leukopenia and thrombocytopenia recovered after treatment with the combination of everolimus and venetoclax, likely reflecting their therapeutic effects on the disease.

In conclusion, we have shown that mTOR inhibitors can enhance the anti-MM effects of venetoclax via up-regulation of BCL-2 expression mainly through mTORC1 inhibition. Farber *et al.*[15] reported that ATP-competitive dual inhibitors of mTORC1/2 augmented the effects of navitoclax via MCL-1 down-regulation in colon and lung cancer cells. The difference between the two observations may stem from the different cellular context or patterns of mTOR inhibition. The combined effect of mTOR inhibitors and venetoclax *in vitro* was reproduced in a murine model without obvious hematological and gastrointestinal toxicity. The efficacy and safety of this combination are worthy of investigation in clinical settings.

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