β adrenergic signaling regulates hematopoietic stem and progenitor cell commitment and therapy sensitivity in multiple myeloma

Multiple myeloma (MM) development is dependent upon critical interactions with the bone marrow (BM) niche.[1] The contribution of catecholamines and adrenergic signaling from the highly innervated BM niche[2] to MM development is under-explored. MM patients demonstrate an elevated conserved transcriptional response to adversity (CTRA), indicative of stress that correlates with poor survival.[3] A retrospective study evaluating the effects of the non-selective β adrenergic receptor (AR) blocker propranolol in immunomodulatory drug-treated MM found propranolol to improve progression-free survival (PFS) and overall survival (OS).[4] MM patients exhibit reduced megakaryocyte–erythrocyte progenitors (MEP) and increased monocytic myeloid-derived suppressor cells (MDSC) ($CD14^{+}HLADR^{low}$) in the BM, suggestive of increased myeloid bias.[5] Introduction of MM precursor monoclonal gammopathy of undetermined significance (MGUS) cells into humanized IL-6 MIS(KI)TRG6 mice promotes progression to MM, suggesting the sufficiency of extrinsic BM niche elements in fostering MM development.[6] Consistent with this, administration of propranolol in MM patients undergoing hematopoietic stem cell transplant (HSCT) demonstrates a significant reduction of not only the CTRA response, but also marked reductions in myeloid lineage bias.[3] How targeting adrenergic signaling regulates hematopoietic stem and progenitor cell (HSPC) commitment in MM remains poorly understood. Our study provides mechanistic rationale for the application of propranolol to resolve both microenvironmental and MM-specific tumor promoting biology.

For this study, samples from MM patients, ranging from newly diagnosed to those with relapsed refractory MM were analyzed, exhibiting a range of reduced hemoglobin (Hgb) levels (characteristics in the *Online Supplementary Table S1*) and anemia. BM aspirates were obtained from consenting patients following an Emory University Institutional Review Board-approved protocol. Research was conducted in accordance with the Declaration of Helsinki. Ficoll gradient isolated mononuclear cells were cultured for phase I expansion in serum-free expansion medium (SFEM) containing granulocyte macrophage colony-stimulating factor (GMCSF), stem cell factor (SCF), interleukin 3 (IL-3 – pluripotent hematopoietic colony-stimulating factor) or further expanded in phase II (day 6-16) by culture in SCF, HT (holo-transferrin), EPO (erythropoietin), and SFEM for all assays involving progenitor cells as previously described.[7,8] Cell death/viability were assessed by AnnexinV/DAPI flow cytometry. The CoMMpass MM trial (clinicaltrials gov. Identifier: NCT0145429) data was downloaded from Genomic Data Commons. All other assays are as previously reported.[8,9]

Analysis of the proportion of granulocyte-monocyte-progenitors (GMP) (Lin^{neg} $CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}$) *versus* MEP ($Lin^{neg}$ $CD34^{+}CD38^{+}CD123^{-}CD45RA^{-}$) reconfirmed the significantly reduced number of MEP[10] and importantly for the first time, identified a higher proportion of GMP in MM BM (n=10) *versus* control bone marrow (CBM) (Figure 1A).

Myeloid-biased hematopoiesis contributes to anemia and a protumorigenic BM marrow niche in MM. Strategies targeting the myeloid lineage skew can potentially prevent MM progression and development of fatal refractory disease. V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B transcription factor (MAFB) and globin transcription factor 1 (GATA1) are central regulators of myeloid *versus* erythroid lineage commitment. We previously showed that the development of anemia in human burn patients and scald burn mice is driven by high MAFB *versus* GATA1 expression in CMP. Similarly, we found CMP from MM BM (n=10) expressed significantly higher MAFB and reduced GATA1 expression compared to CMP from CBM (Figure 1B), which inversely correlated with the percentage of MEP. Notably, both reduced MEP/GMP ratio and elevated MAFB/GATA1 expression in MM CMP were maintained after phase I expansion of HSPC, suggesting that the skew in lineage specification is intrinsically driven (*Online Supplementary Figure S1A* and *B*). Genetic suppression of MAFB expression reduced GMP and increased MEP in the MM BM samples (n=4) (Figure 1C), suggesting that reduction of MAFB was sufficient to block the myeloid bias detected in MM.

Concordant with reduced MM MEP specification, MM BM also exhibited significantly lower total erythroblasts compared to CBM (Figure 1D). Although variability in early erythroblast (EEB) numbers were noticed, late erythroblasts (LEB) were uniformly lower in all MM BM samples (n=10), indicating erythropoietic arrest in MM (Figure 1D).

We have previously shown in human burn patients and scald burn mice that conditions resulting in high catecholamines instigate myelo-erythroid reprioritization and anemia.[8] As stress and catecholamines are known to modulate the BM microenvironment in MM,[11] we investigated whether adrenergic signaling regulated MEP/GMP specification in MM BM.

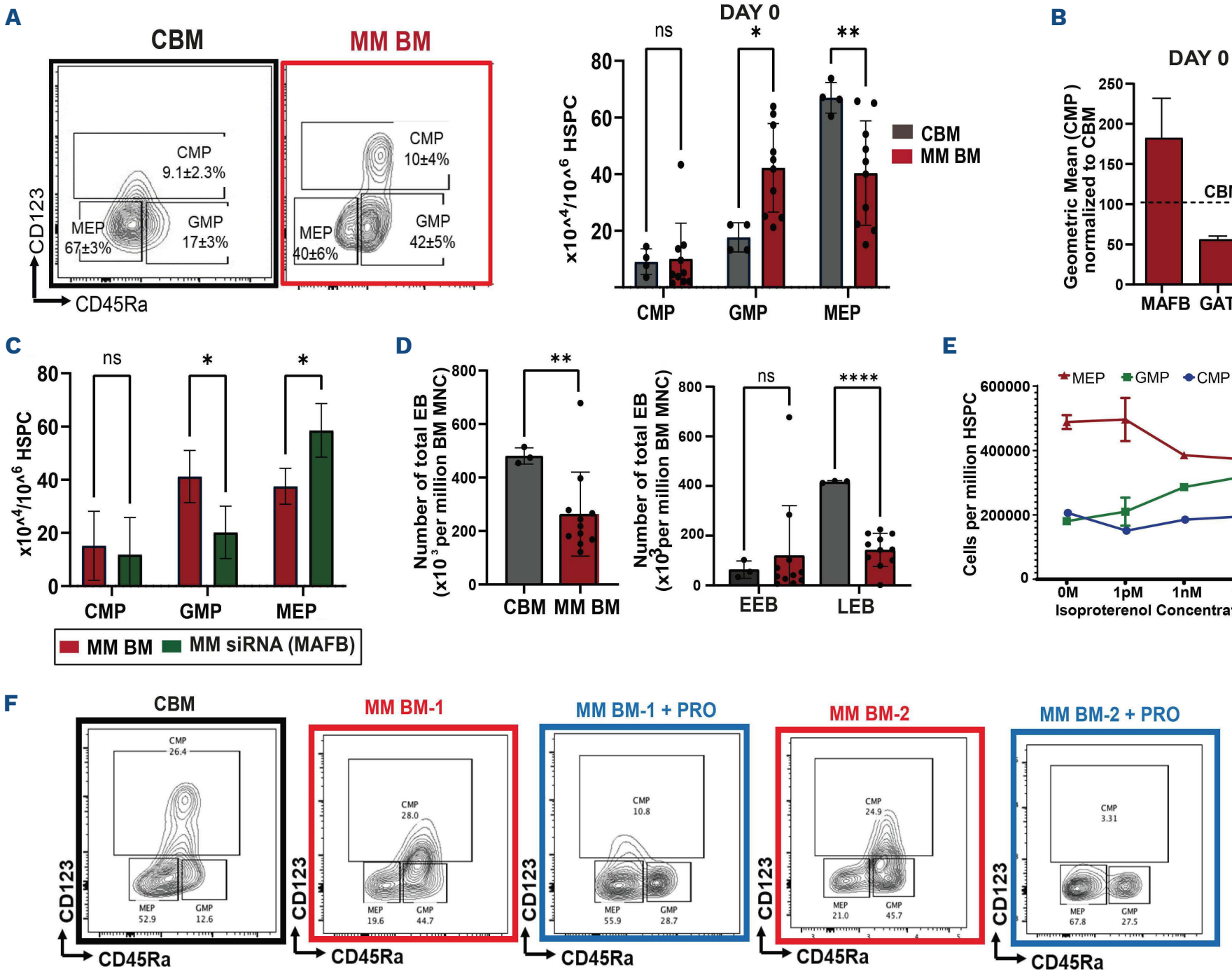
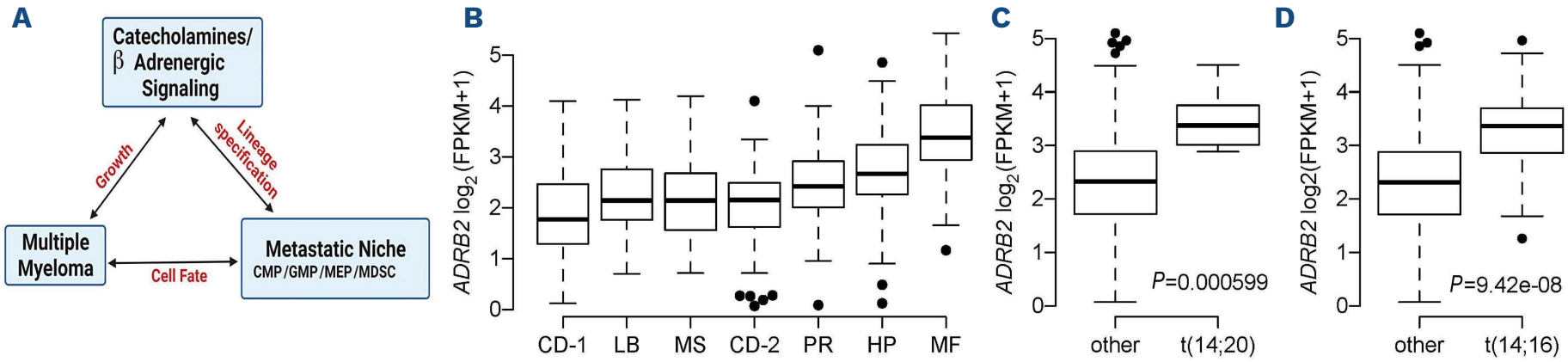
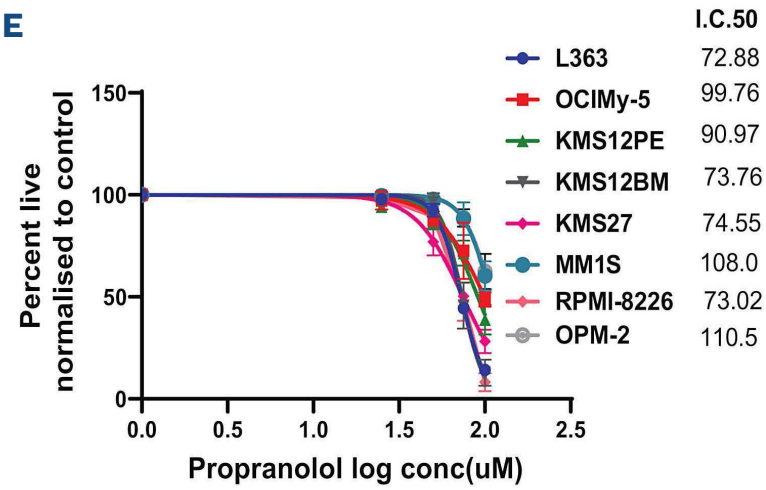


Figure 1. Multiple myeloma patient samples exhibit reduced megakaryocyte–erythrocyte progenitors, elevated MAFB/GATA1 and reduced late erythroblast development that is reversed with propranolol treatment. (A) Representative contour plots of control bone marrow (CBM) and multiple myeloma (MM) sample mononuclear cells in forward *vs.* side scatter (left panel), from flow cytometry day 0 CBM and MM BM evaluated for common myeloid progenitors (CMP)/ granulocyte-monocyte-progenitors (GMP)/megakaryocyte–erythrocyte progenitors (MEP) frequencies (n=10) (right panel). (B) Mean fluorescence intensity (MFI) of MAFB and GATA1 expression in CMP from 10 MM patients at day 0, evaluated by flow cytometry. (C) Introduction of non-targeting and MAFB-directed small interfering RNA (siRNA) in MM BM samples (n=4). Frequencies of CMP, GMP and MEP in MM *vs.* MM siRNA-transfected samples are shown. (D) Evaluation of total erythroblasts (EB) ($CD71^+CD235a^{-/+}$) in MM *vs.* CBM $P<0.001$ (n=10) (left panel). Late erythroblasts (LEB) ($CD71^+CD235a^+$) are significantly decreased ($P<0.0001$) compared to early erythroblasts (EEB) ($CD71^+CD235a^-$) suggesting erythropoietic arrest in MM (right panel). (E) MEP, GMP and CMP quantified in hematopoietic stem and progenitor cell (HSPC) in phase 1 cultures treated with increasing doses of isoproterenol (ISO). (F) CMP, GMP and MEP frequencies quantified in in CBM and MM treated *ex vivo* with propranolol (PRO).

We first evaluated basal expression levels of the α and β AR by flow cytometry. HSPC of MM BM and CBM were found to exhibit similar expression of both α (α_1, α_2) and β (β_1, β_2, β_3) AR (*data not shown*). Stimulation of CBM HSPC with increasing concentrations of isoproterenol, a specific agonist of β AR, increased GMP with a corresponding reduction of MEPs (Figure 1E). Isoproterenol, importantly, increased MAFB expression in CMP and GMP and reduced GATA1 expression in CMP, GMP and MEP (*Online Supplementary Figure S1C* and *D*). Phenylephrine, an α AR specific agonist, had no effect on HSPC specification towards GMP *versus* MEP (n=4) or on MAFB/GATA1 expression, suggesting β adrenergic stimulation specifically regulates myeloid bias (*Online Supplementary Figure S1E*). Inhibition of β AR with propranolol suppressed MAFB expression in CMP (*Online Supplementary Figure S1C*). Importantly, β AR inhibition with propranolol was also able to reverse the low MEP:GMP ratio in MM BM (Figure 1F). These results, in sum, demonstrate that in MM, i) β adrenergic signaling can regulate HSPC specification; ii) propranolol reverses the





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SAMPLE ID	I.C.50 BTZ (nM)	I.C.50 BTZ(nM)+PRO (50μM)	I.C.50 BTZ (nM)+PRO (100μM)
PS10002043	17.86	10.35	2.302
PS10002013	17.66	12.84	3.798
PS10002039	4.16	4.427	2.333
PS10001562	>50	6.758	0.4065

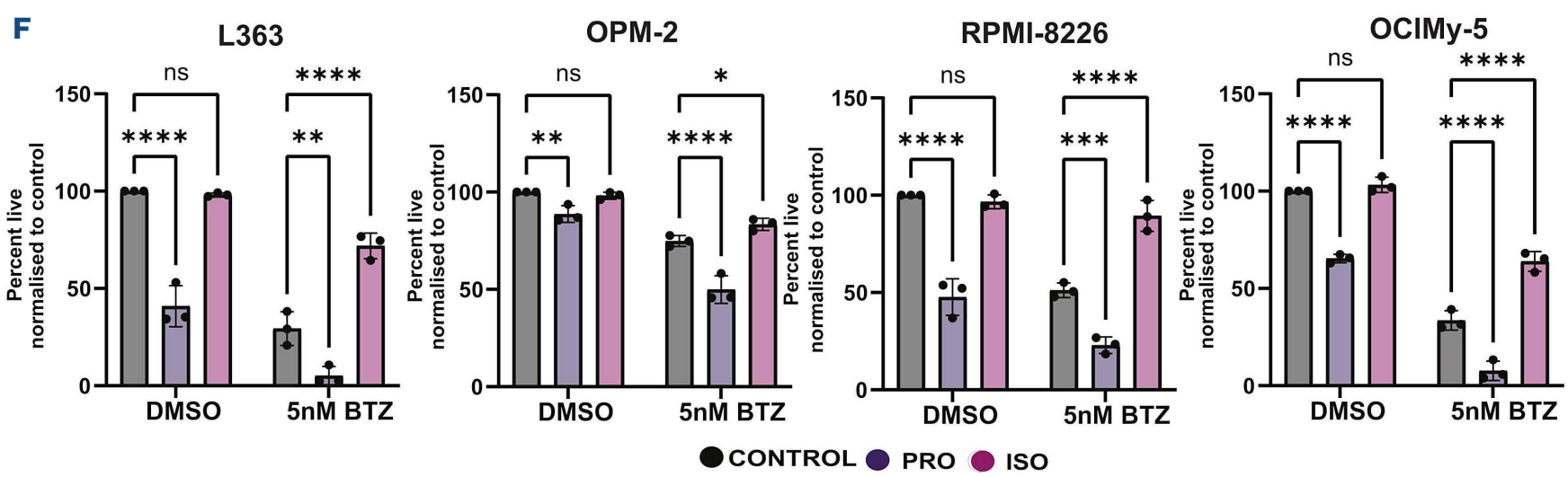

Figure 2. Isoproterenol and propranolol regulate multiple myeloma sensitivity to the proteasome inhibitor bortezomib. (A) Model of how β adrenergic signaling establishes a tumor promoting triad between multiple myeloma (MM) and the skewed common myeloid lineage specification in the BM (bone marrow) niche. (B) *ADRB2* expression in MM gene expression subtypes from the CoMMpass study (IA17). CD-1: Cyclin D1; LB: low bone disease; MS: MMSET; CD-2: Cyclin D1 and CD20; PR: proliferation; HP: hyperdiploid; MF: MAF. (C) *ADRB2* expression in patients with t(14;20) (IGH-MAFB) (D) and t(14;16) (IGH-MAF) translocations. *P*-values were determined by two-sided *t*-test. (E) MM lines treated with dose range of propranolol (PRO) for 24 hours (h) assessed for viability using Annexin V/DAPI flow cytometric staining. (F) Cell lines treated with 75 μM PRO or isoproterenol (ISO) as indicated for 24 h assessed for viability using Annexin V/DAPI flow cytometric staining. (G) MM patient BM mononuclear cells treated with 50 or 100 μM PRO in combination with bortezomib (BTZ) assessed for BTZ half maximal inhibitory concentration (I.C.50) using AnnexinV/DAPI flow cytometric staining.

effects of β agonist-stimulated elevation in GMP and lastly; iii) propranolol regulates MAFB/GATA1 expression to restore MEP commitment in MM (Figure 2A).

Ectopic MAFB expression in mouse HSC promotes acquisition of a tumoral plasma cell fate without induction of MAF in tumor cells.[12] MAF also has a role in promoting MM growth and its expression correlates with poor OS.[13] MAF thus has both cell extrinsic and intrinsic roles in shaping myeloma genesis, warranting development of strategies to target MAF for MM therapy. Evaluation of RNA sequencing in primary MM samples from the CoMMpass trial[14] (clinical trials gov. Identifier: NCT0145429) indicated *ADRB2* was significantly higher in the MAF (MF) gene expression subtype (Figure 2B). Consistent with this, *ADRB2* expression was elevated in MM samples harboring the high risk-associated t(14;20) or t(14;16) translocations that

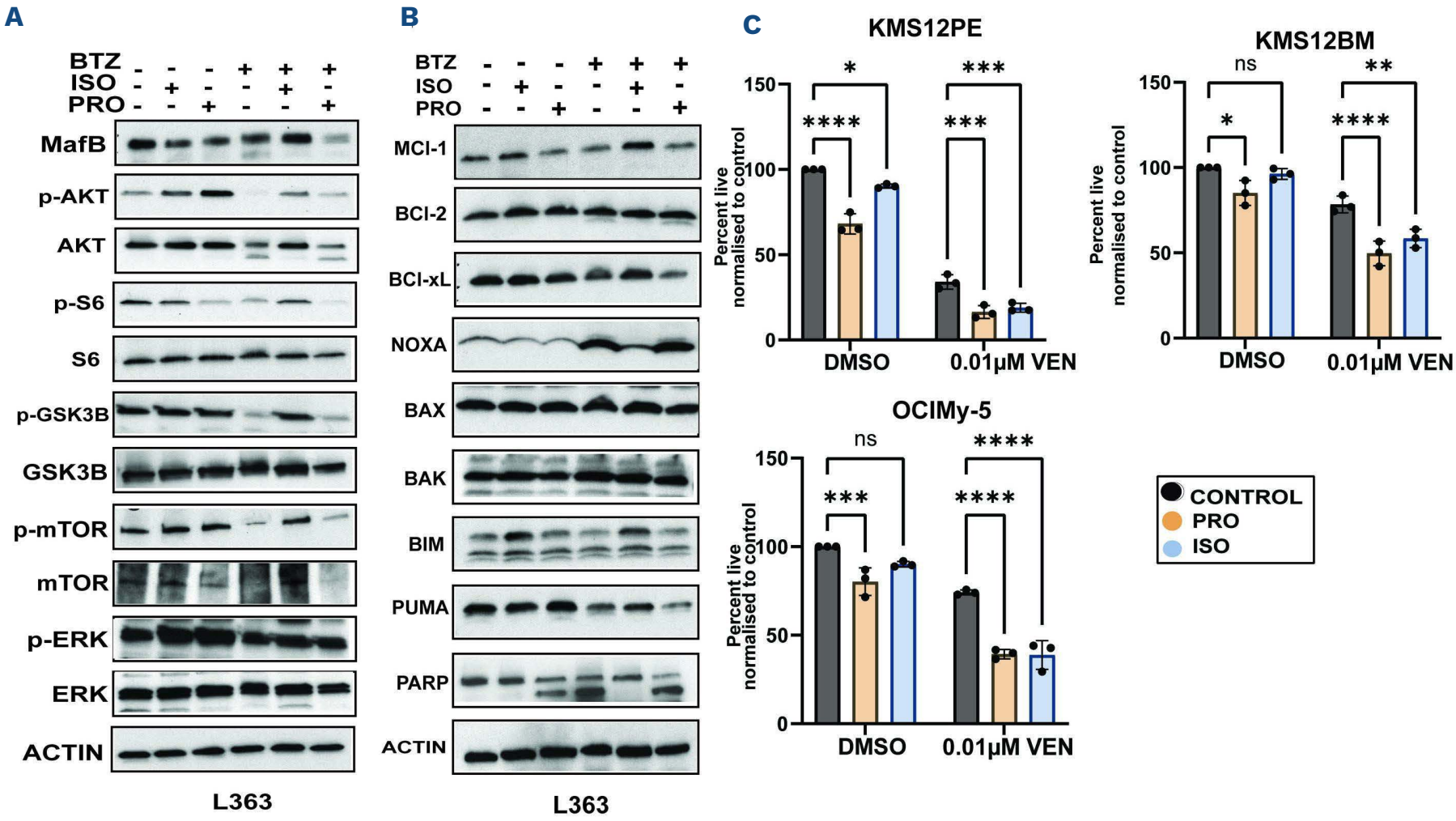

Figure 3. Propranolol increases sensitivity to the BCL-2 antagonist venetoclax. (A and B) L363 cells treated with bortezomib (BTZ) and/or isoproterenol (ISO)/propranolol (PRO) as indicated for 18 hours (h), assessed for indicated proteins by immunoblot analyses. (C) Cell lines treated with 75 μM PRO or 75 μM ISO in combination with 0.01 μM venetoclaX (VEN) for 24 h assessed for viability. All cell death/viability assessed by AnnexinV/DAPI flow cytometry. (D) Model: β adrenergic signaling elevates MAFB *vs.* GATA1 expression in common myeloid progenitors (CMP) leading to increased granulocyte monocyte progenitor (GMP) *vs.* megakaryocyte erythrocyte progenitor (MEP) specification in multiple myeloma, establishing a feed forward loop. Model is created in BioRender.com. MDSC: myeloid-derived suppressor cells; HSPC: hematopoietic stem and progenitor cells; βAR: β adrenergic receptor.

juxtapose the IgH enhancer to drive elevated levels of *MAFB* and *MAF* expression, respectively (Figure 2C and D). *ADRB1* and *ADRB3* expression was low to undetectable in most MM samples (*data not shown*). Additionally, ADRB2 was detected in all MM cell lines tested (*Online Supplementary Figure S1F*). These observations prompted us to examine the effects of propranolol on MM cells.

Propranolol has potent anti-cancer effects attributed to both tumor intrinsic and extrinsic effects.[15] We found propranolol to elicit cytotoxicity in MM cell lines (Figure 2E). Proteasome inhibitors (PI) like bortezomib (BTZ) are backbone MM therapeutics, however, most MM patients become refractory to PI. We found treatment of MM cell lines with lower doses of propranolol enhanced, while isoproterenol reversed, sensitivity to BTZ, irrespective of MAFB expression status (Figure 2F). In order to clinically validate our observations, we tested the effects of isoproterenol, propranolol and BTZ treatments in MM patient samples (n=4). As seen in cell lines, isoproterenol promoted resistance to BTZ, while isoproterenol increased sensitivity to BTZ in the MM primary cells (Figure 2G). We found isoproterenol in the context of BTZ treatment to elevate MAFB, pAKT, p-S6 and pmTORC1 (Figure 3A). While treatment with BTZ is known to stabilize MAFB expression,[16] propra-

nolol significantly reduced MAFB expression in BTZ treated cells, underscoring the utility of using propranolol to target MAFB and overcome β adrenergic-stimulated activation of the AKT-mTOR axis (Figure 3A).

Since most chemo-resistance occurs because of impaired ability to execute apoptosis consequent to reduced "priming" i.e., suboptimal quantities of BH3 activators bound to anti-apoptotics,[9,17,18] we evaluated the expression of the major BCL-2 family proteins (Figure 3B). BTZ cytotoxicity is dependent upon increased NOXA expression that, upon binding MCL-1, allows for pro-apoptotic BIM release. Interestingly, we found BTZ-induced elevation of NOXA and reduction of MCL-1 and BCL-xL is suppressed by isoproterenol (Figure 3A). Co-treatment with propranolol and BTZ maintains NOXA induction, MCL-1 suppression, and PARP cleavage with further reduction of BCL-xL and PUMA. Lastly, we found propranolol increases sensitivity to the BCL-2 antagonist venetoclax (VEN) and importantly, isoproterenol stimulation did not reverse propranolol-induced sensitivity to VEN (Figure 3C).

In conclusion, our results suggest neurotransmitters elevate MAFB in MM CMP to augment pro-tumorigenic GMP-MDSC commitment, as summarized in the schematic (Figure 3D), that can be reversed with propranolol, restricting myelopoiesis in MM. Additionally, we show that β adrenergic stimuli selectively increase resistance to proteasome inhibitors, while targeting β adrenergic signaling with propranolol increases sensitivity to BTZ and VEN. We acknowledge the limited patient sample size for the current study and the need for greater mechanistic understanding of how β adrenergic signals regulate intra- and inter-cellular signaling to promote niche remodeling and drug sensitivity. Our results, in sum, underscore the importance of further interrogation of early application of propranolol and other β blockers in MM therapy.

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AKN has significant financial interest in Janssen Pharmaceuticals and has participated on advisory boards and received honoraria from Janssen, Takeda, Amgen, BMS/Celgene, Glaxo SmithKline, Sanofi, Oncopeptides, BeyondSpring, Karyopharm, SecuraBio, and Adaptive Technologies. SL is a consultant for Takeda, Celgene, Novartis, BMS, Amgen, ABBVIE, and Janssen and on the Board of directors with stock for TG therapeutics. LHB receives research funding from AstraZeneca (2019), consultancy, and honoraria from AstraZeneca; and performs consultancy for Genentech (2019) and Abbvie. All other authors declare no competing financial interests.

Contributions

RN, VS, KM, MS conceived and designed the research; RN and VS performed all experimentation; Bioinformatic analyses were performed by BGB. SMM and VAG assisted with patient sample purification; AKN, SL oversaw myeloma patient sample collection; LHB, BGB, VAG, AKN, SKM and KM provided helpful critiqu; KM provided edits to the manuscript; RN and MS wrote the manuscript.

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Data-sharing statement

All technical information pertaining to the experimentation performed is available on request.

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