Proliferative events ameliorate DNA damage accumulation without affecting function in hematopoietic stem cells

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Short title: Proliferative events affect DNA damage accumulation in HSCs

Abstract

Proliferation and resulting accumulation of DNA damage have been causally linked with age-associated functional decline in hematopoietic stem cells (HSCs). Using serial bleeding as a proliferative cue, we here show that increased HSC proliferation has little effect on age-associated functional decline. A surprising decrease in the DNA double-strand breaks demonstrates a probable benefit of blood donation during lifetime and warrants a relook at the effects of proliferation on HSC function.

Main

The number of long-term (LT-) repopulating HSCs with multi-lineage engraftment potential decreases drastically in aged mice and humans[1]. Increased proliferation rates, such as in the case of deletion of cell cycle regulators p21, p27, and Bmi1, resulted in pre-mature aging like phenotype[2]. DNA damage accumulation has been implicated with age-associated and proliferation induced functional decline in HSCs[3]. Mice deficient in *Atm* expression (*Atm*$^{-/-}$), that mediates response to DNA double strand breaks (DSBs), showed progressive bone marrow (BM) failure due to poorly functioning HSCs[4]. The exit from quiescence in HSCs was directly linked to DNA damage and functional decline[5,6]. Contrarily, aged HSCs exhibited activation of DNA repair mechanisms and clearance of DNA damage upon cell cycle entry[7]. Here, we sought to examine the effect of repeated proliferative events during the lifetime on age-associated functional decline in HSCs and DNA damage accumulation.

As blood loss is a known inducer of HSC proliferation[8], we used a serial bleeding-based regimen to test the effect of physiological demand driven proliferation on HSC aging (Schematic in Figure 1A). After confirming cell cycle entry in HSCs following repeated blood withdrawals (supplemental Figure 1A,B), we compared the blood cell counts in aged mice that underwent serial bleeding (old donors) with the age-matched (old controls) and young control mice. At 12 months, we did not observe any difference in WBC, lymphocyte, granulocyte, hematocrit, monocytes, eosinophil, RBC and hemoglobin levels between old control and old donor groups (supplemental Figure 1C-J). However, a decrease in WBC, granulocyte and monocyte counts was noticeable in the two groups of old mice when compared with young controls (supplemental Figure 1C,E,G). Even after 18 months of age, we did not notice any change in blood cell counts from old donors when compared with old control (supplemental Figure 1K-R). Again, both old mice groups showed significant decline in WBC, lymphocyte, hematocrit, monocytes and hemoglobin levels (supplemental Figure 1K,L,N,O,R) and a significant increase in granulocyte and eosinophils when compared with the young group (supplemental Figure 1M,P). At 24 months of age, an unchanged number of monocytes and granulocytes was observed with a higher eosinophil count (supplemental Figure 1S-U). Higher eosinophil numbers could be linked with pro-inflammatory state known to setup with aging in mice and humans[9]. At 24 months of age, we continued to see a decreased number of WBCs reflected also in lymphocyte counts, which was significantly more robust in old donor mice than old controls (Figure 1B,C). As reported before[10,11], we could also note a consistent decline in erythropoietic activity as there was a decrease in RBC numbers, along with hematocrit (Figure 1D,E) and hemoglobin levels (supplemental Figure 1V). Overall, we found little effect of serial bleeding on blood cell production.

Along with altered blood cell production, function and composition of hematopoietic stem and progenitor cell (HSPC) population changes with aging[1,12]. A robust increase in the HSC population identified as $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) side population[13], $CD34^{-}$LSK cells[14] as well as $CD48^{-}CD34^{-}$ $EPCR^{+}CD150^{+}$LSK cells has been reported[15] with no change in short-term (ST-) HSCs ($CD34^{+}Flk2^{-}$ LSK cells)[10]. Therefore, we next analyzed if serial bleeding regimen had any effect on the age-associated functional changes in HSC populations (supplemental Figure 2A). As compared with the young mice, both groups of old mice showed robust increase in the frequency of LSK cells, LT-HSCs, MPP2, ST-HSCs, and MPP3/4 (Figure 1F-H, supplemental Figure 2B-G) populations. No effect of serial bleeding was observed when old donor mice were compared with old controls. In aged hematopoietic system, myeloid bias linked with altered differentiation potential of HSCs[16], and changes in lineage-committed progenitor populations[17] has been extensively reported. Our experiments also showed robust increase in the myeloid cell population in the BM of the aged mice groups (Figure 1I). Concomitantly, we noted a significant decrease in B-cell population (Figure 1J), while no change was observed in the T-cell population (Figure 1K). Hence, the serial bleeding regimen did not alter the lineage composition of the hematopoietic system within the BM.

In vivo data supported by long-term engraftment studies has also established a myeloid biased, poor hematopoietic reconstitution capacity of aged HSCs[15]. We used whole BM MNCs in our repopulation assays to assess if repeated hematopoietic insult induced by serial bleeding had any adverse impact on HSC aging (Figure 2L). While we did not observe any decline in donor-derived engraftment resulting from aged HSPCs up to a period of 8-weeks (Figure 1M,N), 12-week onwards we noted a significant decrease without any effect of serial bleeding regimen (Figure 1O,P). We also noted a myeloid biased long-term engraftment following transplantation of aged HSPCs (supplemental Figure 2I-K). These results are consistent with earlier studies that attributed this phenotype to an altered clonal diversity[18] and enrichment of myeloid committed progenitors[19].

Previous studies that reported DNA damage accumulation in aged HSCs[20], used comet assays performed under alkaline conditions for higher sensitivity[21]. To examine the effect of extensive HSPC proliferation on accumulation of DSBs specifically, we chose to perform neutral comet assays[22]. The proportion of LSK cells with comet tail representing DSBs was expectedly higher in the aged mice groups, but remained unaffected by serial bleeding regimen (Figure 2A,B). However, comparison of DSBs per cell between different groups in terms of Olive tail moment revealed a significant clearance of DNA damage following serial bleeding regimen (Figure 2C). To link DDR pathways with HSC proliferation, we used available single-cell transcriptomic data from aged HSCs (GSE147729)[23]. GSEA was performed on quiescent and proliferative HSCs identified using CellCycleScoring function of R-package Seurat. Analysis showed a robust increase in the overall enrichment of DDR genes (Figure

2D, supplemental Table 1) and Hallmark pathways (supplemental Figure 3A, supplemental Table 2) in proliferative HSCs compared to quiescent HSCs in aged mice. Interestingly, higher expression of DNA repair genes remained consistent for proliferative HSCs from young mice as well (Figure 2D). Reactome pathway analysis (supplemental Table 3) of upregulated DDR genes revealed a significant enrichment of DDR pathways in proliferative HSCs from aged mice (Figure 2E). Our previous results also have described enhanced DDR pathways and DNA repair in proliferative fetal liver HSCs than adult BM HSCs[24]. Next, we examined if proliferation induced by short-term serial bleeding regimen in the aged mice could have an impact on DDR gene expression and the status of accumulation of DNA damage. We selected a few DDR genes up-regulated in the proliferative HSCs in young as well as aged mice (supplemental Figure 3B, supplemental Table 1) and examined their expression post-proliferative regimen by quantitative RT-PCR on HSPCs (Figure 2F-I). Results clearly showed elevated levels of most of the genes across the DDR pathways (Figure 2F-I) tested. Finally, to confirm the effect of serial bleeding induced activation of DDR pathways on clearance of DSBs, we performed neutral comet assays on sorted LSK cells (Figure 2J). These experiments provided striking results as we noted a massive decrease in the Olive tail moment showing clearance of DSBs (Figure 2J,K). Overall, these results are in contrast to the prevalent notion that higher proliferation rates in HSCs can exacerbate DNA damage accumulation. Evidence also shows that the level of DNA damage accumulation might not be directly linked with age-associated functional decline. Serial bleeding as a simple regimen to clear DNA damages can have clinical implications.

Methods

Mice

Six weeks to twenty-four months old C57BL/6J (CD45.2) and B6.SJL-PTPRCA (CD45.1) mice were bred and maintained in the animal facilities at KU Leuven, Belgium and IISER Thiruvananthapuram, India. During the experiments, mice were maintained in isolator cages at humidified constant temperature with a 12 h light-dark cycle. The mice were fed with autoclaved water and irradiated food (Safe diet, France) ad libitum. All animal experiments were approved by the Institutional Animal Ethics Committees for the respective animal facilities. At IISER Thiruvananthapuram, the animals were maintained as per guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Blood withdrawal

To induce excessive rounds of proliferation in HSCs during the lifetime, a long-term serial bleeding regimen was followed. Mice aged six weeks were used to draw 200µl blood via tail clipping method. Blood was drawn weekly for 12-weeks, followed by fortnightly bleeding for 14-weeks and monthly bleeding till 1 year of age. The mice were thereafter maintained for a period of two years and HSCs analysis was performed at 12 months, 18 months and 24 months of age. A short-term serial bleeding regimen of three blood withdrawals in 10-days period with three days intervals was used in aged mice (24 months of age) to induce HSC proliferation. At every time point, peripheral blood analysis was performed on Erba hematology analyzer for detailed blood cell counts. Detailed functional analysis of the hematopoietic system was performed at 24 months of age.

Bone marrow aspiration

Mice were sacrificed via cervical dislocation and hindlimb bones were harvested. Adjacent muscle tissues were removed and bones were flushed with 1X PBS using a syringe with 26G needle. The resulting cell suspension was passed through a 41µm cell strainer (Corning, USA). The filtered cell suspension was diluted with 1X PBS and centrifuged at 600xg for 5 min at 4°C. The BM mononuclear cells were carefully resuspended in 1mL 1X PBS and were counted manually using a Neubauer hemocytometer (Neubauer, Germany).

Transplantation

Frequency and function of the HSCs from young control, old control and old donor mice was examined using long-term hematopoietic reconstitution assays. Freshly isolated 250,000 whole BM cells ($CD45.2^{+}$) were transplanted along with 750,000 competitor WBMCs ($CD45.1^{+}$) into lethally

irradiated (9Gy) 8-12 weeks old female mice. All irradiated mice were fed on enrofloxacin (Baytril) containing water. Peripheral blood chimerism and multilineage engraftment analysis was performed every 4 weeks by flow cytometry.

Flow cytometry

Analysis of donor-derived chimerism, multi-lineage engraftment and characterization of hematopoietic system was performed by flow cytometry. For chimerism and multi-lineage engraftment, donor and recipient cells were identified as CD45.2$^+$ and CD45.1$^+$ cells, respectively. Within the CD45.2$^+$ donor derived cells, lineage-committed cells were identified as myeloid (CD11b$^+$/Gr-1$^+$), T-cell (CD3e$^+$) and B-cell (B220$^+$) populations. On the basis of the expression of SLAM markers, CD150 and CD48, lin$^-$c-kit$^+$Sca-1$^+$ (LSK) population was subdivided into four subpopulations: CD150$^+$CD48$^-$ (LT-HSCs), CD150$^+$CD48$^+$ (MPP2), CD150$^-$CD48$^+$ (MPP3/4), and CD150$^-$CD48$^-$ (ST-HSCs) were characterized by allophycocyanin-conjugated anti-lineage antibody cocktail, PE-conjugated anti-mouse c-kit antibodies, BB700-conjugated anti-mouse Sca-1 antibodies, FITC-conjugated anti-mouse CD48 antibodies, and PECy7-conjugated CD150 antibodies. Suitable isotype controls for each antibody were used in all experiments. A complete list of antibodies used for these experiments is provided in the Table S4.

Cell cycle analysis was performed on BM derived MNC population to examine the effect of serial bleeding regimen on HSPC proliferation. The BM MNCs were first immuno-labelled for identification of LSK cells, as described above. After cell surface staining with FITC conjugated anti-lineage antibody cocktail, BB700 conjugated anti-mouse Sca-1 and PE conjugated anti-mouse c-kit antibodies, the cells were fixed using BD Cytofix/Cytoperm buffer. The cells were then washed with Perm/Wash buffer and incubated with AF647 conjugated Ki-67 antibodies. The cells were further washed and labelled with DAPI for 30 mins on ice. The samples were acquired using FACS Aria III (BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR).

Neutral comet assay

This method was used to perform comet assay to assess DNA damage in freshly sorted BM derived HSPCs under neutral pH. FACS sorted LSK cells were resuspended in low-melting agarose (Sigma; type VII). The suspension was poured onto agarose-coated comet slides dropwise and incubated at 4°C. After agarose solidifies, slides were immersed in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, pH 10) for 1h. The slides are then washed with pre-chilled neutral electrophoresis buffer and incubated for 30 minutes. The slides are then transferred for electrophoresis in pre-chilled 1X neutral electrophoresis buffer (10X buffer contains 1M Trizma base,

3M Sodium acetate trihydrate, pH is set at 9 using glacial acetic acid). After electrophoresis (~60 min, 21 V), air-dried and precipitated slides (10X precipitation buffer contains 7.5M ammonium acetate, which is diluted to 1M using 100% ethanol) were stained with SYBR-Gold solution (1:1000). Olive Tail Moment was scored for 100-200 cells/sample by using Open Comet plugin in ImageJ software.

Quantitative RT-PCR

Hematopoietic progenitors were isolated with EasySep™ mouse hematopoietic progenitor isolation kit. Total RNA was prepared using TRIzol™ Reagent. The purity and the concentration of RNA were assessed using a micro-volume spectrophotometer (Colibri, Berthold Technologies GmBH & Co. KG Germany). Two micrograms of RNA from each sample was used to synthesize cDNA using PrimeScript RT reagent kit (Takara Biotechnology Co. Ltd. Dalin, China) according to the manufacturer's protocol. Quantitative PCR was carried out using TB Green Premix (Takara Biotechnology Co. Ltd. Dalin, China). The PCR reactions were carried out in a CFX96 detection system (Thermal-Cycler C1000, Bio-Rad Laboratories, Hercules, CA, USA). The list of primers used is given in Table S5.

Single-cell RNA Seq analysis

Single cell RNA seq libraries of BM HSCs isolated from young and aged mice were retrieved from the Gene Expression Omnibus (GEO; GSE147729)[23]. The gene count expression matrix was analyzed using the Seurat v.4.1.1 in RStudio. Cells containing fewer than 100 genes and fewer than 500 unique molecular identifiers were excluded from subsequent analysis. Normalization of raw counts was performed using the NormalizeData function in Seurat. Variable genes were identified using the FindVariableGenes function. Expression values in the dataset were scaled and centered for dimensional reduction using the ScaleData function in Seurat, with default parameters. Principal Component Analysis (PCA) was performed following analysis of cell cycle phase for individual cell was performed with CellCycleScoring function of Seurat, employing a core gene set as previously described[25], distinguishing between cells in different stages. As HSCs at G_o stage are transcriptionally closer to G_1 than S or G_2/M cells we marked them quiescent, while HSCs in the S and G_2/M phases were identified as proliferative. Differential gene expression analysis was performed with FindMarkers function. Subsequently, Hallmark and Reactome pathway enrichment analysis was performed with the differential gene expression dataset, employing a fast gene set enrichment analysis package. Pathways demonstrating statistical significance, indicated by a p-value ≤ 0.05, were

considered noteworthy. For further details, a supplementary table containing all detected pathways is provided for reference (supplementary table 1).

Quantification and statistical analysis

Data are represented mean±SEM or as box and whisker plots (Min to Max). Comparisons between samples from two groups with normally distributed data with equal variance were made using the unpaired two-tailed Student's t-test. Mann Whitney test was used for comparing two groups where data were non-normally distributed. Statistical analyses were performed with Microsoft Excel, GraphPad Prism 6 and RStudio. For all analyses, p-values <0.05 were accepted as statistically significant.

Acknowledgements

This work was supported by the DBT/Wellcome Trust India Alliance Fellowship (IA/I/15/2/502061) awarded to SK and intramural funds from Indian Institute of Science Education and Research Thiruvananthapuram (IISER TVM). IISER TVM Institutional animal facility is supported by funds from the Department of Science and Technology, Government of India (under FIST scheme; SR/FST/LS-II/2018/217). The authors wish to thank Prof. Catherine Verfaillie, KU Leuven for providing material support and hosting SK for performing experiments on animals. S.H.M. and H.A. are supported by IISER TVM. I.M.R. was supported by the senior research fellowship from University Grants Commission (UGC), India.

Contribution

S.K. conceptualized, designed and supervised the study, wrote the manuscript. S.H.M. and I.M.R. performed the experiments and analyzed the results. H.A. performed computational analysis on single cell RNA-Seq data. S.S. provided technical support for experiments on animals.

Data availability

The published datasets used for the analysis were retrieved from the GEO using the accession number GSE147729[23].

Code availability

The code for the bioinformatics analysis, alongside information on software and package versions, is available at https://github.com/stemcellbiologylab.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Figures

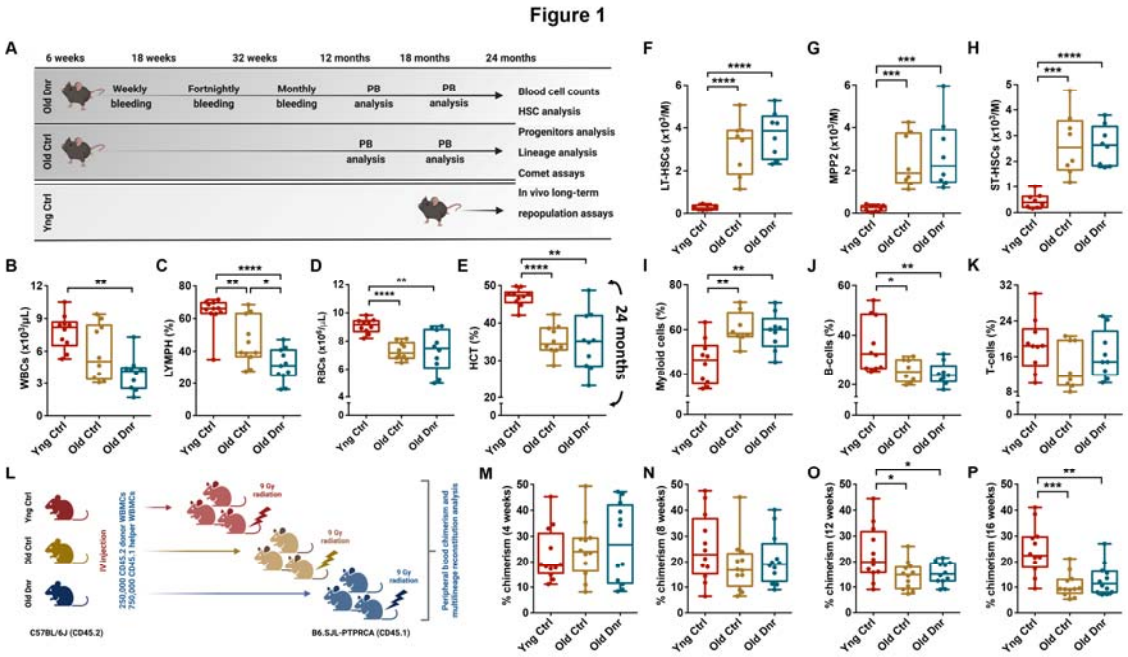


Figure 1. Serial bleeding does not affect the function of HSCs over aging

(A) Schematics depicting the experimental design. Six-weeks old mice were subjected to a long-term serial bleeding regimen. Detailed analysis of the hematopoietic system was followed to assess the effect of enhanced proliferative events on age-associated functional loss in HSCs. (B-E) 24-months old control and donor groups of mice were compared with 6-weeks old control mice for; (B) WBCs, (C) lymphocytes, (D) RBCs, and (E) hematocrit. (F-H) Flow cytometry based analysis of various HSPC populations in the BM. Old donor mice that underwent serial bleeding regimen were compared with young and old control mice for the frequency of; (F) LT-HSCs ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{-}$ cells), (G) MPP2 ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{+}$), and (H) ST-HSCs ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{-}CD48^{-}$). (I-K) The three groups of mice compared for the proportion of; (I) CD11b/Gr-1^{+} myeloid cells, (J) B220^{+} B-cells, and (K) CD4/CD8^{+} T-cells in the BM. (L) Schematic representation of the long-term hematopoietic reconstitution assays performed to compare the function of HSCs from old donor mice with young and old control mice. Donor derived (CD45.2) 250,000 whole BM cells were transplanted along with 750,000 (CD45.1) whole BM supporting cells into lethally irradiated (CD45.1) mice. Donor-derived peripheral blood chimerism was compared among the three groups of mice after; (M) 4 weeks, (N) 8 weeks, (O) 12 weeks, and (P) 16 weeks of transplantation. (n=8-10), unpaired two-tailed Student t test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

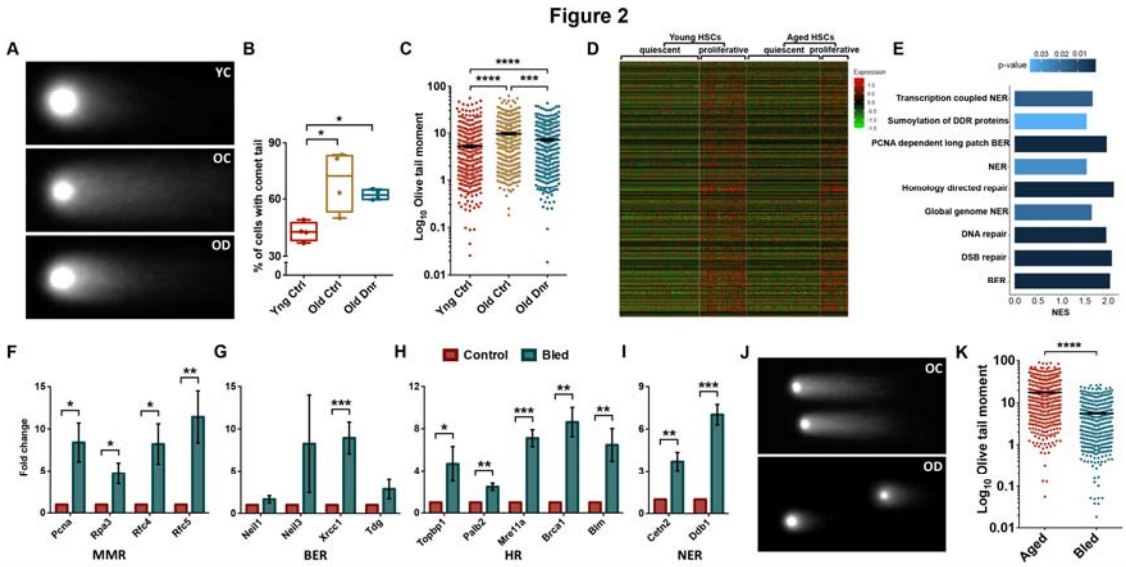
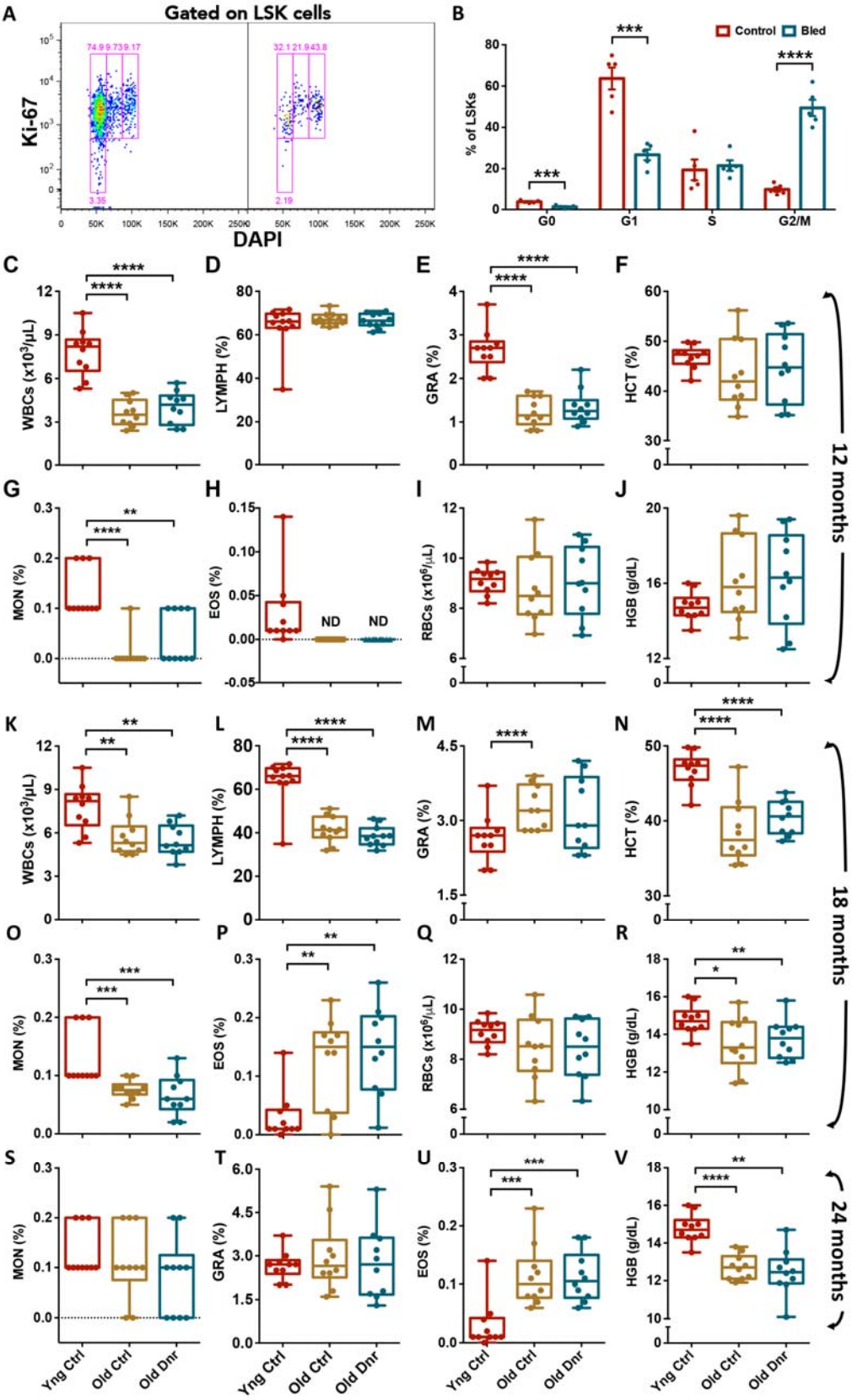


Figure 2. Proliferative events help in the repair of aging induced DNA damage

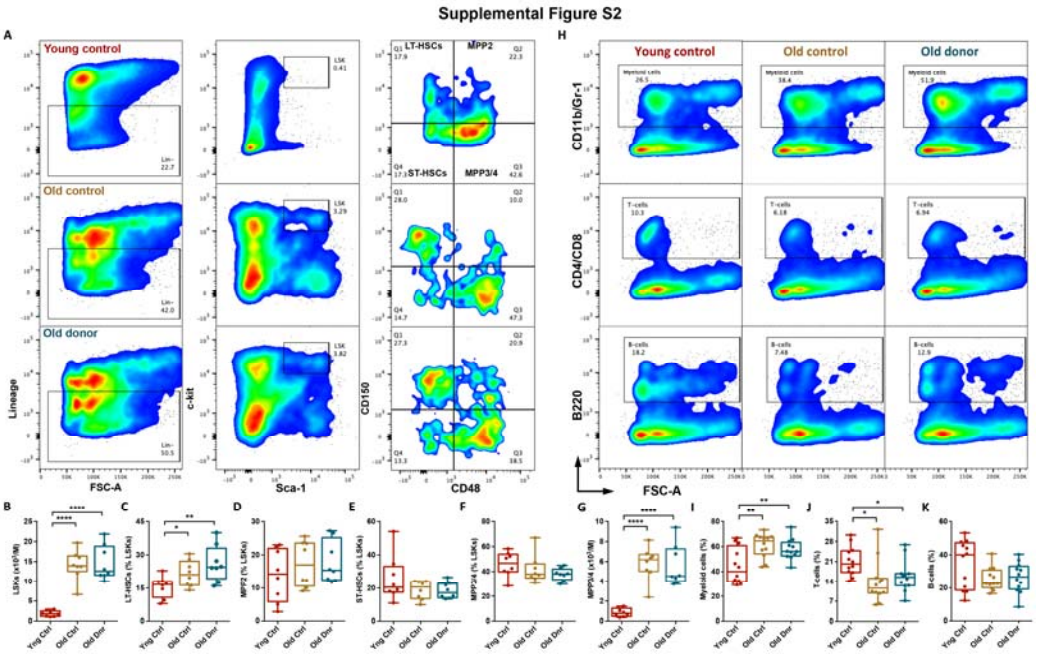
(A-C) Neutral comet assays performed to examine the level of DNA double-strand breaks (DSBs) in HSPC population from old mice that underwent long-term serial bleeding regimen in comparison to young and old controls. (A) Representative comet images from freshly sorted LSK cells from the BM of mice from each group. (B) Proportion of LSK cells from the three groups of mice that showed comet tail. (C) Comparison of Olive tail moment as a measure of sheared DNA per cell. (D,E) Single cell RNA-Seq analysis to compare the gene expression and pathways enriched in quiescent versus proliferative HSCs using R-package Seurat. Data on FACS sorted $Flt3^{-}Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells from young and aged mice was acquired from public database[23]. (D) Heatmap depicting expression of 409 genes associated with DNA damage response (DDR) in quiescent and proliferative HSCs from young and old mice. (E) Differentially expressed Reactome DDR pathways identified using GSEA. The bars depict the normalized enrichment scores (NES) with $p<0.05$. NES>0 indicates signature enrichment in proliferative compared to quiescent HSCs from aged mice. (F-I) Quantitative RT-PCR based analysis to examine the change in the expression of selected DDR pathway genes in BM derived MACS sorted lin^{-} cells following a short-term bleeding regimen in aged mice. The expression of genes with function in; (F) mismatch repair or MMR (*Pcna*, *Rpa3*, *Rfc4* and *Rfc5*), (G) base excision repair or BER (*Neil1*, *Neil3*, *Xrcc1* and *Tdg*), (H) homologous recombination or HR (*Topbp1*, *Palb2*, *Mre11a*, *Brca1* and *Blm*), and (I) nucleotide excision repair or NER (*Cetn2*, *Ddb1*). (J,K) Neutral comet assay performed on FACS sorted LSK cells from aged mice with or without short-term bleeding regimen followed. (J) Representative comet images showing the level of DNA damage due to DSBs. (K) Comparison of Olive tail moment from neutral comet assays performed on LSK cells from the two groups of mice. (n=3-5), unpaired two-tailed Student t test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Supplemental Figure 1



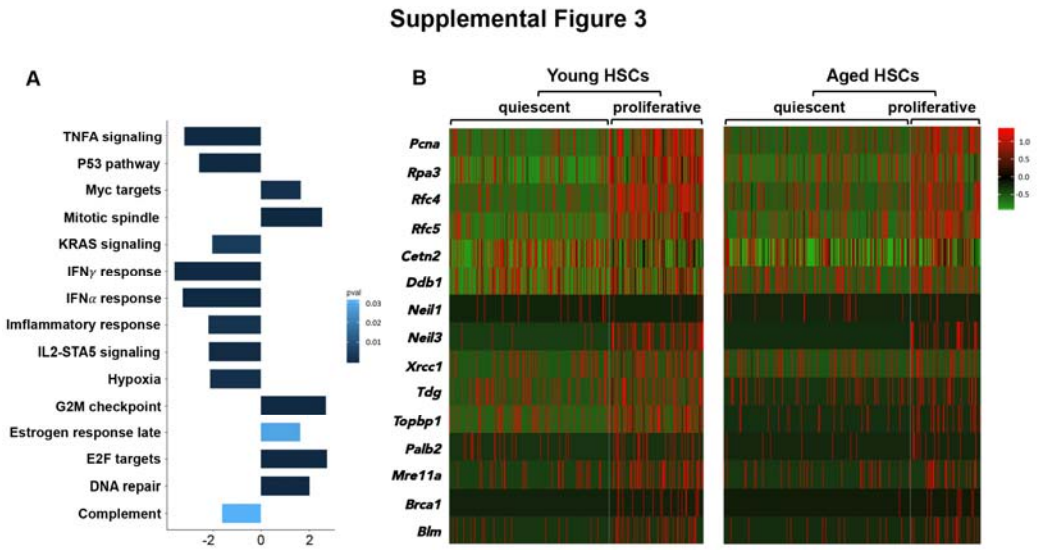
Supplemental Figure 1. Serial bleeding induces HSC proliferation without affecting blood cell production

(A,B) Confirmation of the effect of blood withdrawal on cell cycle entry in BM HSPCs. Young adult mice underwent three blood withdrawals within a period of 10 days followed by cell cycle analysis. The mononuclear cells from the BM were harvested, stained for c-kit, Sca-1 and lineage markers followed by labelling with DAPI and immuno-staining for Ki-67. (A) The cells were gated for $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ fraction and were further analyzed for DAPI and Ki-67 staining for the quantification of the frequency of cells in G_0, G_1, S and G_2/M stages of cell cycle. (B) Comparison of the proportion of LSK cells in various stages of cell cycle. (C-V) Peripheral blood cell counts from old donor (Old Dnr) mice in comparison to the young (Yng Ctrl) and old control (Old Ctrl) mice. (C-J) Peripheral blood cell counts from the two groups of 12-months old mice compared with young adults. The peripheral blood from the mice was analysed for (C) WBCs, (D) lymphocytes, (E) granulocytes, (F) hematocrit, (G) monocytes, (H) eosinophils, (I) RBCs, and (J) hemoglobin. (K-R) Peripheral blood cell counts from 18 months old control and donor groups in comparison to the 6-weeks old mice. The peripheral blood was used to compare; (K) WBCs, (L) lymphocytes, (M) granulocytes, (N) hematocrit, (O) monocytes, (P) eosinophils, (Q) RBCs, and (R) hemoglobin. (S-V) Peripheral blood cell counts from 24 months aged old control and donor groups were analyzed along with six-weeks old mice for; (S) monocytes, (T) granulocytes, (U) eosinophils, and (V) hemoglobin. (n=5-10), Unpaired two-tailed Student t test was performed. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $**** < 0.0001$.



Supplemental Figure 2. Serial bleeding does not affect the composition of hematopoietic stem and progenitor cell populations

(A-G) Bone marrow derived MNCs from 24 months old control and donor mice was compared with young adult (6-weeks old) mice. Mice from the three groups of mice were analyzed for the frequency in the MNCs population as well as within the LSK population. (A) Flow cytometry profiles to analyze LT-HSC ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{-}$), ST-HSCs ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{-}CD48^{-}$), MPP2 ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{+}CD48^{+}$) and MPP3/4 ($Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD150^{-}CD48^{+}$) populations. (B) Frequency of LSK cells within the total BM MNC population from the three groups. (C-F) The LSK population in the BM MNCs further analyzed for the frequency of (C) LT-HSCs, (D) MMPP2, (E) ST-HSCs, and (F) MPP3/4. (G) The frequency of MPP3/4 within the total BM MNC population. (H-K) The NM marrow derived MNCs from old control and donor mice groups were compared with the young adult mice for the proportion of lineage committed cells. (H) Flow cytometry analysis of the BM MNC population to quantify myeloid ($CD11b/Gr\text{-}1^{+}$; upper panel), T-cells ($CD4/CD8^{+}$; middle panel), and B-cells ($B220^{+}$; lower panel). (I-K) Comparison of the three groups of mice for the proportion of (I) $CD11b/Gr\text{-}1^{+}$ myeloid cells, (J) $CD4/CD8^{+}$ T-cells, and (K) $B220^{+}$ B-cells. n=8-10, Unpaired two-tailed Student t test was performed. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.



Supplemental Figure 3. Induction of proliferation in HSCs enhances DDR

(A) Hallmark Pathway analysis in proliferative compared to non-proliferative HSCs based on single-cell RNA Seq data. Gene set enrichment analysis for MSigDB Hallmark 2023.2 pathways associated with aged HSCs was performed. The bar depicts the normalized enrichment scores (NES) with $p<0.05$. NES>0 indicates signature enrichment in proliferative aged HSCs compared to quiescent aged HSCs, while NES<0 indicates enrichment in quiescent HSCs. (B) Heatmap depicting the feature gene expression of the selected 15 genes associated with DNA damage repair pathways in quiescent and proliferative HSCs from young and aged mice. The selected genes belonged to different DDR pathways; mismatch repair (*Pcna*, *Rpa3*, *Rfc4* and *Rfc5*), base excision repair (*Neil1*, *Neil3*, *Xrcc1* and *Tdg*), homologous recombination (*Topbp1*, *Palb2*, *Mre11a*, *Brca1* and *Blm*) and nucleotide excision repair (*Cetn2* and *Ddb1*).