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Assessing The Role of Spleen in Development of Myeloid Bias Amongst Haematopoietic Stem Cells

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Abstract

Myeloproliferative disorders are common in aged individuals, and ageing is associated with a skewing of haematopoiesis towards myeloid lineage. The spleen houses a distinct resident population of HSC primed for myelopoiesis which retain this myeloid bias even after migration to bone marrow niches. The importance of spleen in the phenotype of myeloid bias with ageing has therefore been investigated here.

Two animal models were studied using $CD150^{hi}$ expression as an indicator of long term (LT)-HSC with a myeloid-biased phenotype. A steady-state model investigated a role for spleen in the age-related increase in myeloid-biased HSC by comparing splenectomised aged female mice with age-matched normal animals. This study showed no significant reduction in the proportion of $CD150^{hi}$ myeloid-biased LT-HSC in splenectomised aged mice compared with age-matched controls, discounting a role for spleen. The second model tested whether extramedullary haematopoiesis in spleen during pregnancy accelerates the redistribution of HSC and increases the myeloid-bias amongst bone marrow LT-HSC. The model utilised exbreeder mice were tested and shown to contain higher numbers of LT-HSC in bone marrow than did age-matched controls, indicating an important role for pregnancy in myeloid bias. The importance of spleen on myeloid bias detected in exbreeder mice was also tested. Young female mice were splenectomised and then exposed to multiple rounds of pregnancy. However, no significant role for spleen was indicated in myeloid bias. A further study compared the number of migratory $CD150^{hi}$ myeloid biased LT-HSC in bone marrow in young and old mice, and in old mice which had undergone splenectomy. The results showed that old mice had no more $CCR2^{+}$ LT-HSC than young mice, and no significant difference was detected between old and splenectomised old mice, indicating that spleen was not important in expanding the number of migratory $CCR2^{+}$ LT-HSC.

This study identifies a subset of quiescent, bone marrow resident primitive LT-HSC which acquire myeloid bias with ageing. These are present in higher number in old mice, and in exbreeder mice which have undergone multiple rounds of pregnancy leading to extramedullary hematopoiesis. Data shown here also indicate that spleen plays no significant role in the maintenance of this subset in bone marrow or in the peripheral migration of these cells.

Keywords: *Haematopoiesis, haematopoietic stem cells, spleen, bone marrow, ageing, myeloid bias*

Introduction

Myeloid bias in ageing HSC

Haematopoietic stem cells (HSC) can possess distinct differentiation outputs, such as balanced haematopoiesis as well as haematopoiesis which is biased towards production of myeloid cells, lymphoid cells or platelets [1]. Over the lifespan of an individual, a gradual shift away from balanced haematopoietic output to predominantly myeloid output is known as myeloid bias [2, 3]. This increase in myelopoiesis can be attributed to either an increase in the number of myeloid-biased HSC or an increase in the number of myeloid cells produced per individual HSC [4]. The appearance of increased numbers of myeloid-biased HSC with high self-renewal capacity is considered a

central component of ageing [5]. Transplantation studies are a powerful tool to investigate the effects of ageing on HSC. It has been demonstrated that aged HSC have impaired reconstitution ability in secondary transplantations and exhibit decreased haematopoietic recovery [6]. In aged animals, HSC number and cellular activity is increased compared to younger mice [7], and this may represent a compensatory mechanism for the reduced functions of HSC in homing and engraftment with age.

Gene expression profiling has shown that LT-HSC ageing is accompanied by systemic downregulation of genes mediating lymphoid differentiation and function, coupled with an upregulation of myeloid fate and function-specific genes [8]. These changes are carried downstream, with loss of lymphoid-primed multipotent progenitors, and a corresponding increase in myeloid progenitors over time [9]. Transplantation of aged HSC results in increased myeloid engraftment coupled with impaired lymphoid engraftment [7], and this is observed for both young and old transplant recipients [10]. Conversely, young HSC transplanted into an aged microenvironment have been shown to produce more myeloid cells than those located in a young microenvironment, suggesting a role for the niche in myeloid bias observed in aged HSC [9]. Altogether, these studies provide evidence for an increase in myeloid-biased HSC and increased myelopoiesis with age, and clearly demonstrate that HSC ageing can be driven by a combination of cell-intrinsic changes and external microenvironmental signals.

Extrinsic mechanisms contribute to myeloid bias

Stem cell niches regulate HSC survival and function and may contribute to changes in HSC output with ageing. The bone marrow microenvironment is comprised of supporting cell populations including mesenchymal stromal cells, endothelial cells, osteogenic cells, monocytes, macrophages and megakaryocytes [11]. The extracellular matrix acts as an anchor point for HSC in the stem cell niche, and blood vessels enable delivery of nutrients and signals to the niche. Input from the sympathetic nervous system facilitates stem cell mobilisation and demonstrates how neuronal cues are crucial to HSC trafficking [12].

Several studies have now demonstrated how extrinsic bone marrow microenvironmental signals, in particular osteopontin (OPN) and insulin-like growth factor 1 (IGF-1), drive age-associated myeloid bias in HSC. Niche-supporting bone marrow stromal cells in aged mice have decreased OPN expression, and this is directly linked with the aged HSC phenotype [13]. An OPN-deficient mouse model was used to demonstrate directly that OPN impacts primitive hematopoietic stem and progenitor cells (HSPC) with increased numbers in an OPN-deficient environment [14]. OPN knockout models also demonstrate decreased HSC engraftment [15], and loss of apolar distribution of CDC42 and tubulin proteins [13]. Transplantation of aged HSC into a young donor can attenuate the effects of ageing by restoring HSC frequency, protein polarity and lineage differentiation. Treatment of old HSC with OPN fraction D rescues protein polarity status to the level of young HSC [13]. The ageing phenotype is also associated with decreased IGF-1 expression in the bone marrow microenvironment. IGF-1 regulates HSC through PKA signalling and deletion of the IGF-1 receptor on HSC [15, 16], so that transplantation of HSC into IGF-1-deficient mice results in premature acquisition of myeloid bias [17]. Direct stimulation of middle-aged HSC with IGF-1 restores molecular and functional hallmarks of ageing including mitochondrial network connectivity and membrane potential. Transplantation of middle-aged HSC into young animals also reduces their myeloid bias [18]. These various studies highlight the effect of extrinsic signals on HSC age and functional potential.

The splenic microenvironment inherently supports myelopoiesis

HSC localised in spleen are considered long-term residents rather than circulatory cells transiting the spleen. This was evidenced by parabiosis experiments wherein only small numbers of bone marrow and spleen HSC exchanged places with circulating HSC [19]. While it has been suggested that spleen-resident and bone marrow-resident HSC may be functionally similar [20], the number of HSC in spleen is lower than in bone marrow [21]. However, both cell types have similar activity levels, although spleen HSC are primed to enter cell cycle twice as quickly as their bone marrow counterparts. The spleen may thus serve as a reservoir for HSC for relatively immediate use consistent with their role in emergency haematopoiesis [20].

Bone marrow HSC are capable of multi-lineage differentiation. In contrast, splenic HSC appear to be myeloid-biased. Splenic HSC transplanted into external hosts display favoured myeloid differentiation [20]. Adoptive transfer of bone marrow HSPC also leads to the appearance of spleen colonies, which represent clones of a single or mixed myeloid lineage(s) [22]. *In vitro* studies also support this finding in that bone marrow HSC cultured over spleen-derived stromal cells are restricted to myeloid-

lineage development [23-25]. Overall, the spleen microenvironment appears to reprogram multipotent HSC towards a myeloid-biased output.

Most studies investigating HSC ageing have focussed on bone marrow microenvironments, with little consideration of the role of sites such as spleen in haematopoiesis. This is an important consideration since evidence from *in vitro* studies and *in vivo* models of extramedullary haematopoiesis suggest that spleen-resident HSC are primed for myeloid cell production.

HSC migration

HSC can be found in peripheral blood demonstrating constant migration of HSC between bone marrow and peripheral niches under steady-state conditions [26]. Furthermore, thoracic duct-derived HSPC originating from bone marrow have been shown to enter circulation under steady-state conditions, to transiently migrate to several extramedullary tissues, and eventually return to the bone marrow [27]. Parabiotic mice expressing different isoforms of CD45 (CD45.1/CD45.2) have been used to study cross-circulation of HSC [26]. Engraftment of partner-derived HSC was observed in parabionts, which then successfully maintained haematopoiesis following surgical separation. Only 6.6% ± 3.0% of bone marrow-resident HSC were replaced by circulating HSC over 6-12 weeks, which is consistent with slow but steady circulation of HSC between niches.

Since a small proportion of HSC may be constantly migrating, one question to consider then is the fate of myeloid-biased spleen HSC re-entering the bone marrow. This question was experimentally addressed using CD45.1 HSC from spleen transplanted into CD45.2 irradiated recipients, followed by secondary transplantation of recipient bone marrow [20]. Peripheral blood analysis of secondary transplant recipients revealed elevated myeloid cell numbers compared with control animals receiving bone marrow HSC on primary transplant. This suggests that splenic HSC which enter bone marrow must retain a myeloid bias.

In addition to steady-state haematopoiesis described for both bone marrow and spleen, extramedullary haematopoiesis is harnessed during periods of stress and disease over the lifespan of an individual [27]. This involves HSPC mobilisation from bone marrow into the bloodstream followed by localisation in extramedullary tissue niches [28]. A constant flux of HSC across medullary and extramedullary niches may therefore provide an alternative mechanism for the accumulation of myeloid bias amongst HSC with ageing. In such a model normal HSC that enter spleen may become functionally myeloid-biased and retain that phenotype upon subsequent migration to the bone marrow (Fig. 1). Thus, the steady-state circulation of HSC over a lifetime could result in accumulation of myeloid-biased HSC in the bone marrow of aged individuals.

A role for spleen in myeloid bias

Two animal models have been used here to assess whether niches for HSC in spleen contribute to increased myeloid bias amongst bone marrow HSC. The first steady-state model compares the percentage of $CD150^{hi}$ long-term (LT)-HSC in old versus aged-matched splenectomised mice to determine a role for spleen in the development of myeloid bias. This model builds on former evidence that myeloid bias is more evident in old mice [5]. A second steady-state model compares ex-breeder mice that have undergone several rounds of pregnancy with splenectomised age-matched ex-breeders. Extramedullary haematopoiesis occurs during pregnancy due to increased oestrogen levels [29], and multiple rounds of pregnancy are expected to induce and accelerate HSC mobilisation to the spleen. Finally, the role of spleen in myeloid bias is analysed specifically on HSC expressing the migratory marker CCR2 [30, 31].

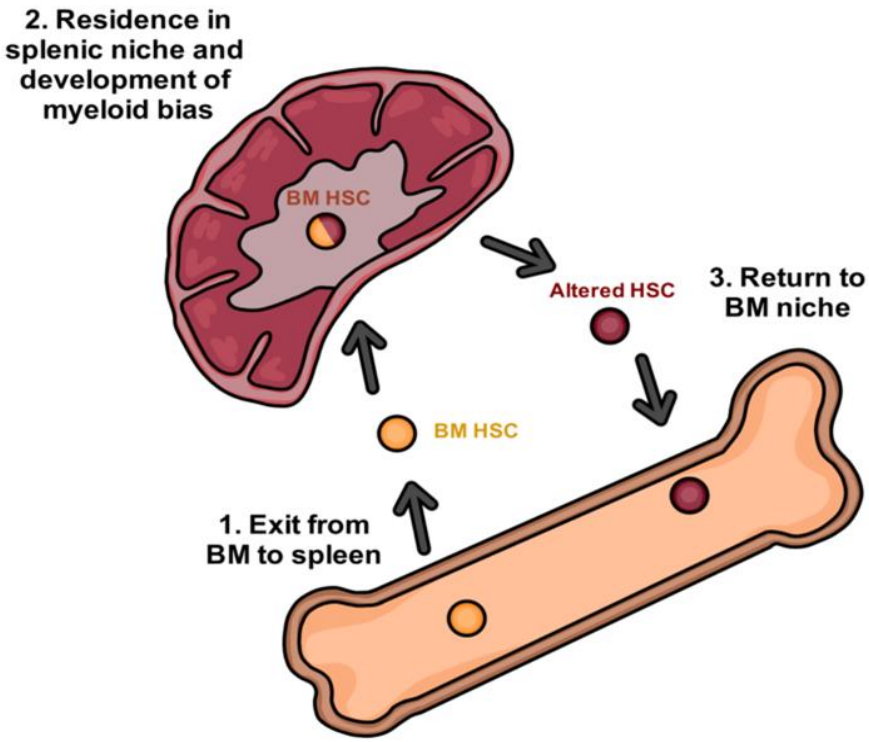

Fig. 1. Proposed development of myeloid bias during steady-state circulation

Schematic of steady-state circulation whereby bone marrow-resident HSC (1) exit the bone marrow niche and home to spleen. Residence in the spleen microenvironment extrinsically contributes to acquisition of a myeloid-biased phenotype (2) which is then carried by HSC for their lifetime and is expressed following return to the bone marrow HSC niche (3).

Materials and Methods

Animals

Specific, pathogen-free male and female C57BL/6JArc (C57BL/6J) mice were obtained from the Animal Resource Centre (Perth, WA, Australia). All experimental methods and treatments of animals were performed in accordance with the guidelines and the regulations as prescribed in *The Australian Code for the care and use of animal for scientific purposes, 8th edition (Australian Research Council and the National Health and Medical Research Council: 2013, updated in 2021).* The study described here has been reported in accordance with ARRIVE guidelines for experimental work involving animals. Adult female mice were used at ages 6- to 16-weeks and 21-months, while adult male mice were used to generate ex-breeder mice and were aged between 10-weeks and 9-months of age. Adult euthanasia was performed by cervical dislocation. Neonatal euthanasia was achieved through hypothermia. Mice were housed in the Bond University Animal Holding Facility and handled according to protocols approved by the Animal Ethics Committee of the University of Queensland (protocol number: UQBR/079/19).

Isolation of haematopoietic cells

To isolate bone marrow cells, the femur and tibia of both hind legs were dissected, and the epiphyses cut off to expose the diaphyses. The central marrow was ejected from the bone into a 14mL conical 2mL ice-cold phosphate buffered saline (PBS) (Sigma-Aldrich Corporation; St. Louis, MO, U.S.A.) using a 23G × 1 ¼" syringe, followed by repetitive pipetting to dissociate marrow into a single cell suspension.

To isolate peripheral blood cells, animals were anaesthetised using a Darvall Stinger Streamline Isoflurane vaporiser (Darvall Vet, Gladesville, NSW, Australia). Animals were placed in the induction chamber under a heat lamp and exposed to isofluorane set at 5%. Animals were then removed from the induction chamber and peripheral blood was collected from the right eye via retro-orbital bleeding using a heparinised 1.1mm × 75mm capillary micro-haematocrit tube (Thomas Scientific, NJ, U.S.A.). Approximately 50µL of blood was collected. For terminal bleeds, approximately 200µL of blood was collected. Flow-through blood was collected in a 1mL Titertube micro test tube marked to measure either 50µL or 200µL (Biorad, CA, U.S.A.).

In preparation for flow cytometry, cells were transferred into a 14mL conical centrifuge tube (TPP Techno Plastic Products AG; Trasadingen, Switzerland), pelleted for 5 minutes at 200G and 4°C and supernatant discarded. To remove red blood

cells (RBC) from cell suspensions, the cell pellet was resuspended in 1mL RBC Lysis Buffer (eBioscience Incorporated; San Diego, CA, U.S.A.) for 5 minutes at room temperature. Cells were then washed in 14mL PBS and resuspended in 1mL of staining buffer (PBS supplemented with 2% bovine serum albumin; Sigma-Aldrich).

Magnetic cell separation

Target progenitor cell populations were enriched by removing all lineage marker-expressing mature cells through magnetic depletion. Enrichment of HSPC was achieved using a lineage-depletion antibody panel. For antibody labelling, bone marrow or spleen cell suspensions were first sedimented and the supernatant discarded. The cell pellet was resuspended in the remaining supernatant, then cells were blocked with purified CD16/32 antibody (Fc block: BioLegend, San Diego, CA, U.S.A) (1µL antibody/10^7 cells) for 10 minutes at 4°C. After incubation, cells were washed with 10mL staining buffer. Supernatant was then discarded, and the biotin-conjugated depletion cocktail added (1µL antibody/10^7 cells). This contained biotinylated antibodies with specificity for CD11a (M17/4), Gr-1 (RB6-8C5), CD11c (N418), NK1.1 (PK136), CD19 (6D5) from BioLegend; and CD3□ (145-2C11) from BD Pharmingen (San Diego, CA, U.S.A.). Following a 10-minute incubation at 4°C and washing with 10mL staining buffer, cells were resuspended in the remaining supernatant. Subsequently, magnetic microbeads (Miltenyi Biotech; Bergisch Gladbach, Germany) were added to cells (20µL/10^7 cells) followed by incubation for 10 minutes at 4°C.

Cell aggregates were first removed by bringing the total volume to 2mL with staining buffer and transferring cells through a 100µm cell strainer into a labelled 12 × 75mm Falcon tube (Becton Dickinson). For magnetic depletion, the tube was placed inside an EasySep™ Magnet (STEMCELL Technologies; Vancouver, BC, Canada) for 10 minutes at room temperature. Following this, the non-antibody binding cell fraction was poured into a fresh Falcon tube and placed into the magnet for 10 minutes for a second round of depletion. The non-binding cell fraction of this second Falcon tube was decanted into a fresh 14mL conical centrifuge tube for centrifugation. Supernatant was discarded and cells resuspended in 1mL staining buffer. Antibody-bound cells from the second Falcon tube were resuspended in staining buffer and retained for use as a control sample for flow cytometry to identify lineage-positive cells. The efficiency of enrichment for progenitor cells was estimated by comparing cell counts before and after depletion.

Cell number was estimated by staining dead cells with trypan blue (12% in PBS) (Sigma-Aldrich) and counting cells using a haemocytometer. Live non-staining cells were counted by phase-contrast microscopy using a Leitz Diavert Inverted microscope (Leica Microsystems GmbH; Wetzlar, Germany).

Flow cytometry

Flow cytometry was used to analyse and characterise cell populations. Cells (up to 10^6 cells/well) were aliquoted into a 96-well U-bottom plate (TPP Techno Plastic Products), sedimented for 5 minutes at 200G and 4ºC, then supernatant discarded. Wells were labelled with 10µL antibody cocktail prepared by diluting each antibody 1:100 in staining buffer followed by incubation for 10 mins at 4°C protected from light. Wells were washed once with 150µL staining buffer before supernatant was discarded and secondary reagents added (1:400 dilution) for a further 10-minute incubation. After a final wash, samples were resuspended into 150µL staining buffer and transferred into 12 × 75mm labelled Falcon tubes (Becton Dickinson).

Fluorochrome-conjugated antibodies used to detect haematopoietic cells included CD201-PE (eBio1560) from eBioscience; c-Kit-APC Cy7 (2B8) from BD Pharmingen; CD150-PE Cy7 (TC15-12/F12.2), Sca-1-BV421 (D7), CD48-FITC (HM48-1), CD43-FITC (S11), CD5-BV510 (53-7.3), B220-PE (RA3-6B2) and CD11b-APC Cy7(M1/70) from BioLegend (San Diego, CA, U.S.A.); CD19-PE Cy7 (eBio1D3), and unconjugated CCR2 antibody from Invitrogen (Waltham, MA, U.S.A.). Secondary conjugates used were SA-BV510 from eBiosciences and Goat anti-Rat-Alexa Fluor 647 from BioLegend.

Splenectomy

Animals aged 5-weeks-old were anaesthetised using a Stinger Streamline Isoflurane vaporiser (Darvall Vet). Animals were placed in the induction chamber under a heat lamp and exposed to isofluorane set at 5% for induction and 3% for abdominal surgery. Mice received a subcutaneous injection of the analgesic Buprenophine (Temgesic, NSW, Australia) at a dose of 0.02mg/kg. Animals were swabbed with 80% ethanol, shaved, and then incised to access the spleen. The incision was approximately 1.5cm in length above the spleen along the transverse plane on the left oblique abdomen. Blunt scissors were used to separate the skin from the peritoneal wall which was then incised before carefully exteriorising the spleen from the peritoneal cavity. The major arteries located under the spleen were tied with Vicryl

5/0 suture thread using a 26mm 1/2c taper suture needle (Ethicon Inc., Somerville, NJ, U.S.A.). The spleen was then removed, and the peritoneal cavity closed using Vicryl 5/0 suture thread using a 26mm 1/2c taper suture needle (Ethicon). Two clips from an Autoclip™ Wound Closing System (Becton Dickinson, San Jose, CA, U.S.A.) were used to close the surgical site. Mice were monitored daily and scored to assess development of adverse effects. Control mice that did not receive splenectomy were housed in the same conditions as the splenectomised mice. Animals were maintained for 9- to 21-months.

Preparation of ex-breeder mice

Female C57BL/6J mice were splenectomised at the age of 4 weeks and then bred for six cycles of litter production, where 1 male mouse was placed in a cage of 4 adult female mice for 1 week. Following 6 reproductive cycles, animals were held until 9-months of age. Normal female C57BL/6J mice (aged 4-weeks) were simultaneously bred for six cycles as age-matched control ex-breeders.

Statistics

All experiments used biological triplicates as a minimum for each experiment. Individual experiments compared one control and one test sample as a minimum. Where applicable, previous data generated in the lab was used to calculate power and sample size in G*Power software (Heinrich Heine University Düsseldorf, Northrhine-Westphalia, Germany). Statistical analyses were performed in GraphPad Prism v9 (GraphPad Software, San Diego, CA, U.S.A.). All error bars represent SEM. Outliers were identified using the ROUT method (Q = 1%). Significance was assessed using unpaired *t*-tests with Welch's corrections. Significantly different values are indicated as * when $p \leq 0.05$, ** when $p \leq 0.005$, *** when $p \leq 0.0005$ and non-significant (ns) when $p > 0.05$.

Results

Identification of bone marrow HSC by flow cytometry

The method used here for gating and identifying myeloid-biased LT-HSC draws on two former published procedures for cell identification. Initial gating for HSC as a $Lin^{-}CD48^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ subset is shown in Fig. 2. Staining for CD201 is then included to identify HSC with self-renewal capacity. E-SLAM/CD201 is a durable marker of LT-HSC that have self-renewing potential [32, 33]. This procedure therefore identifies all self-renewing LT-HSC as a subset of $Lin^{-}CD48^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}CD201^{+}CD150^{+}$ cells. The second component of the procedure then invokes the use of CD150 expression levels to distinguish myeloid biased LT-HSC as $CD150^{hi}$ cells [5, 34]. Our two-step procedure for identifying myeloid-biased LT-HSC therefore differs from previously published methods. It routinely involves CD201 gating to further identify LT-HSC through their self-renewal capacity before gating to find $CD150^{hi}$ myeloid-biased LT-HSC.

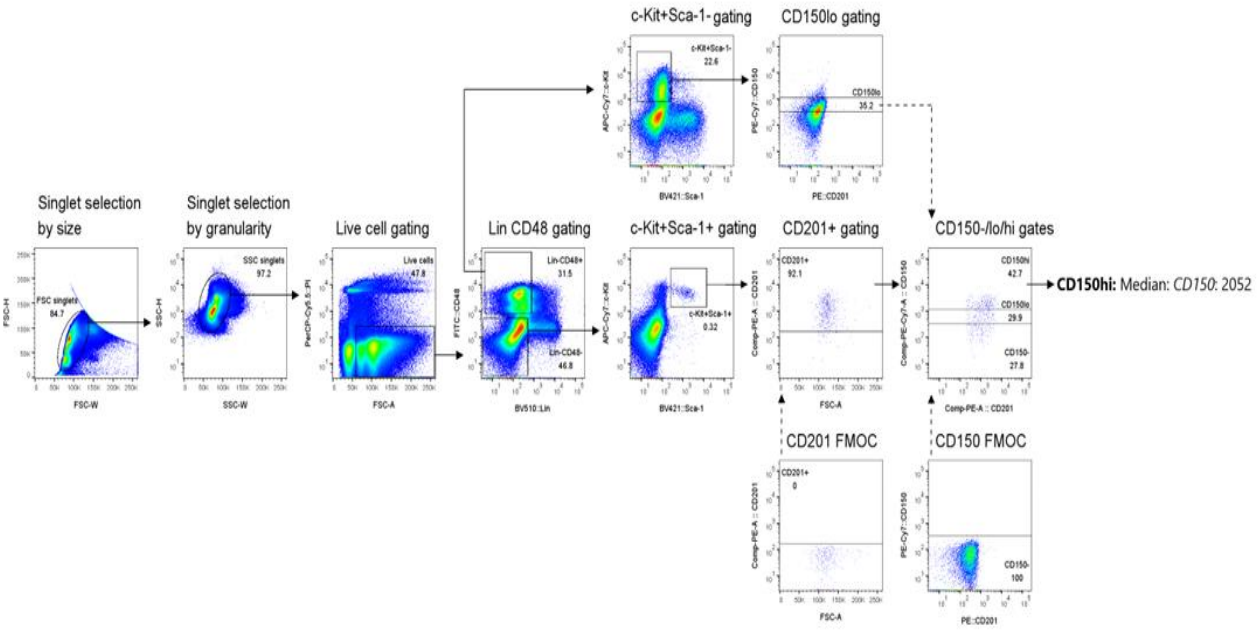

Fig. 2. Separation of HSC subsets by CD150 expression

Bone marrow cells were prepared from a 4-month-old female C57BL/6J mouse and enriched for HSPC through magnetic depletion of $Lineage^{+}$ mature cells through staining with biotinylated antibodies specific for CD3, CD11c, CD19, Gr-1 and NK1.1. Remaining cells were then stained with antibodies specific for CD48 (Alexa Fluor® a488), c-Kit (eFluor®780), Sca-

1 (Brilliant Violet 421), CD150 (PE-Cy7) and CD201 (PE) and analysed flow cytometrically to detect HSC subsets. Live cell discrimination was achieved by staining cells with propidium iodide (PI). Any Lineage$^+$ cells remaining after magnetic depletion were gated out by staining with streptavidin (SA)-BV510 as a secondary reagent. CD48$^+$ cells were also gated out. Fluorescence minus one controls (FMOC) were used to set gates for no CD150 and no CD201 expression. Boundaries for the CD150^{lo} gate were set using an internal control cell population (Lin$^-$CD48$^+$c-Kit$^+$Sca-1$^-$). Live, singlet, Lin$^-$CD48$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells were gated for CD201 expression and then CD150^{hi} expression using FMOCs. These gates allowed analysis of %CD150^{hi} LT-HSC and mean fluorescence intensity (MFI) of CD150^{hi} expression. Percent positive cells is indicated for each gate.

Bone marrow cells were first prepared for analysis by magnetic depletion of mature cells to enrich the progenitor population. Flow cytometry utilised singlet selection followed by live cell gating (PI$^-$). Any Lin$^+$ cells remaining after magnetic depletion were then gated out along with CD48$^+$ cells during flow cytometry. This subset was then gated to identify Lin$^-$ CD48$^-$Sca-1$^+$c-Kit$^+$ cells. Fluorescence-minus-one controls (FMOC) were then used to set positive gating boundaries for CD201 and CD150 expression. To establish a boundary between low and high CD150 expression on LT-HSC, an internal multipotent progenitor control population (Lin$^-$CD48$^+$c-Kit$^+$Sca-1$^-$) was used [5]. This antibody staining and flow cytometry gating protocol separated CD150^{hi} myeloid-biased LT-HSC, CD150^{lo} balanced LT-HSC and CD150$^-$ ST-HSC amongst bone marrow Lin$^-$ CD48$^-$c-Kit$^+$Sca-1$^+$CD201$^+$ cells (*Fig. 2*). One challenge with this gating protocol is that CD150 often presents as a continuum of positive expression on LT-HSC [35, 36]. The median fluorescence intensity (MFI) of CD150 amongst CD150^{hi} myeloid-biased LT-HSC was therefore investigated as an additional parameter. To establish a control reference for young animals, the MFI of CD150 in gated CD150^{hi} myeloid-biased LT-HSC populations of 4-month-old mice was analysed (*Fig. 2*).

Myeloid bias amongst LT-HSC in aged mice

Previous transplantation studies have demonstrated that a high proportion of LT-HSC from aged mice are myeloid-biased, displaying increased CD150^{hi} LT-HSC frequencies in bone marrow compared to younger animals [5]. To verify the gating strategy used in *Fig. 2,* estimates were made of the percentage of myeloid-biased (CD150^{hi}) LT-HSC present in bone marrow of aged (21-months) versus young (8- to 10-weeks) C57BL/6J female mice.

A significantly higher percentage of CD150^{hi} LT-HSC (p = 0.0151) in old mice (46.94% ± 27.34%; n = 16) compared to young mice (27.89% ± 17.00%; n = 13) was confirmed in these studies (Fig. 3A). This outcome was consistent with former reports of transplantation studies, and so verifies the use of flow cytometry in this analysis, providing scope to further analyse the relationship between extramedullary haematopoiesis and age-related myeloid bias amongst LT-HSC [5, 37]. Comparison of CD150 median fluorescence intensity (MFI) in CD150^{hi} LT-HSC showed no difference (p = 0.3254) in aged mice (2886 ± 809.3) compared with young mice (2699 ± 1274) (Fig. 3B).

A role for extramedullary haematopoiesis involving spleen was next tested for its impact on myeloid bias developing over the lifetime of an individual. This was based on evidence that low numbers of HSC circulate in peripheral blood, and because the spleen promotes intrinsic myeloid-biased differentiation in HSC. CD150 expression was analysed on LT-HSC in bone marrow in aged (21 months) mice which had been splenectomised at 5 weeks and compared with age-matched controls (Fig. 3C). If the spleen microenvironment drives a myeloid-biased phenotype with ageing, then splenectomised animals would have a lower proportion of CD150^{hi} bone marrow LT-HSC than age-matched controls. No significant difference (p = 0.3629) was detected in the percentage of bone marrow-derived CD150^{hi} LT-HSC in aged mice (46.94% ± 27.34%, n = 16) compared with splenectomised aged mice (43.13% ± 28.67%, n = 12) (Fig. 3C). CD150 MFI values for Lin$^-$Sca-1$^+$c-Kit$^+$CD150^{hi}CD48$^-$ LT-HSC populations in each of the aged mice (2886 ± 809.3) and splenectomised aged mice (3074 ± 1273) were also compared, but no significant difference was detected (p = 0.3300) (Fig. 3D). Overall, the absence of spleen does not reduce the frequency of myeloid-biased bone marrow LT-HSC in aged mice.

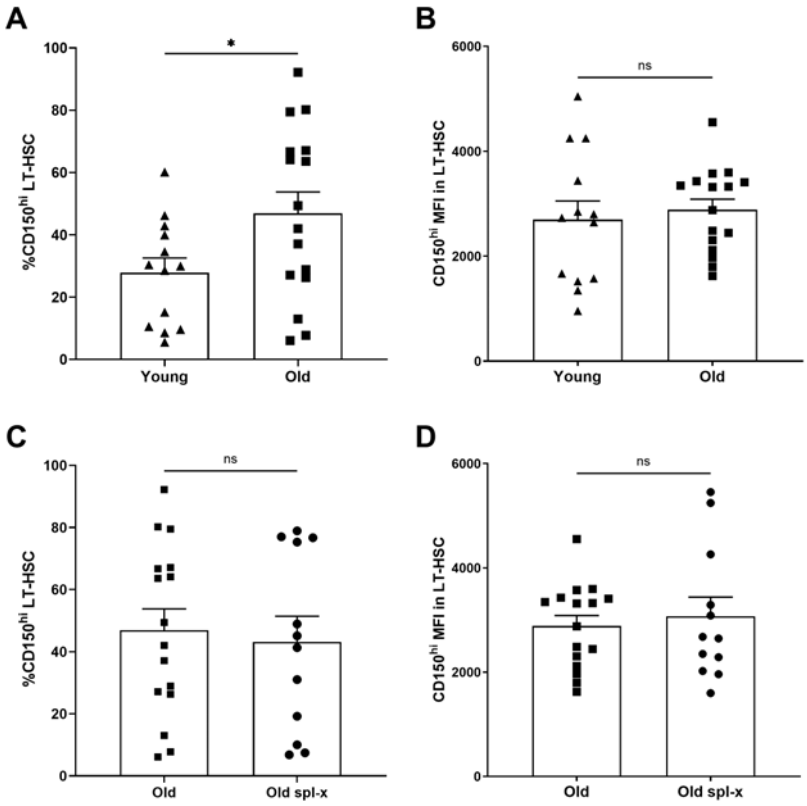

Fig. 3. Comparison of myeloid bias amongst LT-HSC in young, old and asplenic old mice

Bone marrow cells were prepared as described in *Fig. 2*. A) The percentage of myeloid-biased (CD150^{hi}) LT-HSC, and B) the median fluorescence intensity (MFI) for CD150^{hi} on LT-HSC, is shown for young (▲; 8- to 10-weeks-old; *n* = 13) and old (■; 21-months-old; *n* = 16) mice. C) The percentage of myeloid-biased (CD150^{hi}) LT-HSC, and D) the MFI of CD150^{hi} on LT-HSC, from old (■; 21-months; *n* = 16) mice and asplenic (Spl-x) old (●; 21-months; splenectomised at 5-weeks of age; *n* = 12) mice. Data represent the mean ± SEM of multiple (13) independent experiments. Significantly different values are indicated as * when $p \leq 0.05$, ** when $p \leq 0.005$, and non-significant (ns) when $p > 0.05$.

Effect of ageing on blood leukocytes

An increase in the number of myeloid cells over lymphoid cells in the peripheral blood is also associated with an ageing phenotype. This myeloid bias represents the outcome of HSC activity, with reports demonstrating that myelopoiesis is increased in aged mice at the expense of lymphopoiesis [38]. To confirm an ageing phenotype amongst peripheral blood leukocytes, B and T lymphoid and myeloid cell frequency was measured in the peripheral blood of young and aged animals. The gating protocol used to measure prevalence of cell subsets is shown in Fig. 4A. Using this method, the ageing model was confirmed by showing that young mice had a significantly higher frequency of T cells (13.98% ± 3.16%; *n* = 9) compared with old mice (8.29% ± 5.40%; *n* = 10) (p=0.0064; Fig. 4B). However, no significant difference (p = 0.1126) was detected between young and old mice in terms of peripheral myeloid cell frequency (38.27% ± 14.80% and 49.46 ± 23.28%, respectively) (Fig. 4B), although this was highly variable in aged mice. Similarly, no significant difference (p = 0.2640) was observed between the frequency of B lymphocytes in peripheral blood of young and old mice (27.10% ± 16.82% and 21.71% ± 19.64%, respectively) (Fig. 4B).

Peripheral blood cell distribution was then measured in splenectomised aged mice compared with age-matched normal animals (Fig. 4C). However, no significant difference was found between splenectomised aged mice and aged-matched controls for peripheral B cell frequency (22.52% ± 16.80% and 21.71% ± 19.64%; p = 0.0558), peripheral myeloid cell frequency (50.80% ± 14.23% and 49.46 ± 23.28%; p = 0.4427) or for peripheral T lymphoid cell frequency (7.807 ± 4.62% and 8.290 ± 5.40%; p = 0.4230) (Fig. 4C). Interestingly, splenectomy appears to have reduced the variability in myeloid cell frequency noted in aged mice. Overall, the absence of a spleen in aged animals did not change the frequency of mature T, B or myeloid cells in peripheral blood.

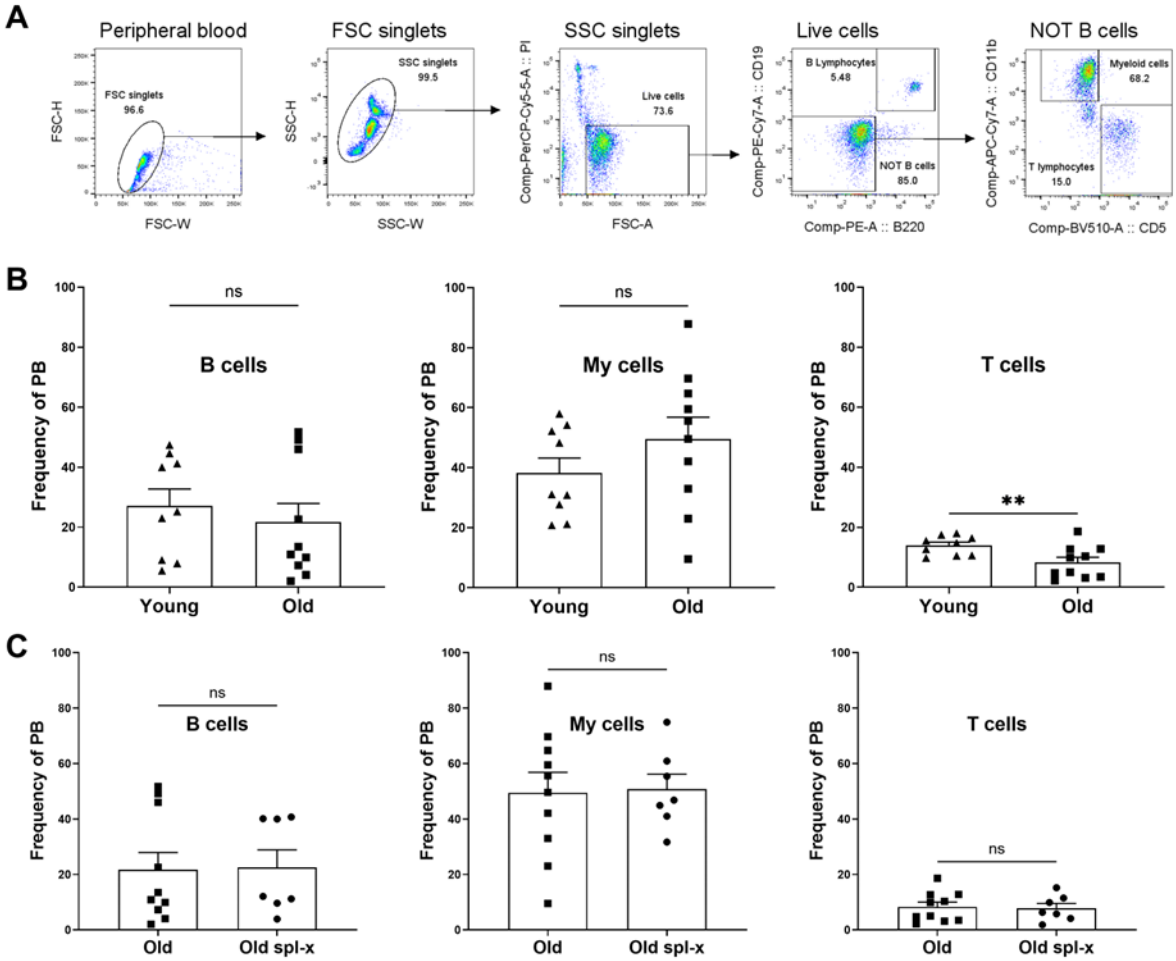

Fig. 4. Frequency of peripheral myeloid and lymphoid cells in young, old and asplenic old mice

Peripheral blood was retro-orbitally harvested from mice and red blood cells lysed. Cells were then stained with antibodies specific for CD43, CD5, CD11b, CD19 and B220. Live cells were identified through absence of PI staining. A) Representative gating strategy to identify B cells, myeloid (My) cells and T cells is shown. B) The frequency of B cells, myeloid (My) cells and T cells amongst red blood cell-lysed peripheral blood was estimated in young (▲; 8- to 10-weeks; n = 9) and old (■; 21-months; n = 10) mice. C) The frequency of B cells, myeloid (My) cells and T cells amongst red blood cell-lysed peripheral blood was estimated in old (■; 21-months; n = 10) and asplenic old mice (●; 21-months; splenectomised at 5-weeks of age; n = 7). Data represent the mean ± SEM of multiple (10) independent experiments. Significantly different values are indicated as * when $p \leq 0.05$, and non-significant (ns) when $p > 0.05$.

The impact of multiple pregnancies on development of myeloid biased HSC

Pregnancy causes increased demand for haematopoiesis stimulating the migration of HSC from bone marrow to spleen and extramedullary haematopoiesis [29]. Increased levels of oestrogen during pregnancy also increases HSC division, frequency, cellularity and erythropoiesis in the spleen [39]. Multiple rounds of pregnancy were therefore used as a model for accelerated HSC migration to determine whether this impacts the development of myeloid bias amongst HSC. As with the ageing model, it was hypothesised that increased HSC traffic to and from the spleen, stimulated by increased haematopoietic demand from multiple pregnancies, would lead to the accumulation of myeloid-biased HSC in the bone marrow. Multiple pregnancies were induced in adult female mice from 8-weeks of age and these ex-breeders were then compared with normal aged-matched control mice for the frequency of myeloid-biased LT-HSC in bone marrow

Analysis of ex-breeder mice showed a significant difference (p = 0.0376) in the percentage of $CD150^{hi}$ HSC amongst LT-HSC populations (60.65% ± 8.30%) compared with control age-matched mice (49.47% ± 10.88%) (*Fig. 5A*). A comparison of CD150 MFI amongst gated $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}CD48^{-}CD150^{hi}$ cells demonstrated that the MFI of CD150 was not statistically different (2709 ± 616.1) from age-matched control mice (2530 ± 451) (p = 0.2902) (Fig. 5B). Results from this study show that multiple rounds of pregnancy drive the development of myeloid bias

in bone marrow HSC.

The role of spleen in development of myeloid bias

Experiments were designed to determine the effect of repeated pregnancy-induced extramedullary haematopoiesis events on the bone marrow HSC composition in mice that had been splenectomised ahead of first pregnancy. These were compared with normal adult ex-breeders. No statistically significant difference (p = 0.1073) was observed in the percentage of myeloid-biased LT-HSC between ex-breeders and splenectomised ex-breeders (60.65% ± 8.30% versus 54.87% ± 7.28%, respectively) (Fig. 5C). Analysis of CD150 MFI of LSK CD48^{-}CD150^{hi} LT-HSC compared with ex-breeder controls (2709 ± 616.1 compared to 2645 ± 499.3) showed no significant difference (p = 0.4126) (Fig. 5D). Overall, no significant difference was detected in the proportion of myeloid-biased bone marrow LT-HSC which develop in the absence of spleen in ex-breeder mice following multiple rounds of pregnancy.

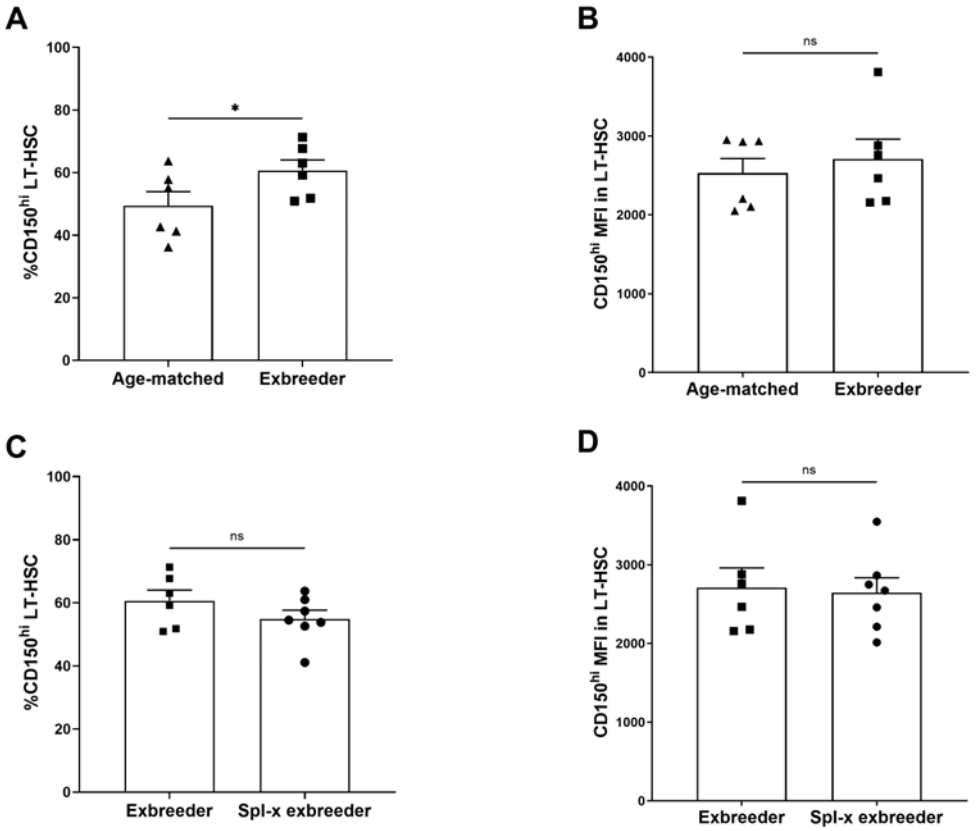

Fig. 5. Myeloid bias amongst LT-HSC in exbreeder and asplenic exbreeder mice

Female C57BL/6J mice underwent 6 rounds of pregnancy and were sacrificed at 15-months of age (exbreeders). Some exbreeder mice had been splenectomised ahead of first pregnancy. Bone marrow cells were prepared as described in *Fig. 2*. A) The percentage of myeloid-biased (CD150^{hi}) LT-HSC, and B) the median fluorescent intensity (MFI) of CD150^{hi} LT-HSC, was estimated for exbreeder (■; 15-months n = 6) and age-matched control mice (▲; 15-months; n = 6). C) The percentage of myeloid-biased (CD150^{hi}) LT- HSC, and D) the median fluorescence intensity (MFI) of CD150^{hi} for myeloid-biased LT-HSC was estimated in exbreeder (■; 15-months; n = 6) and splenectomised (Spl-x) exbreeder mice (●; 15-months; n = 7). Data represent the mean ± SEM of 6 or 7 independent experiments. Significantly different values are indicated as * when $p \leq 0.05$, ** when $p \leq 0.005$, and non-significant (ns) when $p > 0.05$.

The role of spleen in development of myeloid bias amongst migratory LT-HSC

CCR2 is important in HSPC migration [31] and is expressed on a small portion of bone marrow HSPC that display heightened proliferation rates, reduced reconstitution potential and skewing to myeloid gene expression [30]. It was therefore used as a marker to assist in identification of myeloid bias. Lin^{-}CD48^{-}Sca-1^{+}c-Kit^{+} CD150^{hi} cells were gated according to the protocol shown in Fig. 2 and then analysed for CCR2 expression (Fig. 6A).

A comparison of %CCR2^{+}CD150^{hi} LT-HSC in bone marrow of female C57BL/6J young (8-10 weeks) and aged mice (21 months) revealed no significant difference (45.08% ± 41.71% (n = 13) and 39.86% ± 34.37% (n = 16); p = 0.3602) (Fig. 6B). A similar comparison of %CCR2^{+}CD150^{hi} LT-HSC in bone marrow of aged mice (21 months) mice and aged mice previously splenectomised at 5 weeks of age

and analysed at 21 months, revealed no significant difference (39.86% ± 34.37% (n = 16) and 34.96% ± 39.11% (n = 12); p = 0.3665) (Fig. 6C). The results from this study indicate that spleen plays no significant role in supporting myeloid bias on migratory CCR2-expressing LT-HSC located in bone marrow.

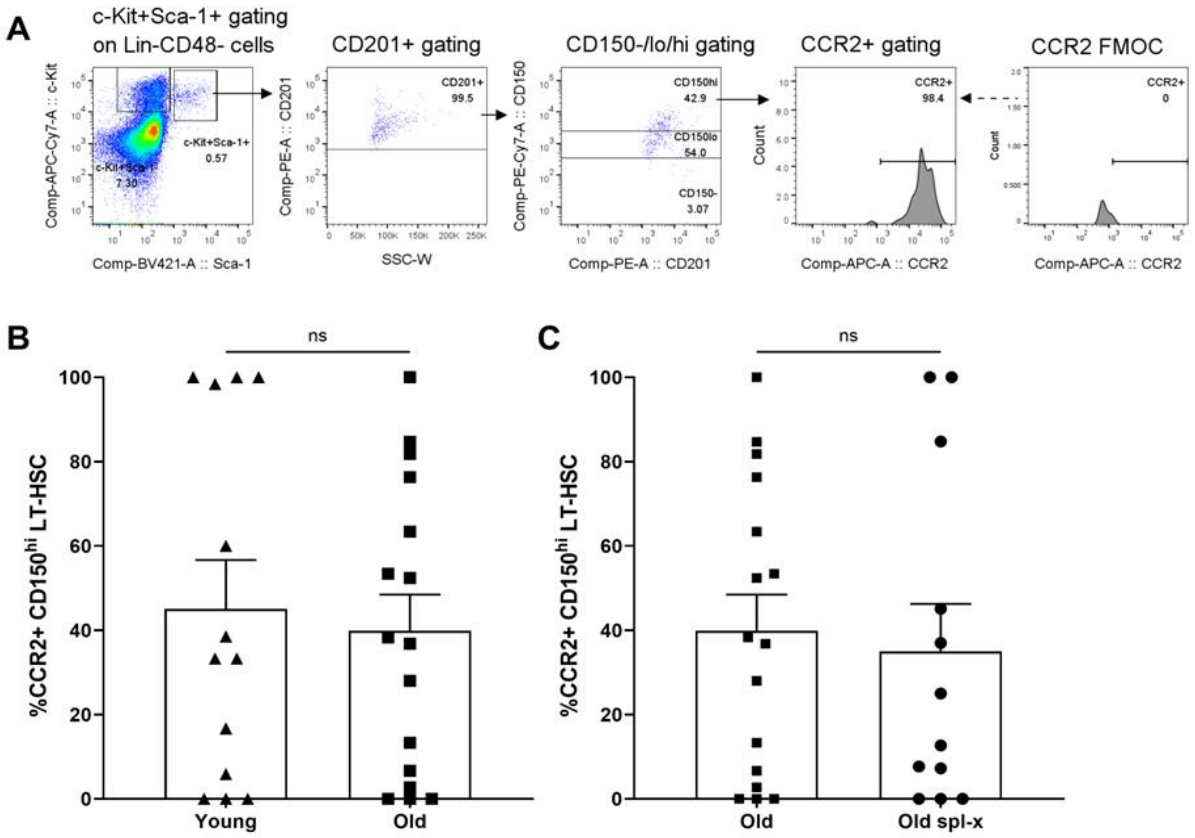

Fig. 6. Importance of spleen in development of myeloid bias amongst CCR2$^+$ migratory HSC

Bone marrow cells were prepared as described in *Fig. 2*. A) The gating strategy employed was the same as shown in Fig. 2. The diagram shows Sca-1 and c-Kit gating imposed on a selected Lin-CD48- subset of bone marrow. This is followed by gating for CD201, CD150 and CCR2 using FMOCs. Boundaries for the CD150^{lo} gate were set using an internal control population (Lin$^-$CD48$^+$c-Kit$^+$Sca-1$^-$). B) Young (▲; 8- to 10-weeks-old; n = 13) and old (■; 21-months-old; n = 16) mice were compared for the percentage of Lin$^-$ CD48$^-$Sca-1$^+$c-Kit$^+$ CD201$^+$CD150^{hi}CCR2$^+$ myeloid biased LT-HSC, and C) Old (■; 21-months-old; n = 16) mice and asplenic old (●; 21-months; splenectomised at 5-weeks of age; n = 12) mice were compared for the percentage of Lin$^-$ CD48$^-$Sca-1$^+$c-Kit$^+$CD201$^+$CD150^{hi}CCR2$^+$ HSC. Data represent the mean ± SEM of multiple (13) independent experiments. Significantly different values are indicated as * when $p \leq 0.05$, ** when $p \leq 0.005$, and non-significant (ns) when $p > 0.05$.

Discussion

The development of myeloid bias is identified here in both aged and multiparous mice. The importance and role of spleen in this process was, however, discounted through quantification of myeloid-biased LT-HSC in bone marrow using phenotypic identification. While we show no role for spleen, our data do support earlier findings that myeloid bias increases in multiparous and old mice. Recent studies in wild mice have, however, identified a more important role for aging than for pregnancy in myeloid bias. Wild mice have a unique distribution of mature and progenitor cell populations in bone marrow compared with laboratory mice [40]. Despite this distinct phenotype, a comparison of 6-month-old retired breeders with 5-week-old young mice revealed the same ratio of myeloid to lymphoid cells in bone marrow [40].

Information collected here adds to our knowledge of 1) a known endogenous redistribution of HSC across medullary and extramedullary haematopoietic tissues including spleen that is ongoing over the lifetime of an individual [25, 27, 29, 41], and 2) a propensity for spleen HSC to retain myeloid-bias following relocation to bone marrow niches [20]. An important caveat to our results is that HSC ageing is a multi-factorial process and that a range of environmental niches exist in bone marrow and in peripheral hematopoietic tissue which can impact the development of HSC in different ways. While myeloid

bias amongst bone marrow LT-HSC is increased during stress and particularly pregnancy, it is not dependent on spleen. This study provides no evidence to support LT-HSC residency in spleen as causative of increased myeloid bias amongst bone marrow LT-HSC following either steady-state hematopoiesis during ageing, or extramedullary hematopoiesis across multiple pregnancies. Further analysis of $CCR2^{+}$ migratory LT-HSC also indicated no dependency on spleen as a specific site supporting migratory LT-HSC and their development of myeloid bias.

Myeloid-biased bone marrow $CCR2^{+}$ HSC have previously been described as a subset of HSC that are primed to migrate to spleen upon inflammatory stimulation [30, 42]. They are thought to be responsible for seeding the inflammatory myelopoietic response which occurs in spleen following myocardial infarction [42]. Despite this former evidence for the trafficking of $CCR2^{+}$ into spleen during inflammation, we have been unable here to identify a role for spleen in influencing the myeloid bias of $CCR2^{+}$ LT-HSC. CCR2 expression has also been identified on bone marrow HSC and other progenitors which had migrated into the peritoneum of an inflammatory mouse model [31]. However, these HSC do not appear to represent primitive self-renewing LT-HSC since they could engraft bone marrow of primary and secondary recipients, although this was much reduced in the secondary hosts [31]. These findings raise the possibility that a distinct subset of HSC may provide extramedullary hematopoiesis under conditions of ageing and inflammation [30, 42]. This is also supported by evidence that bone marrow HSC can express TLR2 and TLR4, which are functional since ligation with LPS leads to differentiation [43].

A potential limitation of this study is the use of cell surface marker phenotype to identify myeloid-biased LT-HSC. However, using this methodology it has been possible to replicate myeloid bias in aged over young mice, and in exbreeders compared with age-matched controls. Other studies that have investigated HSC ageing and myeloid bias employed transplantation studies [3, 5-7, 13], where the outcome identifies the relative hematopoietic outputs of, for example, $CD150^{lo}$ versus $CD150^{hi}$ HSC, young versus old HSC, or even bone marrow versus spleen HSC. However, it has been recently shown that the conditioning process used for HSC transplantation drastically alters the HSC niche and amplifies extremes in HSC lineage bias following reconstitution [38]. This was highlighted through “unconditioned” HSC transplantation where lineage bias could not be detected. Therefore, HSC transplantation that involves pre-conditioning is likely to induce a perturbed or inflammatory state of haematopoiesis. Of note, in unconditioned recipients, transplanted HSC engraft in bone marrow without disrupting or replacing resident host HSC [44]. Interestingly, an unintended consequence of a pre-conditioning step could be that it also presents a more sensitive model for detecting myeloid-biased HSC [3, 5-7, 13]. Therefore, in line with previous reports, secondary HSC transplantation may be required to distinguish the relative haematopoietic outputs of normal aged versus splenectomised aged bone marrow HSC.

There is early precedence for this study and for investigating a role for spleen in the development of myeloid bias in ageing and multiparous mice. Pregnancies are known to affect the age-related size of lymphoid and myeloid cell populations in murine spleen [45], and Barrat and coworkers as early as 1997 found increased production of myeloid cells associated with pregnancies in mice at 15 months and in aged multiparous mice at 23 months [46]. This current study now demonstrates a role for multiple rounds of pregnancy in the development of myeloid bias amongst LT-HSC in bone marrow. In the comparison between ex-breeder and control age-matched animals, bone marrow LT-HSC composition increased after multiple rounds of pregnancy-induced extramedullary haematopoiesis (Fig. 5A). Despite the role of spleen in pregnancy to serve as an emergency reservoir for increased haematopoietic demand [25, 29], it was found that splenectomised ex-breeder animals exhibited no difference compared with normal ex-breeder animals in terms of bone marrow LT-HSC composition.

Possible reasons why a role for spleen was not identified in this study could be the involvement of other extramedullary sites for haematopoiesis (e.g. liver) [28, 47], or increased activity of multipotential progenitors (MPP) or other progenitors that serve to amplify blood cell numbers [48, 49]. Furthermore, it is possible that the replicative stress placed upon the haematopoietic compartment in pregnancy-induced extramedullary haematopoiesis may differ from that of inflammatory challenge used in other studies, such as the Toll-like receptor 3 agonist polyinosinic:polycytidylic acid [50]. One possibility is that a compensatory biological mechanism exists to promote conservation of the haematopoietic system following pregnancy, which is sufficient to ensure haematopoietic robustness despite several rounds of pregnancy in an individual.

Conclusions

This paper reveals no role for spleen in the support or development of specific myeloid-biased HSC populations over time. As with bone marrow HSC, spleen-resident HSC are known to slowly enter the circulation with 1.5-3.6% of HSC in spleen of a parabiosis model found to have partner phenotype through secondary transplantation studies over 6-12 weeks [19]. Single HSC transplantation studies using bioluminescence labelling have also traced the movement of HSC lodged in spleen to other niches [51]. Less is known about the behaviour of spleen HSC, both inside the splenic niche, and particularly following migration to the bone marrow. Inside a spleen microenvironment, there is *in vitro* [23, 24] and *in vivo* [42, 52] evidence for myeloid-restricted HSC differentiation, analogous to myeloid-biased HSC activity. Outside the spleen, one study modelled the outcome of spleen HSC migration to the bone marrow, finding that after secondary transplantation, spleen-originating HSC retained a higher capacity for myeloid lineage differentiation [20]. Perhaps, as splenic HSC can enter the cell cycle more quickly than their bone marrow counterparts, and cell cycling has been shown to drive HSC ageing, comparison of the two via serial transplantation into a secondary host would display increased myelopoiesis amongst splenic HSC, a phenomenon not identifiable in the steady-state models used in this paper.

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Data Availability Statement

All original data is included in this publication. The datasets used and/or analysed supporting the conclusions of the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal study was conducted according to protocols approved by the Animal Ethics Committee of the University of Queensland (protocol number: UQBR/079/19).

Author Contributions

CS: Investigation, Methodology, Data curation, Data analysis, Writing original draft, Writing – review and editing. HO: Data analysis, Supervision, Writing original draft, Writing – review and editing. JT: Conceptualisation, Methodology, Data curation, Data analysis, Supervision, Writing original draft, Writing - review and editing.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Declarations

Clinical Trial Number

Not Applicable

Consent for publication

All authors have approved publication of this paper.

Competing interests

The authors declare that they have no competing interests.

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