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LETTER

Spironolactone inhibits the NLRP1 inflammasome and alleviates defective erythropoiesis in Diamond-Blackfan anemia

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The relevance of the NLR family pyrin domain containing 1 (NLRP1) inflammasome was recently extended to the regulation of the erythroid-myeloid lineage decision in both zebrafish and human hematopoietic stem and progenitor cells (HSPCs), acting independently of dipeptidyl peptidase 9 (DPP9)[1] and being activated upon its phosphorylation by the ZAKα/P38 kinase axis following ribosomal stress.[1] This novel function of the NLRP1 inflammasome may be of clinical relevance, since defects in ribosome biogenesis caused several ribosomopathies, such as Diamond-Blackfan anemia, in which reduced ribosome levels selectively impair translation of a subset of mRNAs, including *GATA1* mRNA.[2] For this reason, we performed a screening testing 768 compounds approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) using HEK293T cells transfected with NLRP1 and ASC-GFP plasmids (Supporting Information S1: Figure S1A). This approach enables the visualization and quantification of NLRP1 inflammasome activation by monitoring the oligomerization of the ASC adaptor protein fused to GFP. Upon inflammasome activation, ASC-GFP forms distinct cytosolic specks, whereas in the absence of activation, it remains evenly distributed throughout the cytoplasm. ASC specks were already visible at 6 h post-transfection (hpt) in cells transfected with both plasmids but not with ASC alone (Supporting Information S1: Figure S1B). However, no specks were observed at 4 hpt (Supporting Information S1: Figure S1B). Therefore, cells were treated at 4 hpt with the compounds at 10 μM and analyzed by fluorescence microscopy at 24 hpt (Supporting Information S1: Figure S1B). Among all the compounds analyzed, flufenamic acid, pantoprazole, spironolactone, and amlodipine decreased NLRP1-dependent ASC speck formation. These compounds were then tested at different concentrations (Supporting Information S1: Figure S2). Flufenamic acid at 100 μM and amlodipine at 50 and 100 μM were toxic for the cells. In addition, although flufenamic acid, pantoprazole and amlodipine hardly decreased the number of specks at the different concentration tested, spironolactone was able to show a dose-dependent inhibitory effect (Supporting Information S1: Figure S2). Importantly, 50 μM spironolactone decreased the number of specks in HEK293T transfected with NLRP1 and NLRC4, while it did not affect the percentage of specks formed in the presence of NLRP3 (Supporting Information S1: Figure S3A,B). In addition, spironolactone failed to inhibit the self-oligomerization of ASC speck in the absence of NLRP1 (Supporting Information S1: Figure S3A,B). Notably, spironolactone was also able to inhibit the oligomerization of ASC promoted by the NLRP1 C-terminal fragment containing UPA and CARD subdomain, which form the platform to recruit ASC (Supporting Information S1:

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Figure S3C).[3] As spironolactone is a mineralocorticoid receptor antagonist, which is indicated to treat heart failure, edema, hyperaldosteronism and hypertension,[4] we then tested the effects of spironolactone on NLRP1 inflammasome in steroid-free culture medium and found that spironolactone was able to inhibit NLRP1-dependent ASC speck formation independently of the presence of steroids (Supporting Information S1: Figure S3D).

These results led us to analyze the effects of spironolactone on hematopoiesis. Spironolactone treatment promoted terminal erythroid differentiation of K562 cells, assayed as accumulation of hemoglobin (Supporting Information S1: Figure S4A), and, as consequence, a decrease in the amount of GATA1 protein levels (Supporting Information S1: Figure S4B,C). Moreover, it also decreased caspase-1 activity at 48 h post-treatment (Supporting Information S1: Figure S4D). Similar results were also obtained in steroid-free culture medium (Supporting Information S1: Figure S4E). As we have previously shown that pharmacological inhibition of ZAKα/P38/NLRP1 axis promoted GATA1 accumulation in K562 cells,[5] we analyzed NLRP1, ZAKα, pP38 and GATA1 at very early time points upon induction of erythroid differentiation with hemin. The results showed that spironolactone induced a robust accumulation of GATA1 during the initial stages of erythroid differentiation; however, it is degraded in untreated cells between 150 and 180 min (Supporting Information S1: Figure S5A). Additionally, while ZAKα and NLRP1 levels rose in spironolactone-treated cells—likely as a compensatory mechanism—spironolactone inhibited P38 phosphorylation, which is critical for NLRP1 activation. Notably, P38 phosphorylation peaked at 120 minutes in untreated cells, just prior to GATA1 degradation (Supporting Information S1: Figure S5A). The effect of spironolactone in accelerate erythroid differentiation of K562 cells was further confirmed by the quicker and/or more robust induction of the transcript levels of GATA1 target genes that encode proteins involved in erythroid differentiation, such as ALAS2 (5′-Aminolevulinate Synthase 2), EPOR (erythropoietin receptor), HBA1 (hemoglobin α1), HBD (hemoglobin δ) and HBZ (hemoglobin θ) (Supporting Information S1: Figure S5B and Supporting Information S3: Table S1).

We next treated zebrafish larvae with different concentrations of spironolactone (Figure 1A), and we observed that the survival decreased at 50 and 100 μM (Figure 1B). Therefore, we selected 10 μM of spironolactone to analyze hematopoiesis in zebrafish larvae. Treatment of larvae with 10 μM spironolactone phenocopied the effect of Nlrp1 deficiency[1]; that is, larvae had significantly reduced caspase-1 activity (Figure 1C), decreased neutrophil counts (Figure 1D,E) and increased erythrocyte number (Figure 1F,G). Notably, HSPCs, macrophages, T lymphocytes and thrombocytes were hardly affected by spironolactone (Figure 1H,I and Supporting Information S1: Figure S6A–E). Importantly, forced expression of mRNA encoding wild type Caspa, but not catalytically inactive Caspa ($Caspa^{C270A}$), reversed the neutropenia and erythrocytosis induced by spironolactone (Figure 1J,K and Supporting Information S1: Figure S7A,B), confirming that spironolactone regulates hematopoiesis through the inflammasome.

As spironolactone seemed to specifically inhibit the NLRP1 inflammasome and phenocopied the effects of NLRP1 inhibition in both K562 cells and zebrafish, we analyzed its effects on the differentiation of human HSPCs from healthy donors and patients with DBA, a ribosomopathy in which reduced ribosome levels selectively impair translation of a subset of mRNAs, including *GATA1* mRNA,[2] and thus, inhibition of the NLRP1 inflammasome is expected to increase GATA1 protein amount in HSPCs and then promotes erythropoiesis. We first differentiated $CD34^+/CD133^+$ HSPCs from two healthy donors during 7 days with EPO in the presence of spironolactone from 3 to 7 days. Interestingly, spironolactone accelerated erythropoiesis, as assayed as the plasma membrane glycophorin A expression by flow cytometry (Supporting Information S1: Figure S8A,C), increased the differentiation score, that is, the ratio between intermediate precursors ($CD71^+/CD235A^+$) and early progenitors ($CD71^+/CD235A^-$) (Supporting Information S1: Figure S8D), and the transcript levels of GATA1-dependent erythroid genes (Supporting Information S1: Figure S8E). As expected, spironolactone treatment also robustly inhibited CASP1 activity during erythroid differentiation (Supporting Information S1: Figure S8F).

We next analyzed the effects of spironolactone on both zebrafish DBA models and DBA patient's HSPCs. The results showed that spironolactone was able to alleviate the anemia of Rps19-deficient zebrafish larvae (edition efficiency of 89%), assayed as their hemoglobin contents (Figure 2A and Supporting Information S3: Table S2). Notably, overexpression of human *RPS19* mRNA restored hemoglobin levels in Rps19-deficient larvae (Supporting Information S1: Figure S9), validating the model for studying DBA. Similarly, spironolactone robustly increased the number of BFU-E colonies derived from mononuclear cells from five out of six DBA patients, while no statistically significant effects were found in the formation of CFU-GM colonies (Figure 2B and Supporting Information S3: Table S3). In addition, spironolactone also alleviated defective erythropoiesis of RPS19-deficient human $CD34^+$ HSPCs (Figure 2C and Supporting Information S1: Figure S10).

To analyze the mechanism involved in the inhibition of the NLRP1 inflammasome by spironolactone, we used its analog eplerenone. We found that eplerenone failed to decrease NLRP1-dependent ASC speck formation (Supporting Information S1: Figure S11A). In addition, eplerenone failed to decrease the number of neutrophils in zebrafish larvae (Supporting Information S1: Figure S11B,C), and to promote the differentiation of K562 cells (Supporting Information S1: Figure S11D) and decrease GATA1 protein levels (Supporting Information S1: Figure S11E,F).

Our chemical screening revealed that spironolactone specifically inhibited the formation of ASC speck in HEK293T cells and facilitated erythroid differentiation of K562 cells and human primary HSPCs, phenocopying the effects of genetic inhibition of *caspa* and *nlrp1* in zebrafish.[1,6] Spironolactone seemed to inhibit the ZAKα/P38/NLRP1 axis, which is activated by ribosomal stress in erythroid progenitors, promoting GATA1 inactivation and impairing erythropoiesis[5] (Supporting Information S1: Figure S12). Consisting with this mechanism of action, spironolactone robustly alleviated the impaired erythropoiesis of Rps19-deficient zebrafish larvae and RPS19-deficient $CD34^+$ HSPCs, as well as HSPCs from five out of DBA patients. Since spironolactone is an FDA/EMA-approved drug, it is attractive for repurposing in the treatment of DBA. The therapeutical options for DBA patients are rather limited and most of them receive either corticosteroids or chronic red blood cell transfusions,[7] both treatments having important side effects. Although the impact of spironolactone must be evaluated in more DBA patients having different mutations, our results are quite encouraging.

Another important issue that needs to be clarified is the mechanism of action of spironolactone, since its analog eplerenone failed to inhibit the NLRP1 inflammasome and to regulate hematopoiesis. Consequently, it is crucial to determine if the structural differences between these two compounds determine their different activity on NLRP1 inflammasome. Currently, spironolactone and eplerenone are used to treat hypertension, heart failure, edema, hyperaldosteronism and nephrotic syndrome,[8] since they are antagonists of the mineralocorticoid (aldosterone) receptor promoting sodium and water excretion. Spironolactone has been used in the clinic for more than 50 years, but some side effects have being observed related to its non-specificity and it affinity to the androgen and progesterone receptors.[9] Eplerenone is derivative of spironolactone with increased

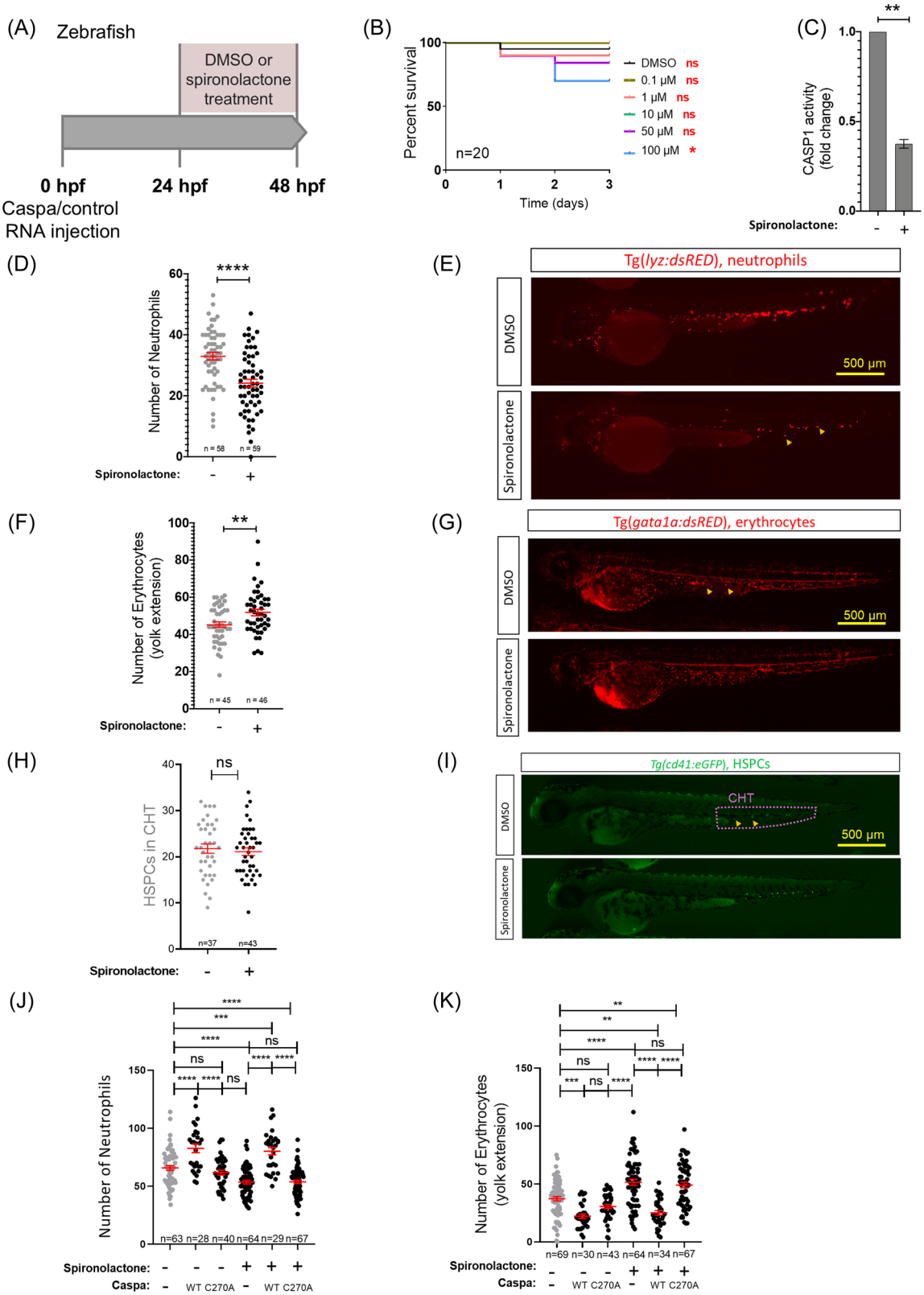

FIGURE 1 **Spironolactone regulates hematopoiesis in zebrafish**. Experimental design **(A)**, survival **(B)**, caspase-1 activity **(C)** and number of neutrophils **(D, J)**, erythrocytes **(F, K)** and HSPCs **(H)** of 2 day-postfertilization (dpf) *Tg(lyz:dsRED)*, *Tg(gata1a:dsRED)* and *Tg(cd41:eGFP)* larvae, respectively, injected with RNAs encoding wild type (WT) or catalytic-dead (C270A) Caspa at the one-cell stage and treated from 1 to 2 dpf by bath immersion with either 10 µM spironolactone or DMSO. Representative images of neutrophils **(E)**, erythrocytes **(G)** and HSPCs **(I)** (labelled with arrowheads) are also shown. Each dot represents one individual and the mean ± SEM for each group is also shown. *p* values were calculated using Log-Rank test **(B)**, Student's *t*-test **(C, D, F, H)** and ANOVA followed by Tukey multiple comparison test **(J, K)**. n.s., non-significant; CHT, caudal hematopoietic tissue; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

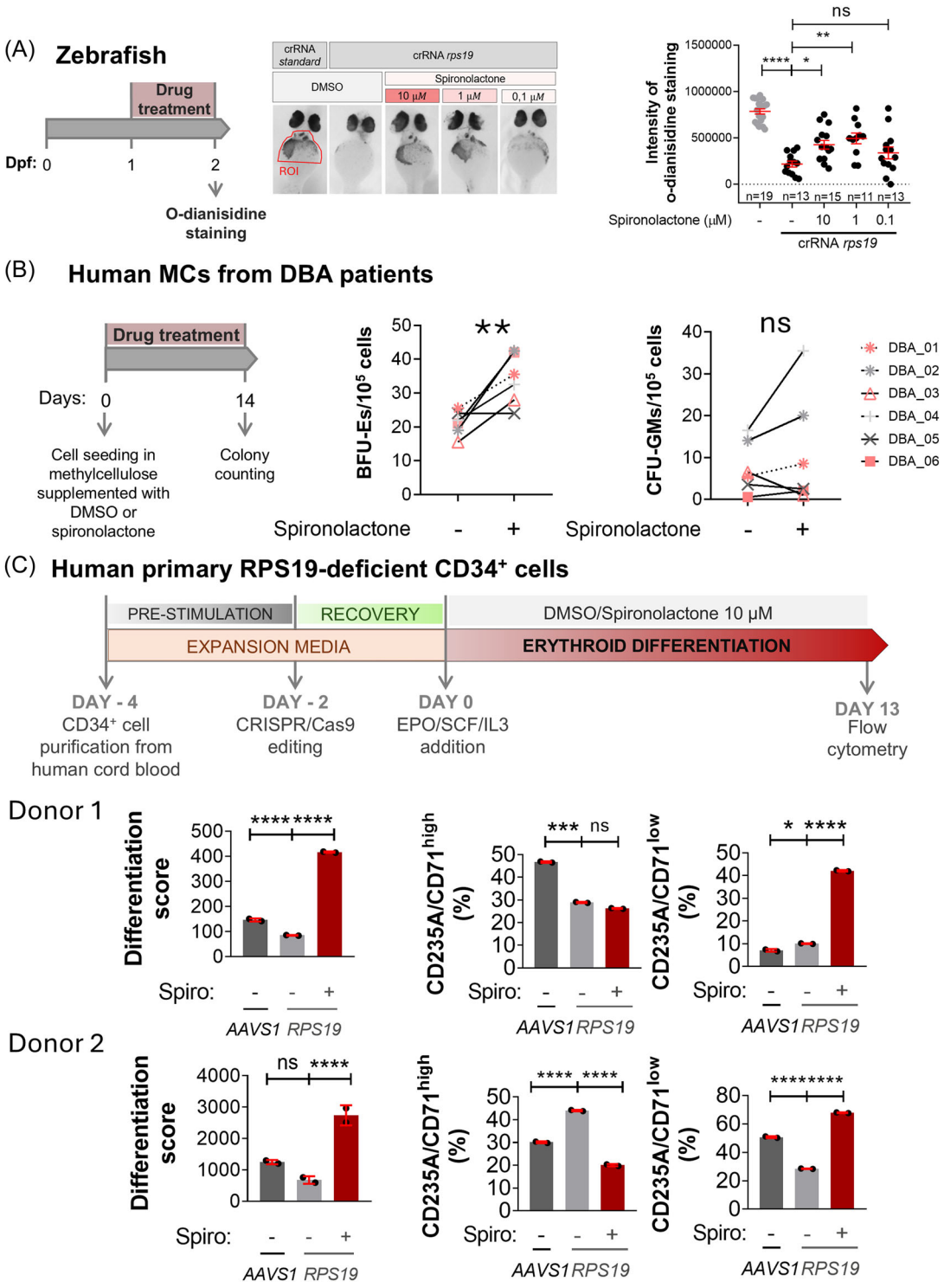

FIGURE 2 **Spironolactone alleviates erythropoiesis defects in zebrafish and human DBA models, as well as in HSPCs from DBA patients. (A)** Hemoglobin staining in 2 dpf crRNA *rps19*/Cas9 complexes-injected larvae treated from 1 to 2 dpf by bath immersion with 10 μM of spironolactone or DMSO. **(B)** BMMCs and PBMCs from DBA patients were incubated for 2 weeks in human methylcellulose complete medium at 37°C in the presence or absence of 10 μM spironolactone, and BFU-E and CFU-GM colonies counted using standard morphological criteria. Note that BMMCs were used from patient 1 and PBMC from the rest of patients. Each dot represents one individual and the means ± SEM for each group is also shown. **(C)** Primary human $CD34^+/CD133^+$ HSPCs from human cord blood were edited with either *AAVS1* or *RPS19* gRNA/Cas9 complexes and differentiated for 13 days with EPO in the presence of either DMSO or 10 μM spironolactone. Cells were stained with anti-CD235A-APC (Glycophorin A) and anti-CD71-FITC (Transferrin Receptor) and erythroid differentiation was then analyzed by flow cytometry. The differentiation score (ratio between $CD71^+/CD235A^+$ (intermediate erythroid progenitors and reticulocytes) and $CD71^+/CD235A^-$ (early erythroid progenitors), and the percentage of $CD235A^+/CD71^{high}$ (erythroblasts) and $CD235A^+/CD71^{low}$ (reticulocytes) are shown. *p* values were calculated using Student´s *t*-test **(B)** and ANOVA followed by Tukey multiple comparison test **(A, C)**. ns, non-significant; ROI, region of interest; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

specificity for the mineralocorticoid receptor.[10] Hence, eplerenone has 100 times lower affinity for androgen, progesterone and glucocorticoid receptors.[5] It has been observed different effects between eplerenone and spironolactone, which may be related to their effects on progesterone.[11] However, our experiments using steroids-free culture medium in HEK293 with reconstituted NLRP1 inflammasome and in K562 cells upon erythroid differentiation, ruled out that spironolactone mediates an indirect inhibition of NLRP1 inflammasome through engaging steroid receptors. Therefore, it is plausible that spironolactone, but not eplerenone, is able to binds and inhibits the NLRP1 inflammasome due to structural differences. Further experiments need to be performed to understand the different effects of these two compounds on NLRP1 inflammasome and its usefulness to treat blood disorders associated to chronic inflammatory diseases and congenital anemias, such as DBA.

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AUTHOR CONTRIBUTIONS

Victoriano Mulero and Sylwia D. Tyrkalska conceived the study. Lola Rodríguez-Ruiz, Juan M. Lozano-Gil, María Ocaña-Esparza, Ana M. Conesa-Hernández, and Ana B. Pérez-Oliva performed the research. Lola Rodríguez-Ruiz, Juan M. Lozano-Gil, María Ocaña-Esparza, Ana M. Conesa-Hernández, Ana B. Pérez-Oliva, José L. Fuster, Andrés Jérez, Laura Murillo-Sanjuán, Cristina Diaz-de-Heredia, Guzman Lopez-de-Hontanar, Julian Sevilla, Diana García-Moreno, María L. Cayuela, Alicia Martínez-López, Sylwia D. Tyrkalska, and Victoriano Mulero analyzed the data. José L. Fuster, Andrés Jérez, Laura Murillo-Sanjuán, Cristina Díaz-de-Heredia, Guzmán López-de-Hontanar, and Julián Sevilla provided essential material. Sylwia D. Tyrkalska and Victoriano Mulero wrote the original draft. All authors edited the final version, and read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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