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Beta adrenergic signaling regulates HSPC commitment and therapy sensitivity in multiple myeloma

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Author contributions

R.N., V.S., K.M., M.S. conceived and designed the research. R.N. and V.S. performed all experimentation. Bioinformatic analyses were performed by B.G.B. S.M.M. and V.A.G. assisted with patient sample purification. A.K.N., S.L. oversaw myeloma patient sample collection. L.H.B., B.G.B. V.A.G., A.K.N., S.K.M. and K.M provided helpful critique. K.M provided edits to the manuscript. R.N. and M.S. wrote the manuscript.

Competing interests

A.K.N. 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. L.H.B. 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.

Data sharing statement

All technical information pertaining to the experimentation performed is available on request.
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Ethics approval and consent to participate

BM aspirates were collected from consenting myeloma patients following an Emory University Institutional Review Board (IRB)-approved protocol. Research was conducted in accordance with the Declaration of Helsinki.

To the editor:

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 βAR blocker propranolol (PRO) in immunomodulatory drug-treated MM found propranolol to improve progression free survival (PFS) and overall survival (OS) (4). MM patients exhibit reduced MEPs (megakaryocyte–erythroid progenitors) and increased monocytic-MDSCs (myeloid-derived suppressor cells) (CD14+HLADRlow) in the BM, suggestive of increased myeloid bias (5). Introduction of MM precursor MGUS (monoclonal gammopathy of undetermined significance) 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 Hgb levels (characteristics in Supplementary Table 1) and anemia. BM aspirates were obtained from consenting patients
following an Emory University Institutional Review Board-approved protocol. Ficoll gradient isolated mononuclear cells were cultured for Phase I expansion in serum free expansion medium (SFEM) containing GMCSF (granulocyte macrophage colony-stimulating factor), SCF (stem cell factor), IL-3 (interleukin 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 described in (7, 8). Cell death/viability were assessed by AnnexinV/DAPI flow cytometry. The CoMMpass MM trial (NCT0145429) data was downloaded from Genomic Data Commons. All other assays are as reported in (8) and (9).

Analysis of the proportion of granulocyte/monocyte progenitors (GMPs) (Lin\textsuperscript{neg} CD34\textsuperscript{+}CD38\textsuperscript{+}CD123\textsuperscript{-}CD45RA\textsuperscript{+}) vs MEPs (Lin\textsuperscript{neg} CD34\textsuperscript{+}CD38\textsuperscript{+}CD123\textsuperscript{-}CD45RA\textsuperscript{-}) re-confirmed the significantly reduced number of MEPs (10) and importantly for the first time, identified a higher proportion of GMPs in MM BM (n=10) vs CBM (control bone marrow) (Figure 1A).

Myeloid-biased hematopoiesis contributes to anemia and a pro-tumorigenic bone marrow niche in MM. Strategies targeting the myeloid lineage skew can thus 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 vs. erythroid lineage commitment. We previously showed that the development of anemia in human burn patients and burn scald mice is driven by high MAFB vs GATA1 expression in CMPs (8). Similarly, we found CMPs from MM BM (n=10) expressed significantly higher MAFB and reduced GATA1 expression compared to CMPs from CBM (Figure 1B), which inversely correlated with the percentage of MEPs. Notably, both reduced MEP/GMP ratio and elevated MAFB/GATA1 expression in MM CMPs were maintained after phase I expansion of HSPCs, suggesting that the skew in lineage specification is intrinsically driven (Supplementary Figure 1 A, B). Genetic suppression of MAFB expression reduced GMPs and increased MEPs 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 (EEBs) numbers were noticed, late erythroblasts (LEBs) 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.

We first evaluated basal expression levels of the alpha- and beta-adrenergic receptors by flow cytometry. HSPCs of MM BM and CBM were found to exhibit similar expression of both α (α1, α2) and β (β1, β2, β3) receptors (data not shown). Stimulation of CBM HSPCs with increasing concentrations of isoproterenol (ISO), a specific agonist of β-ARs, increased GMPs with a corresponding reduction of MEPs (Figure 1E). Isoproterenol, importantly, increased MAFB expression in CMPs and GMPs and reduced GATA1 expression in CMPs, GMPs and MEPs (Supplementary Figure 1C, D). Phenylephrine, an α-AR specific agonist, had no effect on HSPC specification towards GMP vs MEPs (n=4) or on MAFB/GATA expression, suggesting β adrenergic stimulation specifically regulates myeloid bias (Supplementary Figure 1E). Inhibition of β-AR with propranolol suppressed MAFB expression in CMPs (Supplementary Figure 1C). 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, 1) β adrenergic signaling can regulate HSPC specification; 2) propranolol reverses the effects of β agonist-stimulated elevation in GMPs and lastly; 3) propranolol regulates MAFB/GATA1 expression to restore MEP commitment in MM (Figure 2A).

Ectopic MAFB expression in mouse HSCs 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-seq in primary MM samples from the CoMMpass trial (14) (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 juxtapose the IgH enhancer to drive elevated levels of MAFB and MAF expression, respectively (Figure 2C,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 (Supplementary Figure 1F). 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 reviewed here (15). We found propranolol to elicit cytotoxicity in MM cell lines (Figure 2E). Proteasome inhibitors (PIs) inhibitors like bortezomib (BTZ) are backbone MM therapeutics,
however, most MM patients become refractory to PIs. 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). 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 propranolol 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), propranolol significantly reduced MAFB expression in BTZ treated cells, underscoring the utility of using propranolol to target MAFB and overcome beta 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. 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 CMPs 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 beta-adrenergic stimuli selectively increase resistance to proteasome inhibitors, while targeting β-adrenergic signaling with propranolol increases sensitivity to BTZ and venetoclax. We acknowledge the limited patient sample size for the current study and the need for greater mechanistic understanding of how beta-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 beta blockers in MM therapy.
Abbreviations

GMPs- Granulocyte/monocyte progenitors
CMPs- Common myeloid progenitors
MAFB- v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B transcription factor
GATA1- globin transcription factor 1
MEPs- myeloid erythroid progenitors
PRO- propranolol
ISO- isoproterenol

References


Figure Legends

Figure 1: MM patient samples exhibit reduced MEPs, elevated MAFB/GATA and reduced late erythroblast development that is reversed with propranolol (PRO) treatment A. Representative contour plots of CBM and MM sample mononuclear cells in forward vs. side scatter (left panel), from flow cytometry. (right) Day 0 CBM and MM BM evaluated for CMP/GMP/MEP frequencies (n=10); B. MFI of MAFB and GATA1 expression in CMPs from 10 MM patients at Day 0, evaluated by flow cytometry; C. Introduction of non-targeting and MAFB-directed SiRNA in MM samples (n=4). Frequencies of CMPs, GMPs and MEPs 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 EB (CD71^+CD235a+) are significantly decreased (p<0.0001) compared to Early EB (CD71^+CD235a^-) indicating erythropoietic arrest in MM (right panel); E. MEP, GMP and CMP quantified in HSPCs in Phase 1 cultures treated with increasing doses of ISO; F. CMP, GMP and MEP frequencies quantified in MM treated ex vivo with PRO.

Figure 2: Isoproterenol (ISO; non-selective β adrenoceptor agonist) and propranolol (PRO) regulate MM sensitivity to the proteasome inhibitor bortezomib (BTZ): A. Model of beta adrenergic signaling establishes a tumor promoting triad between multiple myeloma and the skewed common myeloid lineage specification in the 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 PRO for 24 hrs assessed for viability using Annexin V/DAPI flow cytometric staining; F. Cell lines treated with 75μM PRO or ISO as indicated for 24 hrs 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 BTZ assessed for viability using AnnexinV/DAPI flow cytometric staining.
Figure 3: Propranolol increases sensitivity to the BCL-2 antagonist venetoclax (VEN). A, B. L363 cells treated with BTZ and/or ISO as indicated for 18 hrs, assessed for indicated proteins by immunoblot analyses; C. Cell lines treated with 75μM PRO or ISO in combination with 0.01μM VEN for 24 hrs assessed for viability; All Cell death/viability assessed by AnnexinV/DAPI flow cytometry; D. Model: Beta adrenergic signaling elevates MAFB vs GATA1 expression in common myeloid progenitors (CMPs) 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.
## Supplementary information

### Table 1: Patient sample details

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Supplementary Figure 1: A. Day 6 expanded CBM and MM BM evaluated for CMP/GMP/MEP frequencies (n=10); B. MFI of MAFB and GATA1 expression in CMPs from 10 MM patients at Day 6, evaluated by flow cytometry; C. MAFB and GATA1 quantification of samples from panel E (1 representative data set shown, n=4); Geometric mean intensities were measured for MAFB and GATA1 in CMPs and normalized to the no stimulation condition that was arbitrarily defined as 100; D. Geometric mean intensities were measured for MAFB and GATA1 in GMPs and MEPs normalized to no stimulation condition which was arbitrarily defined as 100; E. MEP, GMP and CMP quantified in HSPCs in Phase 1 cultures treated with increasing doses of phenylephrine; F. Expression of ADRB2 and MAFB in indicated MM lines with actin evaluated as loading control.