Published in final edited form as:

Leukemia. 2021 November ; 35(11): 3319–3323. doi:10.1038/s41375-021-01145-0.

Role of MBD3-SOX2 axis in residual myeloma following pomalidomide

Rakesh Verma¹, Andrew R. Branagan², Mina L. Xu^{3,4}, Richard A. Flavell^{5,6}, Kavita M. Dhodapkar^{7,8}, Madhav V. Dhodapkar^{8,9}

¹Department of Medicine, Yale University, New Haven, CT, USA

²Division of Hematology and Oncology, Massachusetts General Hospital, Boston, MA, USA

³Department of Pathology, Yale University, New Haven, CT, USA

⁴Department of Laboratory Medicine, Yale University, New Haven, CT, USA

⁵Department of Immunobiology, Yale University, New Haven, CT, USA

⁶Howard Hughes Medical Institute, Yale University, New Haven, CT, USA

⁷Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Emory University, Atlanta, GA, USA

⁸Winship Cancer Institute, Emory University, Atlanta, GA, USA

⁹Department of Hematology and Medical Oncology, Emory University, Atlanta, GA, USA

To the Editor:

Combination therapies based on immune-modulatory drugs (IMiDs^R) such as thalidomide, lenalidomide, and pomalidomide (Pom) have led to high rates of tumor regression in myeloma (MM) patients. These patients subsequently receive prolonged IMiD-based maintenance therapies. However, the great majority ultimately experience disease relapse, highlighting the unmet need to understand the mechanisms regulating the persistence and growth of residual myeloma. Binding of IMiDs such as Pom to the CRL4^{CRBN} E3 ubiquitin ligase complex leads to proteasomal degradation of IKZF1 and (AIOLOS) IKZF3. Degradation of these targets is both necessary and sufficient for anti-proliferative effects of Pom on myeloma cells and also mediates immune-activation by enhancing IL2 expression in T cells [1–5]. However CRL4^{CRBN} E3 ubiquitin-IKZF1/IKZF3 pathway does not explain the persistence of residual MM cells and apparent biological paradox that IMiDs mediate synergistic anti-myeloma effects with proteasome inhibitors in the clinic. Here we describe a

[✉]Rakesh Verma, rakesh.verma@aya.yale.edu; Madhav V. Dhodapkar, madhav.v.dhodapkar@emory.edu.

Author contributions

RV designed and performed experiments and wrote the manuscript. AB and KD performed clinical research and provided access to specimens. MLX provided help with immunohistochemical analyses. RAF provided access to mice used in the study. MVD provided oversight for the project and wrote manuscript. All authors contributed to interpretation of data and approved the final manuscript.

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41375-021-01145-0>.

Conflict of interest The authors declare that they have no conflict of interest.

novel MBD3-SOX2 pathway active in residual MM cells after Pom and its role in regulating growth and clonogenicity of residual MM following IMiD therapy and implications for IMiD-PI synergy.

Methods

Study design

In order to study the biology of residual MM following Pom, MM cells were exposed to the drug (100–300 ng/mL) or DMSO control in vitro and then utilized for subsequent experiments with indicated doses equivalent to the therapeutic concentrations achieved in vivo [6]. INA-6 cells xenografted into RGS6 mice [7] were utilized to study growth of MM cells in vivo using the intra-femoral delivery of MM cells as described earlier (Supplementary Fig. S1) [8]. Biospecimens obtained under the auspices of a clinical trial of pomalidomide (NCT01319422) in relapsed MM [9] were utilized to evaluate the effects of Pom on primary MM cells in vivo. All biospecimens were obtained following informed consent approved by Yale IRB and all animal studies were performed in accordance with protocols approved by Yale IACUC. Details of methods are noted under supplementary methods.

Results and discussion

In order to better understand the biology of residual MM after Pom, we evaluated the growth of residual MM cells after Pom exposure (see supplementary methods). Residual MM cells (MM cell lines and primary tumor cells from MM patients) after Pom exposure demonstrated enhanced clonogenic growth in vitro as compared to DMSO controls (Fig. 1a). INA6 MM cells residual after in vitro exposure to Pom also exhibited enhanced growth potential following injection into RGS6 mice in vivo (Fig. 1b, c). To gain further insights into the underlying mechanism, we compared pre/post treatment gene expression profiles of purified tumor cells from three patients after 2 cycles of pomalidomide therapy (Supplementary Table S1). Gene-set enrichment analysis revealed enrichment of embryonal stem cell pathways as one of the top pathways altered in these cells (Supplementary Fig. S2, Supplementary Table S2). Embryonal stem cell (ES) pathways including ES gene SOX2 have been previously implicated in regulating clonogenic growth of human MM [10–12]. SOX2 depletion by RNAi led to inhibition of clonogenic growth of human MM cells in vitro (Supplementary Fig. S3a) and in vivo in MM xenografts (Fig. 1d, Supplementary Fig. 3b–d). Similarly, MM cells residual after in vitro exposure to Pom also exhibited significant gain in SOX2 transcripts (Fig. 1e) and RNAi-mediated inhibition of SOX2 abrogated elevated clonogenic potential in Pom-exposed residual MM cells (Fig. 1f). Together, these data demonstrate that residual MM after Pom exposure has greater capacity for clonogenic growth in a SOX2-dependent manner.

SOX2 is well established as an embryonal stem cell (ES) pluripotency gene and MBD3 has emerged as a critical component of the pluripotency repressor complex [13]. We hypothesized that as with other IMiD-mediated effects that involve drug-induced degradation of substrates, Pom mediated induction of SOX2 may involve drug-induced depletion of a proximate co-repressor factor such as MBD3. Immunoblotting of Pom-

exposed residual MM cell lines revealed significant loss in the MBD3 protein (Fig. 2a, Supplementary Fig. S4a) and concurrent increase in several ES genes like SOX2, OCT4, KLF4, and NANOG (Supplementary Fig. S4b). Similarly, in primary patient MM cells, Pom exposure led to the depletion of IKZF1 and MBD3, along with significant gain in SOX2 and H3K4me2 (Supplementary Fig. S4c). In vivo, analysis of paired marrow biopsies before and after 2 cycles of Pom therapy from two MM patients with residual MM also demonstrated clear loss of MBD3 expression by immuno-histochemistry, concurrent with an increase in SOX2 transcripts (Fig. 2b and Supplementary Fig. S4d). Together these data demonstrate that Pom exposure is associated with depletion of MBD3 and enrichment of SOX2 in MM cells both in vitro and in vivo.

RNAi-mediated inhibition of MBD3 also led to an increase in SOX2 and clonogenic growth of human MM cells (Fig. 2c, Supplementary Fig. S4e). To directly evaluate the role of MBD3 depletion in the enhanced clonogenicity observed in Pom-residual cells, we overexpressed MBD3 in MM1s cells (Supplementary Fig. S4f, g). Overexpression of MBD3 abrogated enrichment of clonogenicity in Pom exposed MM cells (Fig. 2d). Together these data demonstrate that enhanced clonogenicity of Pom-residual disease is dependent on drug-induced MBD3 depletion.

Pull-down assays for MBD3 demonstrated direct interaction of MBD3 with ubiquitin (Supplementary Fig. S4h), and treatment with proteasome inhibitor (PI) MG132 led to an increase in IKZF1 and MBD3 expression, consistent with a role for K48-proteasome mediated degradation in the turnover of these proteins [14]. Concurrent exposure to PI MG132 and Pom abrogated Pom-induced depletion of MBD3 (Fig. 2e). Interestingly, concurrent PI therapy blunted but still permitted IKZF1 depletion in Pom exposed MM cells, consistent with prior studies [15]. Drug induced depletion of MBD3 is also observed with lenalidomide, but not dexamethasone, suggesting that this may be a class-effect of multiple IMiDs (data not shown). Together, these data demonstrate that Pom-induced MBD3 depletion is proteasome-dependent and that combination of Pom and PI abrogates this pathway in Pom-residual disease.

In summary, these data demonstrate that residual MM cells following Pom exposure are characterized by activation of stemness genes such as SOX2. The underlying mechanisms involve MBD3 depletion in Pom exposed MM cells and downstream activation of key pluripotency genes (Supplementary Fig. S4i). Activation of stemness genes in residual disease may not only impact the persistence of residual disease, but in principle, also drug resistance and risk of second malignancies [10]. Concurrent proteasome inhibition prevents Pom-mediated depletion of MBD3, providing a novel mechanism for observed synergy between these agents. Further studies are needed to understand the mechanisms underlying MBD3 depletion. Future studies in additional patients may also identify which patients and MM subtypes have greater activation of MBD3-SOX2 axis in residual MM, as these patients may be better candidates for PI-IMiD combinations as opposed to single agent IMiDs for maintenance strategies. Small molecules or immunebased strategies targeting MBD3-SOX2 axis may synergize with IMiDs to eradicate residual disease in MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

MVD is supported in part by funds from NCI R35 CA197603, Specialized Center for Research and translational research program of Leukemia and Lymphoma Society and the Multiple Myeloma Research Foundation (MMRF). Authors would like to thank patients and clinical colleagues at Yale, and Zifeng Mai, Lin Zhang, Dr. Mehmet H Kocoglu, Carla Weibel, Jon Alderman, Grzegorz Wylezinski, Dr. Roni Nowarski for their support.

References

1. Lu G, Middleton RE, Sun H, Naniong M, Ott CJ, Mitsiades CS, et al. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins. *Science*. 2014;343:305–9. [PubMed: 24292623]
2. Kronke J, Udeshi ND, Narla A, Grauman P, Hurst SN, McConkey M, et al. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science*. 2014;343:301–5. [PubMed: 24292625]
3. Gandhi AK, Kang J, Havens CG, Conklin T, Ning Y, Wu L, et al. Immunomodulatory agents lenalidomide and pomalidomide costimulate T cells by inducing degradation of T cell repressors Ikaros and Aiolos via modulation of the E3 ubiquitin ligase complex CRL4(CRBN.). *Br J Haematol*. 2014;164:811–21. [PubMed: 24328678]
4. Lopez-Girona A, Mendy D, Ito T, Miller K, Gandhi AK, Kang J, et al. Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. *Leukemia*. 2012;26:2326–35. [PubMed: 22552008]
5. Zhu YX, Braggio E, Shi CX, Bruins LA, Schmidt JE, Van Wier S, et al. Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide. *Blood*. 2011;118:4771–9. [PubMed: 21860026]
6. Xu Y, Li J, Ferguson GD, Mercurio F, Khambatta G, Morrison L, et al. Immunomodulatory drugs reorganize cytoskeleton by modulating Rho GTPases. *Blood*. 2009;114:338–45. [PubMed: 19417207]
7. Yu H, Borsotti C, Schickel JN, Zhu S, Strowig T, Eynon EE, et al. A novel humanized mouse model with significant improvement of class-switched, antigen-specific antibody production. *Blood*. 2017;129:959–69. [PubMed: 28077418]
8. Das R, Strowig T, Verma R, Koduru S, Hafemann A, Hopf S, et al. Microenvironment-dependent growth of preneoplastic and malignant plasma cells in humanized mice. *Nat Med*. 2016;22:1351–7. [PubMed: 27723723]
9. Sehgal K, Das R, Zhang L, Verma R, Deng Y, Kocoglu M, et al. Clinical and pharmacodynamic analysis of pomalidomide dosing strategies in myeloma: impact of immune activation and cereblon targets. *Blood*. 2015;125:4042–51. [PubMed: 25869284]
10. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. *Cancer Res*. 2008;68:190–7. [PubMed: 18172311]
11. Tanno T, Lim Y, Wang Q, Chesi M, Bergsagel PL, Matthews G, et al. Growth differentiating factor 15 enhances the tumorinitiating and self-renewal potential of multiple myeloma cells. *Blood*. 2014;123:725–33. [PubMed: 24345755]
12. Spisek R, Kukreja A, Chen LC, Matthews P, Mazumder A, Vesole D, et al. Frequent and specific immunity to the embryonal stem cell-associated antigen SOX2 in patients with monoclonal gammopathy. *J Exp Med*. 2007;204:831–40. [PubMed: 17389240]
13. Rais Y, Zviran A, Geula S, Gafni O, Chomsky E, Viukov S, et al. Deterministic direct reprogramming of somatic cells to pluripotency. *Nature*. 2013;502:65–70. [PubMed: 24048479]

14. Liu Y, Huang X, He X, Zhou Y, Jiang X, Chen-Kiang S, et al. A novel effect of thalidomide and its analogs: suppression of cereblon ubiquitination enhances ubiquitin ligase function. *FASEB J*. 2015;29:4829–39. [PubMed: 26231201]
15. Shi CX, Kortum KM, Zhu YX, Jedlowski P, Bruins L, Braggio E, et al. Proteasome inhibitors block Ikaros degradation by lenalidomide in multiple myeloma. *Haematologica*. 2015;100:e315–7. [PubMed: 25975838]

Fig. 1. Enhanced clonogenic potential and growth of pomalidomide-residual disease is SOX2 dependent.

a Significant increase in clonogenic potential of residual human MM cells (both MM cell lines and primary MM cells) exposed to Pom compared to DMSO controls. Values shown are mean $\pm$ s.e.m. **b** Enhanced engraftment of GFP + human MM cells (INA6) in xenografts with Pom exposed residual MM cells vs DMSO controls as analyzed via In situ immunofluorescence imaging. **c** Elevated soluble human IL6 receptoralpha levels (sHuIL6Ra) in blood of mice xenografted with Pom-exposed residual MM cells vs DMSO controls. Values

shown are mean $\pm$ s.e.m. **d** Left panels—Immune-fluorescence imaging analysis showing decreased level of engraftment in mice xenografted with SOX-2 depleted human MM cells (GFP + INA6) vs controls. Right panel—Significantly decreased levels of sHuIL6Ra levels in blood of mice xenografted with SOX2 depleted human MM cells (INA6) vs controls. Values shown are mean $\pm$ s.e.m. **e** Significant increase in SOX2 transcripts (RQ-PCR) in Pom exposed residual MM cell line (MM1.S and ARK) and primary MM cells. Values shown are mean $\pm$ s.e.m. **f** SOX2-depleted MM (MM1.S) cells showing abrogation of enhanced clonogenic potential in Pom exposed residual MM cells vs siRNA-NT controls. Values shown are mean $\pm$ s.e.m.

Fig. 2. MBD3 mediated SOX2 enrichment of human MM cells in Pom exposed residual MM cells.

a Depletion of MBD3 in Pomresidual MM: Immunoblotting and quantitative analysis utilizing LICOR image analysis software, showing pom-residual disease MM cells with loss of MBD3 protein in human primary MM & MM cell lines. Values shown are mean $\pm$ s.e.m.

b Changes in MM cells following exposure to Pom in vivo. Bone marrow specimens were analyzed after exposure to Pom in vivo for 2 cycles of therapy. Immunohistochemistry (IHC) analysis showing depletion of MBD3 protein in vivo after 2 cycles of Pom therapy in the bone marrow biopsies of MM patients vs pre-therapy biopsy samples.

c Increase in clonogenic potential of MBD3-depleted MM cells (siRNA-MBD3) vs NT controls. Values

shown are mean $\pm$ s.e.m. **d** Abrogation of enhanced clonogenicity after pom exposure in MBD3-overexpressing cells as compared to empty vector controls. Values shown are the mean $\pm$ s.e.m. **e** Immunoblot results showing abrogation of MBD3 depletion in Pom exposed residual MM cells (ARK cells) with combination treatment of MG132 (proteasome inhibitor, PI) vs degradation of IKAROS.