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

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Methods

Culture of HUDEP cells

Briefly, cells were maintained in expansion medium consisting of StemSpan SFEM (Stem Cell Technologies, catalog number:09650) supplemented with 50ng/mL Stem Cell Factor (SCF) (Peprotech, catalog number: 300-07), 3U/mL Erythropoietin (EPO) (NeoRecormon 2000U/0.3mL), 1µM dexamethasone (Sigma Aldrich, catalog number: D4902-100MG) and 1µg/mL doxycycline (Sigma Aldrich, catalog number: I2643-50MG). Cells were maintained at a concentration of 100,000 cells/mL. Differentiation was induced by seeding the cells at 2 x 10^5 cells/mL in differentiation medium containing 10ng/mL SCF, 1ng/mL Interleukin-3 (IL-3) (Stem Cell Technologies, catalog number: 78194.1), 3U/mL EPO, 1U/mL Penicillin – Streptomycin (Gibco, catalog number: 15140122) and 1µg/mL doxycycline. After day 2, cells were reseeded at 3.5 x 10^5cells/mL. After day 4 of differentiation, cells were reseeded at 5 x 10^5cells/mL without doxycycline. On day 8, cells were cultured in a differentiation medium containing holotransferrin (0.5mg/mL) (Sigma Aldrich, catalog number: T4132), 3U/mL EPO and 1U/mL Penicillin – Streptomycin and maintained at 1 x 10^6cells/mL with complete media change every other day.

Culture of $CD34^+$cells

Briefly, cells were cultured with 100ng/mL of Interleukin-6 (IL-6) (Miltenyi Biotech, catalog number: 130-093-929), 10ng/mL of IL-3 (Stem Cell Technologies, catalog number: 78194.1) and 50ng/mL of SCF (Peprotech, catalog number: 300-07) in a base medium composed of Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, catalog number: 31980-048) supplemented with 15% BIT 9500 (Stem Cell Technologies, catalog number: 9500), 100 U/mL Penicillin Streptomycin (Gibco, catalog number: 15140122) and 2mM L-Glutamine (Gibco, catalog number: 25030-024). Medium was topped up every other day. After 9 days, the cells were cultured in the second phase medium containing 10ng/mL IL-3, 50ng/mL SCF and 2U/mL of EPO. Cells were maintained at a concentration of 200,000 cells/mL. Note that when appropriate, cultures were treated with Ac-YVAD-cmk, caspase 1 non-reversible inhibitor, at a concentration of 50µM (diluted in DMSO) at every media change during phase 2 of differentiation. Note that control condition was treated with similar volume of DMSO alone.

Antibodies

The antibodies used for the flow cytometry analysis are: BV421-conjugated anti-glycophorin A (GPA) (BD Bioscience), APC-conjugated anti-CD49d (BD Bioscience), FITC conjugated

anti-Band3 (Bristol Institute for Transfusion Sciences), AlexaFluor 647 anti-GATA1 (Cell Signaling). Cell stains 7-Aminoactinomycin D (7AAD) was purchased from BD Bioscience. For mouse experiments, APC anti-Ter119 and PE anti-CD71 were purchased from BD Bioscience.

The following antibodies were used for Western blot analysis: β-Actin Rabbit monoclonal antibody, FoxO3a Rabbit monoclonal antibody, GATA-1 (D24E4) Rabbit monoclonal antibody, NRF2 Rabbit monoclonal antibody. All antibodies were obtained from Cell Signaling. Anti-Rabbit IgG, HRP-linked secondary antibody was also obtained from Cell Signaling. Catalog numbers for all antibodies are listed in Supplementary Table 2.

Flow Cytometry

Differentiation Kinetics for human cells

The differentiation kinetics of CD34+ cells during phase 2 of culture were monitored every other day. Briefly, 10^5 cells were resuspended in 20µl of 1x PBS supplemented with 0.5% Bovine Serum Albumin (BSA) and incubated with GPA, CD49d and Band 3 (1/20 dilution) for 30 minutes in the dark. After two washes with 1x PBS /0.5% BSA, the cells were incubated with 7AAD for 5 minutes and analysed using the DIVA Software (version 9) on the FORTESSA Flow Cytometer (BD Bioscience). Compensation was performed using the BD Biosciences compensation beads, following the manufacturer instructions.

Reactive Oxygen Species (ROS) stain

The ROS Assay Kit 520nm (Invitrogen by Thermo Fisher Scientific, catalog number: 88-5930-74) was used according to the manufacturer's instructions. Briefly, 10^5 cells were stained for 60 minutes at 37^{o}C in 1x ROS Assay Stain diluted in ROS Assay Buffer. Cells were then analysed using the DIVA Software (version 9) on the FORTESSA Flow Cytometer (BD Bioscience).

GATA1 antibody staining

Cells (20^5) were stained with GPA for 30 minutes in the dark at 4^{o}C. Cells were then washed twice with 1xPBS 1/0.5% BSA. The Transcription Factor Buffer Set (BD Bioscience) was used to fix and permeabilize the nucleus prior to GATA1 staining, as per the manufacturer's instructions. Stained cells were incubated with 200µl of 1x Fix/Perm Buffer for 50 minutes at 4^{o}C, followed by 2 washes with 500µl of the 1x Perm/Wash Buffer. Cells were then stained with GATA1 (1 in 10 dilution) antibody for 50 minutes at 4^{o}C, followed by two washes with

1x Perm/Wash Buffer (cells were centrifuged at 400g for 6 minutes). Cells were analyzed using the DIVA Software (version 9) on the FORTESSA Flow Cytometry (BD Bioscience).

FAM-FLICA Caspase 1 Assay

Spleen and bone marrow cells ($1x10^6$ cells/tube in RPMI 1640; 100 µL) were used in assays. Antibody staining solution was prepared and added to each sample tube (1µL Ter119 PE, Invitrogen, Catalog number: 12-5921-83; 1µL CD71 PerCP-Cy5, BD Bioscience, Catalog number: 562858; 3.3 µL FAM-FLICA 30 x, ImmunoChemistry Technologies, Catalog number: FAM-FLICA Caspase 1 kit; 94.7 µL RPMI 1640 cell culture medium, Vitrocell, São Paulo, Brazil). Samples were incubated for 30 minutes, 37 °C. After the incubation, each sample was washed with 2 mL of 1x Apoptosis Wash Buffer and centrifuged at 200 x g, 5 minutes, 22°C. The samples were then fixed with 200 µL of FAM-FLICA fixing buffer (ratio of 1:5) and were immediately analyzed by FACS-Calibur flow cytometer (BD FACSCalibur™, BD Biosciences, Jan Jose, CA, USA), acquiring 10 000 events using the 488 nm laser. For analysis of FAM-FLICA, FlowJo VX software was used. Gating of erythroid progenitor cells for analysis by flow cytometry was performed as described by Shimet al. 2020[22]. The erythroid progenitor population is identified in mice as the CD71+Ter119+ population and includes the Pro E ($Ter119^{low}$) population and downstream progenitor populations. $Ter119^{high}$ cells were gated and differentiated by size into $Ter119^{+}CD71^{+}$ Ery A and Ery B. Ery C represents nucleated mature red blood cells (RBCs) that retain Ter119 but lose CD71 expression.

Microscopy

Cytospin was performed using 100,000 cells. Cells were washed twice with PBS and spun on slides using the Cytospin 2 centrifuge (Shandon). Slides were stained with May-Grunwald-Giemsa (MGG) stain following the manufacturer's instructions (Sigma). Slides were then washed with deionized water and left to dry. Slides were covered by a coverslip (Menzel – Glaser) Cells were imaged using an inverted microscope (Rebel by ECHO) at 20x and 40x magnification. Analysis was performed using the ImageJ software[34].

Protein extraction and western blot

Nuclear and cytoplasmic extracts were prepared from 3 x 10^6 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's instruction (Thermo Scientific). 15µg of nuclear extract was analysed by SDS-PAGE using 10% polyacrylamide gels, followed by Western immunoblotting. Antibodies used include GATA1, FoxO3a,

NRF2and β-actin. Blots were developed using the Clarity and Clarity Max enhanced chemiluminescence (ECL) kits (Bio-Rad) and visualized on the Chemidoc MP imaging system (Bio-Rad). Analysis was performed using the Image Lab software (Bio-Rad). Antibodies are listed in the section above.

Isolation of murine bone marrow cells

Bone marrow cells were collected from euthanized AS and SS Berkeley mice under UK Home Office regulations. Bone marrow isolation was performed under sterile conditions. Bone marrow femurs were dissected, and the bone marrow was flushed out using an insulin syringe (INSU/LIGHT, #INS1ML27G13) and filter-sterile 1x PBS. Single-cell suspension was obtained by pipetting the bone marrow tissue up and down. The cell suspension was filtered through a 70μm cell strainer (BD Falcon).

Isolation of murine spleen cells

Each dissected spleen was placed in 3mL of 1x PBS and spun in the gentleMACS Dissociator (Miltenyi Biotec) which allows dissociation of tissues into a single-cell suspension. The cell suspension was passed through a 40μm Cell Strainer (BD Falcon) to ensure a single cell suspension.

Hemoglobin measurement in mice

Blood was drawn from mice under anesthesia using a syringe treated with heparin, and the samples were then placed into BD microtainer tubes with EDTA (Cat No 459036, Greiner Bio-one). The counts of red blood cells and hematocrit levels were assessed. Hemoglobin (Hb) concentrations were measured using the Hemocue Hb 201+ system. Comprehensive blood counts analyses for all samples were conducted at the Central Diagnostics Facility of the University of Cambridge.

Histology of bone marrow and spleen tissue

Bone marrow and spleen tissues from both treated and untreated animals were preserved using 10% formalin solution (Catalog Number P126-33 from Macron Fine Chemicals) and subsequently sent to Central Diagnostic Services, Queen's Vet School Hospital at the University of Cambridge. The long bone and muscle tissues underwent a decalcification process to eliminate calcium deposits. After decalcification, the tissues were embedded in paraffin wax. This included dehydration, clearing, and infiltration with wax to maintain the

structural integrity and preserve the microscopic details of the tissues. The embedded tissues were then thinly sliced and placed onto glass microscope slides. Finally, these sections were stained with Hematoxylin and Eosin (H&E)[35]. Images were taken using ECHO Rebel microscope.

Murine hematopoietic stem cell (HSC) isolation and *ex vivo* differentiation

Murine HSCs were isolated using the Lineage Cell Depletion Kit mouse (Miltenyi Biotec, catalog number: 130-090-858) and the MACS separator, following the manufacturer's instructions. Isolated HSCs were pelleted at 800g for 10 minutes at 4°C and resuspended in IMDM differentiation medium, supplemented with 20% FBS (Thermo Scientific, catalog number: 10270-106), 10 units/ml EPO (obtained from King's College Hospital Pharmacy, NeoRecormon 2000U/0.3mL) , 10 ng/ml SCF (Peprotech, catalog number: 300-07), 10 µM dexamethasone (Sigma Aldrich, catalog number: D4902-100MG), 100 ng/ml Insulin Growth Factor 1 (IGF1, R&D systems, catalog number: 791-MG), 2 mM L-glutamine (Gibco, catalog number: 25030081), 50 units/ml penicillin G and 50 µg/ml streptomycin (Sigma Aldrich, catalog number: P4333), 10^{-4}M β-mercaptoethanol (Sigma Aldrich, catalog number: M3148-25ML), 10 µg/ml recombinant human insulin (Merck #11061-68-0), 1% detoxified BSA (Sigma Aldrich, catalog number: 10735086001,), 200 µg/ml holotransferrin (Sigma Aldrich, catalog number: T4132) at a concentration of 0.1 x 10^6 / mL as described by (Shuga *et al.*, 2007). Differentiation kinetics were monitored by flow cytometry and microscopy as described herein.

Imaging Flow Cytometry staining in mice progenitor cells

Cells (50^5) were stained with Ter119 antibody for 30 minutes in the dark. Cells were then washed twice with 1x PBS/0.5% BSA. The Transcription Factor Buffer Set (BD Bioscience) was used to fix and permeabilize the nucleus prior to staining with GATA1 antibody, following manufacturer's instructions. Stained cells were incubated with 200µl of 1x Fix/Perm Buffer for 50 minutes at 4°C, followed by 2 washes with 500µl of the 1x Perm/Wash Buffer. Cells were then stained with GATA1 antibody (1/10 dilution) for 50 minutes at 4°C. After two washes with 500µl 1x Perm/Wash Buffer, the cells were processed using the INSPIRE software on the AMNIS Imagestream (Luminex). Analysis was performed using the IDEAS software (version 6.2). All analysis strategies are explained in Supplementary Figure 6.

Cell sorting of Ter119+ cells from the bone marrow and spleen of mice

Cells (10 million) were stained with Ter119 antibody for 30 minutes in the dark. Cells were then washed twice with 1x PBS/0.5% BSA. 4',6-diamidino-2-phenylindole (DAPI) was added as a dead/live stain. Cells were sorted into 15mL Falcon collection tubes containing 1mL of 1x PBS1/50%FBS, using the Aria cell sorter (BD Bioscience) with the collection chamber being at 4°C. The gating strategy for the sorting is explained in Supplementary Figure 5.

Cell staining for Cytof analysis

Cells (1.5 million) were thawed in IMDM medium and kept in the incubator at 37°C for one hour for recovery. Cells were then washed twice with Maxpar PBS (Standard Biotools) and centrifuged at 300g for 5 minutes. The cell pellet was then incubated with 2x Cell-ID Cisplatin-195Pt (Standard Biotools) for 5mins at 37°C. After incubation, cells were quenched and washed with Maxpar Cell Staining buffer (Standard Biotools) using 5x of packed cell volume, followed by centrifugation at 300g for 5 minutes. A blocking step was performed with 10µl of Human TruStain FcX (Standard Biotools) for 10minutes at room temperature and cells were pelleted. Antibodies against cell surface markers (Supplementary Table 1) were added to the cell pellet in 40µl of mastermix with antibodies added at a concentration of 1/100 to a total volume of 100µl), followed by incubation for 30 minutes at room temperature. Following the incubation, cells were washed twice with the Maxpar Cell Staining Buffer (Standard Biotools). For nuclear antibody staining, cells were permeabilized with 1mL of Maxpar Nuclear Antigen Staining Buffer (Standard Biotools), followed by incubation for 30 minutes at room temperature. Cells were then washed twice with 2mL of Maxpar Nuclear Antigen Staining Perm and centrifuged at 800g for 5 minutes. Antibodies against internal markers (Supplementary Table 1) were added to the cell pellet in 50µl of mastermix with antibodies added at a concentration of 1/100 in a total volume of 100µl, followed by incubation for 30 minutes at room temperature. This was followed by 2 washes (500µl) using the Maxpar Nuclear Antigen Staining Perm. A final incubation with the secondary antibodies was performed for 30 minutes at room temperature in a total volume of 100µl (50µl cell suspension and 50µl antibody mix). Cells were then washed with 2mL Nuclear Antigen Staining Perm buffer and centrifuged at 800g for 5 minutes. Cells were then fixed using 1.6% Formaldehyde (Sigma Aldrich). After fixation, cells were resuspended in 1mL intercalation solution and incubated overnight at 4°C. Cells were then analyzed on the Helios (A CyTOF System, Fluidigm). Analysis of data was performed using the OMIQ software.

Analysis of gene expression

Total RNA was extracted from 1 million erythroblasts using the RNeasy Midi Kit (Qiagen) following the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg of total RNA with SuperScript III RNase H^- Reverse Transcriptase (Invitrogen) and oligo(dT)18 primer at 50°C for 50 minutes. Real-time PCR was performed using an ABI PRISM 7500 instrument (Applied Biosystems) with Power SYBR Green Master Mix. The reaction mixtures were incubated for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, and a final step of 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. Gene expression for each mRNA was normalized to PABPC1 in each sample.

Supplementary Figure Legends

Supplementary Figure 1: **Differentiation of WT and SS HUDEP-2**. (A) A contour plot of GPA+ cells from one WT (red) and one SS (blue) HUDEP-2 culture, showing the distribution of cell populations with respect to the expression of Band-3 FITC on the x-axis and CD49d-APC on the y-axis. Data are represented from day 0, 2, 4, 6 and 8 of differentiation. Pro: Proerythroblast, EB: Early Basophilic, LB: Late Basophilic, Poly: Polychromatic, Ortho: Orthochromatic erythroblasts. (B) Bar graph representing the percentage of proerythroblasts, early basophilic, late basophilic, polychromatic, and orthochromatic erythroblasts at day 2 of differentiation in WT (red) and SS (blue) HUDEP-2 cell line (n=4) (mean ± standard deviation). (C) Bar graph representing the percentage of polychromatic and orthochromatic erythroblasts at day 6 of differentiation in WT (red) and SS (blue) HUDEP-2 cell line (n=4). Images of WT and SS HUDEP-2 cells at (D) day 2 and (E) day 6 of differentiation, after MGG staining and visualization by light microscopy.

Supplementary Figure 2: **Schematic overview of the 2-step differentiation system of CD34+ hematopoietic stem and progenitor cells from sickle (SS) patients and healthy individuals and of the downstream analysis carried out at different stages of erythroid differentiation.**

Supplementary Figure 3: **Distribution of GPA in HD and SS differentiation.** (A) A line plot representation of the percentage of GPA positive cells observed during phase 2 of differentiation of CD34+ progenitor cells from one healthy donor (HD, red) and one SS patient (blue). (B) A bar graph showing the percentage of GPA positive cells at days 2, 4, 6 and 8 of phase 2 of differentiation of HD (red) and SS (blue) (n=4) (mean ± standard deviation). *$p<0.05$ Mann-Whitney test.

Supplementary Figure 4: **Erythroid differentiation of CD34+ cells isolated from the peripheral blood of Healthy donor (HD) and SS individuals.** (A) A contour plot of GPA+ cells from phase 2 of differentiation of one HD (red) and one SS (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 presented for day 0 to day 12 of phase 2. Pro: Proerythroblast, EB: Early Basophilic, LB: Late Basophilic, Poly: Polychromatic, Ortho: Orthochromatic erythroblasts. (B) Bar graphs representing the percentage of proerythroblasts, early basophilic, late basophilic, polychromatic, and orthochromatic erythroblasts at day 2 (D2), day 4 (D4), day 6 (D6) and day 8 (D8) of differentiation (n=4) (mean ± standard

deviation). (C) Images of HD and SS cells stained with MGG at day 2, day 4, day 6 and day 8. *p<0.05, Mann-Whitney test.

Supplementary Figure 5: **Reactive oxygen species levels in WT and SS HUDEP-2 cell lines.** (A) Histogram showing reactive oxygen species (ROS) levels in WT (red) and SS (blue) HUDEP-2 cells on days 2 and 6 of differentiation. (B) Bar graph representing the fold change in mean fluorescence intensity (MFI) of ROS staining in WT and SS HUDEP-2 cell on days 2, 4, 6 and 8 of differentiation (n=4), (mean ± standard deviation).

Supplementary Figure 6: **mRNA levels of GATA1 in Healthy donor (HD) and SS individuals at day 6 of phase 2 of erythroid differentiation.** (n=4) (mean ± standard deviation).

Supplementary Figure 7: **GATA1 expression in WT and SS HUDEP-2 cell lines.** (A) Western blot images of GATA1 (upper panel) and Actin (lower panel) proteins in nuclear extracts from WT and SS HUDEP-2 cells on days 0, 2, 4 and 6 of differentiation. (B) Bar graph representing the quantification of GATA1 protein levels in the nuclear extracts of WT and SS HUDEP-2 cells on days 2 and 6, relative to Actin protein levels (n=3), (mean ± standard deviation).

Supplementary Figure 8: **A schematic representation of the experimental design for the AS and SS Berkeley mice.**

Supplementary Figure 9: **Histological analysis of the bone marrow and spleen of AS and SS Berkeley mice.** (A) Bar graph representing the hemoglobin (g/dL) levels in the peripheral blood of AS (n=5) and SS (n=8) mice. (B) A bar graph representing the animal weight (g) of AS (n=6) and SS (n=7) mice. (C) Left panel: An image showing the difference in size between AS and SS spleens; Right panel: bar graph representing the weight (g) of the AS (n=6) and SS (n=5) spleens. (D) Histopathology of bone marrow slices from AS (left) and SS (right) mice stained with HE. (E) Histopathology of spleen slices from AS (left) and SS (right) mice, stained with HE (upper panel) and Perls staining (stain of free iron deposit) (lower panel). *p<0.05, **p<0.01, ***p<0.001 Mann-Whitney test.

Supplementary Figure 10: **Erythropoiesis in the AS and SS Berkeley mice.** Density plot of Ery A (basophilic), Ery B (orthochromatic) and Ery C (reticulocytes) cell populations in the (A) bone marrow and (B) spleen of one AS and one SS mouse.

Supplementary Figure 11: **Flow cytometry gating strategy for the cell sorting of Ter119+ cells from the spleen and bone marrow of AS and SS mice**. The cells were first gated based on Forward Side Scatter – Area (FSC-A) and Side Scatter- Area (SSC-A). This was followed by single cell gating for gating and sorting Ter119+ cells using the FACS Aria 3 cell sorter (BD Bioscience).

Supplementary Figure 12: **Gating strategy for the GATA1 Low Erythroblasts and GATA1 High Erythroblasts using the IDEAS Software**. Cells were gated using the Aspect Ratio and Area features. Then the cells were gated based on focused images using the Gradient RMS feature. Focused cells were then gated based on Ter119 expression. An analysis mask was created next to distinguish between Ter119+ erythroblasts (with nucleus) and Ter119+ red cells (without nucleus). This was performed using the Perimeter and H-variance features of the cells.

The Erythroblasts were then used to create a new analytical pipeline that distinguishes the GATA1 Low Erythroblasts from the GATA1 High Erythroblasts, using the Max Pixel_MC_Ch02_GATA1.

Supplementary Figure 13: **Inhibition of caspase 1 activity.** (A) A bar graph representing GATA1 MFI level at day 6 in HD (red), SS (dark blue) and SS + Ac-YVAD-cmk (50µM) (light blue) erythroblasts. (n=3). (B) Bar graph representing MFI ROS levels in SS erythroblasts (dark blue) and SS+ Ac-YVAD-cmk (50µM) erythroblasts (light blue) at day 6 and 8 of differentiation (n=3).

Supplementary Tables

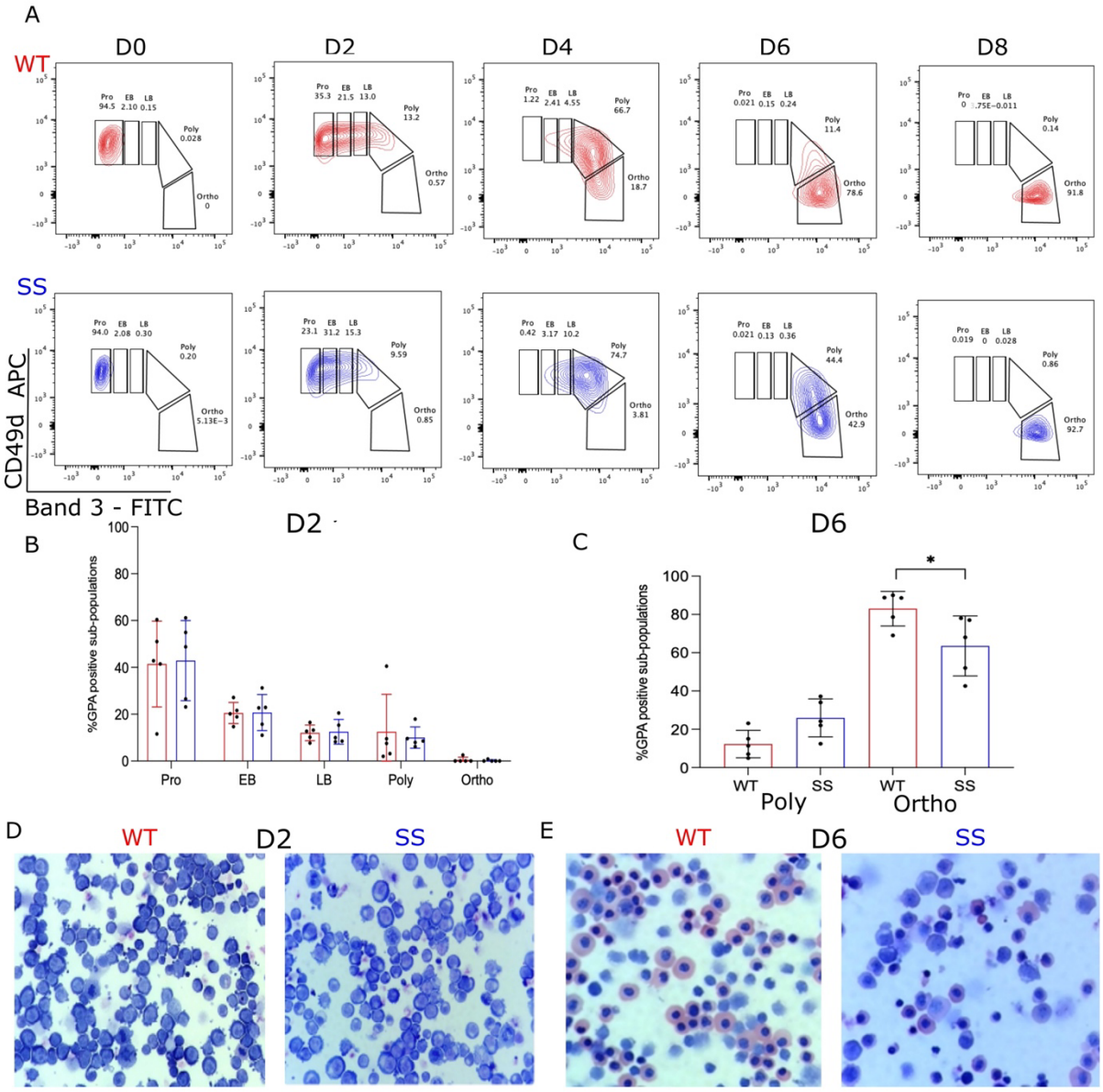
Supplementary Table 1: **Antibodies used for Cytof experiment.**

Product Name	Catalog Number	Surface / internal
Anti-human CD235a HIR2-141Pr	3141001B	Surface marker
Anti-Human CD117/c-kit (104D2)-143Nd	3143001B	Surface marker
Anti-Human CD34 (581)-148Nd	3148001B	Surface marker
Anti-Human CD36 (5-271)-155Gd	3155012B	Surface marker
Anti-Human CD49d (9F10)-174Yb	3174018B	Surface marker
Anti-Human CD71 (OKT-9)-175Lu	3175011B	Surface marker
Anti-PE (PE001)-156Gd	3156005B	Secondary antibody
Anti-APC (APC003)-176Yb	3176007B	Secondary antibody

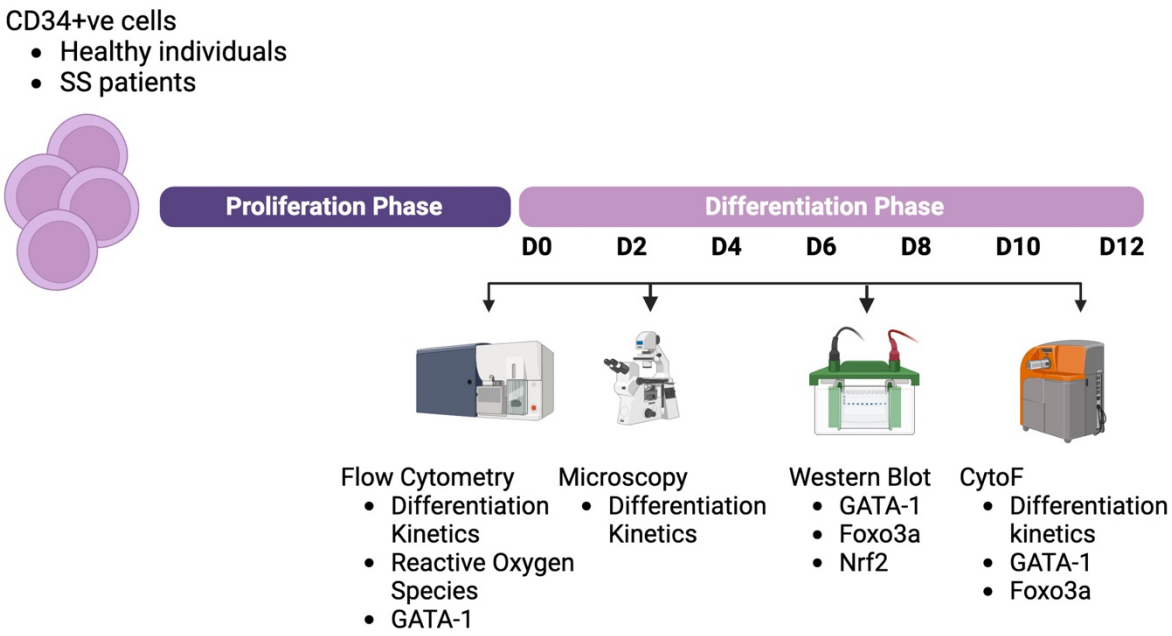
Supplementary Table 2: **Antibodies used for western blot and flow cytometry.**

Product Name	Catalog Number	Manufacturer
BV421-conjugated anti-glycophorin A (GPA)	562938	BD Bioscience
APC-conjugated anti-CD49d	559881	BD Bioscience
FITC conjugated anti-Band3	5439FI	Bristol Institute for Transfusion Sciences
AlexaFluor 647 anti-GATA1	89084S	Cell Signaling
Cell stains 7-Aminoactinomycin D (7AAD)	559925	BD Bioscience
APC conjugated anti-Ter119	557909	BD Bioscience
PE conjugated anti-CD71	567206	BD Bioscience
β-Actin Rabbit monoclonal antibody	4970S	Cell Signaling
FoxO3a Rabbit monoclonal antibody	2497	Cell Signaling
GATA-1 (D24E4) Rabbit monoclonal antibody	4589S	Cell Signaling
NRF2 Rabbit monoclonal antibody	12721	Cell Signaling
Anti-Rabbit IgG, HRP-linked secondary antibody	7074	Cell Signaling

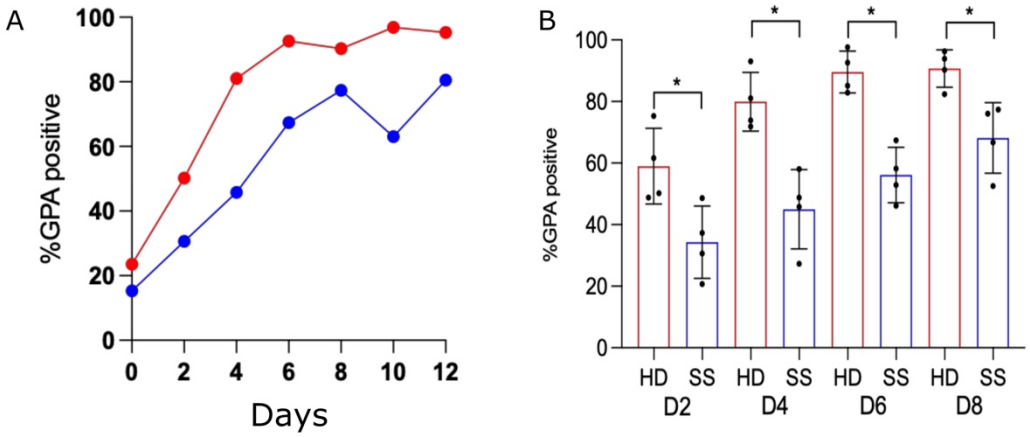
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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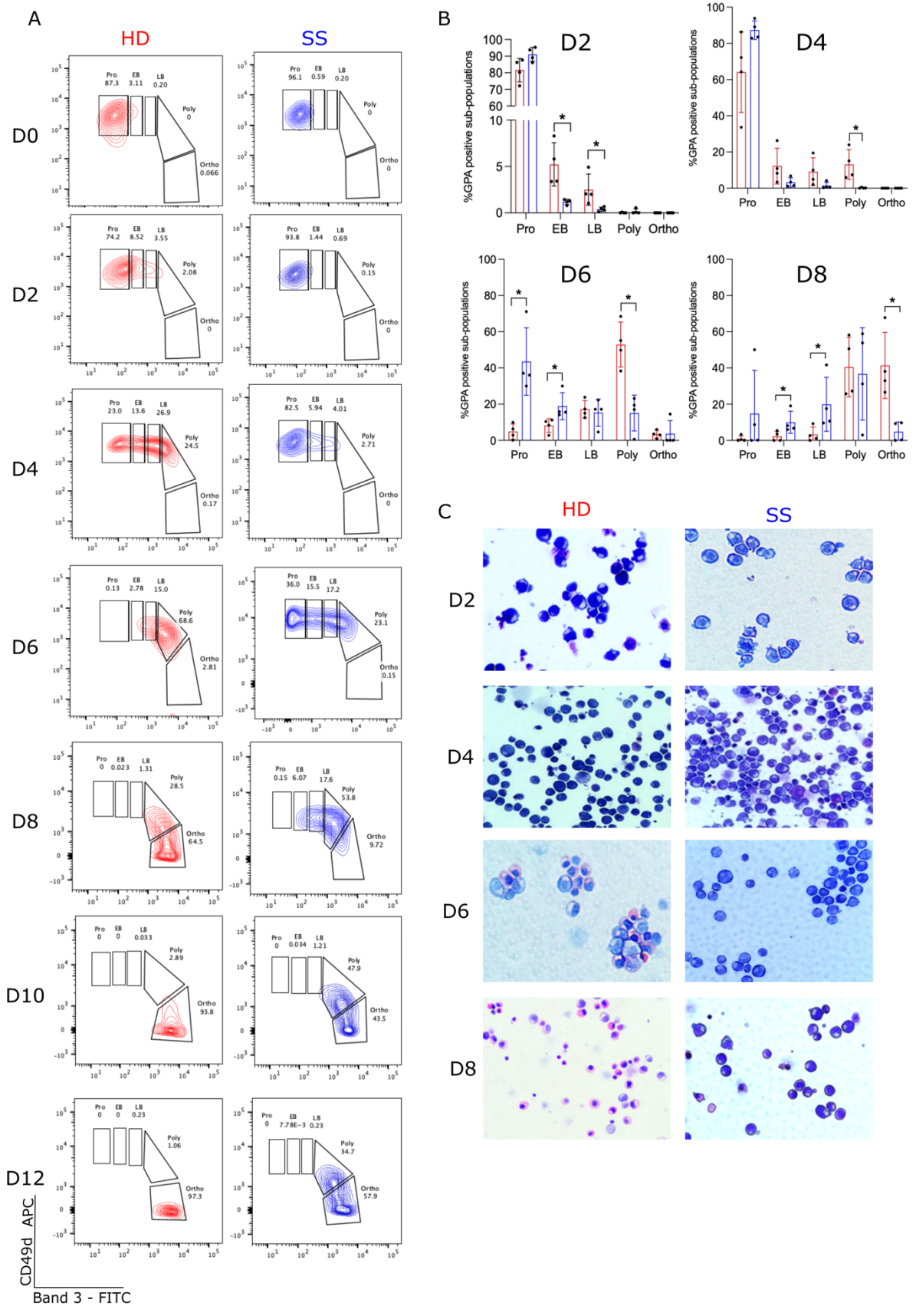

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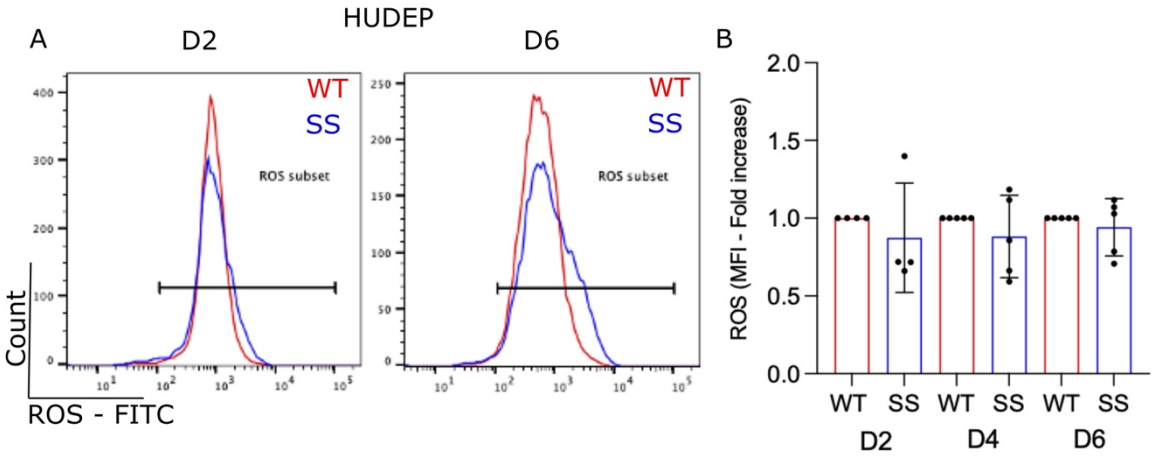
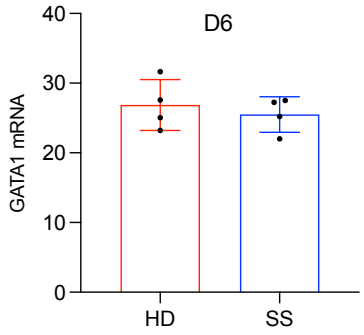
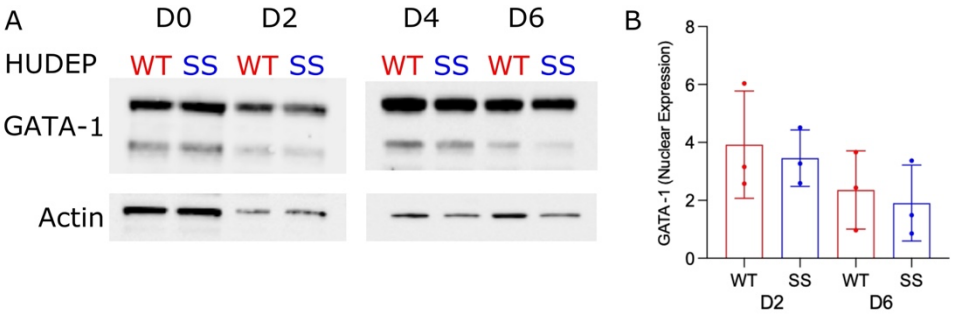


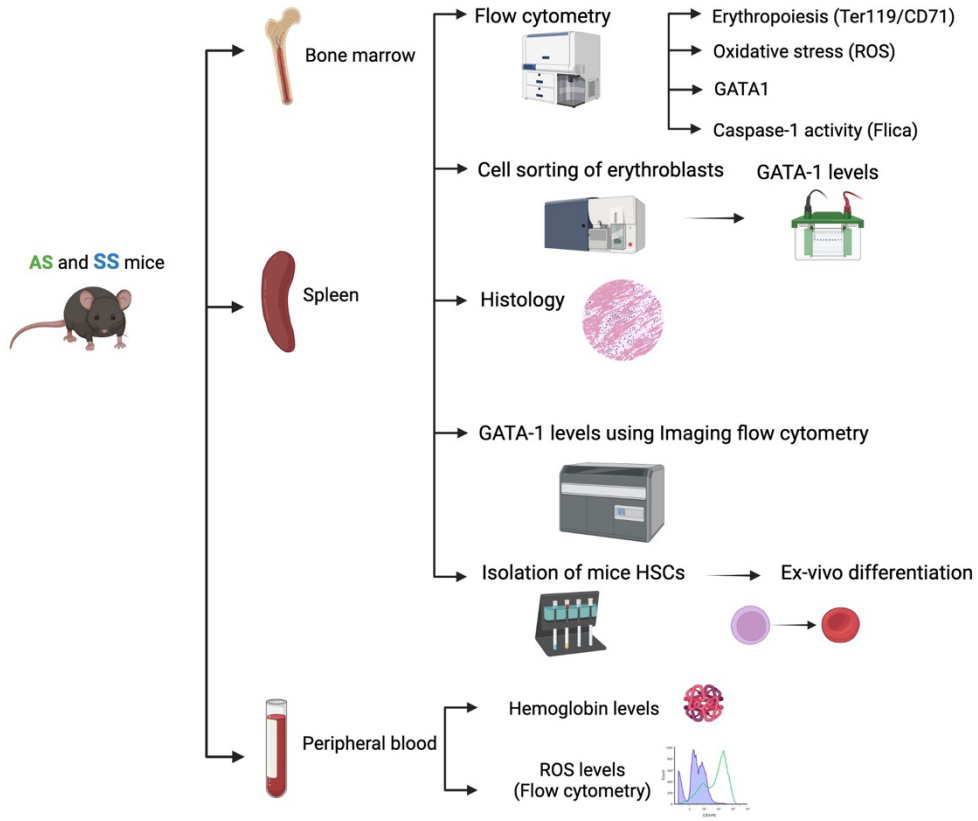
Supplementary Figure 6



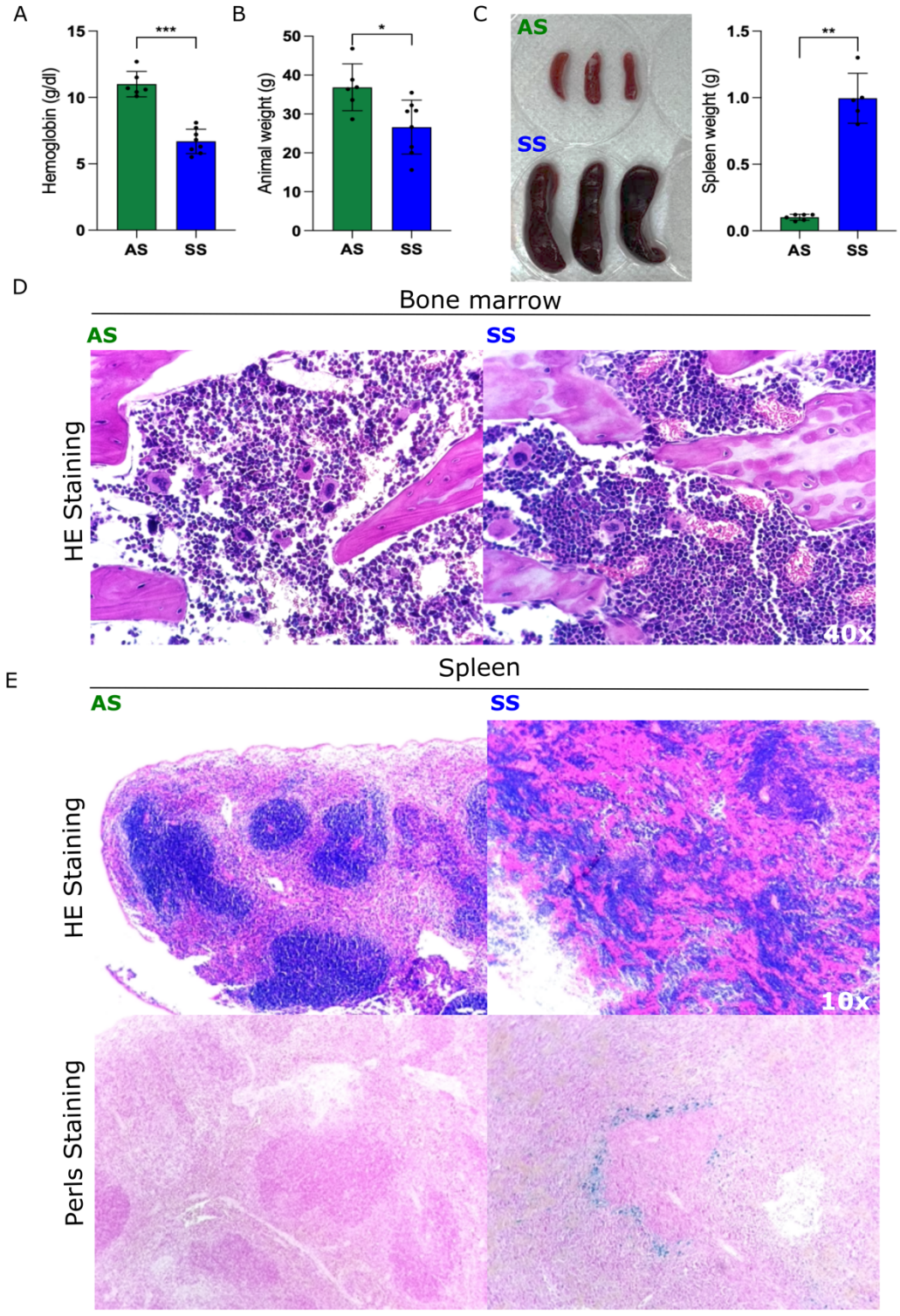
Supplementary Figure 7



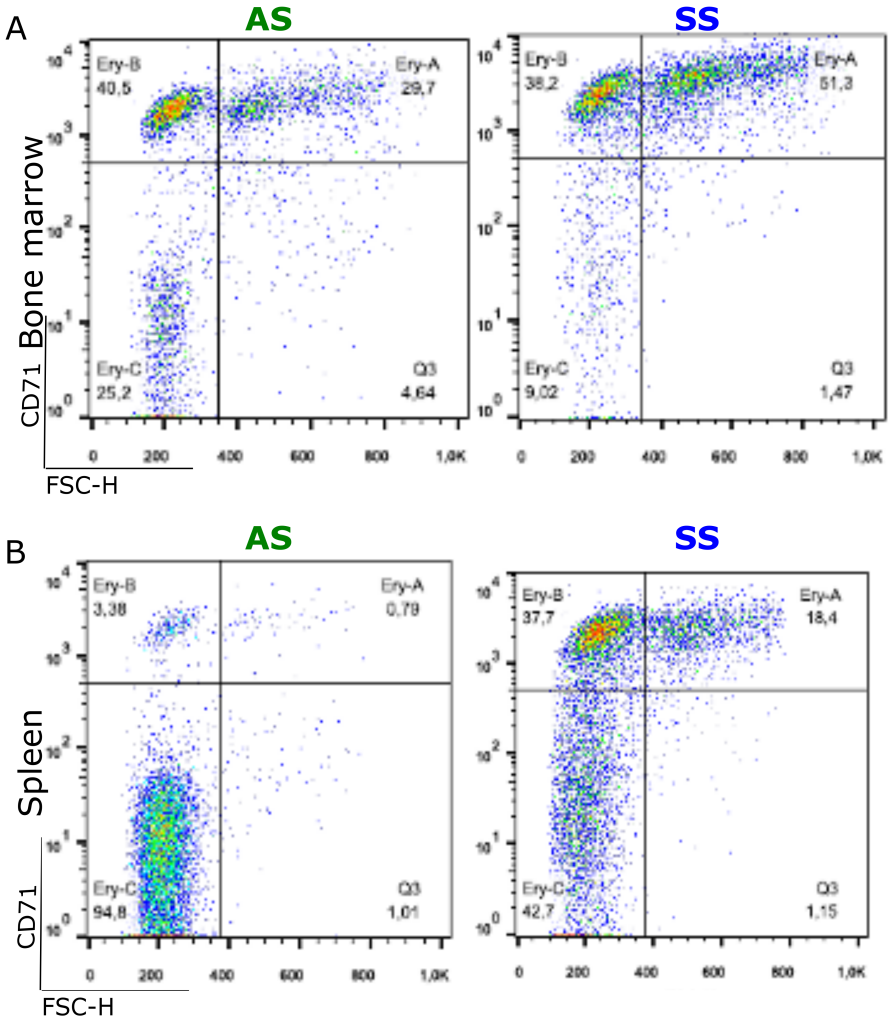
Supplementary Figure 8



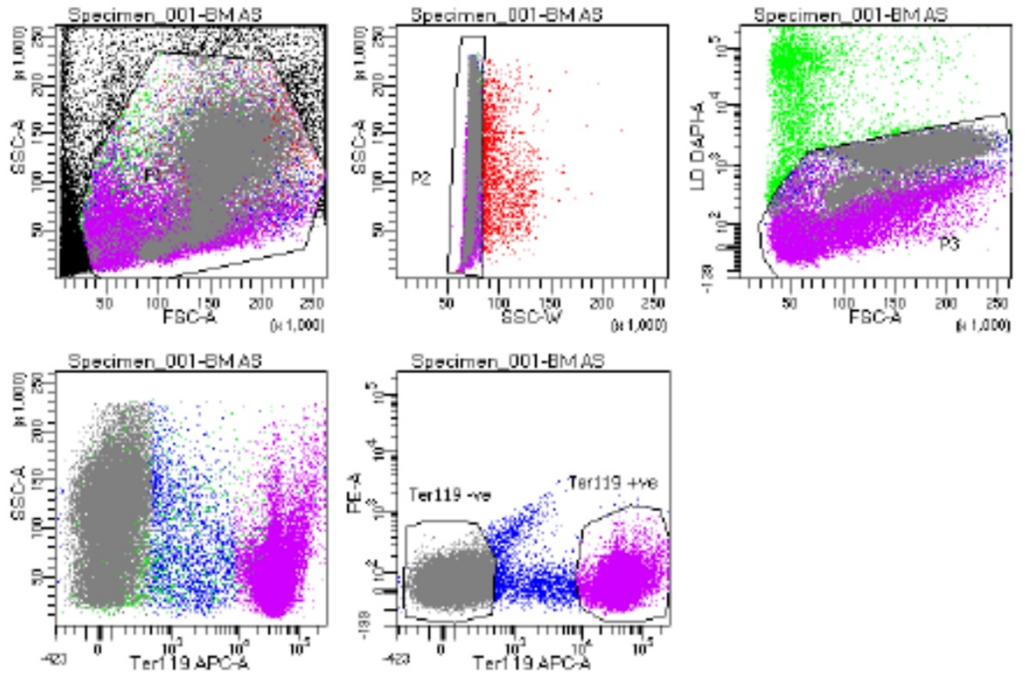
Supplementary Figure 9



Supplementary Figure 10



Supplementary Figure 11

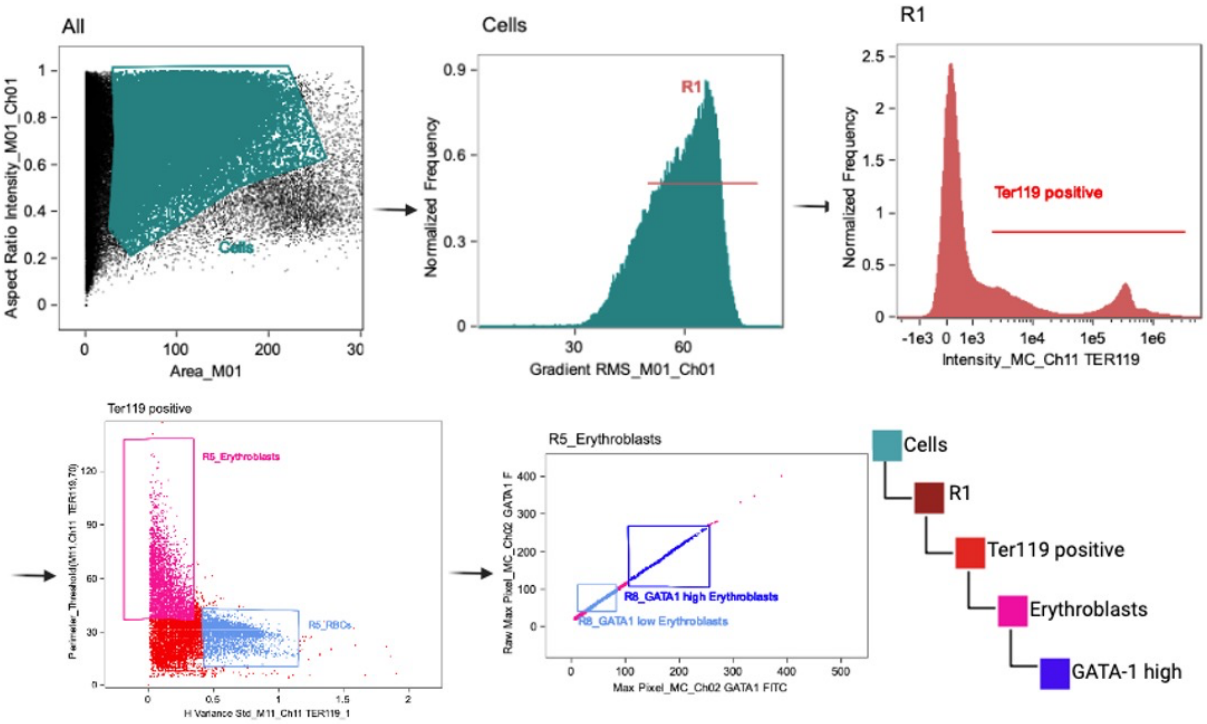


Sample	Ter119-	Ter119+
BM AS	4,003,986	4,145,218
BM SS	1,897,169	2,409,096

Tube: BM AS

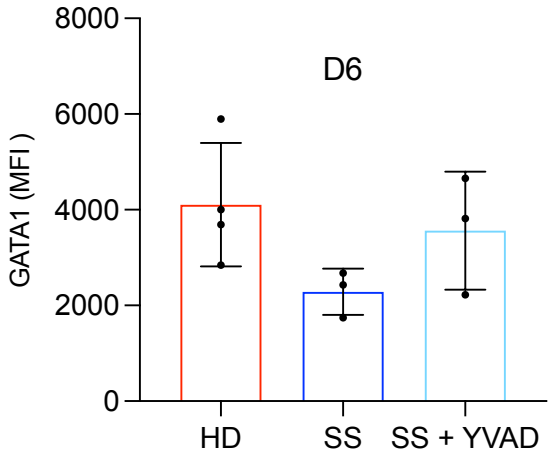
Population	#Events	%Parent	%Total
All Events	75,746	####	100.0
P1	56,006	73.9	73.9
P2	54,139	96.7	71.5
P3	50,267	92.8	66.4
Ter119 +ve	23,645	47.0	31.2
Ter119 -ve	24,571	48.9	32.4

Supplementary Figure 12



Supplementary Figure 13

A



B