Reduced GATA1 levels are associated with ineffective erythropoiesis in sickle cell anemia

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Received: June 4, 2024, 2024.
Accepted: November 27, 2024.
Early view: December 5, 2025.

https://doi.org/10.3324/haematol.2024.286010

Abstract

Ineffective erythropoiesis (IE) is defined as the abnormal differentiation and excessive destruction of erythroblasts i n the bone marrow, accompanied by an expanded progenitor compartment and relative reduction in the production of reticulocytes. It is a defining feature of many types of anemia, including β-thalassemia. GATA1 is an essential transcription factor for erythroid differentiation, known to be implicated in hematological conditions presenting with IE, including β-thalassemia and congenital dyserythropoietic anemia. However, little is known about the role of GATA1 in the erythropoietic defects recently described in sickle cell anemia (SCA). In the present study, we performed a detailed characterization of the role of GATA1 and ineffective erythropoiesis in SCA using both *in vitro* and *in vivo* assay systems. We demonstrate a significant decrease in GATA1 protein levels during SCA erythropoiesis and a concomitant increase in oxidative stress. Furthermore, we found that an increase in the activity of the inflammatory caspase, caspase 1, was driving the decrease in GATA1 levels during SCA erythropoiesis and that, upon inhibition of caspase 1 activity, SCA erythropoiesis was rescued and GATA1 levels partially restored. Our study further elucidates the defect in erythropoiesis in SCA, and may therefore help in the development of novel approaches to normalize the bone marrow niche prior to stem cell transplantation, or facilitate the production of healthy stem cells for gene therapy.

Introduction

Sickle cell anemia (SCA) is a recessively inherited disease and one of the most common severe monogenic disorders worldwide[1] affecting more than 8 million people. SCA is due to a single mutation in the β-globin chain (β^S) of human hemoglobin resulting in HbS. This abnormal hemoglobin polymerizes in the deoxygenated state and causes the pathognomic red blood cell (RBC) sickling. Although every person with SCA has the same underlying genotype, being homozygous for the β-globin variant (HBB; c.20A>T,p.Glu7Val), it is a remarkably variable condition, with a wide range of clinical manifestations.

Ineffective erythropoiesis is known as the abnormal differentiation of erythroid progenitors accompanied by an expanded progenitor compartment, increased destruction of abnormal erythroblasts and a paucity in the production of mature erythroid cells. It is recognized as an important factor in many types of anemia, including β-thalassemia.[2,3] However, it has not been systematically investigated in SCA. In a recent study, it was shown that erythroblasts from the bone marrow of sickle mice had increased reactive oxygen species (ROS) production and an increased level of apoptosis, both of which are characteristics of ineffective erythropoiesis.[4] In another study using both *in vivo* human-derived erythroblasts and *in vitro* erythroblasts produced from SCA patients, the extent of ineffective erythropoiesis in SCA was evaluated.[5] By mimicking the hypoxic environment of the bone marrow niche, this work showed that hypoxia induces high levels of apoptosis during eryth-

ropoiesis in SCA, suggesting that disordered erythropoiesis is a feature of SCA.[5] More recently, using the sickle Townes mouse model, impaired erythropoiesis was observed in the bone marrow, characterized by a remarkable decrease in erythroblast levels in bone marrow when compared to the relevant control.[6]

GATA1, the founding member of the GATA factor family of transcription factors, is critical for the differentiation of the erythroid lineage. Together with orchestrating the expression of erythroid-specific genes, GATA1 controls the growth, differentiation, and survival of the erythroid lineage.[7] GATA1 is known to be downregulated in hematological conditions presenting with abnormal erythropoiesis including β-thalassemia and Diamond-Blackfan anemia.[8,9] However, little is known about the role of GATA1 in the erythropoietic defects observed in SCA.

In the present study, we performed a detailed characterization of the role of GATA1 and ineffective erythropoiesis in SCA using both *in vitro* and *in vivo* assay systems. We documented in both systems a decrease in GATA1 protein levels during SCA erythropoiesis and a concomitant increase in oxidative stress levels. Mechanistically, we found that an increase in the activity of the inflammatory caspase, caspase 1, was behind the decrease in GATA1 levels during SCA erythropoiesis. Notably, upon the inhibition of caspase 1 activity, SCA erythropoiesis was rescued and GATA1 levels partially restored.

Methods

Cell lines

The human umbilical cord blood-derived erythroid progenitor-2 (HUDEP-2) cell line was obtained from the Nakamura laboratory under an MTA agreement.[10] The sickle HUDEP-2 (sHUDEP-2) cell line was obtained from the Tisdale laboratory under an MTA agreement.[11]

Human biological samples

This study was conducted according to the Declaration of Helsinki with approval from the Health Research Authority (HRA) and Health and Care Research Wales (HCRW) (reference number: 21/WS/0117). Blood samples were obtained from sickle cell anemia (SS) patients and healthy donors (HD) after informed written consent at King's College Hospital (KCH, London, UK).

Mouse model

Berkeley mice (The Jackson Laboratory, stock# 003342) were maintained in the King's College London Biological Service Unit (BSU), Guy's campus. Genotyping and breeding were performed as recommended by The Jackson Laboratory. Mice were housed and handled according to UK Home Office guidelines (project licence: P5741188). All animal experiments were approved by the Named Animal Care and Welfare Officer (NACWO). Adult male and female mice aged between 4-5 months were used in this study, and in each experiment equal number of male and female mice were used. Details of mice experiments are listed in the *Online Supplementary Appendix*.

Laboratory methods

HUDEP-2 and sHUDEP-2 cells were cultured as previously described.[12] Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by Percoll fractionation and $CD34^+$ cells were isolated from PBMC using magnetic beads. $CD34^+$ cells were cultured in an *in vitro* two-phase liquid culture system as previously described.[5] Further information, including details of antibodies used, flow cytometry, measurement of reactive oxygen species, GATA1 staining, the caspase assay, analysis of mouse spleen and bone marrow, imaging flow cytometry, and mass cytometry are given in the *Online Supplementary Appendix*.

Statistical analysis

Statistical analysis was performed using Graphpad Prism (version 9). Data was analyzed using the Mann-Whitney unpaired test.

Results

Delayed terminal erythroid differentiation in human sickle cell anemia erythroblasts

We first tested a human HUDEP-2-derived proerythroblast cell line in which the sickle mutation was introduced by gene editing in both β-globin alleles (hereafter referred to as SS HUDEP-2 cells).[11] We induced differentiation of both SS and wild-type (WT) HUDEP-2 cells and monitored differentiation kinetics using flow cytometry and microscopy. At day 0 of differentiation, both WT and SS HUDEP-2 cells are at the proerythroblast stage, shown by Band3 and CD49d staining (*Online Supplementary Figure S1A*). Differentiation kinetics were similar between both WT and SS HUDEP-2 at days 2 and 4 (*Online Supplementary Figure S1A, B, D*). At day 6, however, a slight delay in differentiation was observed in the SS HUDEP-2 cell line, also evidenced by the significantly fewer orthochromatic erythroblasts (*Online Supplementary Figure S1A, C, E*).

We next tested primary $CD34^+$ cells isolated from the peripheral blood of HD and patients with SCA (homozygous for the sickle mutation, SS) and cultured in a two-phase differentiation system.[5] Differentiation kinetics were monitored during phase 2 of the culture (*Online Supplementary Figure S2*). The percentage of glycophorin A-positive (GPA^+) cells was evaluated as a reflection of the commitment of the $CD34^+$ cells to the erythroid lineage. Significantly fewer GPA^+ cells were observed in the SS compared to the HD cultures, indicating an alteration in the commitment of SS progenitors to the erythroid lineage (*Online Supplementary*

Figure S3A, B). We assessed the erythroid differentiation kinetics of the GPA$^+$ cell population only, by profiling the CD49d and Band3 cell surface markers. In SS cultures, a striking delay in differentiation was observed compared to HD cultures, starting from day 2 of phase 2 (*Online Supplementary Figure S4A-C*). Most erythroblasts in the SS cultures were at the proerythroblast stage at day 4, while the HD cells were at the basophilic stage. At day 6 of differentiation, 52.9% (standard deviation [SD] 12.5) of HD cells were at the polychromatic stage, compared to 15.0% (SD 9.9) for the SS cells (*Online Supplementary Figure S4B-C*). Likewise, by day 8, 41.8% (SD 18.2) of HD cells had reached the orthochromatic stage, compared to only 4.8% (SD 5.39) of SS cells (*Online Supplementary Figure S4B-C*). Overall, we observed a marked delay in the differentiation of SS erythroblasts compared to HD, when placed in similar culture conditions. Moreover, we did not see such a marked delay in the differentiation kinetics of the SS HUDEP-2 cells.

Increased oxidative stress in human sickle cell anemia erythroblasts

To assess oxidative stress in SS erythropoiesis, the levels of ROS were monitored during phase 2 of differentiation in the WT and SS HUDEP-2 cell lines. The mean fluorescence intensity (MFI) levels were measured and normalized according to the ROS levels in WT cells at day 2, 4, and 6 of differentiation. From this analysis, no differences in ROS levels were detected when comparing the WT and SS HUDEP-2 cell lines, during differentiation (*Online Supplementary Figure S5A, B*).

Similar analysis was also performed in the HD and SS primary cultures, with ROS levels monitored at days 2, 4, 6 and 8 of phase 2 of differentiation. ROS levels were higher in the SS cultures compared to HD, starting from day 2 and up until day 8, which was the last time point of differentiation tested (Figure 1A, B). Specifically, ROS MFI levels in SS cells were at 15,242 (SD 8,439) compared to 8,040 (SD 2,862) in HD cells at day 2 of differentiation, further increasing in SS cells to 17,149 on day 4 (SD 8,933), compared to 3,594 (SD 1,591) in HD cells. ROS levels remained high through day 6 in SS cells (5,836, SD 2,907) and day 8 of differentiation (2,106, SD 621) when compared to HD cells (day 6: 2,094, SD 1,637; day 8: 914, SD 68) (Figure 1A, B). Notably, increased ROS levels were apparent from early erythroid development, before significant amounts of sickle hemoglobin are present in the cells.

GATA1 levels are decreased in differentiating human sickle cell anemia erythroblasts

Many defects in erythropoiesis are characterized by aberrant GATA1 protein expression.[13-15] Hence, GATA1 protein levels were assessed in the HD and SS primary cultures using classical flow cytometry, western blot analysis and cytometry by time of flight (CyTOF) technology (*Online Supplementary Figure S2*). Using flow cytometry, MFI levels

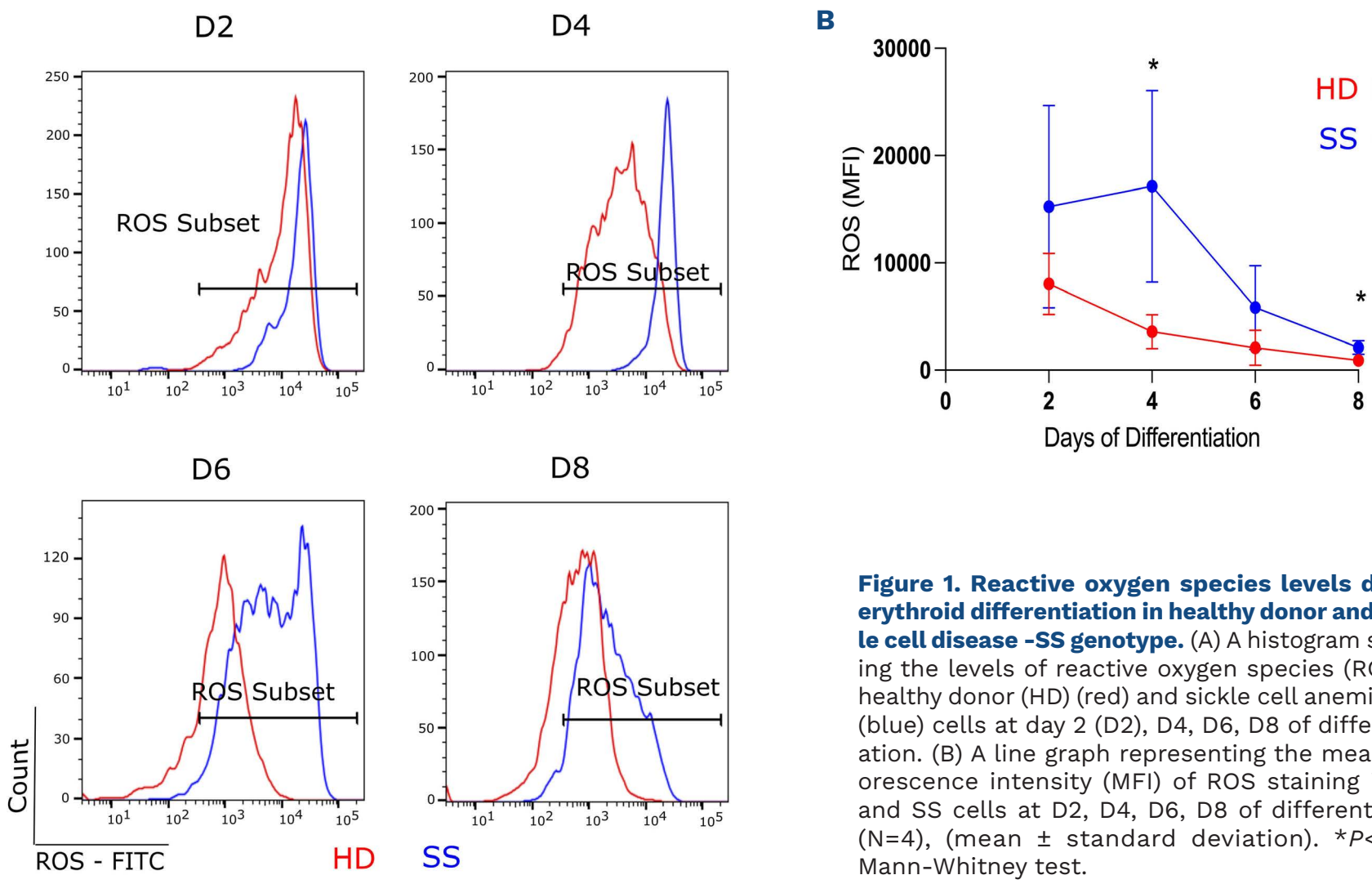
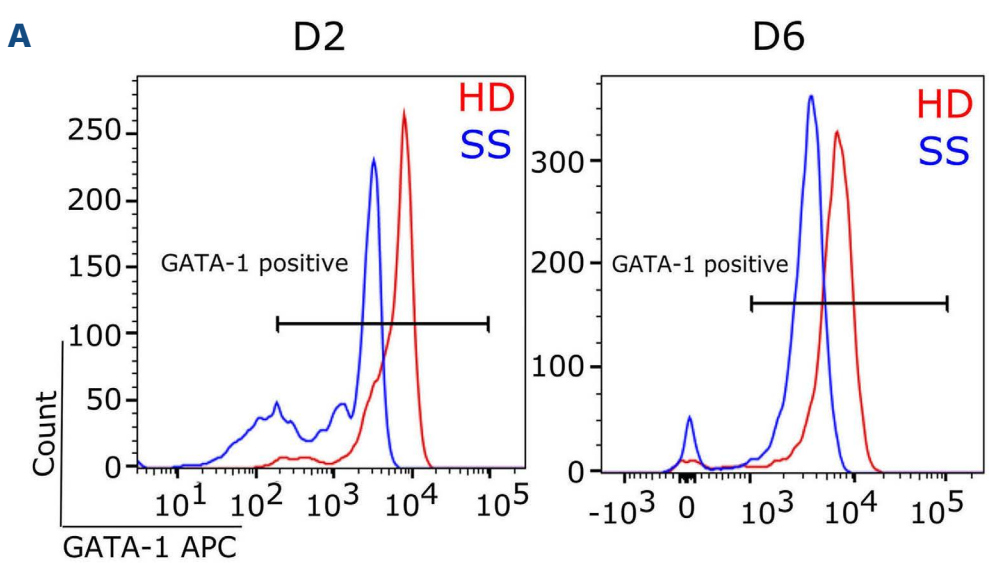
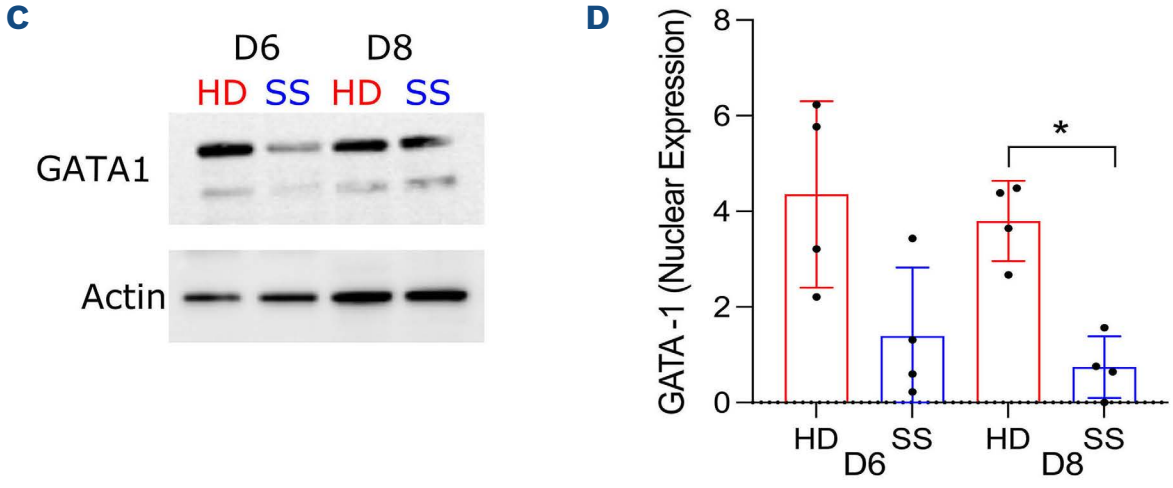
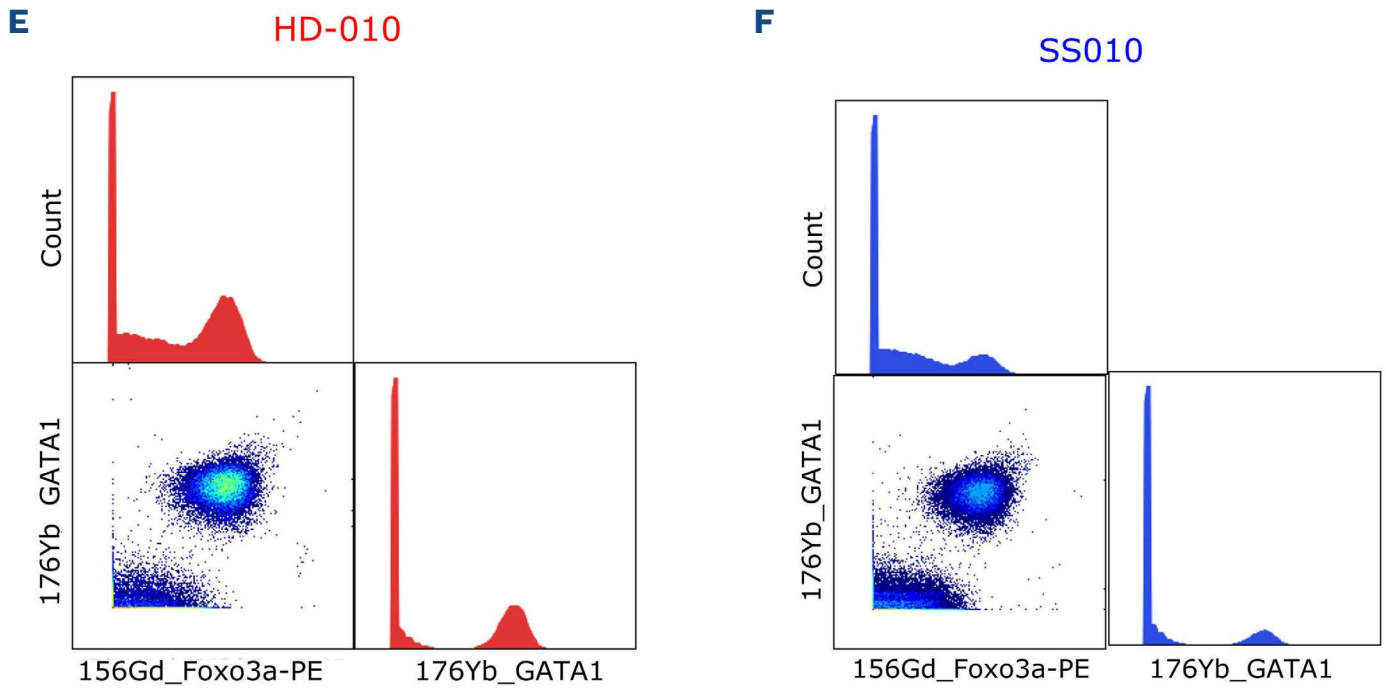


Figure 1. Reactive oxygen species levels during erythroid differentiation in healthy donor and sickle cell disease -SS genotype. (A) A histogram showing the levels of reactive oxygen species (ROS) in healthy donor (HD) (red) and sickle cell anemia (SS) (blue) cells at day 2 (D2), D4, D6, D8 of differentiation. (B) A line graph representing the mean fluorescence intensity (MFI) of ROS staining in HD and SS cells at D2, D4, D6, D8 of differentiation (N=4), (mean ± standard deviation). *P<0.05, Mann-Whitney test.

of GATA1 protein in GPA$^+$ erythroblasts were measured and compared to GATA1 levels in HD erythroblasts (Figure 2A, B). A significant decrease in GATA1 MFI levels was detected in the SS erythroblasts when compared to HD erythroblasts (Figure 2A, B). Specifically, at day 2 the MFI of GATA1 in HD erythroblasts was at a mean of 4,414 (SD 1,066) compared to 1,744 (SD 838) in SS erythroblasts. At day 6 the MFI of GATA1 in HD was 4,107 (SD 1,288) while in SS erythroblasts GATA1 levels were significantly less (1,681, SD 981) (Figure 2A, B). To confirm the low GATA1 levels, nuclear protein extracts were prepared from HD and SS erythroblasts at days 6 and 8 of differentiation and GATA1 protein levels were measured by western immunoblotting analysis and normalized to β-actin levels (Figure 2C, D). GATA1 levels were very low to undetectable in the cytoplasmic fraction we therefore focused our analysis on nuclear extracts of erythroblasts. A decrease in GATA1 was again observed in the SS nuclear extracts when compared to HD on both days, with a more significant decrease observed on day 8 of differentiation (Figure 2C, D). Moreover, GATA1 levels were measured by CyTOF at day 6 in stage-matched HD and SS erythroblasts, identified using the GPA and CD71 surface markers. From this it was evident







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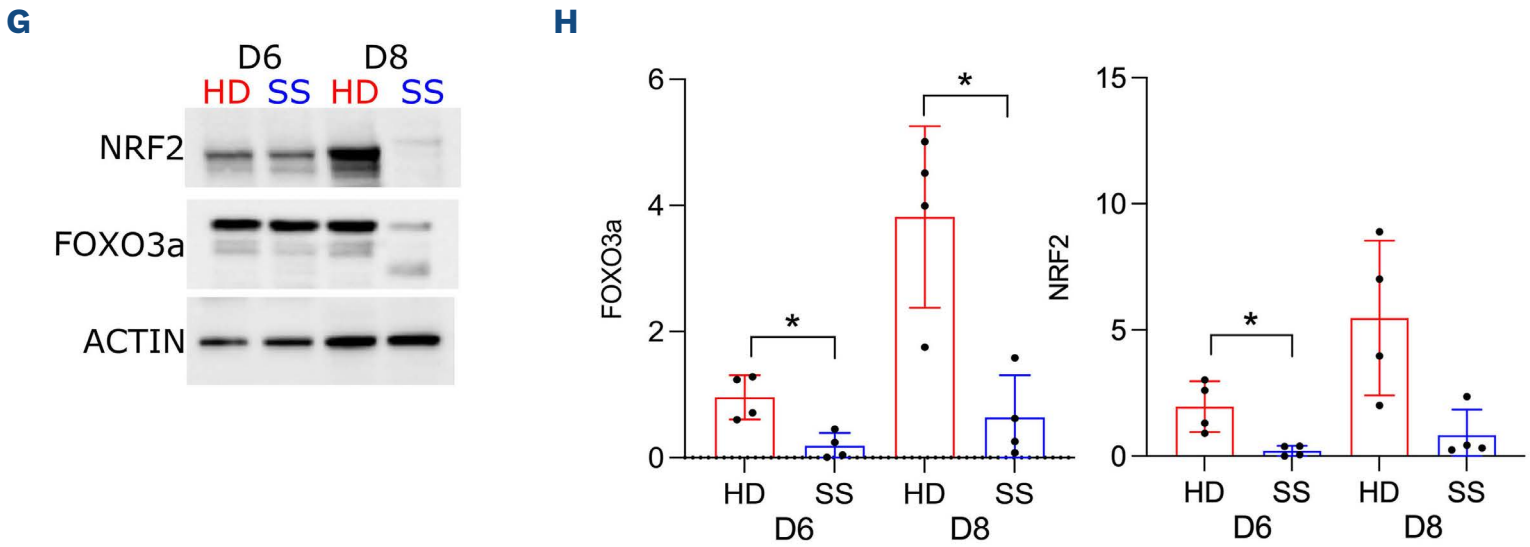

Figure 2. GATA1 protein levels in differentiating healthy donor and sickle cell disease -SS genotype primary cells. (A) A histogram showing the analysis by flow cytometry of glycophorin A-postive (GPA$^+$) cells, assaying for GATA1 protein on day 2 (D2) and D6 of differentiation of healthy donor (HD) (red) and sickle cell anemia (SS) (blue) cells. (B) Bar graph representing the mean fluorescence intensity (MFI) of GATA1 in the GPA$^+$ population at D6 and D8 of HD (red) and SS (blue) cell differentiation (N=4) (mean ± standard deviation) [SD]. (C) Western blot images of GATA1 (upper panel) and actin (lower panel) proteins in nuclear extracts of HD and SS cells at D6 and D8 of differentiation. (D) Bar graph representing the relative quantification by western blotting of GATA1 protein levels in the nuclear extracts of HD and SS cells at D6 and D8. Protein loading per lane for quantification was adjusted relative to actin protein levels (N=4), (mean ± SD). Histogram and density plot representations of GPA$^+$ CD71$^+$ cells showing the levels of protein expression of FOXO3a and GATA1 in (E) HD-010 and (F) SS-010. (G) Western blot analysis of NRF2 (upper panel), FOXO3a (middle pane) and actin (lower panel) in nuclear extracts of differentiating HD and SS cells at D6 and D8 of differentiation. (H) Bar graph showing the quantification of FOXO3a (left) and NRF2 (right) protein levels in nuclear extracts from HD and SS cells at D6 and D8 of differentiation, relative to actin protein levels (N=4), (mean ± SD). *P<0.05, Mann-Whitney test.

that GPA$^+$ CD71$^+$ SS erythroblasts had lower levels of GATA1, indicated by a shift to the left in the cell population, when compared to GPA$^+$ CD71$^+$ HD erythroblasts (Figure 2E, F). Taken together, this confirms that SS erythroblasts express less GATA1 protein when compared to HD erythroblasts. To evaluate whether the decrease in GATA1 is at the post-transcriptional level, we measured *GATA1* mRNA at day 6. *GATA1* mRNA levels were similar between HD and SS erythroblasts (*Online Supplementary Figure S6*).

GATA1 protein levels were also assessed during differentiation of the SS and WT HUDEP-2 cell lines. In line with our observations of no difference in differentiation kinetics, we saw no significant difference by western blot analysis in GATA1 protein levels in WT and SS HUDEP-2 cells throughout differentiation (*Online Supplementary Figure S7A, B*).

NRF2 and FOXOA3 levels are altered in sickle cell anemia erythroblasts

Nuclear factor, erythroid derived 2 (NRF2) and forkhead box O3 (FOXO3a) are transcription factors that are known to be required for the regulation of cellular oxidative stress response genes.[16,17] Since we observed higher ROS levels in the SS cells during differentiation (Figure 1), we investigated how this was reflected in the levels of NRF2 and FOXO3a proteins in the HD and SS erythroblasts at days 6 and 8 of differentiation. We found that, on both days, NRF2 and FOXO3a levels were significantly lower in the SS erythroblasts, as compared to HD (Figure 2G, H). This is particularly evident on day 8 of differentiation, when very little NRF2 and FOXO3a proteins were detectable (Figure 2G). FOXO3a levels were also assayed by CyTOF in the GPA$^+$CD71$^+$ erythroblasts from a HD and an SS patient. Again, we found FOXO3a levels to be significantly lower in GPA$^+$CD71$^+$ SS erythroblasts, compared to HD (Figure 2E, F).

The bone marrow and spleen of sickle cell anemia Berkeley mice exhibit a disrupted architecture

SS Berkeley mice have targeted deletions of the murine α and β globins and carry transgenes containing the human α, β^S, γ^A and γ^G globin genes.[18,19] Thus, these mice exclusively express human sickle hemoglobin.[20] We used Berkeley SS mice to investigate defects in erythropoiesis, with HbAS Berkeley mice used as a control (*Online Supplementary Figure S8*).[21]

As a validation for the model in our hands, hemoglobin levels were measured and found to be lower in the SS mice (mean 6.6 g/dL, SD 0.92) as compared to the heterozygous AS mice (mean 11 g/dL, SD 0.95) (*Online Supplementary Figure S9A*). Animal weight was also lower in the SS mice (mean 26.62 g, SD 6.92) compared to AS mice (mean 36.86 g, SD 6.02) used in this study (*Online Supplementary Figure S9B*). Moreover, spleen weight, which is a well-known characteristic of this mouse model, was much higher in the SS mice (mean 0.99 g, SD 0.18) as compared to the AS mice (mean 0.1 g, SD 0.02) (*Online Supplementary Figure S9C*).

As both the bone marrow and spleen are erythropoietic niches in mice, histological analysis by hematoxylin and eosin (HE) staining was performed on both tissues. Sections of bone marrow from the femur were analyzed and the SS mice showed noticeable clumping of hematopoietic

cells and an increased number of erythroid precursors (*Online Supplementary Figure S9D*). The spleen of SS mice exhibited a disrupted architecture, with a complete loss of well-defined compartments of red and white pulp (*Online Supplementary Figure S9E*). Using Perl's staining, we also observed an increase of iron deposits in the SS mice (*Online Supplementary Figure S9E*). All the above observations are hallmarks of sickle cell pathology and are consistent with previous work on the pathology of the Berkeley SS mice.[20,21]

Delayed erythroid differentiation in the bone marrow and spleen of sickle cell anemia Berkeley mice

We next assessed the levels of Ter119, a specific marker for murine erythropoiesis, in cells isolated from both the bone

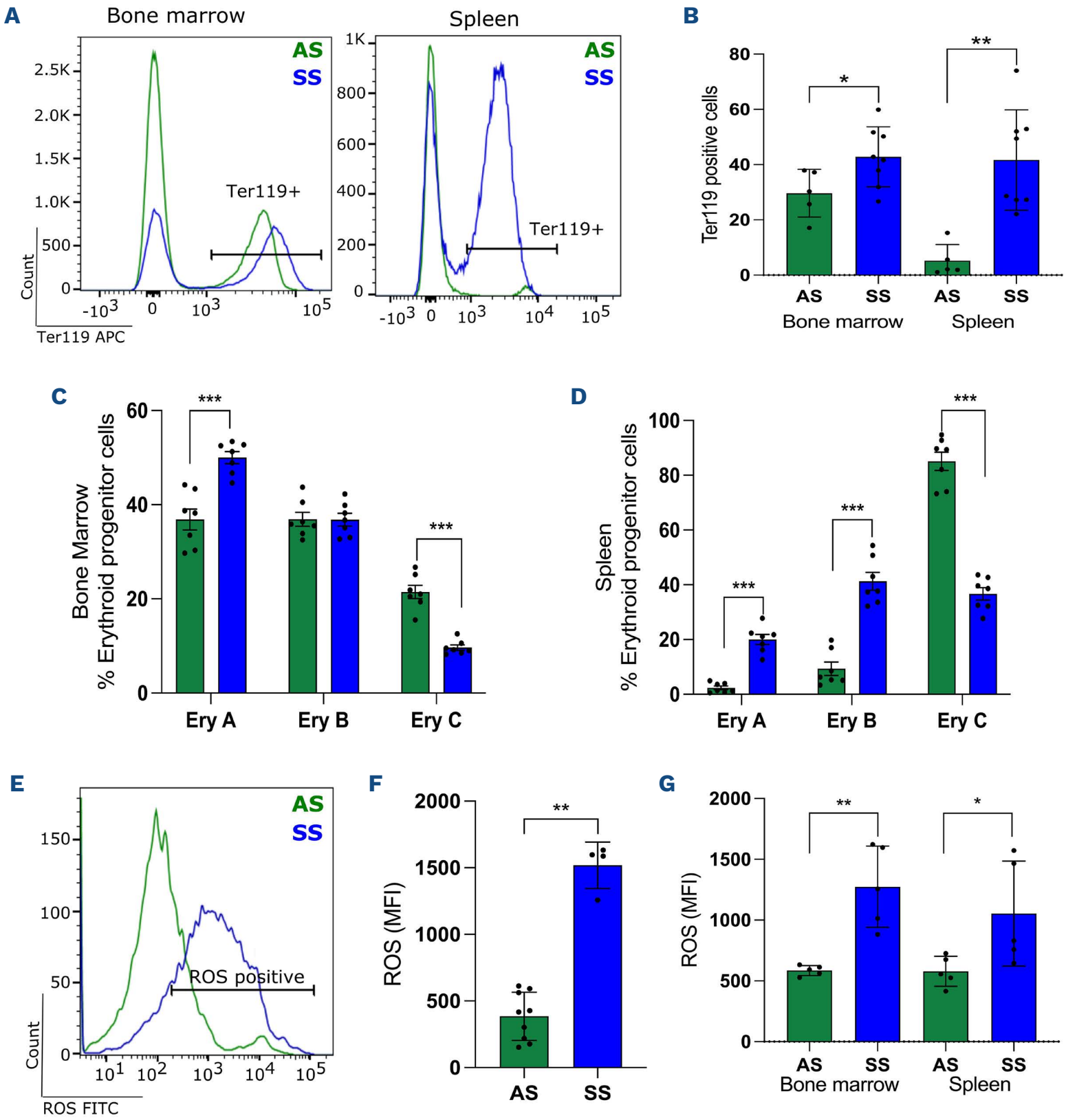

Figure 3. Erythropoiesis and oxidative stress heterozygous AS and homozygous SS Berkeley sickle mice. (A) Histogram representing Ter119 expression in the bone marrow (left) and spleen (right) cells of 1 AS (green) and 1 sickle cell anemia (SS) (blue) mouse. (B) Bar graph representing the percentage of Ter119⁺ cells in the bone marrow and the spleen of AS (N=5) and SS (N=8) mice, (mean ± standard deviation). (C) A bar graph representing the percentage of basophilic erythroblasts (EryA), polychromatic Ery (ryB) and orthochromatic Ery (EryC) cells in the bone marrow of AS and SS mice (N=7). (D) Bar graph representing the percentage of EryA, EryB and EryC cells in the spleen of AS and SS mice (N=7). (E) Histogram representing the levels of reactive oxygen species (ROS) in the peripheral blood of one AS (green) and one SS (blue) mouse. (F) Bar graph showing ROS mean fluorescence intensity (MFI) in the peripheral blood of AS (N=9) and SS (N=4) mice. (G) A bar graph representing ROS MFI in the bone marrow and spleen of AS and SS mice (N=5) (mean ± standard deviation). **P*<0.05, ***P*<0.01, ****P*<0.001 Mann-Whitney test.

marrow and the spleen of the SS and AS Berkeley mice, as a reflection of erythropoietic activity in these tissues. An increased percentage of Ter119$^+$ cells was observed in the bone marrow (mean 42.84%, SD 10.8) and spleen (mean 41.69%, SD 18.16) of SS mice compared to the bone marrow (mean 29.64%, SD 8.6) and spleen of AS (mean 5.21%, SD 5.9) (Figure 3A, B).

We next assessed the differentiation kinetics during erythropoiesis, by characterizing the levels of basophilic erythroblasts (EryA), polychromatic erythroblasts (EryB) and orthochromatic erythroblasts (EryC), that were defined using Ter119 and CD71 staining in hematopoietic cells isolated from the bone marrow and spleen of SS and AS mice.[22] The SS bone marrow cells had a significantly higher per-

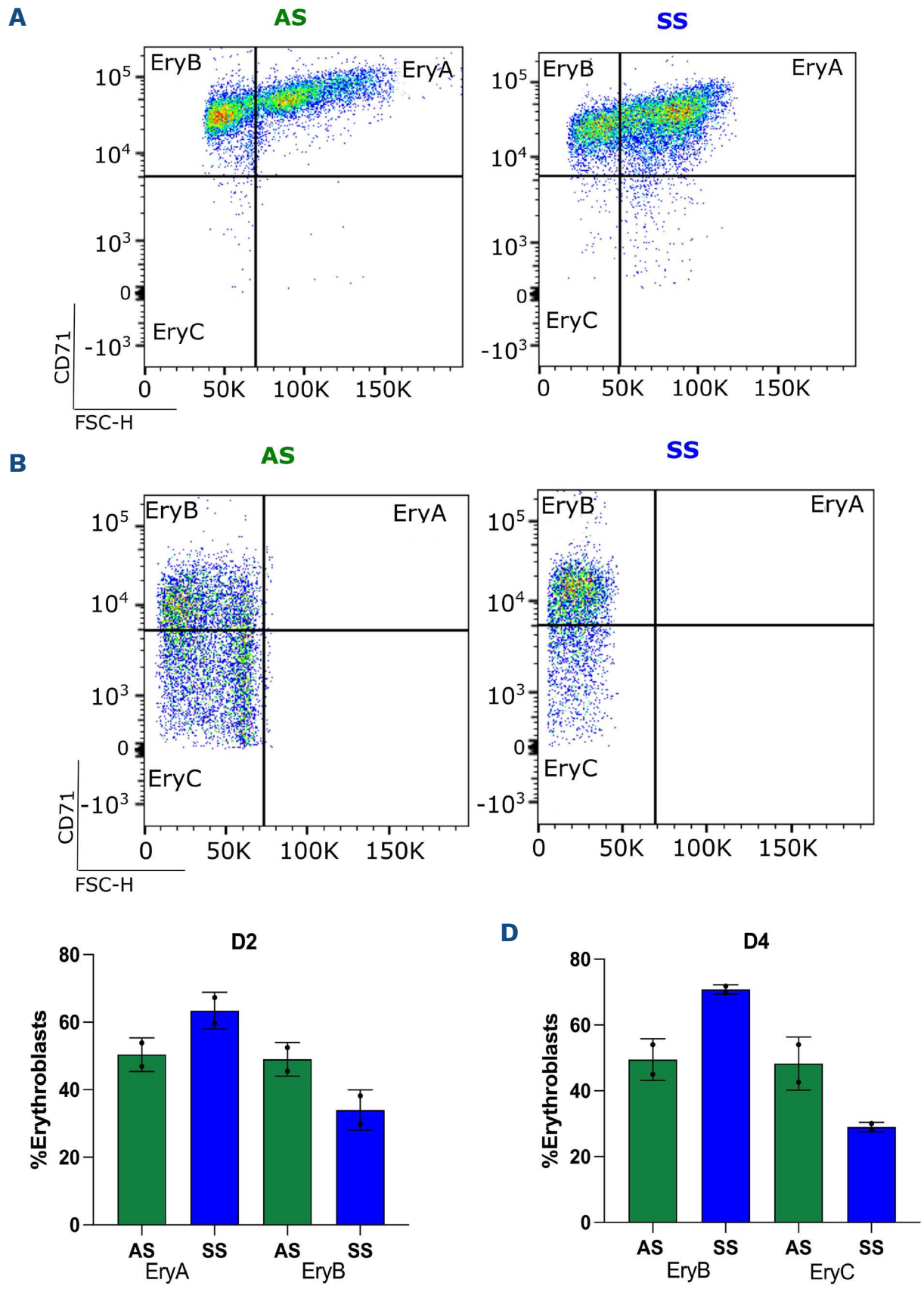

Figure 4. *Ex vivo* erythroid differentiation of Lin-negative cells from the bone marrow of AS and homozygous SS Berkeley mice. A density plot representing the distribution of basophilic erythroblasts (EryA), polychromatic Ery (EryB) and orthochromatic Ery (EryC) cells arising from *ex vivo* differentiated hematopoietic stem cells (HSC) isolated from the bone marrow of 1 AS and 1 sickle cell anemia (SS) mouse, on (A) day 2 (D2) and (B) D4 of culture. The plot represents CD71$^+$ (y-axis) and FSC-H (x-axis). (C) Bar graph representing the percentage of EryA and EryB on D2 of *ex vivo* differentiated bone marrow HSC from AS and SS mice (N=2). (D) Bar graph representing the percentage of EryB and EryC cells at D4 of *ex vivo* differentiated bone marrow HSC from AS and SS mice (N=2) (mean ± standard deviation).

centage of EryA (SS 50.01%, SD 1.27; AS 36.86%, SD 5.88) and a significantly lower percentage of EryC (SS 9.66%, SD 1.5; AS 21.46%, SD 3.7) (Figure 3C; *Online Supplementary Figure S10A*). The spleen isolates of SS mice exhibited a similar profile to that of the bone marrow cells, with a significantly lower percentage of Ery C (36.7%, SD 5.97), when compared to the EryC population in AS (85.16%, SD 8.79) (Figure 3D; *Online Supplementary Figure S10B*). These findings demonstrate that there is a delay in erythroid differentiation similar to that seen in the *ex vivo* analysis of CD34$^+$ cells from sickle patients, described above.

Next, HSC were isolated from the bone marrow of AS and SS mice and placed in an *ex vivo* culture system for 4 days.[23] Differentiation kinetics were monitored at days 2 and 4 of culture using Ter119 and CD71 staining, as described above. At day 2, the SS culture had higher EryA and lower EryB when compared to AS (Figure 4A, C). Moreover, at day 4 the SS culture had higher EryB and lower EryC when compared to AS (Figure 4B, D). Therefore, delayed differentiation was again observed in SS, in keeping with our findings above.

Increased oxidative stress in the bone marrow and spleen of sickle cell anemia Berkeley mice

ROS levels were measured in (i) red cells from peripheral blood, (ii) cells isolated from the bone marrow aspirates and (iii) spleen cells from AS and SS mice. We found that MFI levels were higher in the SS red cells, as compared to AS (Figure 3E, F). ROS MFI levels were also significantly higher in the bone marrow and spleen cells isolated from the SS mice, as compared to the AS (Figure 3G). This is, again, in accordance with the increased oxidative stress seen in the human sickle erythroblasts (Figure 1).

Decreased GATA1 levels in erythroblasts of sickle cell anemia Berkeley mice

GATA1 protein levels were assessed in erythroblasts isolated from the bone marrow and spleen tissues of the AS and SS mice. Isolated cells were stained using Ter119 antibody and sorted using the gating strategy shown in *Online Supplementary Figure S10*. Nuclear extracts were isolated from Ter119$^+$ sorted erythroblasts and GATA1 protein levels were assessed by western blotting. GATA1 levels were decreased in Ter119$^+$ erythroblasts from both SS bone marrow and spleen, as compared to AS cells (Figure 5A-D).

GATA1 protein was also assessed by measuring GATA1 MFI levels in the Ter119$^+$ population by flow cytometry. GATA1 MFI was significantly decreased in SS bone marrow erythroblasts as compared to AS (Figure 5E). A similar pattern was observed in spleen Ter119$^+$ erythroblasts (Figure 5E).

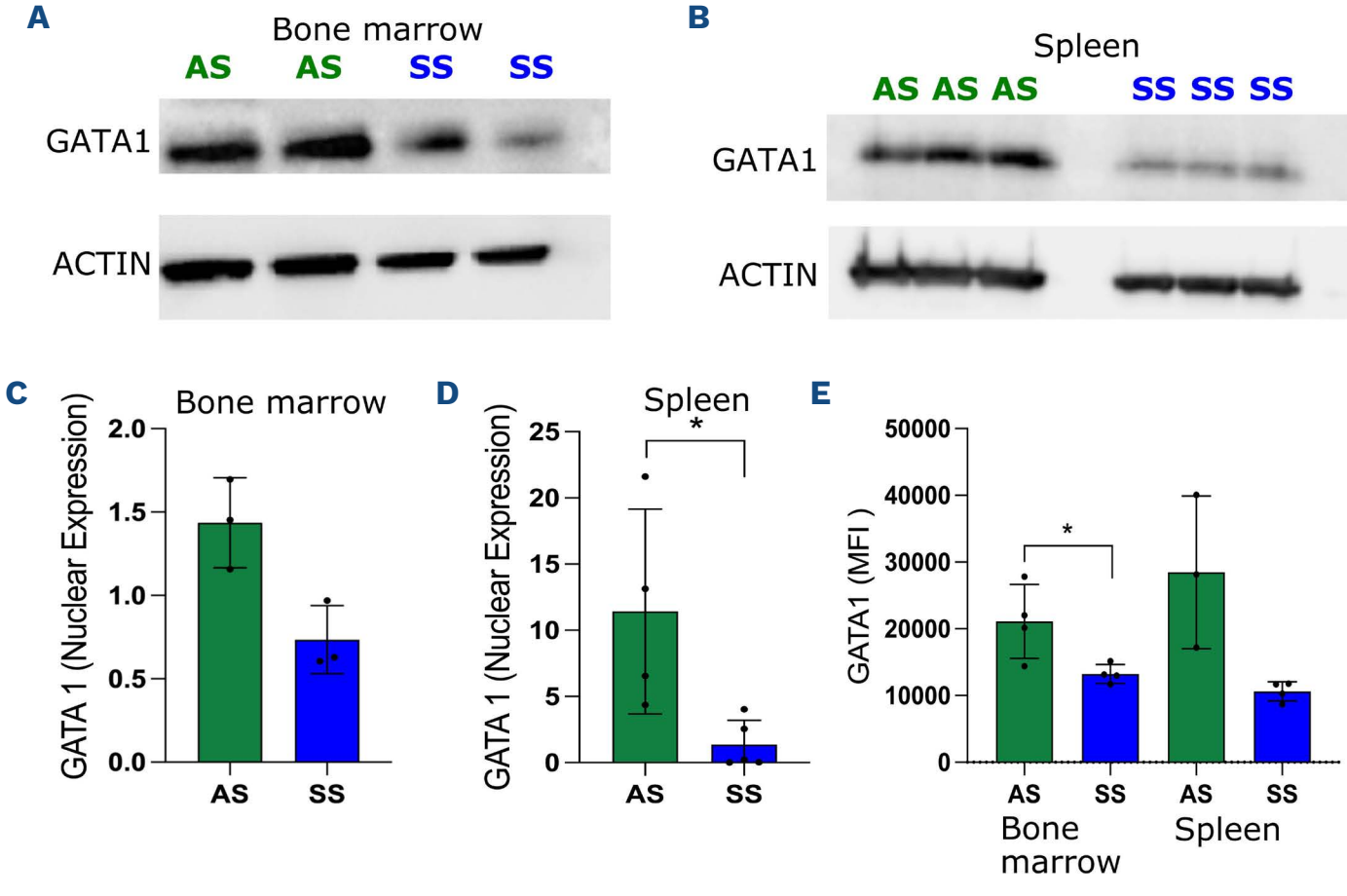

Figure 5. GATA1 protein levels in bone marrow and spleen cells of homozygous SS and heterozygous AS Berkeley mice. (A) Western blot images of GATA1 (upper panel) and actin (lower panel) proteins in nuclear extracts from Ter119$^+$ cells, isolated from AS and sickle cell anemia (SS) bone marrow aspirates. (B) As in (A) using nuclear extracts from Ter119$^+$ cells isolated from AS and SS spleens. (C) Bar graph representing quantification of GATA1 protein levels in the nuclear extracts of Ter119$^+$ cells isolated from AS and SS bone marrow aspirates, relative to actin levels (N=2). (D) Bar graph representing quantification of GATA1 protein levels in the nuclear extracts of Ter119$^+$ cells isolated from AS and SS spleens, relative to actin levels (N=4). (E) Bar graph representing the mean fluorescence intensity (MFI) of GATA1 in the Ter119$^+$ population of the bone marrow (N=4) and spleen (N=4) of AS and SS mice (mean ± standard deviation). *P<0.05 Mann-Whitney test.

To further validate these findings, imaging flow cytometry was used to assess GATA1 levels in SS stage matched erythroblasts. Using a novel analysis pipeline (*Online Supplementary Figure S11*), bone marrow and spleen cells were double stained for Ter119 and GATA1. As Ter119$^+$ cells include erythroblasts, reticulocytes, or mature red cells, we used the IDEAS software to set up an analytical pipeline based on the perimeter and H-variance features, to specifically gate for the erythroblast population (*Online Supplementary Figure S12*). We then discriminated between two populations of GATA1 erythroblasts, the GATA1-low erythroblasts, expressing less GATA1 in the nucleus and the GATA1-high erythroblasts, expressing more GATA1 in the nucleus (Figure 6A-D). Using this approach, we found that there were significantly fewer GATA1-high erythroblasts in the bone marrow and spleen of SS mice, as compared to AS mice (Figure 6C).

Increased caspase 1 activity in the bone marrow and spleen of sickle cell anemia Berkeley mouse

Recently, manipulation of inflammasome components, specifically caspase 1, was shown to alter the differentiation of hematopoietic stem and progenitor cells.[24] It was also reported that GATA1 was susceptible to specific cleavage by caspase 1 in the erythroblastic K562 cell line.[25] As we have shown that inflammation is a characteristic of SS erythropoiesis, we investigated caspase 1 activity in the different erythroblast populations (EryA, EryB and EryC) in the bone marrow and spleen of SS and AS mice. In the bone marrow, caspase-1 activity was higher in all SS erythroblast populations compared to AS, with EryB showing the highest activity (Figure 7A). In the spleen, a similar pattern was observed, with higher levels of caspase 1 activity in the SS erythroblasts as compared to AS (Figure 7B). Note that the EryA population was not studied in the spleen,

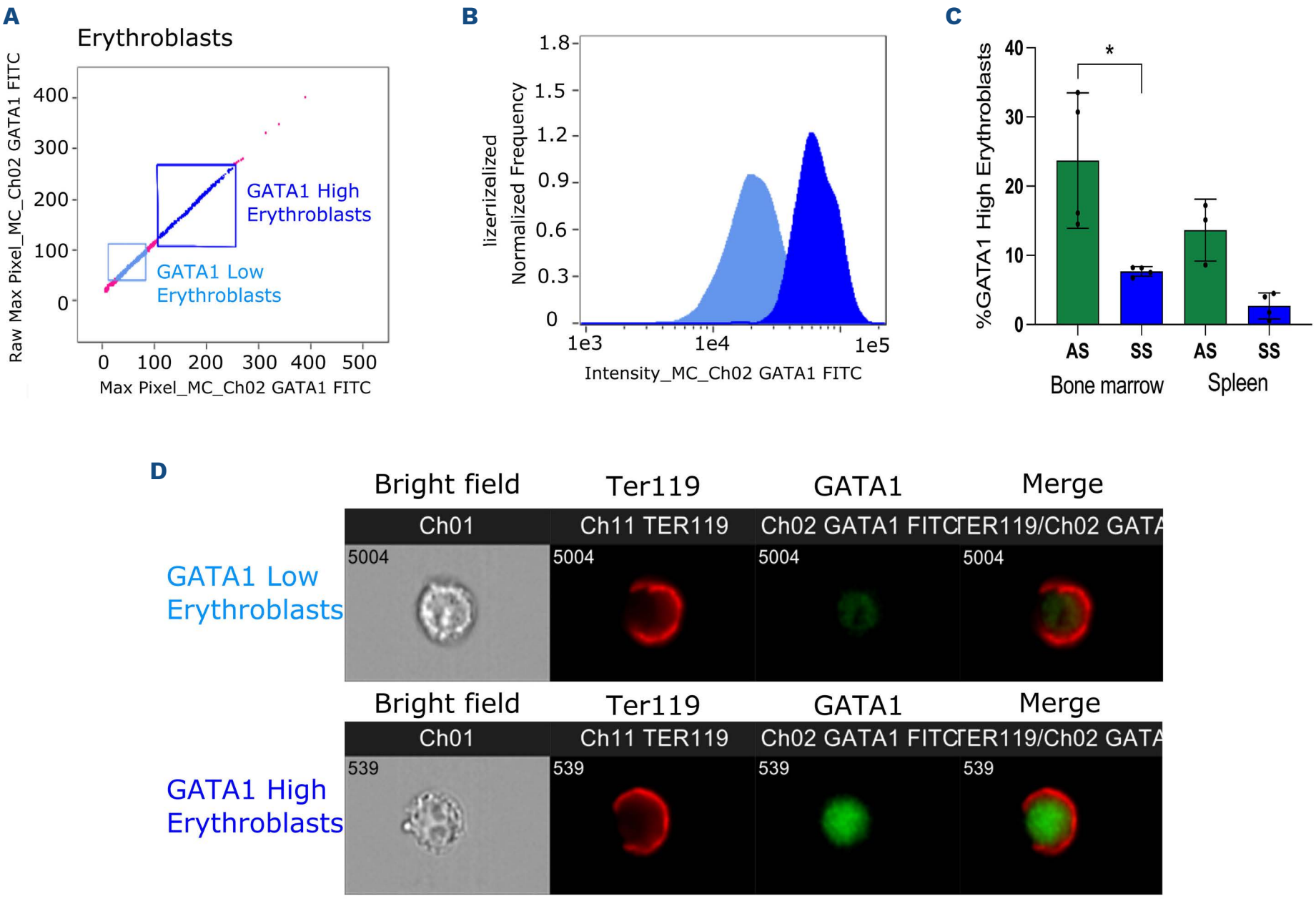

Figure 6. GATA1 protein levels in bone marrow and spleen cells of homozygous SS and heterozygous AS Berkeley mice.(A) Imaging flow cytometry (IFC) analysis graph representing the Max Pixel_MC_Ch02 GATA1 FITC on the x-axis and the Raw Max Pixel_MC_Ch02 GATA1 FITC on the y-axis. This analysis enables the discrimination between stage-matched GATA1-low erythroblasts and GATA1-high erythroblasts, within the Ter119$^+$ erythroblast cell population. (B) Histogram representing the distribution of GATA1 staining in the GATA1-low erythroblasts (light blue) and GATA1-high erythroblasts (dark blue). (C) Bar graph representing the percentage of GATA1-high erythroblasts in the bone marrow (N=4) and spleen (N=3) of AS and SS mice (mean ± standard deviation). (D) Representative images of a GATA1-low erythroblast (upper panel) and a GATA1-high erythroblast (lower panel); images show Bright field, Ter119 (red), GATA1 (green) and the merged image. *P<0.05 Mann-Whitney test.

due to a very low percentage of EryA in spleen of AS mice. Lin-negative cells were isolated from the bone marrow of AS and SS mice and placed in an *ex vivo* culture system for 4 days and caspase 1 activity was measured in the Ter119$^+$ erythroblasts at day 4 of differentiation. SS cultures had higher levels of caspase 1 activity, reflected by higher FLICA levels, an assay that labels active caspase 1, when compared to the AS cultures (Figure 7C).

Inhibition of caspase 1 rescues human sickle cell anemia erythropoiesis

As our findings show that SS erythropoiesis is characterized by increased activity of caspase 1 and decreased GATA1 protein expression, we inhibited caspase 1 activity in SS cultures using the irreversible inhibitor Ac-YVAD-cmk.[26] Upon treatment with Ac-YVAD-cmk (50 μM), the differentiation kinetics of the SS culture were restored compared to the untreated culture, reaching levels closer to those of HD differentiation seen in *Online Supplementary Figure S4* (Figure 8A, B). GATA1 MFI levels were also assessed. An increase in GATA1 MFI levels was observed at both day 6 and day 8 of differentiation in the Ac-YVAD-cmk-treated cultures (Figure 8C, D), in line with the rescued differentiation observed in the treated cultures. Upon treatment with Ac-YVAD-cmk, levels of GATA1 in SS erythroblasts became more comparable to those of HD erythroblasts (*Online Supplementary Figure S12A*). Nevertheless, ROS levels were not restored (*Online Supplementary Figure S12B*) this could be due to several reasons, (i) even though caspase 1 inhibition restores GATA1 levels, GATA1 might not be not solely responsible for oxidative stress regulation in erythropoiesis and thus other factors might be implicated; (ii) caspase 1 inhibition doesn't restore GATA1 to high enough levels to completely restore ROS levels.

Discussion

Defects in SCA erythropoiesis have been previously reported in the literature.[4,5,6,27] In this study we show that SCA erythropoiesis is characterized by a delayed differentiation both in our *in vitro* model of primary cells and our *in vivo* sickle mouse model. The latter is in line with the recent work of Han *et al.*, showing that in the sickle Townes mouse the percentage of more differentiated erythroblasts was significantly less than in controls.[6] However, we did not replicate these findings using the SS HUDEP-2 cell line, a cell line established by generating the sickle mutation in an immortalized

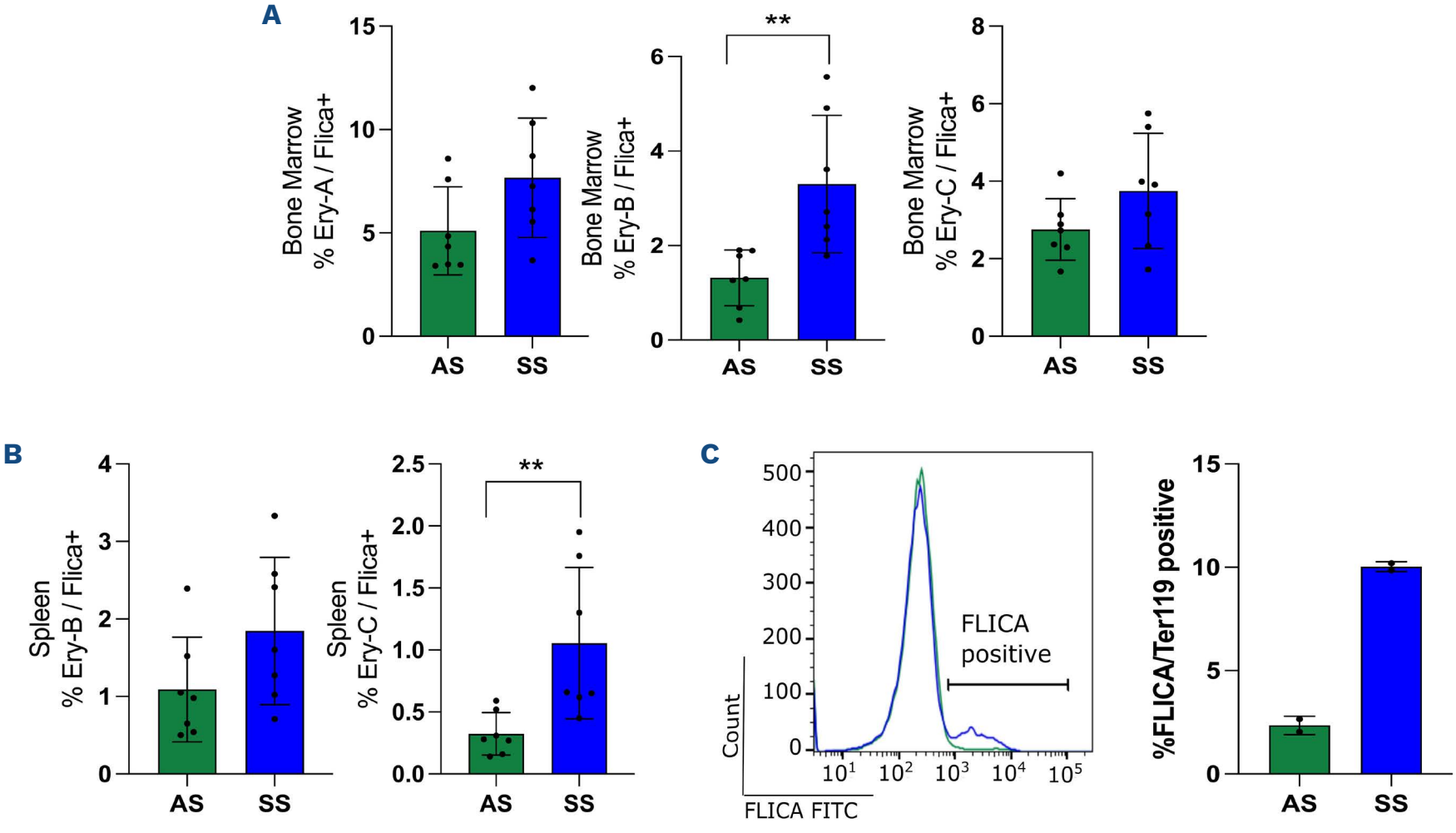

Figure 7. Caspase 1 activity. Bar graph representing the percentage of FLICA-positive (caspase 1 active) cells in (A) the bone marrow and (B) spleen of AS and sickle cell anemia (SS) mice. The percentage of FLICA-positive cells is quantified in the basophilic erythroblasts (EryA, bone marrow), polychromatic Ery (EryB, bone marrow and spleen) and orthochromatic Ery (EryC, bone marrow and spleen) subpopulations (N=7). (C) Left panel: histogram showing FLICA levels in Ter119$^+$ erythroblasts on day 2 of *ex vivo* differentiated Lin$^-$ cells from the bone marrow of 1 AS and 1 SS mouse; right panel: bar graph representing the % FLICA-positive Ter119$^+$ cells on day 2 of *ex vivo* differentiated Lin$^-$ cells from the bone marrow of 1 AS and 1 SS mouse (N=2), (mean ± standard deviation). *P<0.05, **P<0.01, Mann-Whitney test.

human erythroid cell line (HUDEP-2).[11] This important finding suggests that the early abnormalities seen in erythropoiesis in SCA are a result of exposure to an abnormal bone marrow niche, rather than the sickle mutation itself. This may suggest that the effects of inflammation on erythropoietic progenitors persist even after these have been removed from the inflammatory environment, or possibly that the bone marrow niche selectively releases the more damaged stem cells into the circulation. The increased activity of the inflammasome-associated caspase 1 in the bone marrow and

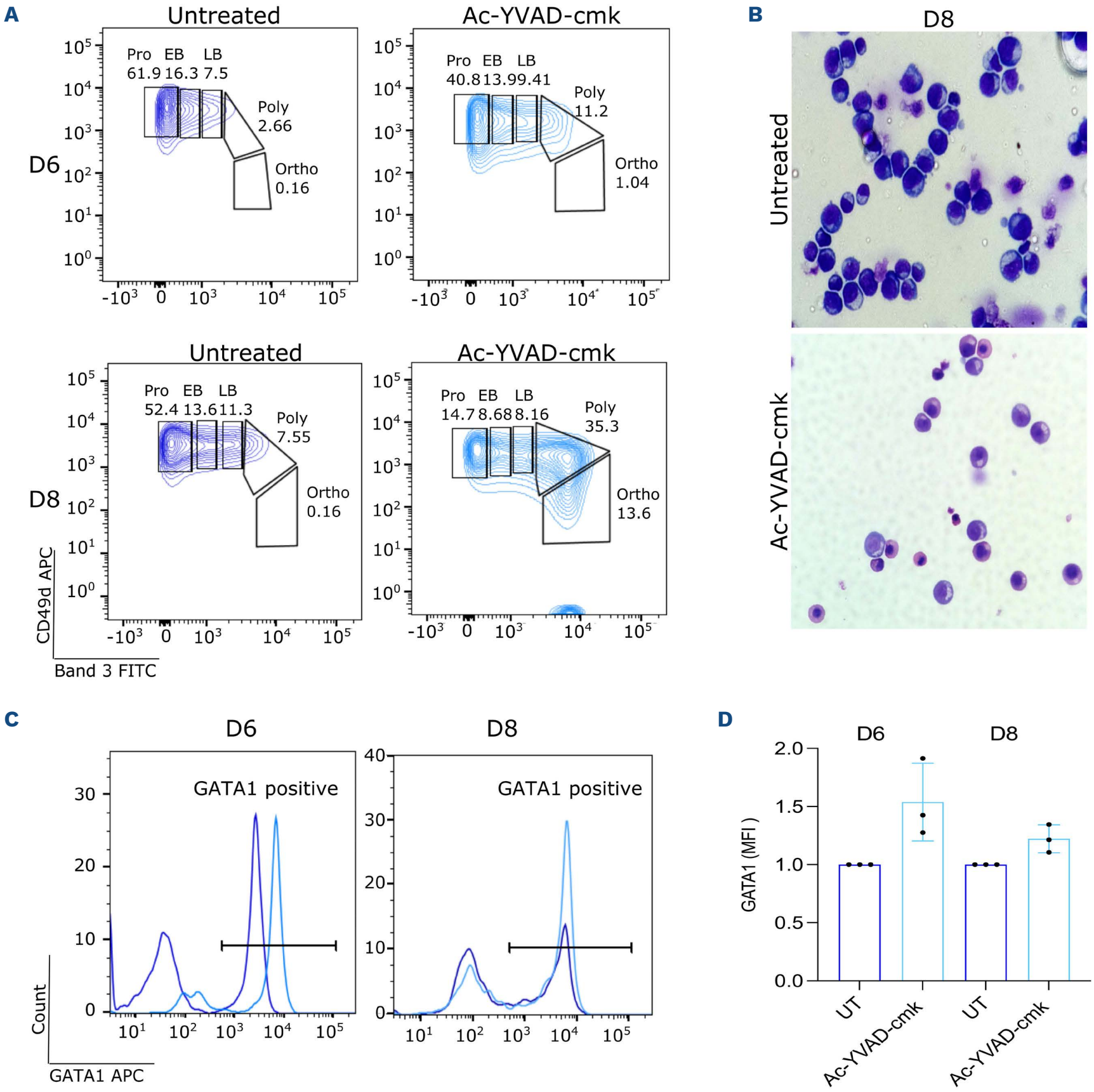

Figure 8. Inhibition of caspase 1 activity. (A) A contour plot of glycophorin A-positive (GPA$^+$) cells from phase 2 of differentiation of sickle cell anemia (SS) untreated (dark blue) and SS treated with Ac-YVAD-cmk (50 μM) (light blue) CD34$^+$ samples, showing the distribution of cell populations with respect to the expression of Band-3 FITC (x-axis) and CD49d-APC (y-axis). Data are represented for day 6 (D6) and D8 of phase 2 of differentiation. Pro: proerythroblast; EB: early basophilic; LB: late basophilic; Poly: polychromatic; Ortho: orthochromatic erythroblasts. (B) Images of SS untreated and Ac-YVAD-cmk treated cells at D8 of culture stained with MGG. (C) A histogram showing the analysis by flow cytometry of GPA$^+$ cells, assaying for GATA1 protein on D6 and D8 of differentiation of SS untreated (dark blue) and SS Ac-YVAD-cmk treated (light blue) cells. (D) Bar graph representing the fold change in mean fluorescence intensity (MFI) of GATA1 in the GPA$^+$ population at D6 and D8 of SS untreated (dark blue) and SS Ac-YVAD-cmk treated (light blue) cells during differentiation (N=3) (mean ± standard deviation). ***P*<0.01 Mann-Whitney.

spleen of the SS mice and the partial rescue of erythropoiesis and of GATA1 protein levels on pharmacological inhibition of caspase 1, suggest that the abnormal erythropoiesis in SCA is, at least in part, caused by inflammation.[28]

Our study also shows that oxidative stress levels are elevated during SCA erythropoiesis, and this likely also contributes to the abnormal erythropoiesis. Oxidative stress in circulating RBC is known to be increased in SCA.[29,30] We found that oxidative stress levels are also elevated in SCA erythroblasts, starting at the proerythroblast stage. Moreover, using the sickle mouse model, increased oxidative stress is observed in both the bone marrow and spleen cells. In line with the increased oxidative stress observed in SCA erythropoiesis, we show that the protein levels of NRF2 and FOXO3a, key transcriptional regulators of the oxidative stress response genes, are markedly reduced in the nuclei of human SS erythroblasts. NRF2 is a master regulator of the antioxidant cell defense system. Activation of NRF2 by the ablation of its negative regulator Keap1 (Kelch-like ECH-associated protein 1), has been shown to significantly improve symptoms in a sickle mouse model and to reduce organ damage.[31] Moreover, FOXO3a was identified as having an essential role regulating the oxidative stress response during erythropoiesis.[17] Expression, nuclear localization, and transcriptional activity of FOXO3a are increased during physiological erythropoiesis.[17,32] Thus, the decrease in the nuclear protein levels of both FOXO3a and NRF2 we observe in SS erythroblasts, could contribute to the ineffective erythropoiesis observed in SCA. Note that, it is possible that other factors such as that KLF1, TAL1 and GATA2 could also be affected and contribute to the abnormal erythropoiesis in SCA.

Our study also shows that erythropoiesis is abnormal in SCA and that acquired GATA1 deficiency plays a major role in this. Both our *in vitro* and *in vivo* models show a significant decrease in GATA1 levels in SCA erythroblasts, when compared to the appropriate control. This decrease in GATA1 was shown by a number of assays and cannot be explained by differences in differentiation stage between SS and HD erythroblasts alone. Instead, we suggest that reduced GATA1 levels may be the cause of the delay in differentiation observed in SCA. Whether decreased GATA1 levels in SCA erythroblasts are responsible for the decrease in NRF2 and FOXO3a levels, is yet to be investigated. Previously, HSP70, a chaperone protein known to protect GATA1 from cleavage by caspase 3 during erythropoiesis, was investigated in SCA erythropoiesis and shown to interact with HbS polymers under hypoxia. Moreover, HSP70 sequestration in the cytoplasm of SCA erythroblasts was also observed.[5] This is similar to observations made in the ineffective erythropoiesis of β-thalassemia. In our study, we identify a novel mechanism responsible for the decrease of GATA1 levels during SCA erythropoiesis, via the activation of caspase 1. Our data show an increase in the activity of caspase 1, an inflammatory caspase, in SCA erythroblasts. Moreover, inhibition of caspase 1 activity partially restored GATA1 levels during SCA erythropoiesis. Therefore, we propose that a decrease in GATA1 levels during SCA erythropoiesis is due to both caspase 3 activation, leading to HSP70 sequestration in the cytoplasm, and caspase 1 activation through the inflammasome. Importantly, we show that the pharmacological inhibition of caspase 1 alleviates ineffective erythropoiesis, thus making it a druggable target in treating SCA. Note that caspase 1 activation, via the formation of inflammasome complex, is known to initiate a pro-inflammatory response through the cleavage of two inflammatory cytokines IL-1b and IL-18, both of which are known to be increased in the plasma of SCA patients.[33-35] Therefore the inhibition of caspase 1 could be restoring erythropoiesis by both increasing levels of GATA1 and decreasing the pro-inflammatory response. Further analyses are required to investigate in depth the role of caspase 1 in inducing inflammation during SCA erythropoiesis.

Overall, our paper contributes to the emerging evidence that ineffective erythropoiesis occurs in SCA, with significant numbers of erythroid precursors failing to mature into circulating red cells.[36] This involves several components: late stage erythropoiesis is impaired by the polymerization of deoxygenated HbS, which damages the cells, prevents maturation and generates oxidative stress and inflammation;[7] this is further amplified by circulating sickle cells infarcting areas of bone marrow and causing sterile inflammation from hemolysis and the release of free hemoglobin. This oxidative stress and inflammation further impair erythropoiesis by activating caspase 1 and causing acquired GATA1 deficiency. Overall, this generates a hostile bone marrow niche and results in the production of abnormal hematopoietic stem cells which fail to mature properly. This is particularly relevant in the context of hematopoietic stem cell-based therapies, including transplantation, gene addition and gene editing, and the need to work with healthy stem cells and a receptive bone marrow niche to optimize outcomes. This study helps to define the precise nature of the defect in sickle erythropoiesis, and may help develop novel approaches to normalize the bone marrow niche prior to stem cell transplantation or facilitate the production of healthy stem cells for gene therapy.

Disclosures

No conflicts of interests to disclose.

Contributions

SEH designed the research study, designed and conducted experiments, analyzed data, wrote and edited the manuscript. PS designed and conducted experiments, analyzed data, and edited the manuscript. JB provided biological samples, discussed data, and edited the manuscript. MEP, CN, LP, HR, EG, LG and PB conducted experiments and ac-

quired data. KN discussed data and edited the manuscript. NC designed experiments analyzed data and edited the manuscript. DR participated in the design of the research, discussed data, and edited the manuscript. JS designed the study, discussed data and edited the manuscript.

Acknowledgments

We sincerely thank the King's College London, Biological Services Unit, the Advanced Cytometry Platform, R&D Department, Guy's and St Thomas' NHS Foundation Trust, Guy's Hospital, London and the Central Diagnostic Services at The Queen's Veterinary School Hospital, University of Cambridge for the support with this project. We would like to thank Dr. Carlo Scala (King's College London) and Dr. Arnaud Chêne (INSERM) for their support. We would also like to sincerely thank Dr. Julien Gernier and Dr. Wassim El Nemer (Etablissement Français du Sang) for their support.

Funding

SEH received funding from the European Union's Horizon 2020 Research and Innovation Program under the Marie Sklodowska-Curie grant agreement number 101024970. Part of this study was funded by the Fetal Medicine Foundation (PS & KN) (registered charity 1037116), and supported by the National Institute for Health Research (NIHR) Biomedical Research Center at Guy's and St Thomas' National Health Service Foundation Trust and King's College London (ISBRC-1215–20006).

Data-sharing statement

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

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