

Alteration of long and short-term hematopoietic stem cell ratio causes myeloid-biased hematopoiesis

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint version 1

March 12, 2024 (this version)

Posted to preprint server

January 20, 2024

Sent for peer review

January 18, 2024

Katsuyuki Nishi, Taro Sakamaki, Akiomi Nagasaka, Kevin S. Kao, Kay Sadaoka, Masahide Asano, Nobuyuki Yamamoto, Akifumi Takaori-Kondo, Masanori Miyanishi 

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan • RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan • Weill Cornell, Rockefeller, Sloan-Kettering, Tri-Institutional MD-PhD Program, New York, NY • Institute of Laboratory Animals, Kyoto University Graduate School of Medicine, Kyoto, Kyoto, 606-8501, Japan • Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017, Japan • Department of Hematology and Oncology, Kyoto University Graduate School of Medicine, Kyoto, Kyoto 606-8507, Japan

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

Myeloid-biased hematopoiesis is a well-known age-related alteration. Several possibilities, including myeloid-biased hematopoietic stem cell (HSC) clones, may explain this. However, the precise mechanisms remain controversial.

Utilizing the Hoxb5 reporter system to prospectively isolate long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), we found that young and aged LT-HSCs co-transplanted into the same recipients demonstrated nearly equivalent myeloid lineage output, contrary to the theory of myeloid-biased HSC clones. Transcriptomics indicated no significant myeloid gene enrichment in aged LT-HSCs compared to their young counterparts. Instead, transplanting reconstituted young HSCs with the ratio of LT/ST-HSCs seen in aged mice can significantly skew the lineage output to myeloid cells. In addition, while the niche environment in the bone marrow minimally affects myeloid-biased hematopoiesis, aged thymi and spleens substantially hinder lymphoid hematopoiesis, resulting in further myeloid-domination. Thus, we demonstrate that myeloid-biased hematopoiesis in aged organisms originates due to alteration of the ratio between LT-HSCs and ST-HSCs rather than in heterogeneous HSC clones with various cell fates.

eLife assessment

This manuscript provides **useful** findings to further explore the heterogeneity of hematopoietic stem cells and myeloid-biased hematopoiesis during aging. The results presented in this study are **incomplete** and additional data is needed to strengthen the conclusions. Some of the methods and data analyses, including the replicates and statistical robustness, remain **inadequate** to support the primary claims.

Introduction

Age-associated changes in individuals are deeply correlated with progressive attenuation of cellular functions in tissue stem cells of organs (Jones and Rando 2011 [↗](#)). In the hematopoietic system, hematopoietic stem cells (HSCs), which possess self-renewal capacity and multipotency (Spangrude et al., 1988 [↗](#); Weissman and Shizuru 2008 [↗](#); Majeti et al., 2007 [↗](#)), are responsible for various hematopoietic alterations with age, such as reduced self-renewal capacity (Morrison et al., 1996 [↗](#)) and myeloid-biased hematopoiesis (Muller-Sieburg et al., 2004 [↗](#); Challen et al., 2010 [↗](#)) due to their functional decline. For example, myeloid-biased hematopoiesis potentially reduces response to infections (Webster, 2000 [↗](#)), reduces vaccination efficacy (Crooke et al., 2019 [↗](#)), and increases myeloid malignancy (Rossi et al., 2008 [↗](#)) in aged individuals. To comprehend these age-associated physiological changes, myeloid-biased hematopoiesis has been studied in considerable detail.

Based on the experimental observation that transplantation of aged HSCs exhibits more myeloid-biased differentiation in young recipient mice than transplantation of young HSCs, this phenotype has been thought to originate in cell-intrinsic changes in the HSC compartment (Geiger et al., 2013 [↗](#); De Haan and Lazare 2018 [↗](#); Schultz and Sinclair 2016 [↗](#)). A myeloid-biased phenotype after aged HSC transplantation leads to the suggestion that myeloid-biased HSC clones selectively expand with age (Cho et al., 2008 [↗](#); Pang et al., 2011 [↗](#)). Transcriptome and epigenetic analyses showing that a set of genes related to myeloid differentiation is significantly enriched in aged HSCs compared to young HSCs (Rossi et al., 2005 [↗](#); Grover et al., 2016 [↗](#)) further supports this hypothesis.

On the other hand, some reports support a different point of view in light of experimental evidence showing that aged lymphoid-biased HSCs still demonstrate the same level of lymphopoiesis as their younger counterparts, despite exhibiting a myeloid-biased gene expression pattern (Montecino-Rodriguez et al., 2019 [↗](#)). This result highlights the limitations of predicting a pattern of HSC differentiation based upon gene expression patterns. Moreover, myeloid progenitors such as granulocyte-macrophage progenitor (GMP) and common myeloid progenitor (CMP) should also increase with age if the selective expansion of myeloid-biased HSCs leads to an increase of myeloid cells in peripheral blood (PB). However, increment of such progenitors with age was not consistently demonstrated in earlier research (Rossi et al., 2005 [↗](#); Nilsson et al., 2016 [↗](#); Min et al., 2006 [↗](#)). Additionally, a mathematical model demonstrated that aging had no effect on the daily production of CMP supplied from multipotent progenitors (MPP) (Busch et al., 2015 [↗](#); Dorshkind et al., 2020 [↗](#)).

As such, while selective expansion of myeloid-biased HSC clones is the most widely accepted hypothesis to explain myeloid-biased hematopoiesis in aging (Mejia-Ramirez and Florian, 2020 [↗](#); SanMiguel et al., 2020 [↗](#)), other mechanisms may exist in light of the inconsistent cellular behavior of progenitor fractions relative to HSCs. To the best of our knowledge, no reports have analyzed kinetics of age-associated changes in HSCs, progenitors, and PB cells simultaneously. Therefore, we started our investigation by examining the correlation between age-related changes in PB and BM to shed light on the mechanism underlying myeloid-biased hematopoiesis that occurs during aging.

Results

The discrepancy of age-associated alternation in peripheral blood and bone marrow calls into question the existence of a myeloid-biased clone

Mouse HSC research on aging has used mice aged 18 months or older (Challen et al., 2010 [↗](#); Cho et al., 2008 [↗](#); D. J. Rossi et al., 2005 [↗](#); Grover et al., 2016 [↗](#); Montecino-Rodriguez et al., 2019 [↗](#); Nilsson et al., 2016 [↗](#)). However, given that continuous accumulation of cellular stress with age causes a gradual decline of cellular functions, a comprehensive analysis from young to old mice is necessary to unravel mechanisms by which age-associated, myeloid-biased hematopoiesis progress. Hence, we analyzed changes in PB (**Fig. S1 A**) at multiple time points from young to old mice. These results showed that the percentage of myeloid cells began to change as early as 6 months in mice and continued to increase at a constant rate until the age of ≥ 23 months (**Fig. 1 A** [↗](#)).

It has been reported that myeloid-biased hematopoiesis is caused by a selective increase of myeloid-biased clones in the immunophenotypically defined (surface-antigen defined) HSC fraction (**Fig. S1 B**) (Beerman et al., 2010 [↗](#)). According to this hypothesis, age-associated myeloid hematopoiesis progression in PB would be paralleled by an increase in myeloid-biased HSC clones. Therefore, we examined the frequency of the HSC and progenitor fractions in the BM at multiple time points (**Fig. S1, B and C**). We found that the frequency of immunophenotypically defined HSC in BM rapidly increased up to the age of 12 months. After the age, they remained plateaued throughout the observation period (**Fig. 1 B** [↗](#)). On the other hand, in contrast to what we anticipated, the frequency of GMP was stable, and the percentage of CMP actually decreased significantly with age, defying our prediction that the frequency of components of the myeloid differentiation pathway, such as CMP, GMP, and MEP would increase in aged mice if myeloid-biased HSC clones increase with age (**Fig. 1 B** [↗](#)).

Finally, we determined the ratio of each fraction in young mice versus aged mice to compare the age-associated transition pattern of components comprising the myeloid differentiation pathway. This analysis revealed that age-associated transition patterns of immunophenotypically defined HSC and CMP in BM were not paralleled with myeloid cell in PB (**Fig. 1 C** [↗](#)). These findings called into question the hypothesis that there is a selective increase of myeloid-biased HSC clones in the aged BM. We then set out to elucidate the mechanism by which myeloid-biased phenotypes arise in the PB of aged mice.

The long-term observation of 2-year-old LT-HSC's differentiation does not indicate the expansion of myeloid-biased clones

Numerous studies have claimed that the myeloid-skewed phenotype observed in PB of aged mice is caused by myeloid-biased HSC clones selectively expanded from HSCs with originally heterogeneous differentiation potentials (Beerman et al., 2010 [↗](#); Dykstra et al., 2011 [↗](#); Sudo et al., 2000 [↗](#)). Other studies have reported that immunophenotypically defined HSCs possess heterogeneity associated with self-renewal capacity, suggesting the existence of at least two different populations in the HSC compartment, LT-HSCs and ST-HSCs (Morrison and Weissman 1994 [↗](#); Spangrude et al., 1995 [↗](#)). Since LT-HSCs have extensive self-renewal capacity, while ST-HSCs lose their self-renewal capacity within a short period, LT-HSCs are thought to persist in the BM throughout life and to enrich age-associated changes compared to ST-HSCs. Therefore, we expect that LT-HSC-specific analyses will help to answer whether myeloid-biased HSC clones exist.

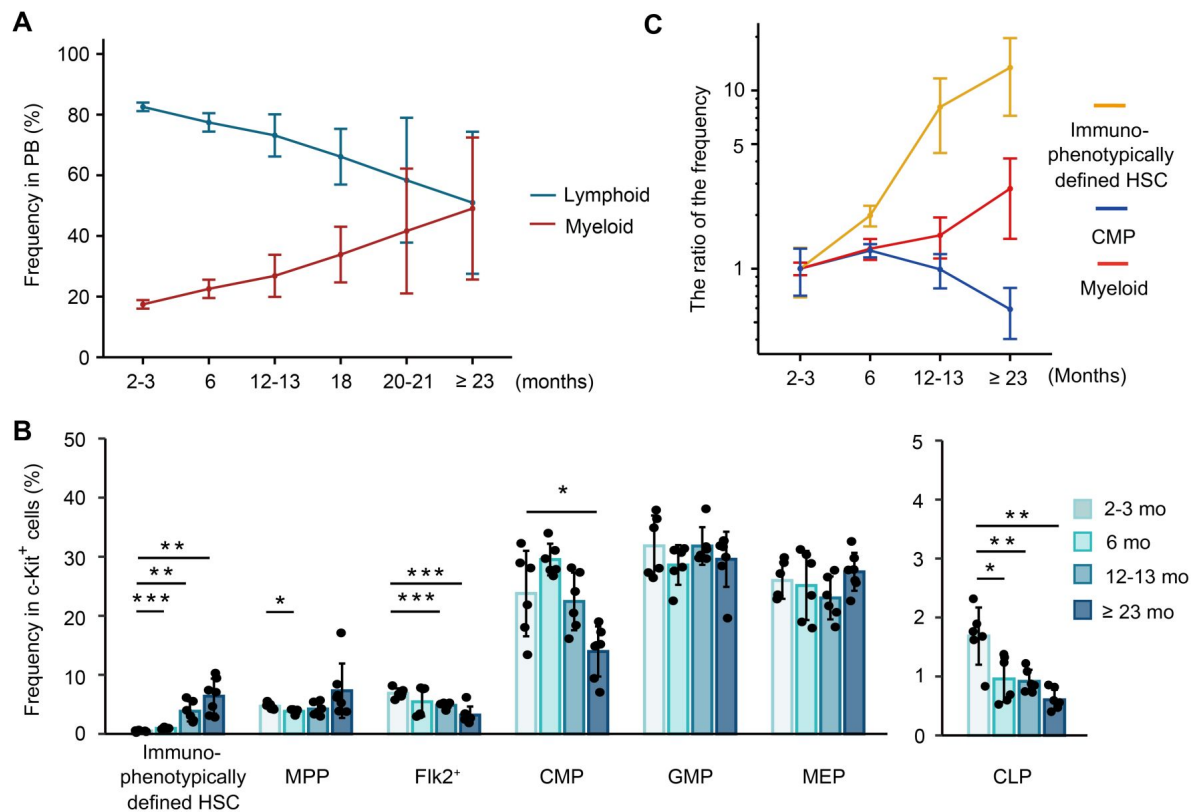


Figure 1.

Comprehensive analysis of hematopoietic alternations with age shows a discrepancy of age-associated changes between peripheral blood and bone marrow

(A) Average frequency of myeloid cells (neutrophils, monocytes) and lymphoid cells (B cells, T cells, and NK cells) in PB at the age of 2-3 months ($n = 6$), 6 months ($n = 6$), 12-13 months ($n = 6$), 18 months ($n = 6$), 20-21 months ($n = 5$), ≥ 23 months ($n = 16$). Abbreviation: PB = Peripheral blood.

(B) Average frequency of immunophenotypically defined HSC and progenitor cells in BM of 2-3-month mice ($n = 6$), 6-month mice ($n = 6$), 12-13-month mice ($n = 6$), ≥ 23-month mice ($n = 7$).

(C) Age-associated changes of immunophenotypically defined HSC and myeloid differentiation components (CMP and myeloid cells in the PB). The ratio of aged to young frequency was calculated as (the fraction frequency at each aged mice (%)) / (the average fraction frequency at 2-3-month mice (%)). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. Data and error bars represent means $\pm$ standard deviation.

We previously reported that *Hoxb5* exclusively marks LT-HSCs in young mice (Chen et al., 2016 [DOI](#)). However, we have not tested in aged mice whether *Hoxb5* specifically labels LT-HSCs and helps to distinguish LT-HSCs and ST-HSCs from immunophenotypically defined HSCs (hereafter, bulk-HSCs). To confirm this, we first analyzed expression of *Hoxb5* in bulk-HSC, MPP, Flk2^+ , and $\text{Lin}^- \text{Sca1}^c\text{-Kit}^+$ populations in aged *Hoxb5*-tri-mCherry mice. We observed that *Hoxb5* was exclusively expressed in bulk-HSCs (Fig. 2 A [DOI](#)). Then, to verify long-term engraftment, we conducted a transplantation assay utilizing *Hoxb5*⁺ HSCs and *Hoxb5*⁻ HSCs, respectively, isolated from 2-year-old mice (Fig. 2 B [DOI](#)). We observed that only recipients receiving aged *Hoxb5*⁺ HSCs exhibited continuous hematopoiesis 16 weeks after primary transplantation (Fig. 2, C [DOI](#) and E [DOI](#); Fig. S2 A). In secondary transplantation analysis, only recipients receiving *Hoxb5*⁺ HSCs exhibited robust hematopoiesis throughout the period of observation, indicating that *Hoxb5* can be used as a specific marker of LT-HSCs in aged mice, as well as young mice (Fig. 2, D [DOI](#) and F [DOI](#); Fig. S2 B).

A serial transplantation assay with a period of observation longer than 8 months should be long enough to observe further myeloid-biased change. If bulk-HSCs isolated from aged mice are already enriched by myeloid-biased HSC clones, we should see more myeloid-biased phenotypes 16 weeks after primary and the secondary transplantation. However, we found that kinetics of the proportion of myeloid cells in PB were similar across primary and the secondary transplantation and that the proportion of myeloid cells gradually decreased over time (Fig. 2 G [DOI](#)). These results suggest the following two possibilities: either myeloid-biased HSCs do not expand in the LT-HSC fraction, or the expansion of myeloid-biased clones in 2-year-old mice has already peaked.

Direct comparison between young vs. aged LT-HSC differentiation reveals that LT-HSCs exhibit unchanged lineage output throughout life

We developed a co-transplantation assay to directly compare the differentiation capacity between young and aged LT-HSCs by co-transplanting ten young GFP⁺ LT-HSCs and ten aged GFP⁻ LT-HSCs into the same recipient mice (Fig. 3 A [DOI](#)). Then, we found that the myeloid lineage proportions from young and aged LT-HSCs were nearly comparable during the observation period after transplantation (Fig. 3, B [DOI](#) and C [DOI](#)). Furthermore, by analyzing the proportion of mature cell types derived from young and aged LT-HSCs in the same donor, we directly compared the capacity for hematopoietic reconstitution in each mature cell type between young and aged LT-HSCs. We confirmed again that the reconstitution ratio was almost the same across all lineages, although bulk hematopoiesis derived from young LT-HSCs predominated (Fig. 3 D [DOI](#)). These results indicate that the differentiation potential of LT-HSCs remains unchanged throughout their lives.

Several reports have demonstrated that transplanting mixed/bulk-HSCs—a combination of LT-HSCs and ST-HSCs—obtained from old animals results in blatantly myeloid-biased hematopoiesis (Beerman et al., 2010 [DOI](#); Dykstra et al., 2011 [DOI](#); Sudo et al., 2000 [DOI](#)). To test this, we co-transplanted bulk-HSCs from young and old mice to corroborate this (Fig. S3 A). As previously described, we observed that aged bulk-HSC exhibited a myeloid-skewed phenotype (Fig. S3 B and C). Additionally, we observed that aged bulk-HSC reconstitution exhibited higher reconstitution of myeloid cells compared to young bulk-HSCs (Fig. S3 D). These findings unmistakably demonstrated that mixed/bulk-HSCs showed myeloid skewed hematopoiesis in PB with aging, while LT-HSCs exhibited unchanged lineage output throughout life.

LT-HSCs never show myeloid-related gene set enrichment during aging

Although the results of co-transplantation of young and aged LT-HSCs did not demonstrate lineage-biased output by LT-HSCs throughout life, enrichment of myeloid genes in aged bulk-HSCs shown by previous studies supports the idea that myeloid-biased hematopoiesis is caused by selective

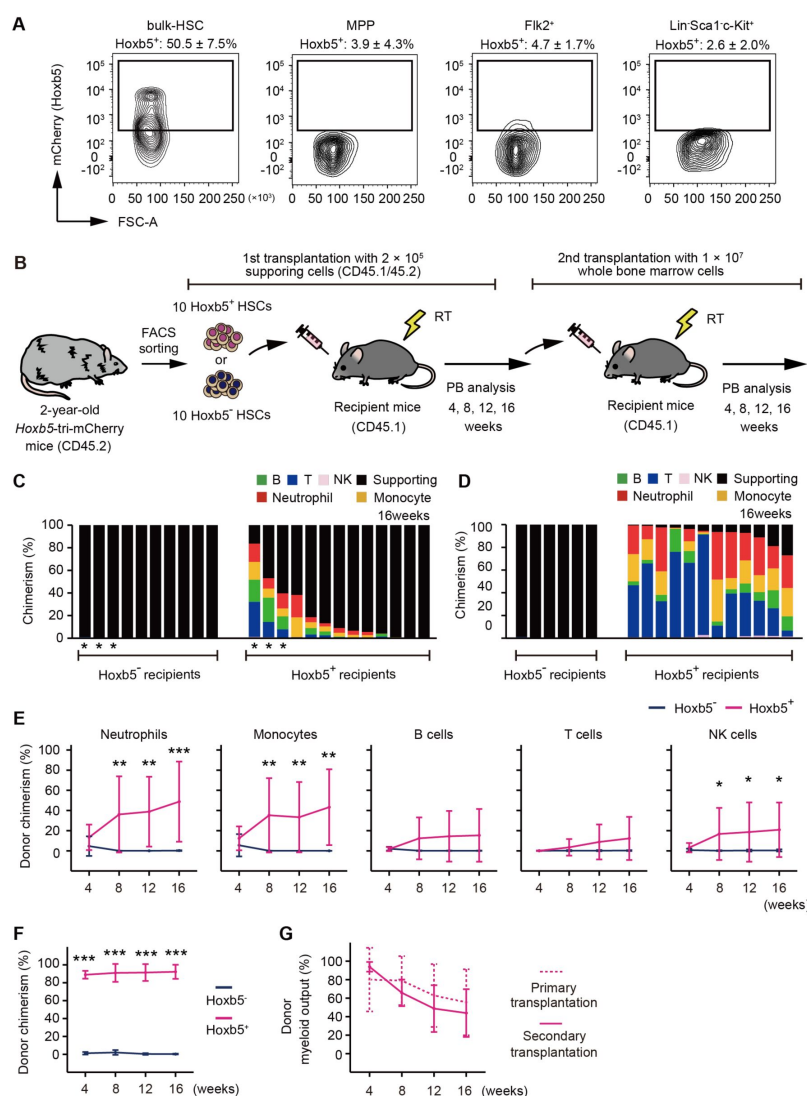


Figure 2.

The expansion of myeloid-biased clones was not observed in 2-year-old LT-HSCs after their transplantation.

(A) Hoxb5 reporter expression in bulk-HSC, MPP, Flk2⁺, and Lin⁻Sca1⁻c-Kit⁺ populations in the 2-year-old *Hoxb5*-tri-mCherry mice. Values indicate the percentage of mCherry⁺ cells ± standard deviation in each fraction (n = 3).

(B) Experimental design to assess the long-term reconstitution ability of Hoxb5⁺ HSCs or Hoxb5⁻ HSCs. Hoxb5⁺ HSCs and Hoxb5⁻ HSCs were isolated from 2-year-old CD45.2 *Hoxb5*-tri-mCherry mice and were transplanted into lethally irradiated CD45.1 recipient mice with 2 × 10⁵ supporting cells (Hoxb5⁺ HSCs, n = 13; Hoxb5⁻ HSCs, n = 10). For secondary transplants, 1 × 10⁷ whole BM cells were transferred from primary recipient mice. Abbreviations: PB = Peripheral blood, RT = Radiation therapy.

(C) Percentage chimerism at 16 weeks after receiving ten aged Hoxb5⁻ HSCs or ten aged Hoxb5⁺ HSCs. Each column represents an individual mouse.

(D) Percentage chimerism at 16 weeks after whole BM secondary transplantation. Donor whole BM cells for secondary transplantation were taken from mice denoted by * in **Figure 2C**.

(E) Kinetics of average donor chimerism in each PB fraction after primary transplantation.

(F) Kinetics of average donor chimerism after secondary transplantation.

(G) Kinetics of average donor myeloid output (myeloid proportion in donor cells) in LT-HSC recipient mice after primary and secondary transplantation. *P < 0.05. **P < 0.01.

***P < 0.001. Data and error bars represent means ± standard deviation.

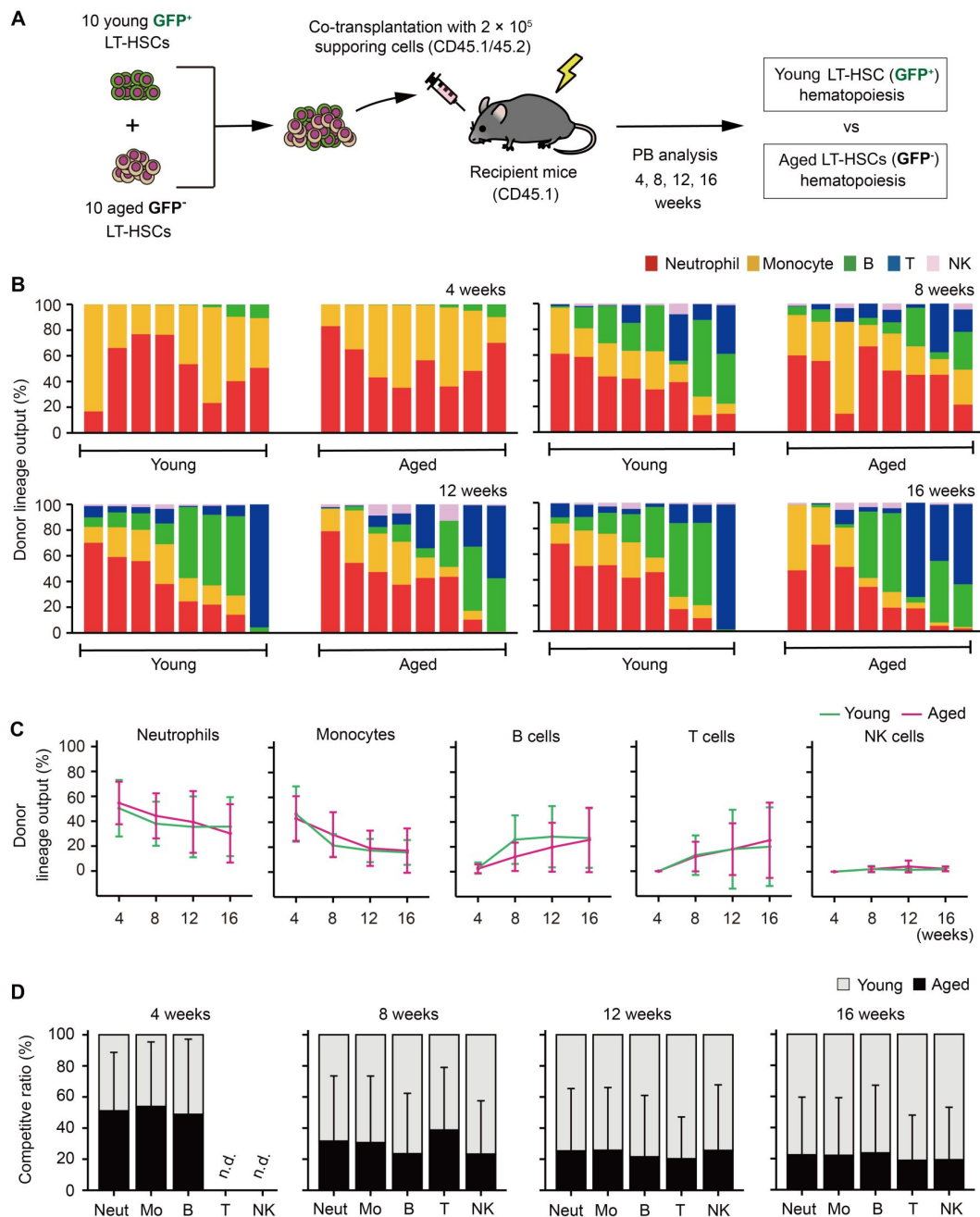


Figure 3.

Aged LT-HSCs show balanced hematopoiesis throughout life

(A) Experimental design for competitive co-transplantation assay using young LT-HSCs sorted from *Hoxb5*-tri-mCherry GFP mice and aged LT-HSCs sorted from *Hoxb5*-tri-mCherry mice. Ten CD45.2⁺ young LT-HSCs and ten CD45.2⁺ aged LT-HSCs were transplanted with 2×10^5 CD45.1⁺/CD45.2⁺ supporting cells into lethally irradiated CD45.1⁺ recipient mice ($n = 8$).

(B) Lineage output of young or aged LT-HSCs at 4, 8, 12, 16 weeks after transplantation. Each bar represents an individual mouse.

(C) Lineage output kinetics of young LT-HSCs or aged LT-HSCs at 4, 8, 12, 16 weeks post-transplant.

(D) Competitive analysis of young LT-HSCs vs. aged LT-HSCs lineage output at 4, 8, 12, 16 weeks post-transplant. Competitive ratio was calculated as the proportion of young LT-HSCs derived cells vs. aged LT-HSCs derived cells in each fraction.

Abbreviations: Neut = Neutrophils, Mo = Monocytes, B = B cells, T = T cells, and NK = NK cells. Data and error bars represent means $\pm$ standard deviation. "n.d." stands for "not detected."

expansion of myeloid-biased HSC clones (D. J. Rossi et al., 2005 [↗](#); Grover et al., 2016 [↗](#)). To compare age-associated myeloid gene enrichment, we isolated bulk-HSC, LT-HSCs, and ST-HSCs from young or aged mice (**Fig. 4 A** [↗](#)).

To ensure the quality of the sorted fraction for subsequent RNA-seq analyses, we verified the *Hoxb5* read counts (**Fig. S4 A**). Cluster dendrograms using the whole transcriptome confirmed that cell fractions isolated from young and aged mice clustered into distinct groups (**Fig. 4 B** [↗](#)). Then, using sets of aging-related genes, such as inflammation (Liberzon et al., 2015 [↗](#); Pietras 2017 [↗](#)), DNA damage (p53 pathway) (Liberzon et al., 2015 [↗](#); D. J. Rossi et al., 2007 [↗](#)), cell cycle progression (E2F target) (Liberzon et al., 2015 [↗](#); Kowalczyk et al., 2015 [↗](#)), and a common aging signature (Flohr Svendsen et al., 2021 [↗](#)), we ran a violin plot analysis on each cell fraction to confirm the occurrence of typical aging-related gene expression changes. We found age-associated changes in inflammation and the DNA damage between cells isolated from young and aged mice, but very similar patterns regardless of cell fraction in young or aged mice. In terms of cell cycle progression and aging signature genes, however, we discovered that only the young LT-HSC fraction differed from other young fractions and tended to represent similar gene expression pattern with aged fractions (**Fig. 4 C** [↗](#)). Then, taking LT-HSC specific gene expression pattern into account, we looked at expression patterns of specific genes that had been highly validated by experiments as being associated with myeloid-biased HSC (Beerman et al., 2010 [↗](#); Flohr Svendsen et al., 2021 [↗](#); Sanjuan-Pla et al., 2013 [↗](#); Mann et al., 2018 [↗](#)). We discovered that expression of these genes is relatively comparable between young and aged LT-HSCs, but not other fractions (**Fig. S4 B**).

A recent comprehensive analysis of mouse HSC aging using multiple RNAseq data sets claimed that almost 80% of differentially expressed genes are poorly reproducible across data sets (Flohr Svendsen et al., 2021 [↗](#)). In fact, we found that almost 80% of genes are not shared when we compare three representative myeloid/lymphoid gene sets (Sanjuan-Pla et al., 2013 [↗](#); Chambers et al., 2007 [↗](#); Pronk et al., 2007 [↗](#)). Additionally, most genes were only used in a single gene set (**Fig. 4, D** [↗](#) and **E** [↗](#)). By using only genes that were shared in these gene sets, we ran a gene set enrichment analysis (GSEA) to see whether myeloid/lymphoid genes were enriched in aged LT-HSCs or other fractions. Neither aged LT-HSC nor aged ST-HSC exhibited myeloid/lymphoid gene enrichment, while shared myeloid-related genes tended to be enriched in aged bulk-HSCs (**Fig. 4, F** [↗](#) and **G** [↗](#)). On the other hand, GSEA analysis using original gene sets, respectively, showed inconsistent results (**Fig. S4, C** and **D**).

A myeloid-biased phenotype in peripheral blood depends on the relative decrease of ST-HSC in the HSC compartment with age

While transplantation of aged bulk-HSCs exhibits a myeloid-biased phenotype in PB shown in previous reports (Beerman et al., 2010 [↗](#); Dykstra et al., 2011 [↗](#); Sudo et al., 2000 [↗](#)) and our results (**Fig. S3**), aged LT-HSCs remain stable in terms of the balance of their differentiation potential between myeloid and lymphoid production (**Fig. 3** [↗](#)). Comparative transcriptome analyses demonstrated that neither LT-HSCs nor ST-HSCs, which are functionally more homogeneous than bulk-HSCs, exhibited myeloid gene set enrichment with age, whereas aged bulk-HSCs tended to show more myeloid gene set enrichment than their young counterparts (**Fig. 4** [↗](#)). We then tried to figure out what causes the myeloid-biased phenotype in PB after transplantation of bulk-HSCs.

Because LT-HSCs in bulk-HSCs exhibit nearly constant level of hematopoiesis throughout life, we hypothesized that ST-HSCs could be the key to discovering mechanisms underlying the myeloid-skewing phenomenon. First, we revisited our previous study (Chen et al., 2016 [↗](#)), which demonstrated that transplanted ST-HSCs maintain lymphocyte production while rapidly losing myeloid lineage production in recipients. This result may potentially indicate the presence of HSC clones with a preference for lymphoid differentiation. Given that donor cells obtained from

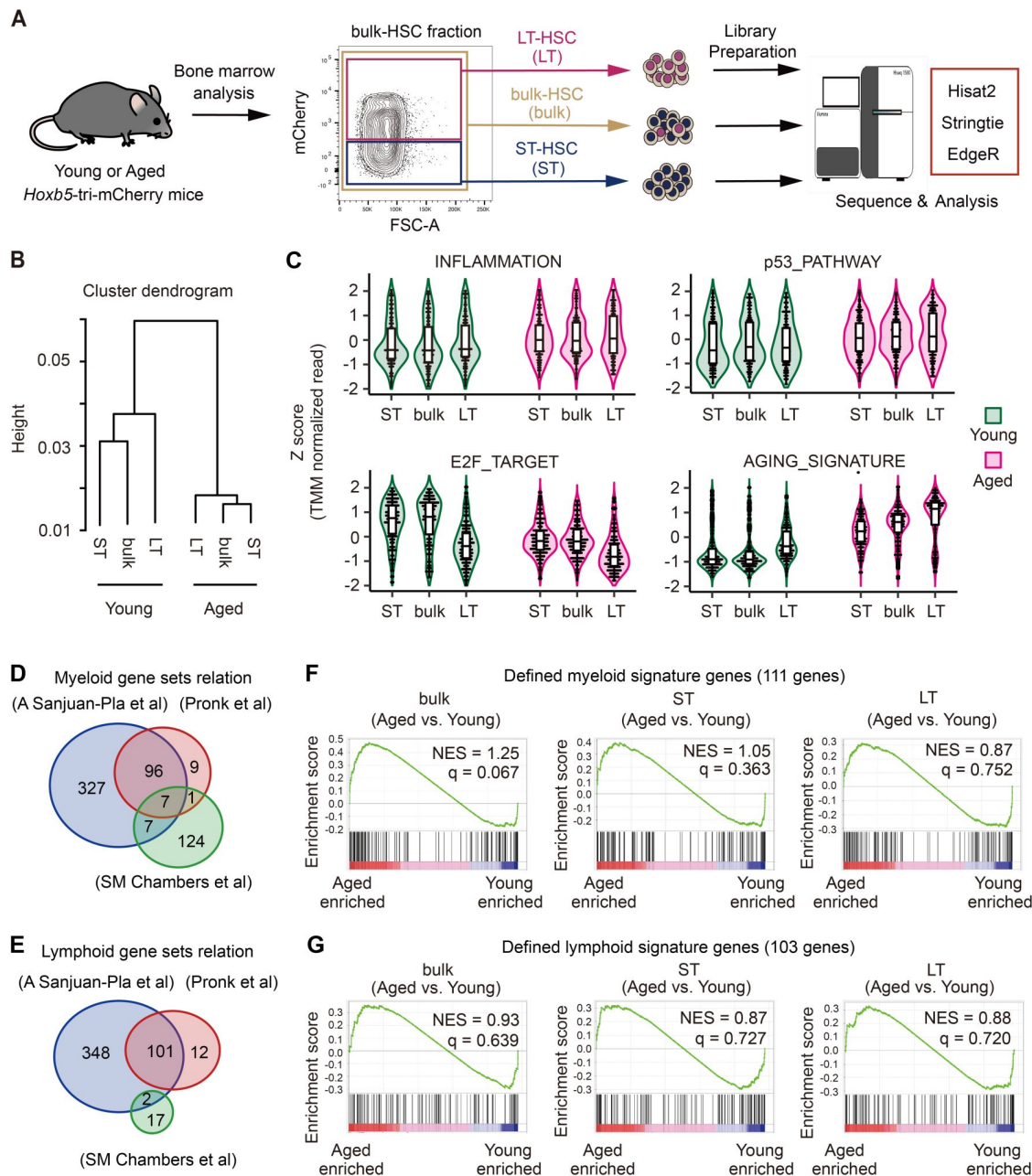


Figure 4.

Myeloid-associated genes were not enriched in aged LT-HSCs compared to their young counterparts

(A) Experimental schematic for transcriptome analysis. LT-HSCs ($n = 3$), ST-HSCs ($n = 3$), and bulk-HSCs ($n = 3$) were sorted from young (2-3 months) or aged (23-25 months) *Hoxb5*-tri-mCherry mice, after which each RNA was harvested for RNA sequencing.

(B) Hierarchical clustering dendrogram of whole transcriptomes using Spearman distance and the Ward clustering algorithm. (C) Violin plots showing normalized gene expression levels for each gene set in young and aged LT-HSCs, ST-HSCs, and bulk-HSCs. Expression values for each gene were standardized independently by applying Z score transformation.

(D, E) Venn diagram showing the overlap of genes between three myeloid signature gene sets, and lymphoid signature gene sets (A Sanjuan-Pla et al., 2013 [\[1\]](#), Pronk et al., 2007 [\[2\]](#), SM Chambers et al., 2007 [\[3\]](#)).

(F, G) Signature enrichment plots from GSEA analysis using defined myeloid and lymphoid signature gene sets that overlapped in the three gene sets. Values indicated on individual plots are the normalized Enrichment Score (NES) and q -value of enrichment.

primary recipients receiving ST-HSCs do not undergo hematopoiesis in secondary transplantation (Chen et al., 2016), it is likely that all ST-HSCs have already lost their ability to self-renew and have disappeared from the HSC fraction in primary recipients. To confirm this, we transplanted ten LT-HSCs or ST-HSCs and then analyzed PB and BM (Fig. 5 A). As we previously reported (Chen et al., 2016), donor-derived hematopoiesis eventually becomes lymphocyte-dominant in PB of ST-HSC recipients over time (Fig. 5 B). Subsequently, it was determined that the vast majority of T cell subsets are memory cells (Fig. 5, C and D). In addition, unlike recipients receiving LT-HSCs, recipients receiving ST-HSCs lacked any bulk-HSCs in their BM (Fig. 5, E and F). These results strongly suggest that lymphoid-biased hematopoiesis observed after transplantation of ST-HSCs is due to persistence of memory-type lymphocytes in PB, rather than to *de novo* lymphopoiesis from lymphoid-biased HSC clones. Persistence of memory-type lymphocytes from ST-HSCs may have led us to misinterpret mechanisms underlying myeloid-biased hematopoiesis after aged bulk-HSC transplantation. To verify this hypothesis, we examined kinetics of the LT-HSC/ST-HSC ratio in the bulk-HSC population, revealing the ratio of ST-HSC to LT-HSC decreased with age (Figure S5). Thus, we hypothesized that the relative decrease in the ST-HSC ratio in the aged bulk-HSC fraction would lead to reduction of memory-type lymphocytes and myeloid-biased hematopoiesis following transplantation of aged bulk-HSCs. To test this hypothesis, we isolated LT-HSCs and ST-HSCs from young donor mice and reconstituted them with a 2:8 ratio (as in young mice) or a 5:5 ratio (as in aged mice) prior to transplanting them (Fig. 6 A). Four weeks after transplantation, PB analysis revealed that both groups had comparable patterns of hematopoiesis. In contrast, recipient mice transplanted with a 5:5 ratio began to exhibit more myeloid-biased hematopoiesis eight weeks after transplantation, and by week 16, they produced significantly more myeloid cells than the other group (Fig. 6, B-D). Additionally, we conducted further investigations to determine whether lymphoid hematopoiesis could be accelerated by alteration of LT-HSC/ST-HSC ratios using cells from aged mice. To verify this, we isolated aged LT-HSCs and ST-HSCs and reconstituted them with a 5:5 ratio or a 2:8 ratio prior to transplantation (Fig. S6 A). Peripheral blood analysis revealed that lymphoid lineage output in the recipient mice transplanted with a 2:8 ratio was significantly greater than those with a 5:5 ratio (Fig. S6 B-D). Based on these findings, we concluded that myeloid-biased hematopoiesis observed following transplantation of aged HSCs was caused by a relative decrease in ST-HSC in the bulk-HSC compartment in aged mice rather than the selective expansion of myeloid-biased HSC clones.

Age-associated extramedullary changes accelerate myeloid-biased hematopoiesis

We found that the fluctuating LT-HSC/ST-HSC ratio in the bulk-HSC compartment corresponded with the myeloid-biased hematopoiesis associated with aging. In contrast, the degree of myeloid bias observed in mice older than 23 months without transplantation (Fig. 1 A; $49.0 \pm 23.4\%$) was significantly greater than in recipient mice receiving young mixed HSCs with a 5:5 ratio 16 weeks after transplantation (Fig. 6 D; $30.8 \pm 30.8\%$). This difference indicated that other intrinsic or extrinsic factors might exist in mice older than 23 months to promote myeloid-biased hematopoiesis. To investigate this further, we transplanted ten GFP⁺ young LT-HSCs into young or 2-year-old recipient mice and examined their PB (Fig. 7 A). We limited the observation period to 12 weeks because many aged recipient mice had died prior to that point (Fig. 7 B). PB analysis revealed that aged recipient mice receiving young LT-HSCs produced significantly more myeloid cells than young recipient mice (Fig. 7, C-E). These results suggested that recipient-dependent extrinsic factors, rather than intrinsic factors of transplanted HSCs, had a greater impact on hematopoietic cell differentiation. Then, we examined BM to determine what extrinsic factors affected differentiation of transplanted HSCs. Percentages of CMP and GMP, downstream components of myeloid differentiation, were stable or significantly lower in aged recipient mice than in young recipient mice, just as they were in non-treated aged mice (Fig. 7 F; Fig. 1 B). In addition, the percentage of CLP, a downstream component of lymphoid differentiation, did not

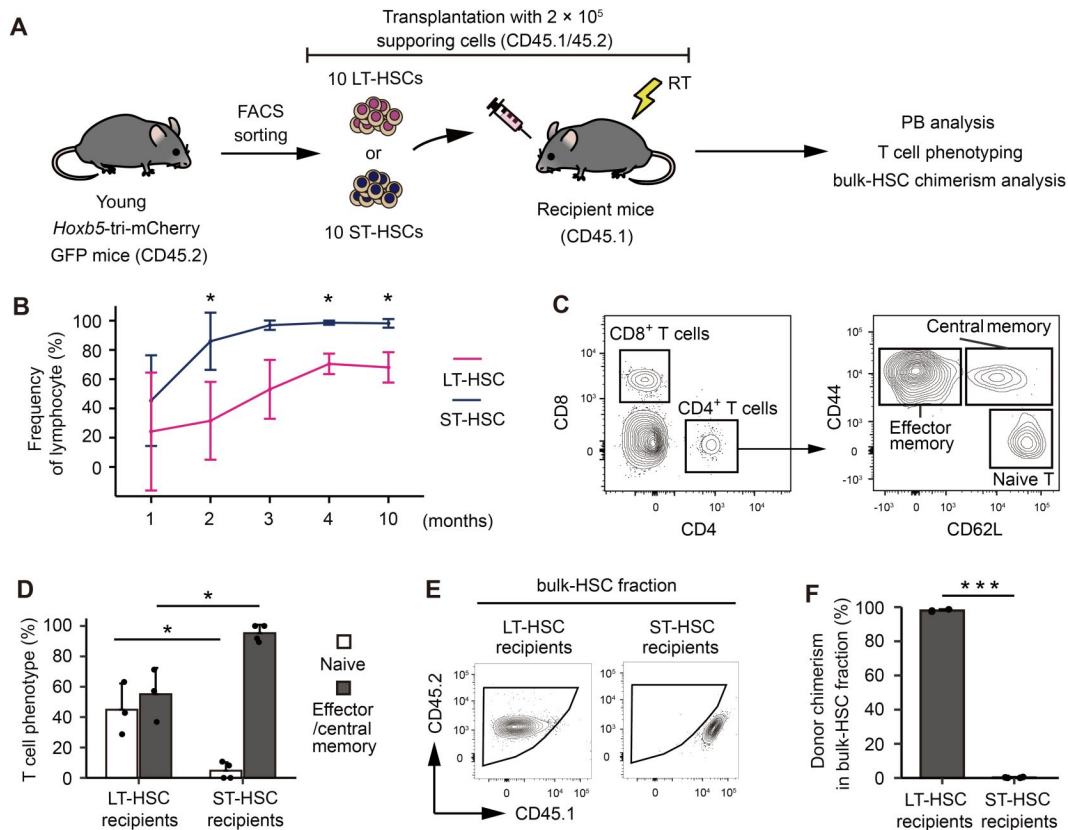


Figure 5.

The memory-type lymphocytes in the peripheral blood make it look as if ST-HSCs are lymphoid-biased HSCs

(A) Experimental design for assessing the lineage output of young LT-HSCs or ST-HSCs. Ten LT-HSCs, or ten ST-HSCs were isolated from 2-month-old CD45.2 *Hoxb5*-tri-mCherry GFP mice and were transplanted into lethally irradiated CD45.1 recipient mice with 2×10^5 supporting cells (LT-HSCs, $n = 3$; ST-HSCs, $n = 4$).

(B) Kinetics of average frequency of lymphoid cells (B cells, T cells, and NK cells) in donor fraction after LT-HSCs or ST-HSCs transplantation.

(C) Gating scheme to identify memory (Central and effector) T cells and naive T cells in the PB after excluding doublets, dead cells, and non-donor cells.

(D) Percentage of memory (Central and effector) T cells and naive T cells in donor CD4⁺ fraction 10 months after LT-HSC or ST-HSC transplantation.

(E) Gating scheme to identify donor cells in bulk-HSC fraction in bone marrow analysis.

(F) Donor chimerism in bulk-HSC fraction 12 months after LT-HSCs or ST-HSCs transplantation. $*P < 0.05$. $***P < 0.001$. Data and error bars represent means $\pm$ standard deviation.

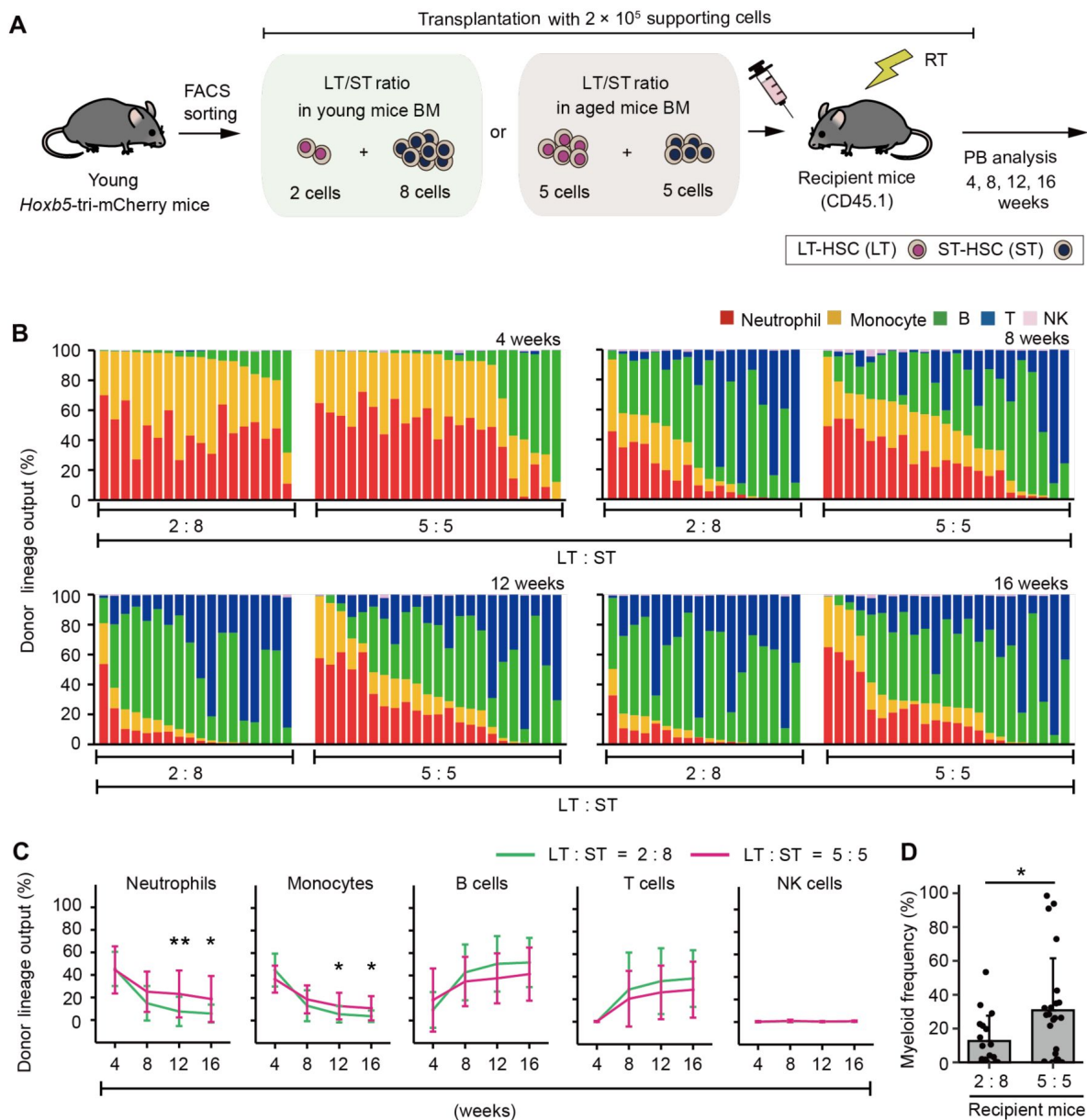


Figure 6.

Hematopoiesis after transplantation inclined either toward myeloid or lymphoid cell production by artificially changing the ratio of LT-HSC/ST-HSC

(A) Experimental design for the transplantation of 2-3-month-old LT-HSCs and ST-HSCs in a 2:8 ratio (the same ratio as in young mice BM) or 5:5 ratio (the same ratio as in aged mice BM). Donor cells were transplanted with 2×10^5 CD45.1⁺/CD45.2⁺ supporting cells into lethally irradiated CD45.1⁺ recipient mice (2:8 ratio, $n = 18$; 5:5 ratio, $n = 23$).

(B) Donor lineage output of young LT-HSCs and ST-HSCs transplanted either in a 2:8 ratio or a 5:5 ratio at 4, 8, 12, 16 weeks post-transplant. Each bar represents an individual mouse.

(C) Kinetics of average lineage output of young LT-HSCs and ST-HSCs in a 2:8 ratio or a 5:5 ratio at 4, 8, 12, 16 weeks post-transplant.

(D) Frequency of myeloid cells in donor cell fraction. * $P < 0.05$. ** $P < 0.01$. Error bars represent standard deviation. Data represent two independent experiments.

differ between young and aged recipients (**Fig. 7 F**). In contrast to previous studies (Pinho et al., 2018; Ergen et al., 2012), these findings suggested that intramedullary age-associated changes may not have a significant impact on LT-HSC differentiation.

Thymi and spleens of recipient mice were then examined, as these are the structures of lymphoid maturation and production of lymphoid progenitors following migration from BM and prior to their appearance in PB. Donor cells were not microscopically detectable in the majority of aged recipient mice (**Fig. 7, G and I**), and quantitative analysis using a flow cytometer revealed that the frequency of donor cells in spleens and thymi of aged recipients was significantly lower than in young recipients (**Fig. 7, H and J**). These results indicated that the process of lymphoid lineage differentiation was impaired in the spleens and thymi of aged mice compared to young mice, resulting in enhanced myeloid-biased hematopoiesis in PB due to a decrease in the *de novo* lymphocyte production.

Discussion

Age-related, myeloid-biased hematopoiesis has been suggested as a potential reason for the decline in acquired immunity in the elderly (Geiger et al., 2013). This process has been linked to an increase in myeloid-biased HSC clones in the bulk-HSC fraction identified by surface antigens through aging. By isolating LT-HSCs and ST-HSCs, respectively, we discovered that age-related changes in the ratio of ST-HSCs to LT-HSCs in bulk-HSCs is responsible for myeloid-biased hematopoiesis.

When aged bulk-HSCs were transplanted into young mice, post-transplant hematopoiesis in recipient mice was significantly biased toward myeloid in PB compared to transplantation of young bulk-HSCs (Beerman et al., 2010; Dykstra et al., 2011; Sudo et al., 2000). Historically, this myeloid-biased hematopoiesis derived from aged bulk-HSCs has been considered as evidence supporting expansion of myeloid-biased HSC clones with aging (Geiger et al., 2013; de Haan and Lazare 2018). Other studies, however, suggest that blockage of lymphoid hematopoiesis in aged mice results in myeloid-skewed hematopoiesis through alternative mechanisms (Montecino-Rodriguez et al., 2019). To clarify the cause for this disparity, we chose to examine the LT-HSC fraction, which persists in the BM for long periods of time and is enriched for aging-related alterations. We discovered that post-transplant hematopoiesis of aged LT-HSCs did not vary from that of young LT-HSCs in terms of differentiation capacity. Expression of myeloid-related genes was not significantly altered when LT-HSCs from young and aged mice were compared. Therefore, we inferred that age-related myeloid-biased hematopoiesis cannot be attributed to an increase in myeloid-biased HSCs, at least among LT-HSCs. Given that bulk-HSCs consist of LT-HSCs and ST-HSCs, and that LT-HSCs exhibit no change in differentiation potential with age, we hypothesized that ST-HSCs may be responsible for the myeloid-skewing phenotype in PB. Transcriptomic analysis revealed no enrichment of myeloid-related genes in the ST-HSC fraction, ruling out the possibility of an increase in myeloid-biased HSCs in the ST-HSC fraction. Alternatively, we observed a proportionate decrease in ST-HSCs in bulk-HSCs accompanying an increase in LT-HSCs. We postulated that this relative decrease in ST-HSC ratio is responsible for myeloid-biased hematopoiesis. When isolated LT-HSCs and ST-HSCs from young mice were reconstituted to replicate an aged BM type in terms of the LT-HSC/ST-HSC ratio, we discovered that PB was strongly skewed towards myeloid cells after transplantation of reconstituted HSCs. In contrast, transplantation of reconstituted HSCs with a young BM type resulted in a significantly lymphoid-skewed PB profile.

On the other hand, it has been reported that myeloid-biased HSCs have a more persistent capacity for self-renewal (Muller-Sieburg et al., 2004). In fact, in our prior investigation comparing PB 16 weeks post-transplant, myeloid cell production in PB was greater when LT-HSCs were transplanted, while ST-HSC transplantation resulted in a considerable increase in lymphoid cell

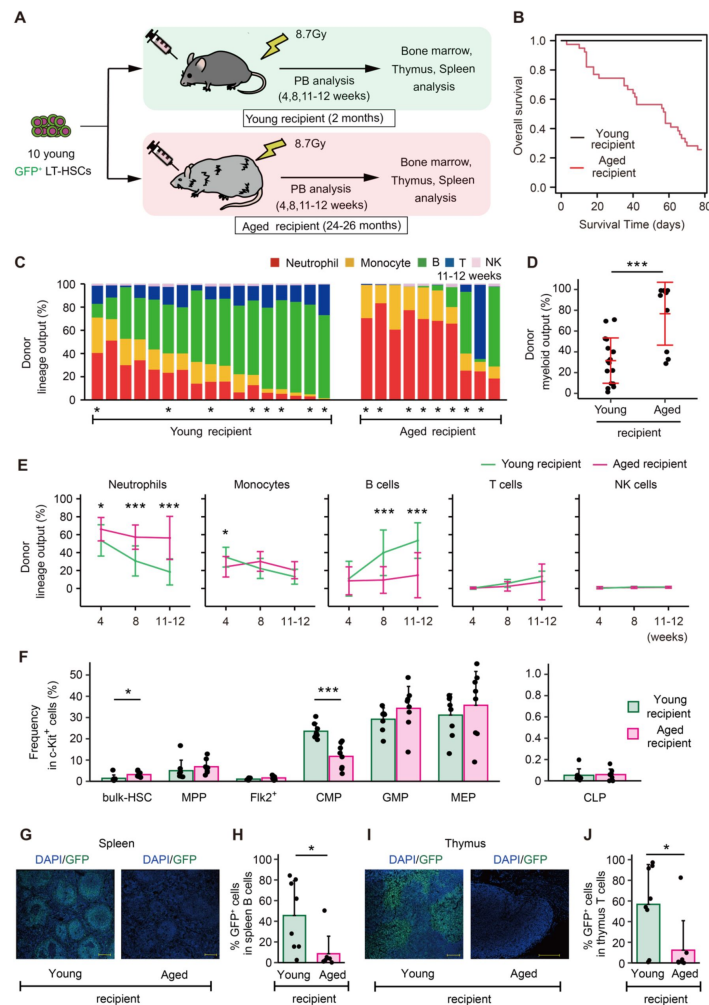


Figure 7.

Age-associated physiological changes drive differentiation of LT-HSCs toward myeloid cells

(A) Experimental design for assessing the impact of age-associated physiological changes on differentiation of LT-HSCs. Ten GFP⁺ LT-HSCs sorted from young (2-3 months) *Hoxb5*-tri-mCherry GFP mice, were transplanted with 2×10^5 CD45.1⁺/CD45.2⁺ supporting cells into lethally irradiated young or aged recipient mice. We defined donor cells as GFP⁺ cells and supporting cells as CD45.1⁺/CD45.2⁺ cells.

(B) Survival rate of recipient mice in each group.

(C) Donor lineage output in young or aged recipient mice 11-12 weeks after transplanting young LT-HSCs (young recipient, $n = 17$; aged recipient, $n = 10$).

(D) Myeloid output (Frequency of donor myeloid cells in donor fraction) in young or aged recipient mice 11-12 weeks after transplantation.

(E) Kinetics of lineage output from donor LT-HSCs in young or aged recipient mice 4, 8, 11-12 weeks after transplantation.

(F) Average frequency of donor bulk-HSC and progenitor cells in donor c-Kit⁺ cells in BM (young recipient, $n = 8$; aged recipient, $n = 8$). BM samples were taken from mice denoted by * in **Figure 7C**.

(G) Representative immunofluorescence images of frozen spleen sections derived from young or aged recipient mice. Green: donor cells (GFP fluorescence); blue: DNA (DAPI); Scale bar: 200 μ m.

(H) Frequency of donor cells in spleen B cells of young or aged recipient mice (young recipient, $n = 8$; aged recipient, $n = 8$). Spleens are taken from mice denoted by * in **Figure 7C**.

(I) Representative immunofluorescence images of frozen thymus sections derived from young or aged recipient mice. Green: donor cells (GFP fluorescence); blue: DNA (DAPI); Scale bar: 200 μ m.

(J) Frequency of donor cells in thymus T cells of young or aged recipient mice (young recipient, $n = 8$; aged recipient, $n = 8$). Thymi are taken from mice denoted by * in **Figure 7C**. * $P < 0.05$. *** $P < 0.001$. Error bars represent standard deviation. Data represent two independent experiments.

production (Chen et al., 2016 [DOI](#)). It is therefore possible that the LT-HSCs and ST-HSCs we isolated utilizing the Hoxb5 reporter system are myeloid- and lymphoid-biased HSCs, respectively. However, the myeloid-producing capacity of LT-HSCs and ST-HSCs 4 weeks post-transplantation is equivalent (Chen et al., 2016 [DOI](#)). In contrast, 16 weeks after transplantation, ST-HSC transplant recipients demonstrate negligible myeloid development and a predominance of lymphocytes, unlike LT-HSC transplant recipients (Chen et al., 2016 [DOI](#)). These characteristics show that early post-transplant ST-HSCs lack the phenotypic of lymphoid-biased HSCs. When ST-HSC-transplanted BM cells are employed as donors for secondary transplantation, no donor-cell-derived hematopoiesis is detected in recipients (Chen et al., 2016 [DOI](#)). In addition, HSCs are not observed in the BM following transplantation of ST-HSCs (Fig. 5 [DOI](#)). These results indicate that ST-HSCs lose their capacity for self-renewal earlier after transplantation than LT-HSCs. In addition, phenotypic examination of lymphocytes in PB after ST-HSC transplantation revealed that nearly all of them were memory cells, and that there was no *de novo* supply of T cells. In other words, ST-HSCs may have been erroneously classified as lymphoid-biased HSCs due to the preservation of memory lymphocytes with a long half-life in the peripheral circulation, despite the absence of *de novo* hematopoiesis following transplantation of ST-HSCs. These findings suggest that the bias toward myeloid and lymphoid lineages in post-transplant PB is not regulated by heterogeneity of multipotency, but by heterogeneity of self-renewal capacity.

Multiple extrinsic factors have been implicated as HSC-independent causes of myeloid-biased hematopoiesis associated with aging. Some findings suggest that cell interaction with niche cells in BM is altered (Pinho et al., 2018 [DOI](#)), whereas others claim that chronic inflammation in aged mice lowers production of lymphoid progenitors and induces myeloid-biased hematopoiesis (Montecino-Rodriguez et al., 2019 [DOI](#); Ergen et al., 2012 [DOI](#)). To identify HSC-independent causes of myeloid-biased hematopoiesis specifically, we transplanted young LT-HSCs into young or aged mice. Cellular differentiation observed in BM revealed that proportions of GMP, MEP, and CLP are unchanged between young and aged recipients. In contrast, an examination of spleens and thymi, the sites of lymphocyte maturation, revealed the absence of donor cells in aged recipients. These findings suggest that myeloid cells increase in aging mice due to a relative decrease in the ratio of mature lymphocytes in PB, resulting from inhibition of lymphocyte maturation outside the BM, such as in the thymus and spleen, rather than a significant change in cell differentiation caused by the intramedullary environment. Attempts have been made in the past to restore thymus function in aged mice by administering keratinocyte growth factor (KGF) (S. W. Rossi et al., 2007 [DOI](#)). Indeed, in aged mice, lymphocyte hematopoiesis recovers, which is consistent with our results that the extramedullary environment influences the myeloid-bias phenotype in PB (S. W. Rossi et al., 2007 [DOI](#)).

It has been clinically documented that recovery of the lymphocyte fraction in PB is delayed in elderly donors, compared to young donors (González-Vicent et al., 2017 [DOI](#); Baron et al., 2006 [DOI](#)). Although it is undeniable that this phenomenon may be due to decreased HSC hematopoietic potential, it may also be due to a change in the ratio of LT-HSC/ST-HSCs in bulk-HSCs, as described in our report, which may give the appearance of a myeloid bias in PB after transplantation. It has also been noted that lymphocyte recovery is delayed in elderly patients compared to younger patients (Berger et al., 2008 [DOI](#); van der Maas et al., 2021 [DOI](#)). Age-related loss in thymus and spleen functions has been recorded in humans, implying that the same mechanism is acting as in our mouse observations. If it is possible to assess and regulate the LT-HSC/ST-HSC ratio in donors and thymus/spleen function in recipients in the future, it will help to develop accurate models for predicting lymphocyte recovery after transplantation as well as new transplantation strategies.

In summary, we have demonstrated for the first time that the ratio of LT-HSC/ST-HSC is important in age-related myeloid skewed hematopoiesis as an HSC-dependent factor and that aging-related thymus and spleen dysfunction also contributes significantly as an HSC-independent factor. Furthermore, here we propose a “self-renewal heterogeneity model” as a new mechanism for hematopoietic heterogeneity, including myeloid-biased hematopoiesis in aged mice (Fig. 8 [DOI](#)). In

addition to this report, we have also shown that cellular differentiation in the PB after transplantation can vary by changing transplantation conditions, ultimately altering self-renewal ability (Sakamaki et al., 2021 [DOI](#); Nishi et al., 2022 [DOI](#)). This highlights the significance of deciphering molecular processes that regulate the heterogeneity of HSC self-renewal capacity, which will help to provide a better understanding of the hematopoietic system and the hematopoietic hierarchy in the future.

Methods

Mice

Mice with an *Hoxb5*-tri-mCherry (C57BL/6J background), derived from our previous work (Chen et al., 2016 [DOI](#)), were harvested for donor cells for transplantation, PB, and BM analysis. CAG-EGFP mice (C57BL/6J background) were bred with *Hoxb5*-tri-mCherry for transplantation experiments. 8-14-week-old C57BL/6-Ly5.1 mice (purchased from Sankyo Labo Service) were used as recipients for transplantation assays. For aged recipients, 2-year-old CD45.2 C57BL/6J mice were purchased from CLEA Japan. Supporting BM cells were collected from 8-12-week-old C57BL/6-Ly5.1 $\times$ C57BL/6J (F_1 mice CD45.1⁺/CD45.2⁺). All mice were housed in specific pathogen-free (SPF) conditions and were carefully observed by staff members. Mice were bred according to RIKEN or Kyoto University. All animal protocols were approved by the RIKEN Center for Biosystems Dynamics Research and Kyoto University.

Flow cytometry, cell sorting, and bone marrow analyses

Flow cytometry and cell sorting were performed on a FACS Aria II cell sorter (BD Biosciences) and analyzed using FlowJo™ software (BD Biosciences). Bone marrow (BM) cells were collected from bilateral tibias, femurs, humeri, and pelvises in Ca²⁺- and Mg²⁺-free PBS supplemented with 2% heat-inactivated bovine serum (Gibco) and 2 mM EDTA (Thermo Fisher Scientific). Cells were passed through 100- μ m, and 40- μ m strainers (Corning) before analysis and sorting. Prior to staining, samples were blocked with 50 μ g/mL rat IgG (Sigma-Aldrich) for 15 min. To enrich HSCs and progenitor populations for sorting and bone marrow analyses, cells were stained with APC-eFluor780-conjugated anti-c-Kit (clone: 2B8) and fractionated using anti-APC magnetic beads and LS columns (both Miltenyi Biotec). c-Kit⁺ cells were then stained with combinations of antibodies as described in Supplemental Table 1. All staining was incubated at 4°C for 30 min, except for CD34 staining, which was incubated for 90 min. Samples were washed twice after staining. Prior to flow cytometry or cell sorting, samples were stained with SYTOX Red Dead Cell Stain (Life Technologies) or 7-aminoactinomycin D (BioLegend). Cells were double sorted for purity.

Transplantation and peripheral blood analyses

12-24 hours prior to transplantation, C57BL/6-Ly5.1 mice, or aged C57BL/6J recipient mice were lethally irradiated with single doses of 8.7 Gy or 9.1Gy. For transplantation assays, donor cells were first combined with 2×10^5 whole bone marrow supporting cells (C57BL/6-Ly5.1 $\times$ C57BL/6J F_1 mice CD45.1⁺/CD45.2⁺) in 200 μ L of PBS with 2% FBS, and then injected into the retro-orbital venous plexus. For evaluation of post-transplant kinetics, PB was collected and analyzed. At each time point, 50 μ L of blood were collected from the tail vein and re-suspended in Ca²⁺- and Mg²⁺-free PBS supplemented with 2 mM EDTA. Red blood cells were lysed twice on ice for 3 min with BD Pharm Lyse Buffer (BD Pharmingen). Identification of leukocyte subsets was performed by staining with antibodies. Antibody information was described in Supplemental Table 1. For evaluation of lineage output, the frequency of each lineage (NK cell, B cell, T cell, neutrophil, and monocyte) was determined in the whole fraction. The analysis of donor lineage output was restricted to donor cells showing $\geq 0.1\%$ donor chimerism at the last PB analysis to allow reliable

Self-renewal heterogeneity model

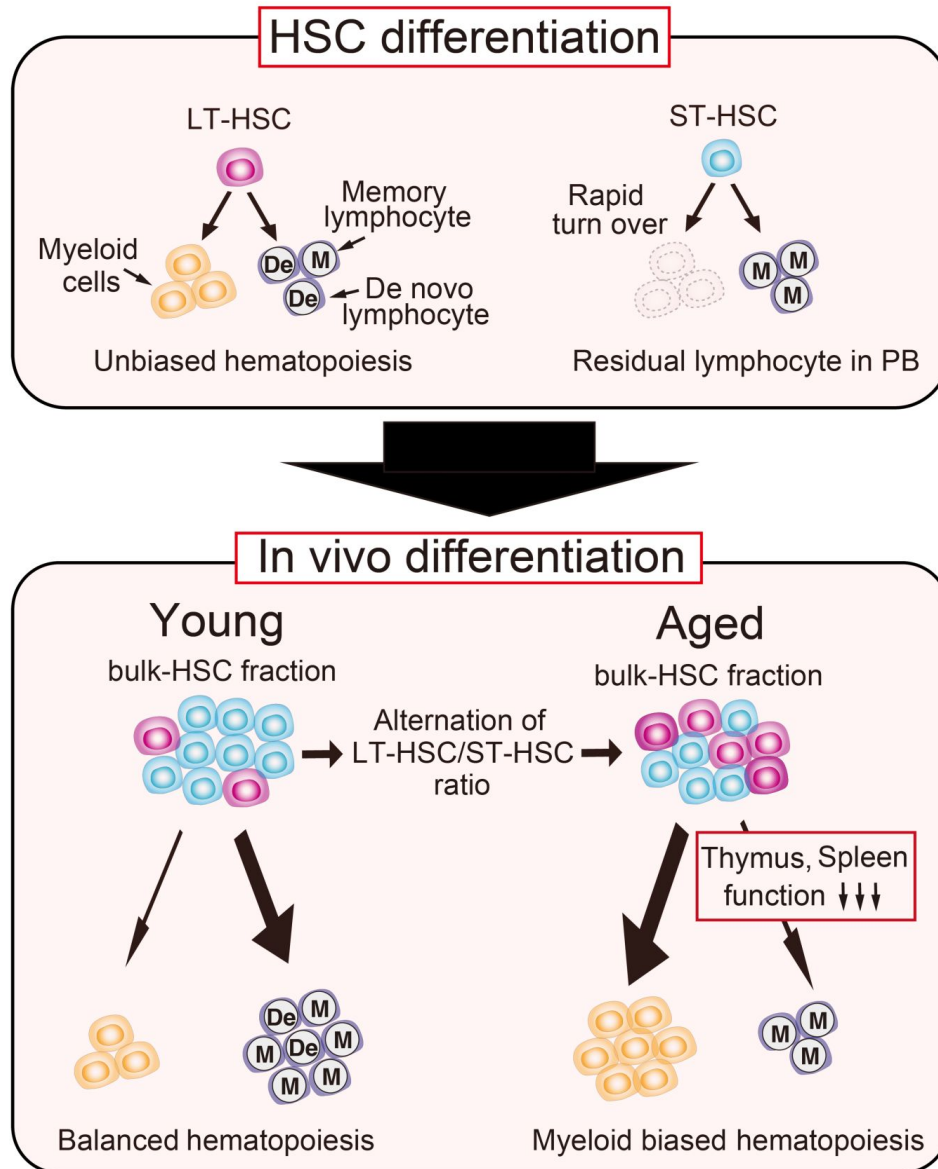


Figure 8.

Our new model: **Self-renewal heterogeneity model**. It has been thought that there were myeloid (My-) or lymphoid biased (Ly-) HSCs, and that clonal selection of My-HSCs caused age-associated myeloid biased hematopoiesis. However, in our model, LT-HSCs represent unbiased hematopoiesis throughout life. ST-HSCs lose their hematopoietic ability within a short period and memory-type lymphocytes remains in the PB after ST-HSC transplantation. These remaining memory-type lymphocytes make it look as if ST-HSCs are lymphoid-biased (The upper section). As a result, the age-associated relative decrease of ST-HSCs in bulk-HSC fraction causes myeloid biased hematopoiesis with age. Additionally, the blockage of lymphoid differentiation at spleen and thymus accelerates further myeloid biased hematopoiesis in aged mice (The lower section).

detection. The percentage of donor chimerism in PB was defined as the percentage of CD45.1⁻CD45.2⁺ cells among total CD45.1⁻CD45.2⁺ and CD45.1⁺CD45.2⁺ cells. PB data represents mice which survived until the last PB analysis.

Thymus and Spleen analysis

Thymi and spleens were harvested and disrupted into a single cell suspension. Cells were passed through 100-μm, and 40-μm strainers (Corning). Isolated thymocytes and splenocytes were incubated on ice for 30 min with appropriately diluted antibodies in staining buffer. Identification of B cells in spleens and T cells in thymi was performed by staining with antibodies described in Supplemental Table 1. Prior to flow cytometry or cell sorting, samples were stained with SYTOX Red Dead Cell Stain (Life Technologies).

Tissue imaging

Freshly dissected spleens and thymi were fixed in 4% PFA (Nacalai tesque) in PBS for 24 h at 4°C. After PFA washout with PBS, tissues were cryoprotected with 30% sucrose in PBS for 24 h at 4°C, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek), and snap-frozen in liquid nitrogen. Serial 10-μm longitudinal cryostat sections were obtained using CryoStar NX50 (Thermo Fisher Scientific). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL, Roche). To reduce autofluorescence, tissue sections were treated with Vector TrueVIEW autofluorescence quenching kit (Vector Laboratories). Images were obtained from Leica TCS SP8 (Leica).

RNA sequencing

Total RNA was isolated with Trizol (Thermo Fisher Scientific) and cleaned up using RNeasy MinElute columns (QIAGEN). cDNA libraries were prepared from bulk-HSCs, ST-HSCs and LT-HSCs using a KAPA RNA HyperPrep kit with RiboErase (HMR) (Kapa Biosystems) and sequenced using a HiSeq 1500 (Illumina) to obtain 2 × 127 base-pair (bp) paired-end reads. Three replicates were sequenced for each population. Raw transcriptome sequence data were mapped to the genome (mm10) using HISAT2 (ver 2.1.0) (Kim et al., 2015). Alignments were then passed to StringTie (ver 1.3.4d), which was used to assemble and quantify transcripts in each sample (Pertea et al., 2016). EdgeR (ver 3.22.5) was then used to compare all transcripts across conditions and to produce tables and plots of differentially expressed genes and transcripts (Robinson et al., 2009). Normalization with the TMM method was performed with the edgeR package in Bioconductor (<https://bioconductor.org/>). Genes (12,808 genes) were selected for hierarchical clustering based on the mean of TMM normalized read counts across all samples' cells (mean ≥ 1). Then, clustering was performed by using the hclust function for R (distance = Spearman's correlation; method = 'ward.D2'). We depicted Venn diagrams and performed GSEA analyses in **Figures 4D-G** and S4C-D using previously published gene sets after excluding genes that could not be annotated by our transcriptome dataset. GSEA was performed using GSEA software (<http://www.broadinstitute.org/gsea>) with default settings (Subramanian et al., 2005).

Quantification and statistical analyses

Statistical analyses were performed using ggplot2 in R (version 4.1.2) or Microsoft Excel. Sample size for each experiment and replicate numbers of experiments are included in figure legends. Statistical significance was determined using Welch's t test. *P* values < 0.05 were considered significant.

Data and materials availability

Sequencing data has been deposited in the Gene Expression Omnibus under accession code GSE226803. Correspondence and requests for materials should be addressed to M.M. (miya75@med.kobe-u.ac.jp).

Acknowledgements

We gratefully acknowledge Hiroshi Kiyonari for animal care and providing recipient mice at RIKEN BDR. Shigehiro Kuraku, Mitsutaka Kadota, and Chiharu Tanegashima provided technical support for RNA sequencing and analysis at RIKEN BDR. Momo Fujii performed imaging experiments at RIKEN BDR. Hitomi Oga, Kayoko Nagasaka, and Masaki Miyahashi provided laboratory management at Kobe University. The authors gratefully acknowledge ongoing support for this work: Masanori Miyanishi was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number JP17K07407 and JP20H03268, The Mochida Memorial Foundation for Medical and Pharmaceutical Research, The Life Science Foundation of Japan, The Takeda Science Foundation, The Astellas Foundation for Research on Metabolic Disorders, AMED-PRIME, AMED under Grant Number JP18gm6110020. Taro Sakamaki was supported by the JSPS Core-to-Core Program, JSPS KAKENHI Grant Numbers JP21K20669, JP22K16334, RIKEN Junior Research Associate Program. Katsuyuki Nishi was supported by JSPS Grant Numbers KAKENHI JP18J13408 and 23K15326.

Authorship Contributions

K.N. conceived, performed, analyzed the experiments. T.S. and K.S. performed transplantation experiments. M.M. conceived, performed, analyzed, and oversaw the experiments. K.N. and M.M. wrote the manuscript. T.S., A.N., K.S.K., K.S., M.A., N.Y., and A.T. provided comments on the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest in relation to the present study.

References

- Baron Frédéric, Storer Barry, Maris Michael B., Storek Jan, Piette Fanny, Metcalf Monja, White Kristen, et al. (2006) **Unrelated Donor Status and High Donor Age Independently Affect Immunologic Recovery after Nonmyeloablative Conditioning** *Biology of Blood and Marrow Transplantation* **12**:1176–87 <https://doi.org/10.1016/j.bbmt.2006.07.004>
- Beerman Isabel, Bhattacharya Deepta, Zandi Sasan, Sigvardsson Mikael, Weissman Irving L., Brydere David, Rossia Derrick J. (2010) **Functionally Distinct Hematopoietic Stem Cells Modulate Hematopoietic Lineage Potential during Aging by a Mechanism of Clonal Expansion** *Proceedings of the National Academy of Sciences of the United States of America* **107**:5465–70 <https://doi.org/10.1073/pnas.1000834107>
- Berger Massimo, Figari O., Bruno B., Raiola A., Dominiotto A., Fiorone M., Podesta M., et al. (2008) **Lymphocyte Subsets Recovery Following Allogeneic Bone Marrow Transplantation (BMT): CD4+ Cell Count and Transplant-Related Mortality** *Bone Marrow Transplantation* **41**:55–62 <https://doi.org/10.1038/sj.bmt.1705870>
- Busch Katrin, Klapproth Kay, Barile Melania, Flossdorf Michael, Holland-Letz Tim, Schlenner Susan M., Reth Michael, Höfer Thomas, Rodewald Hans Reimer (2015) **Fundamental Properties of Unperturbed Haematopoiesis from Stem Cells in Vivo** *Nature* **518**:542–46 <https://doi.org/10.1038/nature14242>
- Challen Grant A., Boles Nathan C., Chambers Stuart M., Goodell Margaret A. (2010) **Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF- β 1** *Cell Stem Cell* **6**:265–78 <https://doi.org/10.1016/j.stem.2010.02.002>
- Chambers Stuart M., Boles Nathan C., Lin Kuan Yin K., Tierney Megan P., Bowman Teresa V, Bradfute Steven B, Chen Alice J., et al. (2007) **Hematopoietic Fingerprints: An Expression Database of Stem Cells and Their Progeny** *Cell Stem Cell* **1**:578–91 <https://doi.org/10.1016/j.stem.2007.10.003>
- Chen James Y., Miyanishi Masanori, Wang Sean K., Yamazaki Satoshi, Sinha Rahul, Kao Kevin S., Seita Jun, Sahoo Debashis, Nakauchi Hiromitsu, Weissman Irving L. (2016) **Hoxb5 Marks Long-Term Haematopoietic Stem Cells and Reveals a Homogenous Perivascular Niche** *Nature* **530**:223–27 <https://doi.org/10.1038/nature16943>
- Cho Rebecca H., Sieburg Hans B., Muller-Sieburg Christa E. (2008) **A New Mechanism for the Aging of Hematopoietic Stem Cells: Aging Changes the Clonal Composition of the Stem Cell Compartment but Not Individual Stem Cells** *Blood* **111**:5553–61 <https://doi.org/10.1182/blood-2007-11-123547>
- Crooke Stephen N., Ovsyannikova Inna G., Poland Gregory A., Kennedy Richard B. (2019) **Immunosenescence and Human Vaccine Immune Responses** *Immunity & Ageing* **16**:1–16 <https://doi.org/10.1186/s12979-019-0164-9>
- Dorshkind Kenneth, Höfer Thomas, Montecino-Rodriguez Encarnacion, Pioli Peter D., Rodewald Hans Reimer (2020) **Do Haematopoietic Stem Cells Age?** *Nature Reviews Immunology* **20**:196–202 <https://doi.org/10.1038/s41577-019-0236-2>

Dykstra Brad, Olthof Sandra, Schreuder Jaring, Ritsema Martha, de Haan Gerald (2011) **Clonal Analysis Reveals Multiple Functional Defects of Aged Murine Hematopoietic Stem Cells** *The Journal of Experimental Medicine* **208**:2691–2703 <https://doi.org/10.1084/jem.20111490>

Dykstra Brad, Olthof Sandra, Schreuder Jaring, Ritsema Martha, De Haan Gerald (2011) **Clonal Analysis Reveals Multiple Functional Defects of Aged Murine Hematopoietic Stem Cells** *Journal of Experimental Medicine* **208**:2691–2703 <https://doi.org/10.1084/jem.20111490>

Ergen Aysegul V., Boles Nathan C., Goodell Margaret A. (2012) **Rantes/Ccl5 Influences Hematopoietic Stem Cell Subtypes and Causes Myeloid Skewing** *Blood* **119**:2500–2509 <https://doi.org/10.1182/blood-2011-11-391730>

Flohr Svendsen Arthur, Yang Daozheng, Kim Kyung Mok, Lazare Seka, Skinder Natalia, Zwart Erik, Mura-Meszaros Anna, et al. (2021) **A Comprehensive Transcriptome Signature of Murine Hematopoietic Stem Cell Aging** *Blood* **138**:439–51 <https://doi.org/10.1182/blood.2020009729>

Geiger Hartmut, De Haan Gerald, Carolina Florian M. (2013) **The Ageing Haematopoietic Stem Cell Compartment** *Nature Reviews Immunology* **13**:376–89 <https://doi.org/10.1038/nri3433>

González-Vicent Marta, Molina Blanca, Deltoro Natalia, Sevilla Julián, Vicario José Luis, Castillo Ana, Ramirez Manuel, Díaz Miguel Ángel (2017) **Donor Age Matters in T-Cell Depleted Haploidentical Hematopoietic Stem Cell Transplantation in Pediatric Patients: Faster Immune Reconstitution Using Younger Donors** *Leukemia Research* **57**:60–64 <https://doi.org/10.1016/j.leukres.2017.03.001>

Grover Amit, Sanjuan-Pla Alejandra, Thongjuea Supat, Carrelha Joana, Giustacchini Alice, Gambardella Adriana, Macaulay Iain, et al. (2016) **Single-Cell RNA Sequencing Reveals Molecular and Functional Platelet Bias of Aged Haematopoietic Stem Cells** *Nature Communications* **7** <https://doi.org/10.1038/ncomms11075>

Haan Gerald de, Lazare Seka Simone (2018) **Aging of Hematopoietic Stem Cells** *Blood* **131**:479–87 <https://doi.org/10.1182/blood-2017-06-746412>

Haan Gerald De, Lazare Seka Simone (2018) **Haan, Gerald De, and Seka Simone Lazare. 2018. “Aging of Hematopoietic Stem Cells.”** www.bloodjournal.org.

Jones D. Leanne, Rando Thomas A. (2011) **Emerging Models and Paradigms for Stem Cell Ageing** *Nature Cell Biology* **13**:506–12 <https://doi.org/10.1038/ncb0511-506>

Kim Daehwan, Langmead Ben, Salzberg Steven L. (2015) **HISAT: A Fast Spliced Aligner with Low Memory Requirements** *Nature Methods* **12**:357–60 <https://doi.org/10.1038/nmeth.3317>

Kowalczyk Monika S., Tirosh Itay, Heckl Dirk, Rao Tata Nageswara, Dixit Atray, Haas Brian J., Schneider Rebekka K., Wagers Amy J., Ebert Benjamin L., Regev Aviv (2015) **Single-Cell RNA-Seq Reveals Changes in Cell Cycle and Differentiation Programs upon Aging of Hematopoietic Stem Cells** *Genome Research* **25**:1860–72 <https://doi.org/10.1101/gr.192237.115>

Liberzon Arthur, Birger Chet, Thorvaldsdóttir Helga, Ghandi Mahmoud, Mesirov Jill P., Tamayo Pablo (2015) **The Molecular Signatures Database Hallmark Gene Set Collection** *Cell Systems* **1**:417–25 <https://doi.org/10.1016/j.cels.2015.12.004>

Maas Nicolaas G. van der, von Asmuth Erik G.J., Berghuis Dagmar, van Schouwenburg Pauline A., Putter Hein, van der Burg Mirjam, Lankester Arjan C. (2021) **Modeling Influencing Factors in B-Cell Reconstitution After Hematopoietic Stem Cell Transplantation in Children** *Frontiers in Immunology* **12**:1–8 <https://doi.org/10.3389/fimmu.2021.684147>

Majeti Ravindra, Park Christopher Y., Weissman Irving L. (2007) **Identification of a Hierarchy of Multipotent Hematopoietic Progenitors in Human Cord Blood** *Cell Stem Cell* **1**:635–45 <https://doi.org/10.1016/j.stem.2007.10.001>

Mann Mati, Mehta Arnav, de Boer Carl G, Kowalczyk Monika S, Lee Kevin, Haldeman Pearce, Rogel Noga, et al. (2018) **Heterogeneous Responses of Hematopoietic Stem Cells to Inflammatory Stimuli Are Altered with Age** *Cell Reports* **25**:2992–3005 <https://doi.org/10.1016/j.celrep.2018.11.056>

Mejia-Ramirez Eva, Florian Maria Carolina (2020) **Understanding Intrinsic Hematopoietic Stem Cell Aging** *Haematologica* **105**:22–37 <https://doi.org/10.3324/haematol.2018.211342>

Min Hyeyoung, Montecino-Rodriguez Encarnacion, Dorshkind Kenneth (2006) **Effects of Aging on the Common Lymphoid Progenitor to Pro-B Cell Transition** *The Journal of Immunology* **176**:1007–12 <https://doi.org/10.4049/jimmunol.176.2.1007>

Montecino-Rodriguez Encarnacion, Kong Ying, Casero David, Rouault Adrien, Dorshkind Kenneth, Pioli Peter D. (2019) **Lymphoid-Biased Hematopoietic Stem Cells Are Maintained with Age and Efficiently Generate Lymphoid Progeny** *Stem Cell Reports* **12**:584–96 <https://doi.org/10.1016/j.stemcr.2019.01.016>

Morrison Sean J., Weissman Irving L. (1994) **The Long-Term Repopulating Subset of Hematopoietic Stem Cells Is Deterministic and Isolatable by Phenotype** *Immunity* **1**:661–73 [https://doi.org/10.1016/1074-7613\(94\)90037-X](https://doi.org/10.1016/1074-7613(94)90037-X)

Morrison Sean J, Wandycz A M, Akashi K, Globerson A, Weissman Irving L (1996) **The Aging of Hematopoietic Stem Cells** *Nature Medicine* **2**:1011–16 <https://doi.org/10.1038/nm0996-1011>

Muller-Sieburg Christa E., Cho Rebecca H., Karlsson Lars, Huang Jing F., Sieburg Hans B. (2004) **Myeloid-Biased Hematopoietic Stem Cells Have Extensive Self-Renewal Capacity but Generate Diminished Lymphoid Progeny with Impaired IL-7 Responsiveness** *Blood* **103**:4111–18 <https://doi.org/10.1182/blood-2003-10-3448>

Nilsson Alexandra Rundberg, Soneji Shamit, Adolfsson Sofia, Bryder David, Pronk Cornelis Jan (2016) **Human and Murine Hematopoietic Stem Cell Aging Is Associated with Functional Impairments and Intrinsic Megakaryocytic/Erythroid Bias** *PLoS ONE* **11**:1–20 <https://doi.org/10.1371/journal.pone.0158369>

Nishi Katsuyuki, Sakamaki Taro, Sadaoka Kay, Fujii Momo, Takaori-Kondo Akifumi, Chen James Y., Miyanishi Masanori (2022) **Identification of the Minimum Requirements for Successful Haematopoietic Stem Cell Transplantation** *British Journal of Haematology* **196**:711–23 <https://doi.org/10.1111/bjh.17867>

Pang Wendy W., Price Elizabeth A., Sahoo Debashis, Beerman Isabel, Maloney William J., Rossi Derrick J., Schrier Stanley L., Weissman Irving L. (2011) **Human Bone Marrow Hematopoietic Stem Cells Are Increased in Frequency and Myeloid-Biased with Age** *Proceedings of the National Academy of Sciences of the United States of America* **108**:20012–17 <https://doi.org/10.1073/pnas.1116110108>

- Pertea Mihaela, Kim Daehwan, Pertea Geo M., Leek Jeffrey T., Salzberg Steven L. (2016) **Transcript-Level Expression Analysis of RNA-Seq Experiments with HISAT, StringTie and Ballgown** *Nature Protocols* **11**:1650–67 <https://doi.org/10.1038/nprot.2016.095>
- Pietras Eric M (2017) **Inflammation: A Key Regulator of Hematopoietic Stem Cell Fate in Health and Disease** *Blood* **130**:1693–98 <https://doi.org/10.1182/blood-2017-06-780882>
- Pinho Sandra, Marchand Tony, Yang Eva, Wei Qiaozhi, Nerlov Claus, Frenette Paul S. (2018) **Lineage-Biased Hematopoietic Stem Cells Are Regulated by Distinct Niches** *Developmental Cell* **44**:634–641 <https://doi.org/10.1016/j.devcel.2018.01.016>
- Pronk Cornelis J.H., Rossi Derrick J., Månsson Robert, Attema Joanne L., Norddahl Gudmundur Logi, Chan Charles Kwok Fai, Sigvardsson Mikael, Weissman Irving L., Bryder David (2007) **“Elucidation of the Phenotypic, Functional, and Molecular Topography of a Myeloerythroid Progenitor Cell Hierarchy.”** *Cell Stem Cell* **1**:428–42 <https://doi.org/10.1016/j.stem.2007.07.005>
- Robinson Mark D., McCarthy Davis J., Smyth Gordon K. (2009) **EdgeR: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data** *Bioinformatics* **26**:139–40 <https://doi.org/10.1093/bioinformatics/btp616>
- Rossi Derrick J., Bryder David, Seita Jun, Nussenzweig Andre, Hoeijmakers Jan, Weissman Irving L. (2007) **Deficiencies in DNA Damage Repair Limit the Function of Haematopoietic Stem Cells with Age** *Nature* **447**:725–29 <https://doi.org/10.1038/nature05862>
- Rossi Derrick J., Jamieson Catriona H.M., Weissman Irving L. (2008) **Stems Cells and the Pathways to Aging and Cancer** *Cell* **132**:681–96 <https://doi.org/10.1016/j.cell.2008.01.036>
- Rossi Derrick J., Bryder David, Zahn Jacob M, Ahlenius Henrik, Sonu Rebecca, Wagers Amy J, Weissman Irving L (2005) **Cell Intrinsic Alterations Underlie Hematopoietic Stem Cell Aging** *Proceedings of the National Academy of Sciences of the United States of America* **102**:9194–99 <https://doi.org/10.1073/pnas.0503280102>
- Rossi Simona W., Jeker Lukas T., Ueno Tomoo, Kuse Sachiyo, Keller Marcel P., Zuklys Saulius, Gudkov Andrei V., et al. (2007) **Keratinocyte Growth Factor (KGF) Enhances Postnatal T-Cell Development via Enhancements in Proliferation and Function of Thymic Epithelial Cells** *Blood* **109**:3803–11 <https://doi.org/10.1182/blood-2006-10-049767>
- Sakamaki Taro, Kao Kevin S., Nishi Katsuyuki, Chen James Y., Sadaoka Kay, Fujii Momo, Takaori-Kondo Akifumi, Weissman Irving L., Miyanishi Masanori (2021) **“Hoxb5 Defines the Heterogeneity of Self-Renewal Capacity in the Hematopoietic Stem Cell Compartment.”** *Biochemical and Biophysical Research Communications* **539**:34–41 <https://doi.org/10.1016/j.bbrc.2020.12.077>
- Sanjuan-Pla Alejandra, Macaulay Iain C., Jensen Christina T., Woll Petter S., Luis Tiago C., Mead Adam, Moore Susan, et al. (2013) **Platelet-Biased Stem Cells Reside at the Apex of the Haematopoietic Stem-Cell Hierarchy** *Nature* **502**:232–36 <https://doi.org/10.1038/nature12495>
- SanMiguel Jennifer M., Young Kira, Trowbridge Jennifer J. (2020) **Hand in Hand: Intrinsic and Extrinsic Drivers of Aging and Clonal Hematopoiesis** *Experimental Hematology* **91**:1–9 <https://doi.org/10.1016/j.exphem.2020.09.197>

- Schultz Michael B., Sinclair David A. (2016) **When Stem Cells Grow Old: Phenotypes and Mechanisms of Stem Cell Aging** *Development (Cambridge)* **143**:3–14 <https://doi.org/10.1242/dev.130633>
- Spangrude Gerald J., Brooks Diane M., Tumas Daniel B. (1995) **Long-Term Repopulation of Irradiated Mice with Limiting Numbers of Purified Hematopoietic Stem Cells: In Vivo Expansion of Stem Cell Phenotype but Not Function** *Blood* **85**:1006–16 <https://doi.org/10.1182/blood.v85.4.1006.bloodjournal8541006>
- Spangrude Gerald J., Heimfeld Shelly, Weissman Irving L (1988) **“Purification and Characterization of Mouse Hematopoietic Stem Cells.”** *Science (New York N.Y)* **241**:58–62 <https://doi.org/10.1126/science.2898810>
- Subramanian Aravind, Tamayo Pablo, Mootha Vamsi K., Mukherjee Sayan, Ebert Benjamin L., Gillette Michael A., Paulovich Amanda, et al. (2005) **Gene Set Enrichment Analysis: A Knowledge-Based Approach for Interpreting Genome-Wide Expression Profiles** *Proceedings of the National Academy of Sciences of the United States of America* **102**:15545–50 <https://doi.org/10.1073/pnas.0506580102>
- Sudo K, Ema H, Morita Y, Nakauchi H (2000) **Age-Associated Characteristics of Murine Hematopoietic Stem Cells [In Process Citation]** *J Exp Med* **192**:1273–80
- Webster Robert G (2000) **Immunity to Influenza in the Elderly** *Vaccine* **18**:1686–89 [https://doi.org/10.1016/S0264-410X\(99\)00507-1](https://doi.org/10.1016/S0264-410X(99)00507-1)
- Weissman Irving L., Shizuru Judith A. (2008) **The Origins of the Identification and Isolation of Hematopoietic Stem Cells, and Their Capability to Induce Donor-Specific Transplantation Tolerance and Treat Autoimmune Diseases** *Blood* **112**:3543–53 <https://doi.org/10.1182/blood-2008-08-078220>

Article and author information

Katsuyuki Nishi

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan, RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan

Taro Sakamaki

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan, RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan

Akiomi Nagasaka

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan, RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan

Kevin S. Kao

Weill Cornell, Rockefeller, Sloan-Kettering, Tri-Institutional MD-PhD Program, New York, NY

Kay Sadaoka

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan, RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan

Masahide Asano

Institute of Laboratory Animals, Kyoto University Graduate School of Medicine, Kyoto, Kyoto, 606-8501, Japan

Nobuyuki Yamamoto

Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017, Japan

ORCID iD: [0000-0002-1006-7394](https://orcid.org/0000-0002-1006-7394)

Akifumi Takaori-Kondo

Department of Hematology and Oncology, Kyoto University Graduate School of Medicine, Kyoto, Kyoto 606-8507, Japan

ORCID iD: [0000-0001-7678-4284](https://orcid.org/0000-0001-7678-4284)

Masanori Miyanishi

Hematopoietic Stem Cell Biology and Medical Innovation (HSCBMI), Department of Pediatrics, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0047, Japan, RIKEN Center for Biosystems Dynamics Research, Kobe, Hyogo 650-0047, Japan

For correspondence: miya75@med.kobe-u.ac.jp

Copyright

© 2024, Nishi et al.

This article is distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use and redistribution provided that the original author and source are credited.

Editors

Reviewing Editor

Jian Xu

St. Jude Children's Research Hospital, Memphis, United States of America

Senior Editor

Utpal Banerjee

University of California, Los Angeles, Los Angeles, United States of America

Reviewer #1 (Public Review):

Summary:

In this study, Nishi et al. claim that the ratio of long-term hematopoietic stem cell (LT-HSC) versus short-term HSC (ST-HSC) determines the lineage output of HSCs and reduced ratio of ST-HSC in aged mice causes myeloid-biased hematopoiesis. The authors used Hoxb5 reporter mice to isolate LT-HSC and ST-HSC and performed molecular analyses and transplantation assays to support their arguments. How the hematopoietic system becomes myeloid-biased

upon aging is an important question with many implications in the disease context as well. However, their study is descriptive with remaining questions.

Weaknesses:

(1) The authors may need conceptual re-framing of their main argument because whether the ST-HSCs used in this study are functionally indeed short-term "HSCs" is questionable. The data presented in this study and their immunophenotypic definition of ST-HSCs (Lineage negative/Sca-1+/c-Kit+/Flk2-/CD34-/CD150+/Hoxb5-) suggest that authors may find hematopoietic stem cell-like lymphoid progenitors as previously shown for megakaryocyte lineage (Haas et al., Cell stem cell. 2015) or, as the authors briefly mentioned in the discussion, Hoxb5- HSCs could be lymphoid-biased HSCs. The authors disputed the idea that Hoxb5- HSCs as lymphoid-biased HSCs based on their previous 4 weeks post-transplantation data (Chen et al., 2016). However, they overlooked the possibility of myeloid reprogramming of lymphoid-biased population during regenerative conditions (Pietras et al., Cell stem cell., 2015). In other words, early post-transplant ST-HSCs (Hoxb5- HSCs) can be seen as lacking the phenotypic lymphoid-biased HSCs. Thinking of their ST-HSCs as hematopoietic stem cell-like lymphoid progenitors or lymphoid-biased HSCs makes more sense conceptually as well. ST-HSCs come from LT-HSCs and further differentiate into lineage-biased multipotent progenitor (MPP) populations including myeloid-biased MPP2 and MPP3. Based on the authors' claim, LT-HSCs (Hoxb5- HSCs) have no lineage bias even in aged mice. Then these LT-HSCs make ST-HSCs, which produce mostly memory T cells. These memory T cell-producing ST-HSCs then produce MPPs including myeloid-biased MPP2 and MPP3. This differentiation trajectory is hard to accept. If we think Hoxb5- HSCs (ST-HSCs by authors) as a sub-population of immunophenotypic HSCs with lymphoid lineage bias or hematopoietic stem cell-like lymphoid progenitors, the differentiation trajectory has no flaw.

(2) Authors' experimental designs have some caveats to support their claims. Authors claimed that aged LT-HSCs have no myeloid-biased clone expansion using transplantation assays. In these experiments, authors used 10 HSCs and young mice as recipients. Given the huge expansion of old HSC by number and known heterogeneity in immunophenotypically defined HSC populations, it is questionable how 10 out of so many old HSCs can faithfully represent the old HSC population. The Hoxb5+ old HSC primary and secondary recipient mice data (Figure 2C and D) support this concern. In addition, they only used young recipients. Considering the importance of the inflammatory aged niche in the myeloid-biased lineage output, transplanting young vs old LT-HSCs into aged mice will complete the whole picture.

(3) The authors' molecular data analyses need more rigor with unbiased approaches. They claimed that neither aged LT-HSCs nor aged ST-HSCs exhibited myeloid or lymphoid gene set enrichment but aged bulk HSCs, which are just a sum of LT-HSCs and ST-HSCs by their gating scheme (Figure 4A), showed the "tendency" of enrichment of myeloid-related genes based on the selected gene set (Figure 4D). Although the proportion of ST-HSCs is reduced in bulk HSCs upon aging, since ST-HSCs do not exhibit lymphoid gene set enrichment based on their data, it is hard to understand how aged bulk HSCs have more myeloid gene set enrichment compared to young bulk HSCs. This bulk HSC data rather suggests that there could be a trend toward certain lineage bias (although not significant) in aged LT-HSCs or ST-HSCs. The authors need to verify the molecular lineage priming of LT-HSCs and ST-HSCs using another comprehensive dataset.

(4) Some data are too weak to fully support their claims. The authors claimed that age-associated extramedullary changes are the main driver of myeloid-biased hematopoiesis based on no major differences in progenitor populations upon transplantation of 10 young HSCs into young or old recipient mice (Figure 7F) and relatively low donor-derived cells in thymus and spleen in aged recipient mice (Figure 7G-J). However, they used selected mice to calculate the progenitor populations in recipient mice (8 out of 17 from young recipients denoted by * and 8 out of 10 from aged recipients denoted by * in Figure 7C). In addition, they

calculated the progenitor populations as frequency in c-kit positive cells. Given that they transplanted 10 LT-HSCs into "sub-lethally" irradiated mice and 8.7 Gy irradiation can have different effects on bone marrow clearance in young vs old mice, it is not clear whether this data is reliable enough to support their claims. The same concern applies to the data Figure 7G-J. Authors need to provide alternative data to support their claims.

<https://doi.org/10.7554/eLife.95880.1.sa2>

Reviewer #2 (Public Review):

Summary:

Nishi et al, investigate the well-known and previously described phenomenon of age-associated myeloid-biased hematopoiesis. Using a previously established HoxB5mCherry mouse model, they used HoxB5⁺ and HoxB5⁻ HSCs to discriminate cells with long-term (LT-HSCs) and short-term (ST-HSCs) reconstitution potential and compared these populations to immunophenotypically defined 'bulk HSCs' that consists of a mixture of LT-HSC and ST-HSCs. They then isolated these HSC populations from young and aged mice to test their function and myeloid bias in non-competitive and competitive transplants into young and aged recipients. Based on quantification of hematopoietic cell frequencies in the bone marrow, peripheral blood, and in some experiments the spleen and thymus, the authors argue against the currently held belief that myeloid-biased HSCs expand with age.

While aspects of their work are fascinating and might have merit, several issues weaken the overall strength of the arguments and interpretation. Multiple experiments were done with a very low number of recipient mice, showed very large standard deviations, and had no statistically detectable difference between experimental groups. While the authors conclude that these experimental groups are not different, the displayed results seem too variable to conclude anything with certainty. The sensitivity of the performed experiments (e.g. Figure 3; Figure 6C, D) is too low to detect even reasonably strong differences between experimental groups and is thus inadequate to support the author's claims. This weakness of the study is not acknowledged in the text and is also not discussed. To support their conclusions the authors need to provide higher n-numbers and provide a detailed power analysis of the transplants in the methods section.

As the authors attempt to challenge the current model of the age-associated expansion of myeloid-biased HSCs (which has been observed and reproduced by many different groups), ideally additional strong evidence in the form of single-cell transplants is provided.

It is also unclear why the authors believe that the observed reduction of ST-HSCs relative to LT-HSCs explains the myeloid-biased phenotype observed in the peripheral blood. This point seems counterintuitive and requires further explanation.

Based on my understanding of the presented data, the authors argue that myeloid-biased HSCs do not exist, as

a) they detect no difference between young/aged HSCs after transplant (mind low n-numbers and large std!); b) myeloid progenitors downstream of HSCs only show minor or no changes in frequency and c) aged LT-HSCs do not outperform young LT-HSC in myeloid output LT-HSCs in competitive transplants (mind low n-numbers and large std!).

However, given the low n-numbers and high variance of the results, the argument seems weak and the presented data does not support the claims sufficiently. That the number of downstream progenitors does not change could be explained by other mechanisms, for instance, the frequently reported differentiation short-cuts of HSCs and/or changes in the microenvironment.

Strengths:

The authors present an interesting observation and offer an alternative explanation of the origins of aged-associated myeloid-biased hematopoiesis. Their data regarding the role of the microenvironment in the spleen and thymus appears to be convincing.

Weaknesses:

"Then, we found that the myeloid lineage proportions from young and aged LT-HSCs were nearly comparable during the observation period after transplantation (Figure 3, B and C)." Given the large standard deviation and low n-numbers, the power of the analysis to detect differences between experimental groups is very low. Experimental groups with too large standard deviations (as displayed here) are difficult to interpret and might be inconclusive. The absence of clearly detectable differences between young and aged transplanted HSCs could thus simply be a false-negative result. The shown experimental results hence do not provide strong evidence for the author's interpretation of the data. The authors should add additional transplants and include a detailed power analysis to be able to detect differences between experimental groups with reasonable sensitivity.

Line 293: "Based on these findings, we concluded that myeloid-biased hematopoiesis observed following transplantation of aged HSCs was caused by a relative decrease in ST-HSC in the bulk-HSC compartment in aged mice rather than the selective expansion of myeloid-biased HSC clones."

Couldn't that also be explained by an increase in myeloid-biased HSCs, as repeatedly reported and seen in the expansion of CD150+ HSCs? It is not intuitively clear why a reduction of ST-HSCs clones would lead to a myeloid bias. The author should try to explain more clearly where they believe the increased number of myeloid cells comes from. What is the source of myeloid cells if the authors believe they are not derived from the expanded population of myeloid-biased HSCs?

<https://doi.org/10.7554/eLife.95880.1.sa1>

Reviewer #3 (Public Review):

In this manuscript, Nishi et al. propose a new model to explain the previously reported myeloid-biased hematopoiesis associated with aging. Traditionally, this phenotype has been explained by the expansion of myeloid-biased hematopoietic stem cell (HSC) clones during aging. Here, the authors question this idea and show how their *Hoxb5* reporter model can discriminate long-term (LT) and short-term (ST) HSC and characterized their lineage output after transplant. From these analyses, the authors conclude that changes during aging in the LT/ST HSC proportion explain the myeloid bias observed.

Although the topic is appropriate and the new model provides a new way to think about lineage-biased output observed in multiple hematopoietic contexts, some of the experimental design choices, as well as some of the conclusions drawn from the results could be substantially improved. Also, they do not propose any potential mechanism to explain this process, which reduces the potential impact and novelty of the study. Specific concerns are outlined below.

Major

(1) As a general comment, there are experimental details that are either missing or not clear. The main one is related to transplantation assays. What is the irradiation dose? The Methods sections indicates "recipient mice were lethally irradiated with single doses of 8.7 or 9.1 Gy". The only experimental schematic indicating the irradiation dose is Figure 7A, which uses 8.7

Gy. Also, although there is not a "standard", 11 Gy split in two doses is typically considered lethal irradiation, while 9.5 Gy is considered sublethal. Is there any reason for these lower doses? Same question for giving a single dose and for performing irradiation a day before transplant.

(2) The manuscript would benefit from the inclusion of references to recent studies discussing hematopoietic biases and differentiation dynamics at a single-cell level (e.g., Yamamoto et al. 2018; Rodriguez-Fraticelli et al., 2020). Also, when discussing the discrepancy between studies claiming different biases within the HSC pool, the authors mentioned that Montecino-Rodriguez et al. 2019 showed preserved lymphoid potential with age. It would be good to acknowledge that this study used busulfan as the conditioning method instead of irradiation.

(3) When representing the contribution to PB from transplanted cells, the authors show the % of each lineage within the donor-derived cells (Figures 3B-C, 5B, 6B-D, 7C-E, and S3 B-C). To have a better picture of total donor contribution, total PB and BM chimerism should be included for each transplantation assay. Also, for Figures 2C-D and Figures S2A-B, do the graphs represent 100% of the PB cells? Are there any radioresistant cells?

(4) For BM progenitor frequencies, the authors present the data as the frequency of cKit⁺ cells. This normalization might be misleading as changes in the proportion of cKit⁺ between the different experimental conditions could mask differences in these BM subpopulations. Representing this data as the frequency of BM single cells or as absolute numbers (e.g., per femur) would be valuable.

(5) Regarding Figure 1B, the authors argue that if myeloid-biased HSC clones increase with age, they should see increased frequency of all components of the myeloid differentiation pathway (CMP, GMP, MEP). This would imply that their results (no changes or reduction in these myeloid subpopulations) suggest the absence of myeloid-biased HSC clones expansion with age. This reviewer believes that differentiation dynamics within the hematopoietic hierarchy can be more complex than a cascade of sequential and compartmentalized events (e.g., accelerated differentiation at the CMP level could cause exhaustion of this compartment and explain its reduction with age and why GMP and MEP are unchanged) and these conclusions should be considered more carefully.

(6) Within the few recipients showing good donor engraftment in Figure 2C, there is a big proportion of T cells that are "amplified" upon secondary transplantation (Figure 2D). Is this expected?

(7) Do the authors have any explanation for the high level of variability within the recipients of Hoxb5⁺ cells in Figure 2C?

(8) Can the results from Figure 2E be interpreted as Hoxb5⁺ cells having a myeloid bias? (differences are more obvious/significant in neutrophils and monocytes).

(9) Is Figure 2G considering all primary recipients or only the ones that were used for secondary transplants? The second option would be a fairer comparison.

(10) When discussing the transcriptional profile of young and aged HSCs, the authors claim that genes linked to myeloid differentiation remain unchanged in the LT-HSC fraction while there are significant changes in the ST-HSCs. However, 2 out of the 4 genes shown in Figure S4B show ratios higher than 1 in LT-HSCs.

(11) When determining the lymphoid bias in ST-HSCs, the authors focus on the T-cell subtype, not considering any other lymphoid population. Could the authors explain this?

(12) Based on the reduced frequency of donor cells in the spleen and thymus, the authors conclude "the process of lymphoid lineage differentiation was impaired in the spleens and thymi of aged mice compared to young mice". An alternative explanation could be that differentiated cells do not successfully migrate from the bone marrow to these secondary lymphoid organs. Please consider this possibility when discussing the data.

<https://doi.org/10.7554/eLife.95880.1.sa0>