nature portfolio

Peer Review File

Lenalidomide-induced pure red cell aplasia is associated with elevated expression of MHC-I molecules on erythrocytes

Corresponding Author: Professor Hsiang-Ying Lee

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Nature Communications, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Hu Q, et al described an interesting myeloma patient who developed PRCA after RVd treatment. Authors mentioned LEN-induced augmentation of MHC-I on erythroid progenitors is associated with the development of PRCA. This report is potentially interesting, however, authors used only one MM patient. This reviewer is not able to accept this draft manuscript in these limited data. Authors should show the convincing data to clarify the mechanisms of LEN-induced PRCA by another MM patients (at least one or two).

Reviewer #2

(Remarks to the Author)
In this report, Qi Hu and colleagues report the findings of a patient with multiple myeloma who developed pure red cell aplasia (PRCA) after RVd therapy. RVd therapy combines lenalidomide, bortezomid and dexamethasone and has become the mainstay for the treatment of multiple myeloma. The authors demonstrate that lenalidomide is, at least in part, involved in the PRCA observed after treatment. Using scRNAseq methods, they confirm impaired erythropoiesis in the marrow of this patient, and unravel hyperactive CD8+ T cells. Finally, erythroid cells from the patient presented with abnormally high expression of MHC-I related genes. Blocking MHC-I or depleting T-cells rescues partially erythropoiesis in vitro, suggesting a potential therapeutic avenue.

Comments:
1. Most of the studies were focused on terminal erythroid differentiation (CD71/GPA) while the defect is clearly present at earlier stages- BFU-e are decreased. It would be beneficial to assess erythropoiesis at the erythroid progenitors' level to understand how lenalidomide acts.
2. In the same view, different reports have involved IMiDs such as Lenalidomide in erythropoiesis and demonstrated a role for cerebron among others. While the scRNAseq is informative, it remains unclear why the authors did not investigate the effects of lenalidomide at the proteomic level since the drug has been involved in the degradation pathway.
3. What was the rationale for the drug concentrations used in the study? Was a combinatorial approach used as in the case of the patient?
4. Figure 4 presents the levels of HLA-A, B and C in culture. Were they also validated ex vivo from the patient's bone marrow?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The author has answered to the reviewer's comments and updated their manuscript accordingly. There are no additional comments so far. This manuscript is acceptable for publication.

Reviewer #2

(Remarks to the Author)
The authors answered my comments

Reviewer #3

(Remarks to the Author)
The authors conducted a comprehensive examination of the mechanisms of lenalidomide-induced PRCA from an erythroid perspective. Their in vitro analysis demonstrated that lenalidomide is a crucial inhibitor of erythropoiesis. Additionally, their single-cell transcriptome analysis revealed the activation of CD8+ T cells and impaired erythropoiesis in the patients' bone marrow. The results demonstrated that lenalidomide treatment led to an unexpected increase in MHC-I expression in the patient's erythrocytes. The blockade of MHC-I or the depletion of T cells was observed to alleviate the erythropoietic defects associated with PRCA. This suggests that the interaction between elevated erythrocytes expressing MHC-I and T cells in the bone marrow may be involved in the pathogenesis of PRCA. In conclusion, their findings have identified a novel mechanism underlying lenalidomide-induced PRCA in cancer patients. The manuscript is of considerable interest and the results are clearly presented. However, the authors should provide further clarification on the following points:

1) The number of cells used for the single-cell analysis in Figure 3 should be specified. The number of cells analyzed is a crucial factor in demonstrating differences between cell subsets. Moreover, the data from multiple patients should be examined to ascertain whether a similar trend was observed in each individual. This will enhance the reliability of the results.

2) To ascertain which cell clusters are different, a UMAP or tSNE figure should be presented in Figure 3B, with HD and PRCA patients separated.

3) In Figure 3, the single-cell assay can demonstrate the erythroid lineage in the bone marrow; however, it is unclear whether this can be observed in the bone marrow. This will enable us to provide a more detailed account of the lineage that is undergoing abnormal differentiation. Additionally, RNA velocity analysis (visualized by UMAP) at that time would reveal the pseudo-developmental trajectory of erythroid clusters, thus allowing for a more detailed examination of cell subset alterations.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The author has answered to my comments and updated their manuscript accordingly. There are no additional comments so far. This manuscript is acceptable for publication.

Point-by-point responses to comments of specific referees are as follows.

Reviewers' comments:

Reviewer #1 multiple myeloma (Remarks to the Author):

Hu Q, et al described an interesting myeloma patient who developed PRCA after RVd treatment. Authors mentioned LEN-induced augmentation of MHC-I on erythroid progenitors is associated with the development of PRCA. This report is potentially interesting, however, authors used only one MM patient. This reviewer is not able to accept this draft manuscript in these limited data. Authors should show the convincing data to clarify the mechanisms of LEN-induced PRCA by another MM patients (at least one or two).

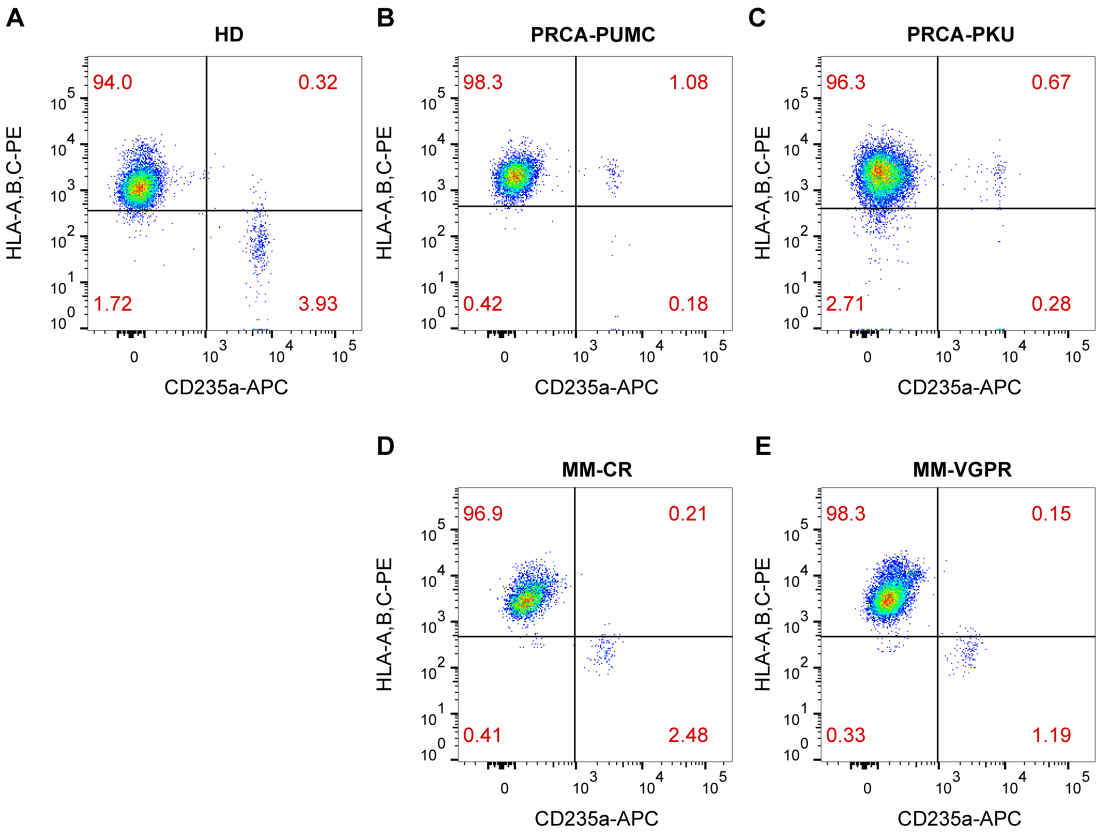
Response:

We appreciate the referee's feedback, particularly your comment, "This report is potentially interesting." We are grateful for this positive evaluation and have addressed the crucial point you raised regarding the number of patients in this study.

Lenalidomide-induced PRCA is a very rare event, with only two such cases reported in the literature, specifically in papers from 2014 and 2018[1, 2]. The mechanisms underlying these cases were not studied. In our study, although we only used one patient, we collected samples at different clinical stages to systematically analyze the cellular and molecular events throughout PRCA's progression, thereby maximizing the value of our study.

Nonetheless, we were fortunate to collect a sample from another multiple myeloma (MM) patient with lenalidomide-induced PRCA, who was previously reported and treated at Peking Union Medical College Hospital[3]. This patient, who also developed PRCA after a series of treatments, had recurrent minimal residual disease (MRD) three months following autologous stem cell transplantation. The patient developed refractory anemia after lenalidomide treatment, which did not respond to stanozolol and a secondary infusion of autologous hematopoietic stem cells. Bone marrow aspiration and biopsy confirmed the diagnosis of PRCA.

With the patient's consent, we collected peripheral blood samples. Peripheral blood contains a small fraction (<2 %) of erythroid progenitor cells which express erythroid surface markers (CD71 and CD235a) and undergo erythroid differentiation in culture[4, 5, 6]. Through flow cytometric analysis, we observed significant MHC-I positivity in this patient's erythroid cells (PBMC-PUMC), a phenomenon similar to that observed in the patient we initially reported in the manuscript (PBMC-PKU) (**Response Figure 1**). This MHC-I positivity was not present in the PBMCs of healthy individuals or in MM patients without lenalidomide-induced PRCA.



Response Figure 1. *Representative flow cytometry results showing MHC-I levels on erythroid cells in the PBMCs from a healthy donor (HD) (**A**), a recent PRCA sample collected from Peking Union Medical College Hospital (PRCA-PUMC) recently (**B**), the PRCA patient initially reported in our manuscript (PRCA-PKU) (**C**), and two multiple myeloma patients treated with three courses of RVd, achieving complete response (CR) (**D**) and very good partial responses (VGPR) (**E**), respectively.*

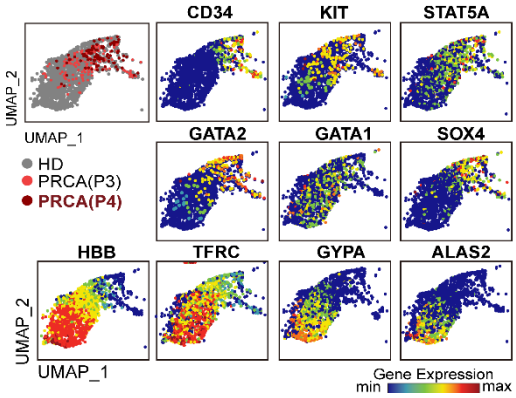
Reviewer #2 multiple myeloma treatment, erythroid development (Remarks to the Author):

In this report, Qi Hu and colleagues report the findings of a patient with multiple myeloma who developed pure red cell aplasia (PRCA) after RVd therapy. RVd therapy combines lenalidomide, bortezomid and dexamethasone and has become the mainstay for the treatment of multiple myeloma. The authors demonstrate that lenalidomide is, at least in part, involved in the PRCA observed after treatment. Using scRNAseq methods, they confirm impaired erythropoiesis in the marrow of this patient, and unravel hyperactive $CD8^+$ T cells. Finally, erythroid cells from the patient presented with abnormally high expression of MHC-I related genes. Blocking MHC-I or depleting T-cells rescues partially erythropoiesis in vitro, suggesting a potential therapeutic avenue.

Comments:

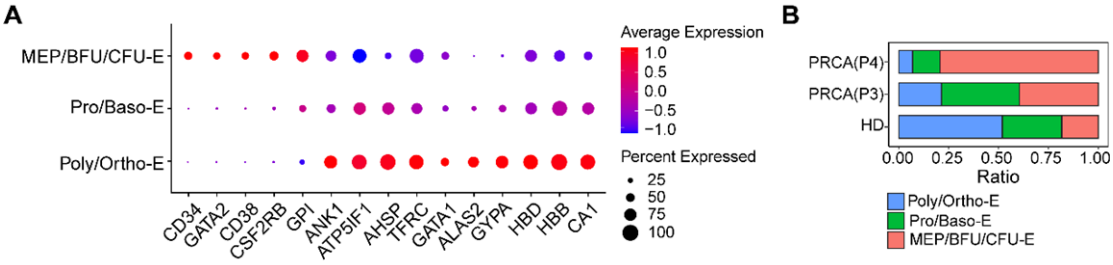
1. Most of the studies were focused on terminal erythroid differentiation (CD71/GPA) while the defect is clearly present at earlier stages- BFU-e are decreased. It would be beneficial to assess erythropoiesis at the erythroid progenitors' level to understand how lenalidomide acts.

Response: Based on the results shown in Figure 3C (**Response Figure 2**), it can be seen that the most pronounced decrease is in the $CD71^+GYPA^+$ terminal erythroblast population in our single-cell RNA-seq data. This led us to initially reason that late erythroblasts (CD71/GYPA) in PRCA likely exhibited defects leading to their destruction. However, we agree with your point that defects in late erythroblasts could be present from the early stages. Therefore, we conducted the following analysis to explore the defects in early-stage cells.



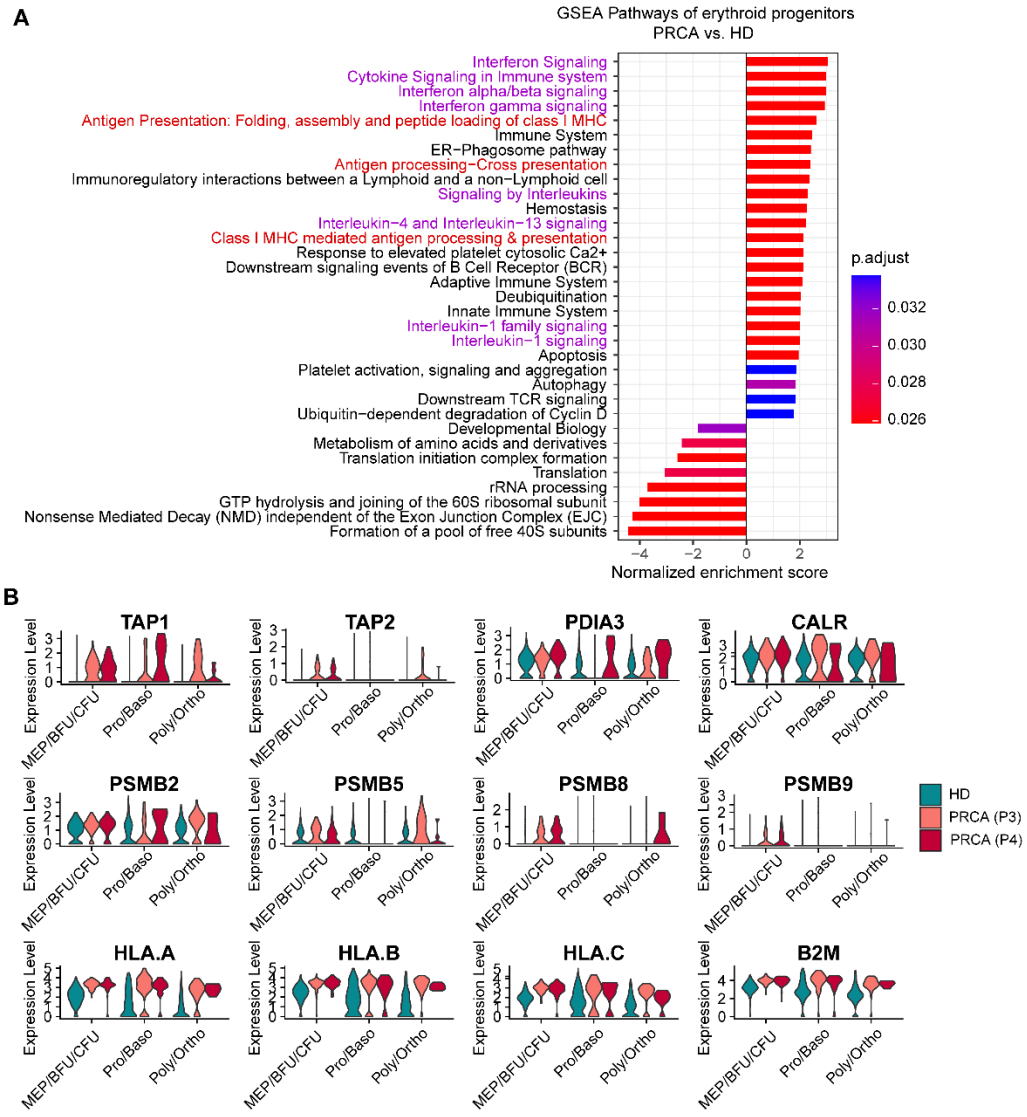
Response Figure 2. *Distribution of erythroid cells in bone marrow. UMAP analysis of erythroid cells in single-cell transcriptome data, combining HD and PRCA samples, with cells colored according to the donor. Expression levels of erythroid markers were used to determine the cell stages.*

First, in single-cell transcriptome analysis, we further subdivided the erythroid lineage into the progenitor population ($CD34^{+}CD38^{+}GATA2^{high}$, containing MEP, BFU-E, and CFU-E), early erythroid precursors ($CD71^{+}CD235^{-}$, containing proerythroblasts and basophilic erythroblasts), and terminal erythroid precursors ($CD71^{+}CD235^{+}$, containing polychromatic and orthochromatic erythroblasts). The corresponding marker gene expression of these three erythroid populations is shown in **Response Figure 3A**. Consistent with our previous observations, in the bone marrow of PRCA patients, especially in the acute phase (P4), the majority of erythroid cells are at the progenitor stages (**Response Figure 3B**), aligning with our *ex vivo* culture results, which show marked developmental defects (Supplemental Figures 1 B&C and 2 in the manuscript).



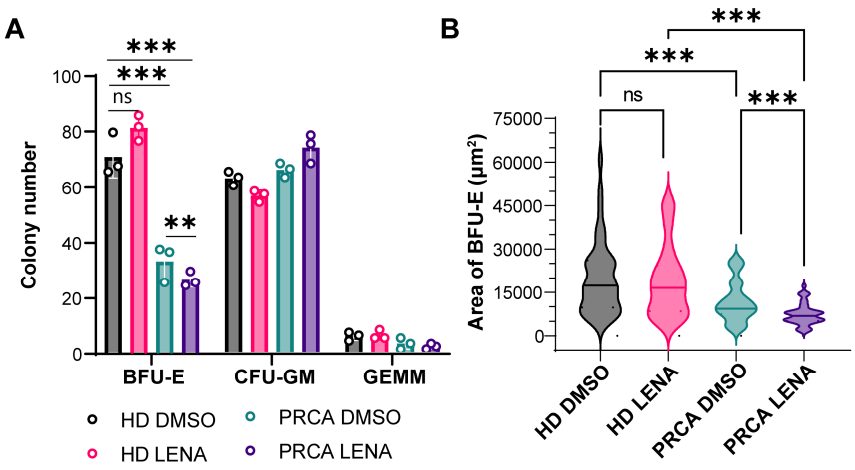
Response Figure 3. A. *Dot plot showing the normalized mean expression levels of discriminative gene sets for each erythroid cell type.* ***B.*** *Distribution of erythroid cells in the bone marrow of different samples.*

Next, we extracted the patient's erythroid lineage progenitor cells and performed differential gene and pathway enrichment analyses. Similar to previous findings, gene set enrichment analysis (GSEA) revealed that development-related, rRNA processing, and translation pathways were downregulated in the erythroid progenitor cells of PRCA patients, indicating aberrant erythroid development and proliferation. In contrast, the patient's erythroid progenitors exhibited a marked activation of immune-related pathways, including those for MHC-I-mediated antigen presentation (**Response Figure 4A**). Furthermore, MHC pathway genes remained significantly elevated in the erythroid cells of patients at both clinical phases (P3 and P4) of the PRCA when we divided the erythroid cells into progenitor and precursor categories (**Response Figure 4B**).



Response Figure 4. A. *GSEA pathway enrichment results for erythroid progenitors. Normalized enrichment scores (NES) are shown, with color denoting P-adjust value.* ***B.*** *Expression levels of antigen processing and peptide loading genes, proteasome and immunoproteasome genes and MHC-I heterodimer genes in MEP/BFU-E/CFU-E progenitors, Pro/Baso-E precursors and Poly/Ortho-E precursors based on scRNA-Seq.*

In addition, we performed a colony forming assay using antibodies to deplete lineage-positive cells. The lineage-negative cells from the patients and healthy controls were plated in a six-well plate at a quantity of 1,000 cells per well and treated with or without lenalidomide. After 14 days of culture, we observed a substantial reduction in both the number and size of burst-forming unit-erythroid (BFU-E) colonies, whereas the numbers of other myeloid colonies (CFU-GEMM, CFU-GM) were unaffected (**Response Figure 5**).



Response Figure 5. A. *Colony-forming assays of lineage negative cells in BMMCs from PRCA or HD with indicated treatments. Quantification of BFU-E, CFU-E, and CFU-GEMM colonies was performed on day 14. Two-tailed paired Student's t-test. (**P < 0.01, ***P < 0.001).* ***B.*** *Area of BFU-E colonies in different samples. Two-tailed paired Student's t-test. (***P < 0.001).*

2. In the same view, different reports have involved IMiDs such as Lenalidomide in erythropoiesis and demonstrated a role for cerebron among others. While the scRNAseq is informative, it remains unclear why the authors did not investigate the effects of lenalidomide at the proteomic level since the drug has been involved in the degradation pathway.

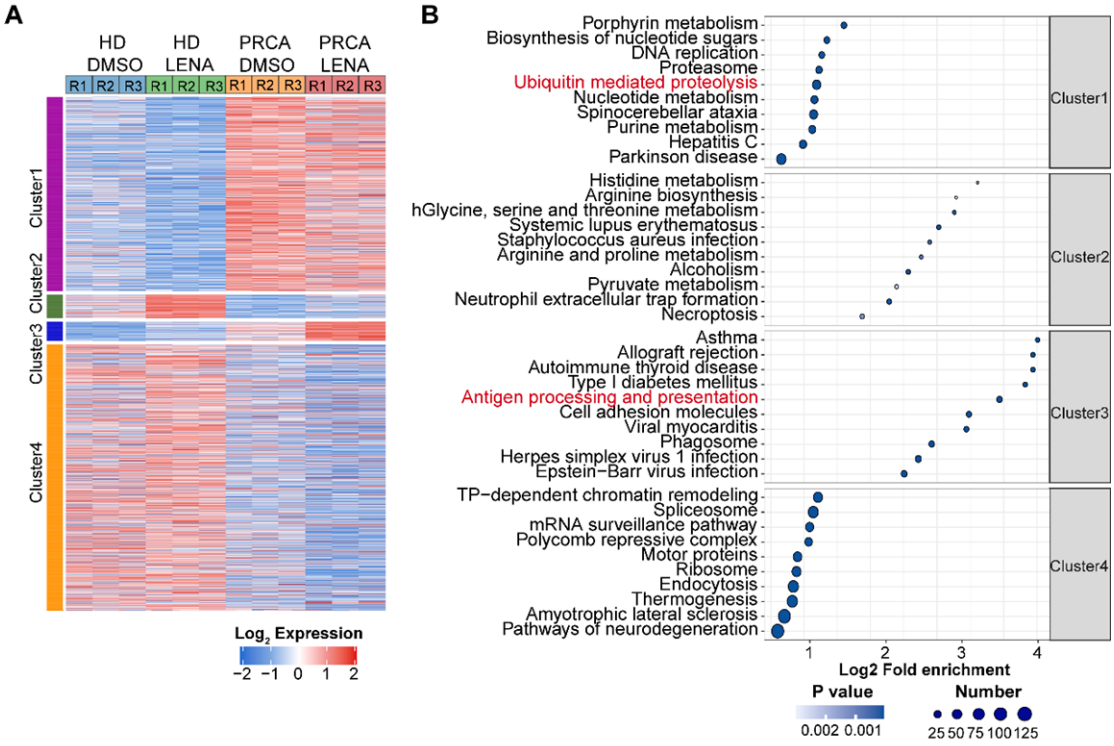
Response: We appreciate your insightful comment. Indeed, lenalidomide has been found to exert its effects by altering the activity of an E3 ubiquitin ligase, leading to the selective ubiquitination and degradation of specific targets:

In multiple myeloma, "Lenalidomide causes selective ubiquitination and degradation of two lymphoid transcription factors, IKZF1 and IKZF3, by the CRBN-CRL4 ubiquitin ligase"[7]. In MDS, lenalidomide was found to "induce the ubiquitination of casein kinase 1A1 (CK1α) by the E3 ubiquitin ligase CUL4–RBX1–DDB1–CRBN (known as $CRL4^{CRBN}$), resulting in CK1α degradation. CK1α is encoded by a gene within the common deleted region for del(5q) MDS and haploinsufficient expression sensitizes cells to lenalidomide therapy"[8].

Therefore, we conducted experiments to investigate whether changes in cellular protein levels underlie the mechanism of lenalidomide in our PRCA case. Specifically, bone marrow mononuclear cells (BMMCs) from healthy donors and the patient were cultured *in vitro* for six days with and without lenalidomide treatment. Erythroid progenitor cells were then sorted, and micro-proteomics analysis was performed.

We performed inter-sample analysis based on the results of mass spectrometry (**Response Figure 6A**). Differential gene clustering analysis of the detected proteins yielded four clusters: Cluster 1 and Cluster 4 contained proteins that were significantly more abundant in PRCA patients and healthy donors, respectively. Cluster 2 and Cluster 3 contained proteins that were significantly more abundant after *in vitro* lenalidomide treatment in healthy donor and PRCA patient cells, respectively.

Further comparison of the differentially enriched proteins in these clusters revealed that the ubiquitin mediated proteolysis pathway is upregulated in PRCA, while the antigen processing and presentation pathway is distinctly upregulated in PRCA after lenalidomide treatment (**Response Figure 6B**).

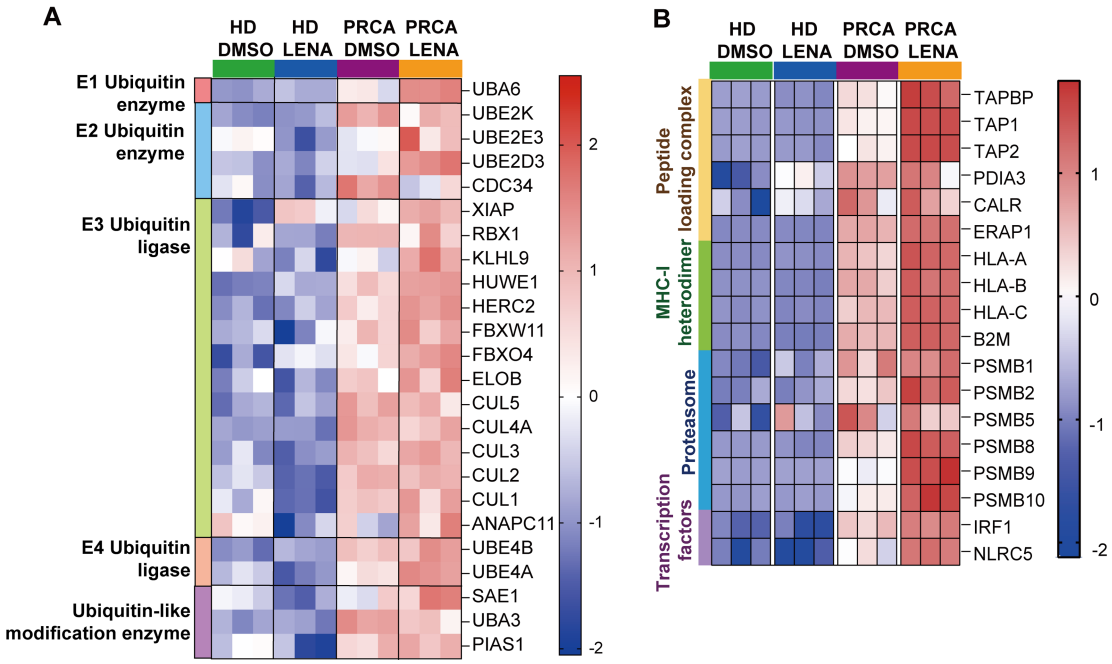


Response Figure 6. A. *Heatmap of proteins detected by micro-proteomics analysis. Clusters 1~4 were identified by hierarchical clustering.* ***B.*** *KEGG pathway enrichment results for different clusters. Dot color represents the P value; dot size represents the number of genes related to the indicated pathway.*

The upregulated proteins in the ubiquitin-related pathway in PRCA include various E1 activating enzyme, E2 ubiquitin-conjugating enzymes, E3 ubiquitin-protein ligases (including the Cullin ubiquitin ligases), and E4 ubiquitin conjugation factors (**Response Figure 7A**).

The MHC-I pathway was enriched in Cluster 3 (**Response Figure 6B**). Specifically, proteins involved in the peptide-loading complex (PLC), MHC-I heterodimer, and proteasome required for peptide degradation, especially those related to the

immunoproteasome (i.e., PSMB8, PSMB9, PSMB10), were significantly enriched. The levels of these proteins were further upregulated in PRCA cells after the addition of lenalidomide, whereas no significant changes were observed between lenalidomide-treated and untreated BMMCs from healthy donors (**Response Figure 7B**).



Response Figure 7. A. *Relative protein levels of ubiquitin-related genes, detected in micro-proteomics analysis.* ***B.*** *Relative protein levels of antigen processing and presentation and the regulatory network for MHC-I related genes, detected in micro-proteomics analysis.*

Collectively, the proteomic analysis reveals higher levels of ubiquitination-related pathway protein expression in PRCA cells compared to HD. Moreover, the analysis shows upregulation of MHC-I pathway protein expression in PRCA cells, which is further induced by lenalidomide treatment, supporting our previous findings from single-cell transcriptome analysis.

3. What was the rationale for the drug concentrations used in the study? Was a combinatorial approach used as in the case of the patient?

Response: Generally, there is no perfect conversion of drug dosages used in clinical treatment to drug concentrations used in cell culture. We tried our best to determine the appropriate drug concentrations for our study by referring to pharmacokinetic studies and in vitro culture data from various publications. We also conducted preliminary experiments testing a range of dosages to cover the published concentration ranges. We selected drug concentrations that reflect previously reported effects in cells, animals or humans, without causing adverse effects such as cell death.

- Dexamethasone: Pharmacokinetic studies (Response Figure 8) indicate that therapeutic doses of dexamethasone result in plasma concentrations ranging from 5 μM to less than 25 nM. Additionally, research shows dexamethasone may promote BFU-E colony formation[10]. Based on these studies, we tested dexamethasone at 20 nM, 100 nM, 250 nM, and 1000 nM. While all concentrations promoted erythroid lineage proliferation, 1000 nM caused excessive inhibition of differentiation. Therefore, we selected 250 nM as the optimal concentration.

[figure redacted]

Response Figure 8. *Pharmacokinetics of dexamethasone. Plasma levels of dexamethasone phosphate (black) and dexamethasone (white) after intravenous administration of 20 mg dexamethasone phosphate. The curves represent the mean of the pharmacokinetics parameters of the individual data (n = 10); the points represent the mean plasma levels. The original plot was cited from Rohdewald P et al.[9], and we added the red fonts to indicate the corresponding molar concentration based on our calculations.*

- Lenalidomide: Pharmacokinetic studies (Response Figure 9) show that the drug is metabolized rapidly in vivo, with plasma concentrations ranging from ~2000 nM to ~386 nM within 12 hours after administration[11]. Additionally, we referred to research which indicates lenalidomide can increase CFU-E colony formation[10]. In preliminary experiments, we tested lenalidomide at 1 nM, 50 nM, 250 nM, and 1000 nM. Observing no effects on both normal and patient cells at low concentrations, we selected 250 nM as suitable for further experiments.

[figure redacted]

Response Figure 9. *Pharmacokinetics of lenalidomide. Representative plasma concentration–time and urine excretion–time profiles of lenalidomide in healthy volunteers. Data are shown as mean ± standard deviation. The original plot was cited from Chen et al. [11], and we added the red fonts to indicate the corresponding molar concentration based on our calculations.*

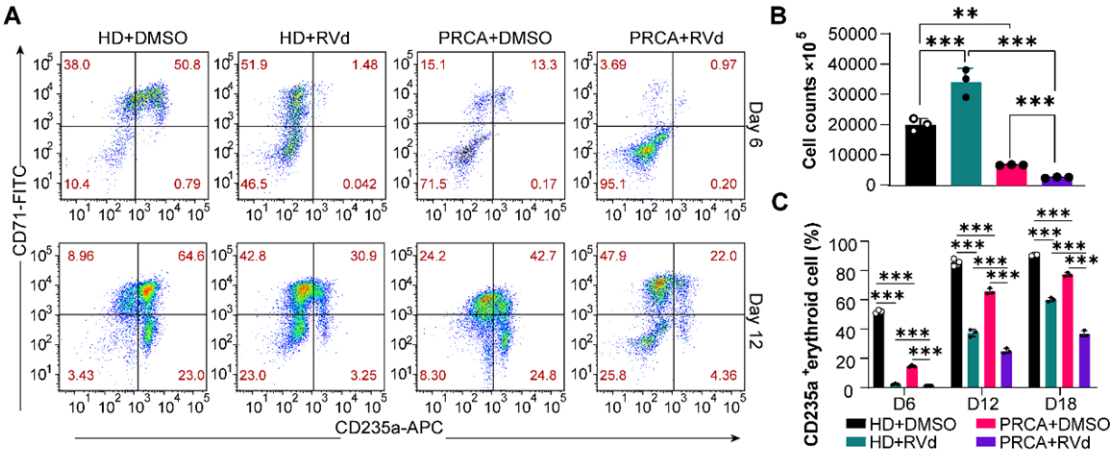
- Bortezomib: Bortezomib's in vivo concentration ranges from 10 nM to 260 nM (Response Figure 10). Determining the optimal in vitro concentration is challenging due to its rapid metabolism and 72-hour maintenance time[12]. We referred to studies on bortezomib's effects on osteoclasts (0.1 nM to 100 nM) and $CD34^+$ cells in neutropenia (10 nM)[13, 14]. Based on these, we tested concentrations of 1 nM, 10 nM, and 50 nM in vitro. Observing no phenotype at 1 nM and severe impairment at 50 nM, we chose 10 nM for further experiments.

[figure redacted]

Response Figure 10. *Pharmacokinetics of bortezomib. Mean plasma concentration-time profile of bortezomib. The red font represents the corresponding molar*

concentration. The original plot was cited from Huang et al. [12], and we added the red fonts to indicate the corresponding molar concentration based on our calculations.

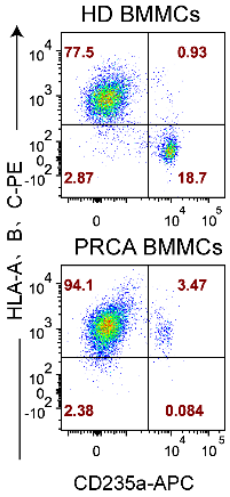
With RVd combination (dexamethasone+lenalidomide+bortezomib), both healthy individuals and patients exhibited a delay in erythroid lineage differentiation compared to the control (DMSO treated). However, HD cells eventually underwent erythroid differentiation, achieving a significantly higher number of erythroid cells compared to the control. In contrast, PRCA cells showed severely impaired cell proliferation and differentiation (**Response Figure 11**). Therefore, we conducted further experiments to identify the specific drug(s) responsible for these effects in the manuscript.



Response Figure 11. A. *BMMCs from HD (left) and PRCA (right) were cultured in serum-free erythroid medium, with or without RVd. Dexamethasone was administered at 250 nM, lenalidomide at 250 nM, and bortezomib at 10 nM. Flow cytometry analyses were performed on days 6 and 12.* ***B.*** *Quantification of the impact of drugs on erythroid proliferation. Cell numbers were counted on day 18.* ***C****. Quantification of CD235a$^+$ erythroid cells on day 6, 12, and 18. Two-tailed paired Student's t-test. (**P < 0.01, ***P < 0.001).*

4. Figure 4 presents the levels of HLA-A, B and C in culture. Were they also validated ex vivo from the patient's bone marrow?

Response: The results of ex vivo analysis of patient and healthy bone marrow samples are shown in Figure 3F (**Response Figure 12**). These assay results are consistent with our scRNA-seq analysis findings.



Response Figure 12. *Representative flow cytometry results showing MHC-I levels on erythroid cells in the bone marrow from HD (upper) and the PRCA patient (lower).*

References

1. Dolai TK, Dutta S, Mandal PK, Saha S, Bhattacharyya M. Lenalidomide-Induced Pure Red Cell Aplasia. *Turkish Journal of Hematology* **31**, 99-100 (2014).

2. Ito T*, et al.* Secondary pure red cell aplasia in multiple myeloma treated with lenalidomide. *Leukemia Research Reports* **10**, 4-6 (2018).

3. Jin X*, et al.* Pure red cell aplasia and minimal residual disease conversion associated with immune reconstitution in a patient with high-risk multiple myeloma. *Chronic Diseases and Translational Medicine* **9**, 341-344 (2023).

4. van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM. The majority of the in vitro erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica* **95**, 1594-1598 (2010).

5. Filippone C*, et al.* Erythroid progenitor cells expanded from peripheral blood without mobilization or preselection: molecular characteristics and functional competence. *PLoS One* **5**, e9496 (2010).

6. Heshusius S*, et al.* Large-scale in vitro production of red blood cells from human peripheral blood mononuclear cells. *Blood Adv* **3**, 3337-3350 (2019).

7. Krönke J*, et al.* Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science* **343**, 301-305 (2014).

8. Krönke J*, et al.* Lenalidomide induces ubiquitination and degradation of CK1α in del(5q)

MDS. *Nature* **523**, 183-188 (2015).

9. Rohdewald P, Möllmann H, Barth J, Rehder J, Derendorf H. Pharmacokinetics of dexamethasone and its phosphate ester. *Biopharmaceutics & Drug Disposition* **8**, 205-212 (2006).

10. Narla A*, et al.* Dexamethasone and lenalidomide have distinct functional effects on erythropoiesis. *Blood* **118**, 2296-2304 (2011).

11. Chen N, Zhou S, Palmisano M. Clinical Pharmacokinetics and Pharmacodynamics of Lenalidomide. *Clin Pharmacokinet* **56**, 139-152 (2017).

12. Huang SY*, et al.* Pharmacokinetic study of bortezomib administered intravenously in Taiwanese patients with multiple myeloma. *Hematol Oncol* **36**, 238-244 (2018).

13. von Metzler I*, et al.* Bortezomib inhibits human osteoclastogenesis. *Leukemia* **21**, 2025-2034 (2007).

14. Gupta K*, et al.* Bortezomib inhibits STAT5-dependent degradation of LEF-1, inducing granulocytic differentiation in congenital neutropenia CD34(+) cells. *Blood* **123**, 2550-2561 (2014).

Point-by-point responses to comments of specific referees are as follows.

Reviewers' comments:

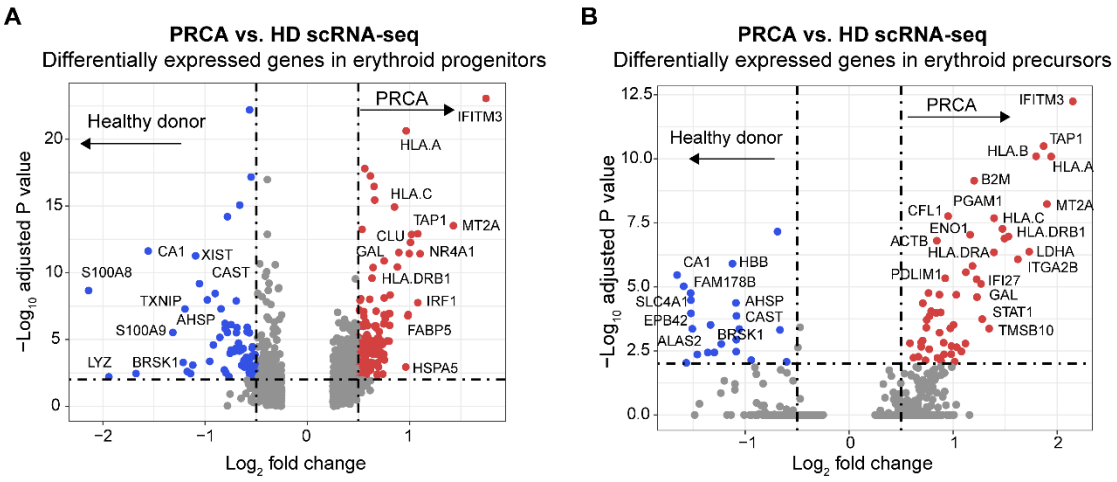
Reviewer #3 (Remarks to the Author):

The authors conducted a comprehensive examination of the mechanisms of lenalidomide-induced PRCA from an erythroid perspective. Their in vitro analysis demonstrated that lenalidomide is a crucial inhibitor of erythropoiesis. Additionally, their single-cell transcriptome analysis revealed the activation of CD8+ T cells and impaired erythropoiesis in the patients' bone marrow. The results demonstrated that lenalidomide treatment led to an unexpected increase in MHC-I expression in the patient's erythrocytes. The blockade of MHC-I or the depletion of T cells was observed to alleviate the erythropoietic defects associated with PRCA. This suggests that the interaction between elevated erythrocytes expressing MHC-I and T cells in the bone marrow may be involved in the pathogenesis of PRCA. In conclusion, their findings have identified a novel mechanism underlying lenalidomide-induced PRCA in cancer patients. The manuscript is of considerable interest and the results are clearly presented. However, the authors should provide further clarification on the following points.

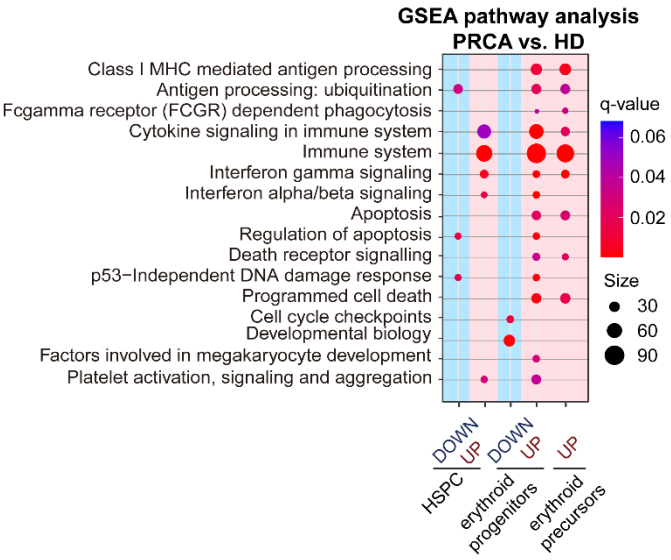
Comments:

1a. The number of cells used for the single-cell analysis in Figure 3 should be specified. The number of cells analyzed is a crucial factor in demonstrating differences between cell subsets.

Response: We appreciate your insightful comment. To achieve a more accurate comparison of transcriptional differences between HD and PRCA samples, we reduced the number of cells in the HD samples to match the cell number in the PRCA samples, and updated the differential gene analysis results accordingly in Figure 3. Specifically, we used the “downsample” function in Seurat to randomly reduce the HD sample to 300 HSC cells, 200 erythroid progenitor cells and 60 erythroid precursor cells, aligning with the cell numbers in the PRCA samples (**Response Figure 1, updated in Supplemental Figure 5B, C**). Based on the updated differential gene analysis results, we have revised the GSEA pathway enrichment analysis accordingly (**Response Figure 2, updated in Figure 3D**). The description regarding cell number specification has been added to the “Unsupervised clustering, marker identification, and cell type annotation” paragraph in the Methods section.



Response Figure 1. ***A****, Volcano plot showing differentially expressed genes in erythroid progenitors (CD71+CD235a-) from PRCA patient compared to healthy donors (HD). Genes with log2(fold change) > 0.5 or < -0.5 are shown in red or blue, respectively.* ***B****, Volcano plot showing differentially expressed genes in erythroid precursors (CD71+CD235a+) from PRCA patient compared to HD. Genes with log2(fold change) > 0.5 or < -0.5 are shown in red or blue.*



Response Figure 2. *GSEA pathway enrichment results for different cell types. Dot color represents the q-value, and dot size represents the number of genes associated with the indicated pathway.*

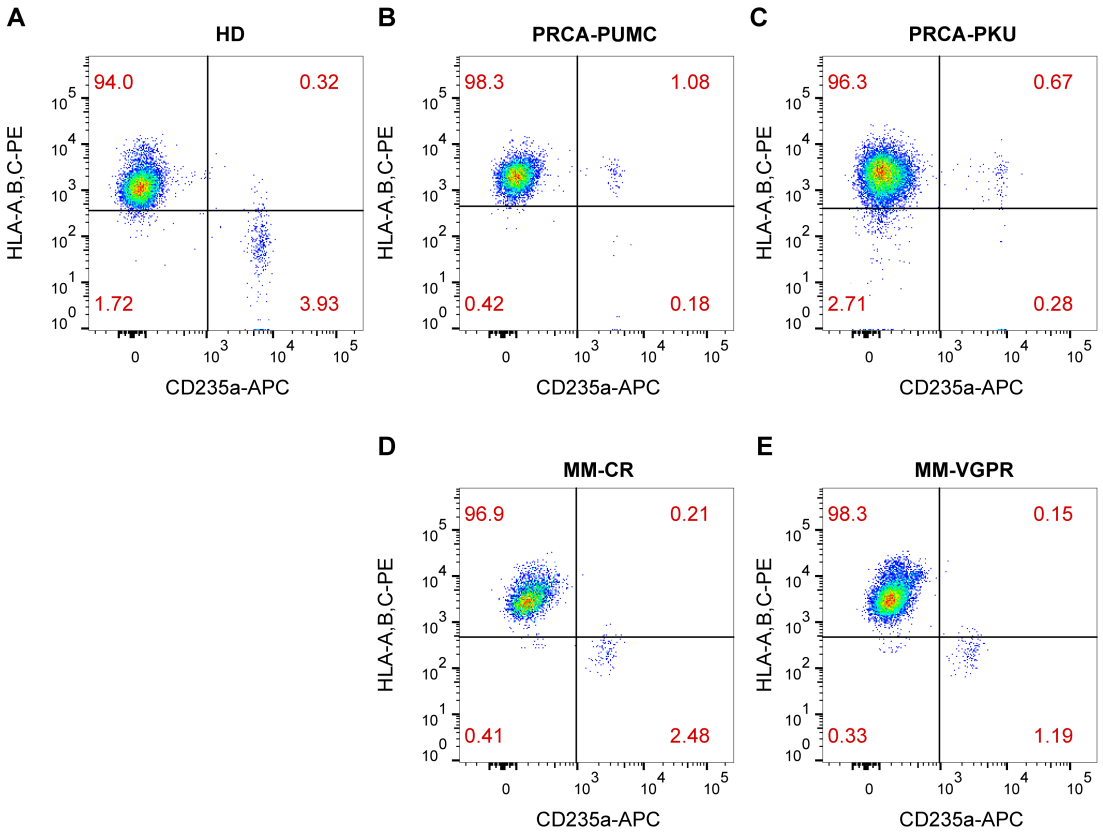
1b. Moreover, the data from multiple patients should be examined to ascertain whether a similar trend was observed in each individual. This will enhance the reliability of the results.

Lenalidomide-induced PRCA is a very rare event, with only two such cases reported in the literature, specifically in papers from 2014 and 2018[1, 2]. The mechanisms underlying these cases were not studied. In our study, although we only used one

patient, we collected samples at different clinical stages to systematically analyze the cellular and molecular events throughout PRCA's progression, thereby maximizing the value of our study.

Nonetheless, we were fortunate to collect a sample from another multiple myeloma (MM) patient with lenalidomide-induced PRCA, who was previously reported and treated at Peking Union Medical College Hospital[3]. This patient, who also developed PRCA after a series of treatments, had recurrent minimal residual disease (MRD) three months following autologous stem cell transplantation. The patient developed refractory anemia after lenalidomide treatment, which did not respond to stanozolol and a secondary infusion of autologous hematopoietic stem cells. Bone marrow aspiration and biopsy confirmed the diagnosis of PRCA.

With the patient's consent, we collected peripheral blood samples. Peripheral blood contains a small fraction (<2 %) of erythroid progenitor cells which express erythroid surface markers (CD71 and CD235a) and undergo erythroid differentiation in culture[4, 5, 6]. Through flow cytometric analysis, we observed significant MHC-I positivity in this patient's erythroid cells (PBMC-PUMC), a phenomenon similar to that observed in the patient we initially reported in the manuscript (PBMC-PKU) (**Response Figure 3**). This MHC-I positivity was not present in the PBMCs of healthy individuals or in MM patients without lenalidomide-induced PRCA.

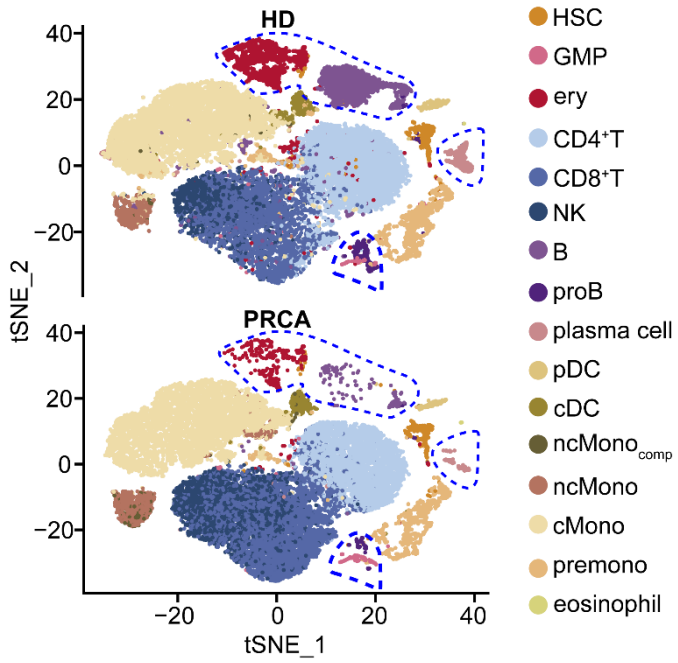


Response Figure 3. *Representative flow cytometry results showing MHC-I levels on erythroid cells in the PBMCs from a healthy donor (HD) (**A**), a recent PRCA sample*

*collected from Peking Union Medical College Hospital (PRCA-PUMC) (**B**), the PRCA patient initially reported in our manuscript (PRCA-PKU) (**C**), and two multiple myeloma patients treated with three courses of RVd, achieving complete response (CR) (**D**) and very good partial responses (VGPR) (**E**), respectively.*

2. To ascertain which cell clusters are different, a UMAP or tSNE figure should be presented in Figure 3B, with HD and PRCA patients separated.

Response: We appreciate your thoughtful suggestion. We have now presented the tSNE plots of PRCA and HD samples separately, as shown in Response Figure 4, and updated Figure 3B in the manuscript accordingly. The clusters of cells exhibiting the most notable differences in cell proportions are highlighted by blue dashed lines. These altered populations include a reduced erythroid lineage, which is strongly associated with PRCA. Additionally, the B-cell lineage, including proB, B cells, B cells, and plasma cells, shows significant reductions, likely associated with drug therapy for multiple myeloma (**Response Figure 4**).



Response Figure 4. *Overview of cell clusters in integrated single-cell transcriptomes derived from BMMCs of the healthy donors (HD, top) and PRCA patient (bottom). Clusters were named based on cluster-specific gene expression patterns. (HSPC, hematopoietic stem and progenitor cell; GMP, granulocyte-monocyte progenitors; ery, erythroid cells; NK, natural killer cells; pDC, plasmacytoid dendritic cells; cDC, conventional (classical) dendritic cells; $ncMono_{comp}$, $C1QA^{+}/CD16^{+}$ complement-expressing non-classical monocytes; ncMono, $CD14^{dim}CD16^{+}$ non-classical monocytes; cMono, $CD14^{++}CD16^{+}$ classical monocytes).*

3a. In Figure 3, the single-cell assay can demonstrate the erythroid lineage in the bone marrow; however, it is unclear whether this can be observed in the bone marrow. This will enable us to provide a more detailed account of the lineage that is undergoing abnormal differentiation.

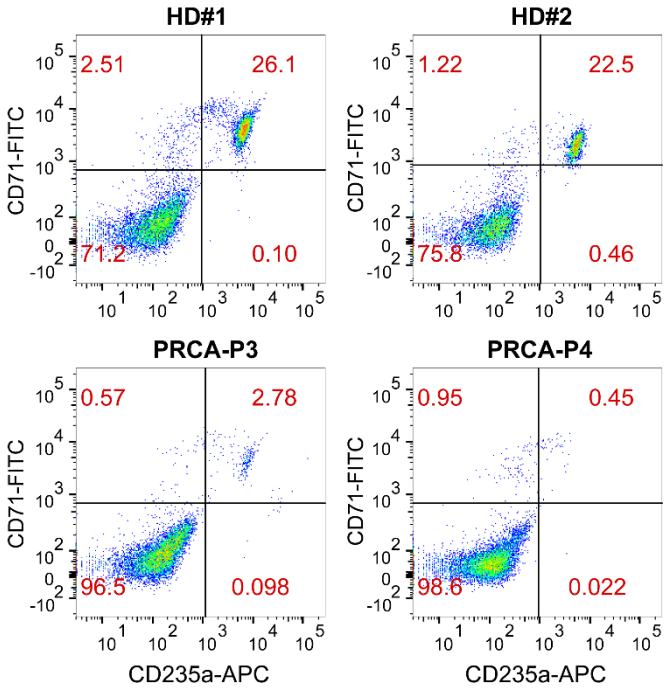
Response: We appreciate your insightful comment. TFRC and GYPA are key markers for erythroid differentiation. During this process, CD71(TFRC) expression initially increases and then declines, while CD235a (GYPA) begins to be expressed in the later stages of erythroid differentiation (**Response Figure 5**).[7] Using these two markers, we analyzed the proportion and sub-population distribution of erythroid cells in the bone marrow across different samples.

[figure redacted]

Response Figure 5. *An overview of human erythropoiesis. The early phase involves the expansion of erythroid progenitors, primarily supported by interleukin-3 (IL-3) and stem cell factor (SCF). The late phase focuses on the maturation of erythroid precursors leading to enucleation, driven mainly by erythropoietin (EPO), though SCF continues to support erythroid precursor expansion early in this phase. Throughout erythroid differentiation, changes in the expression patterns of surface markers changes enable tracking via flow cytometry.*[7]

Through flow cytometric analysis, we observed a marked decrease in erythroid cells, particularly in the bone marrow of patients in the acute phase. Among these, CD235a+ erythroid precursor cells, showed the most pronounced decline— their proportion of reached 20% in healthy donors, while they were significantly reduced (<3%) or nearly absent in the PRCA samples (**Response Figure 6**). In summary, the phenomena we

observed in the bone marrow are consistent with the results obtained from the single-cell transcriptome analysis.



Response Figure 6. *Flow cytometry results showing erythroid cells in the bone marrow mononuclear cells (BMMCs) from 2 healthy donors (top), and from the PRCA patient at stage P3 and P4, respectively.*

3b. Additionally, RNA velocity analysis (visualized by UMAP) at that time would reveal the pseudo-developmental trajectory of erythroid clusters, thus allowing for a more detailed examination of cell subset alterations.

The RNA velocity framework is built on an explicit model of transcriptional processes (transcription, splicing, degradation), offering the potential to address some of the limitations of static measurements.

Upon reviewing the literature, we found that in previous studies, **velocity-inferred trajectories during red blood cell maturation opposed the true differentiation path**. [8]This phenomenon has been observed in both mouse yolk sac erythropoiesis and human fetal liver erythropoiesis, attributed to **a concerted increase in transcription rate of a subset of erythroid genes midway through the red blood cell maturation trajectory** (**Response Figures 7 & 8**).

[figure redacted]

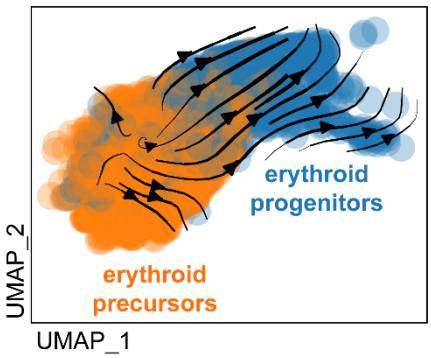
Response Figure 7. *Inferring differentiation trajectories at organismal scale. (**A**) Pijuan-Sala et al.[9] layout displaying single-cell transcriptomes from mouse E6.5 to E8.5, colored by sampled timepoint (left) and by cell-type (right). Overlaying arrows represent inferred developmental trajectories generated using the scVelo pipeline for the entire embryonic dataset, with arrowheads highlighting the erythroid branch, where scVelo trajectory predictions are inconsistent with real-time sampling. (**B**) Pijuan-Sala et al.[9] layout highlighting single-cell transcriptomes from E7.5 (left) and E8.5 (right), colored by cell type (see legend in panel A). Overlaying arrows show inferred developmental trajectories from the scVelo pipeline applied to these individual timepoints, with arrowheads again marking the erythroid branch.[8]*

[figure redacted]

Response Figure 8. *The concept of dual kinetics in gene expression is also observed in human fetal liver hematopoiesis.*[8] *(**A**) UMAP representation of human fetal liver erythroid cell populations. Overlaying arrows indicate inferred developmental trajectories from the scVelo pipeline using all genes (left) and after excluding MURK genes (right). The bottom UMAPs are colored by scVelo-inferred latent time. MEMP: megakaryocyte-erythroid-mast cell progenitor.*

Based on this background, we conducted RNA velocity analysis (visualized by UMAP) on erythroid cells in our single-cell transcriptome datasets. Consistent with previous findings, the RNA velocity direction was reversed, moving from more mature erythroid precursors to earlier erythroid progenitors (**Response Figure 9**). The plot in Response Figure 9 includes all single-cell transcriptome datasets combined for analysis, encompassing both HD and PRCA samples. We attempted to generate separate plots for HD and PRCA, but this was not feasible for the PRCA sample, as it contains erythroid progenitor cells, while precursor cells are largely absent.

In summary, RNA velocity analysis, integrated with single-cell transcriptome data and flow cytometry results, provided more detailed examination and additional validation of cell subset alterations.



Response Figure 9. *UMAP representation of erythroid cell populations from all single-cell transcriptome datasets in this study, with overlaid arrows generated using the Velocyto pipeline.*

Reference

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9. Pijuan-Sala B, et al. A single-cell molecular map of mouse gastrulation and early organogenesis. Nature 566, 490-495 (2019).