Comparative study of hematopoietic stem and progenitor cells between sexes in mice under physiological conditions along time

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Abstract

Hematopoietic stem and progenitor cells (HSPCs) are attractive targets in regenerative medicine, although the differences in their homeostatic maintenance between sexes along time are still under debate. We accurately monitored hematopoietic stem cells (HSCs), common lymphoid progenitors (CLPs), and common myeloid progenitors (CMPs) frequencies by flow cytometry, by performing serial peripheral blood extractions from male and female B6SJL wild-type mice and found no significant differences. Only modest differences were found in the gene expression profile of Slamf1 and Gata2. Our findings suggest that both sexes could be used indistinctly to perform descriptive studies in the murine hematopoietic system, especially for flow cytometry studies in peripheral blood. This would allow diminishing the number of animals needed for the experimental procedures. In addition, the use of serial extractions in the same animals drastically decreases the number of animals needed.

Keywords: animal number reduction; hematopoietic stem and progenitor cells; hematopoietic system; serial monitoring; sexual dimorphism

Introduction

Sex bias in biomedical research is a very common feature, usually showing a strong male sex bias in many disciplines (Beery and Zucker, 2011). The greatest barrier to eliminate this sex bias in animal research is the belief of female rodents being more variable than males because of the estrous cycle, but for most applications, female mice tested throughout their hormone cycles display no more variability than males do (Clayton and Collins, 2014; Prendergast et al., 2014). However, testing both sexes in animal research is essential to avoid biased results (Clayton and Collins, 2014), as well as to ensure that only the necessary number of animals are used for the experimental procedures, thus complying the “Three Rs” ethic guides (Fenwick et al., 2009).

Regarding the hematopoietic system, few studies are focused on mice compared to the studies conducted in humans. Also, we found that some studies were focused on sexual dimorphism of the hematopoietic system in the bone marrow and spleen, but not on peripheral blood (Nakada et al., 2014; Jafri et al., 2017). Hematopoietic stem cells (HSCs) are multipotent cells with self-renewal capacity which give rise to every blood cell type. Their closest progeny are hematopoietic progenitor cells (HPCs), namely common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) which are oligopo- tent cells with limited self-renewal capacity. CLPs and CMPs give rise to the lymphoid and myeloid lineages, respectively (Bryder et al., 2006). HSCs and HPCs are called, as a whole, hematopoietic stem and progenitor cells (HSPCs) (Shizuru et al., 2005; Mazo et al., 2011). These cells are responsible for hematopoietic homeostasis and they also have an important role in pathological conditions by rapidly supplying HPCs which can, in turn, give rise to a big number of specialized immune cells (Massberg and von Andrian, 2009).

Differences in HSPC differentiation between sexes were recently reported in mice. Nakada et al. (2014) demonstrated that female HSPCs in the bone marrow of an animal model for the study of hormonal effects over hematopoiesis divided more frequently than male HSPCs due to estrogen effect. However, there was no real increase in the absolute number of HSPCs in females with respect to males. It was

suggested that female HSPCs undergo asymmetric divisions more frequently than male HSPCs, thus maintaining stable the total number of HSPCs even though they are more active than their male counterparts. However, this was not tested in peripheral blood.

The aim of the present study was to investigate HSC, CLP, and CMP frequencies in peripheral blood under physiologi- cal conditions in male and female wild-type (WT) mice on a B6SJL genetic background at ages coincident with the developmental stages of mice. For this purpose, we set up an innovative experimental procedure based on blood serial extractions from tail vein, using small blood volumes. The reproducibility of this procedure allowed an accurate and time-dependent characterization of these progenitor cells in mice. The surface markers selected for the study of HSPCs were lineage markers characterizing mature blood cells to exclude them from the quantification and to select only the lineage negative HSPC cells (Shizuru et al., 2005); Sca-1 which is expressed by HSCs, CLPs and CMPs (Shizuru et al., 2005); c-Kit, highly expressed by HSCs and CMPs but with a lower presence in CLPs (Shizuru et al., 2005; Challen et al., 2009) and, finally, CD127 to differentiate CMPs from CLPs (Challen et al., 2009).

In addition to the study of HSPC frequencies, we studied the expression levels of six genes related to the hematopoiesis process, which provided extended data about the state of HSPCs in peripheral blood: Ly6a (Sca-1), Slamf1, Gata2, Id1, Ikzf1, and Cebpa. Ly6a (Sca-1) plays an essential role in the regulation of the HSC cycle and their commitment to the hematopoietic progenitors (Bradfute et al., 2005). Slamf1 is highly expressed in long-term HSCs (LT-HSCs), which show a higher self-renewal potential and are more prone to commit to the myeloid lineage (Beerman et al., 2010). Gata2 is related to HSC survival and self-renewal, promoting cell quiescence to maintain the HSC pool in detriment of differentiation (Rodrigues et al., 2012), while Id1 regulates HSC proliferation and blocks their differentiation to more committed stages (Suh et al., 2008, 2009). Finally, Ikzf1 expression is necessary for the commitment of the Multipotent Progenitor (MPP) to the lymphoid lineage (i.e., it controls the lymphopoiesis process) (Schwickert et al., 2014), while Cebpa instructs the MPP to differentiate toward the myeloid lineage (Wolfler et al., 2010).

Materials and methods

Animals

WT mice on a B6SJL genetic background (B6SJL-Tg- (SOD1-G93A)1Gur/J) were used for this study and obtained from The Jackson Laboratory. Food (pellets) was purchased from Harlan Laboratories Animals and administered ad libitum along with tap water. Animals were housed in isolated cages with bedding and individual ventilation (Techniplast) in the Unidad Mixta de Investigación of the University of Zaragoza under a 12 h light/ night cycle, a stable temperature of 21–23◦C and a relative humidity of 55%. The mice were routinely screened for pathogens following a standard procedure. All the experimental procedures were approved by the Ethic Committee for Animal Experiments of the University of Zaragoza. Animal care and experimentation were per- formed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE. The methodology allowed an accurate characterization of the cells in a small volume of whole peripheral blood, which is in accordance with previous studies (Breslin et al., 2013). Notwithstanding, earlier stages than P30 were not manageable to avoid animal suffering and stress due to their small size.

Blood extraction and flow cytometry

For the flow cytometry study, thirty WT mice (B6SJL) (15 males and 15 females) were used. Blood extractions were performed serially at P30, P50, P75, P105, and P120. The selected ages corresponded to the development progression of the mice. Blood obtained from the tail was collected with Microvette1 CB 300 (Sarstedt, Nu€mbrecht, Germany) capillary tubes containing EDTA. The amount of blood collected ranged between 20 and 100 mL. For identification of HSCs, samples were stained with the following antibodies: Mouse Hematopoietic Lineage eFluor 450 Cocktail con- taining CD3 (17A2), CD45R (B220), CD11b (m1/70) TER-119 (TER-119), and Ly-G6 (Gr-1) (RB6-(C5) antibodies; PE-conjugated anti-Ly-6A/E (Sca-1) antibody and APC- conjugated anti-CD117 (c-Kit) antibody (eBioscience, San Diego, CA, USA). For identification of CLPs and CMPs, samples were stained with the aforementioned antibodies plus Pe-Cy7-conjugated anti-CD127 (IL7RA) antibody (BD Biosciences, Franklin Lakes, NJ, USA) to differentiate CMPs

from CLPs. Erythrocytes were lysed and samples were analyzed with Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Collected data were analyzed using Kaluza Flow Analysis Software (Beckman Coulter). Compensation controls for every antibody used were run to correct spectral overlap.

Real time-PCR

For the Real-Time PCR analysis, four WT mice (B6SJL) from each sex were used at P30, P50, P75, P105, and P120. The mice were sacrificed by CO2 anesthesia at each studied age. Blood samples were obtained by heart puncture and collected in 2 mL EDTA tubes. RNA extraction was performed with Purelink Total RNA Blood Purification Kit (InvitrogenTM, Life Technologies; Waltham, MA, USA) and treated with Turbo DNA-free kit (Ambion1, Life Technologies, Waltham, MA, USA) to eliminate genomic DNA. Finally, reverse transcription was carried out using SuperScript First-Strand Synthesis System kit (InvitrogenTM). Ly6a (Sca-1) (Ly6a, Mm00726565_s1), Slamf1 (Slamf1, Mm00443316_m1), Gata2 (Gata2, Mm00492301_m1), Id1 (Id1, Mm00775963_g1), Ikzf1 (Ikzf1, Mm01187882_m1), and Cebpa (Cebpa, Mm00514283_s1) probes were supplied by Applied Biosystems1 (Life Technologies; Foster City, CA, USA). The endogenous gene Gapdh (GAPDH: Mm03302249_g1, Applied Biosystems) was used for normalization of the data. Target gene expression was then normalized and relative gene expression was assessed using the 2—DDCT method (Livak and Schmittgen, 2001).

Statistics

The statistical analysis was performed with SPSS Statistics 19.0 software (IBM, USA). Independent two-tailed Student's t-tests were used to assess statistical significance between groups of means. The data are presented as means, and error bars represent the standard error of the mean (SEM) for flow cytometry results, while error bars for RT-PCR results were calculated as described in the 2—DDCT method (Livak and Schmittgen, 2001). The longitudinal evolution of each cell type along time for both sexes was assessed using one-way repeated measures ANOVA. The statistical significance was established at P-values under 0.05.

Results and discussion

Many studies have demonstrated the existence of sexual dimorphisms affecting both physiological and pathological mechanisms (Whitacre, 2001; Cahill, 2006; Beery and Zucker, 2011). However, these differences cannot be completely explained by sexual hormone influences, and may be due to other factors inherent to the sex (Cahill, 2006). Regarding hematopoiesis, there is little knowledge as to how sex affects the process in physiological conditions in mice peripheral blood. Nakada et al. (2014) stated that estrogen plays an important role in mice hematopoiesis in the bone marrow, while they concluded that it is not the only nor the most important factor affecting hematopoiesis, as it affects HSCs self-renewal, but not HSCs frequency. However, our work shows that only slight significant differences could be found in the hematopoietic process between sexes in peripheral blood, and we present, for the first time in the literature, a follow-up study of HSPC frequencies in peripheral blood along mice development by using serial blood extractions. Mice were considered globally mature from postnatal (P) day 50 on and therefore the analysis of the findings obtained was summarized in immature (P30 and P50) and mature stages (P75, P105, and P120) for a better understanding.

Our first step was to study HSC, CLP and CMP profiles by flow cytometry in WT mice. For this purpose, serial blood samples from male and female mice at P30, P50, P75, P105, and P120 were obtained and accurately analyzed. The rationale behind the selection of the markers studied was comprehensively described above and corresponded to the surface markers widely used for HSPCs identification and isolation. HSCs were identified as Lin- c-Kit+ Sca-1+, CLPs as Lin- c-KitlowSca-1lowCD127+, and CMPs as Lin- c-Kit+ Sca-1- CD127- (Shizuru et al., 2005).

Overall, the serial analysis of flow cytometry data revealed that there were no statistical differences between sexes for any of the three cell types studied at any of the time points selected (Figures 1B–1D). The analysis flow performed can be seen in Figure 1A. As for the time evolution of each cell type, in

general, the highest HSC percentage was found at P30 and P50 in both sexes and it decreased at the mature stages (Figure 1B). A repeated measures ANOVA deter- mined that there were statistically significant differences between time points ($F(4,56) = 5.211$ for males and $F(4,56) = 5.493$ for females, $P < 0.05$) for both sexes. Post hoc tests using the Bonferroni correction revealed that the immature stages (P30 and P50) had a statistically and significantly higher population than that of the mature stages ($P < 0.05$). In relation to CLPs, their frequency was lower in both sexes at the immature stages (P30 and P50), reached the highest percentage at P75 and then decreased until P120 (Figure 1C). The statistical analysis indicated that the observed differences along time were statistically significant, with the immature stages presenting statistically lower percentages than that of the mature stages ($F(4,56) = 8.708$ for males and $F(4,56) = 8.155$ for females, $P < 0.05$; Bonferroni: $P < 0.05$). Finally, CMP percentage showed a light decreasing tendency along time in both sexes from P75 to P120 (Figure 1D) $F(4,56) = 2.945$ for males and $F(4,56) = 3.158$ for females, $P < 0.05$; Bonferroni: $P < 0.05$). Despite the lack of statistical differences for any of the three cell types studied between sexes, HSC mean frequency was higher at the immature stages of mice development. The higher frequency of HSC at P30 and P50 is in accordance with studies showing that the frequency of HSCs is higher at the immature stages of fast development and declines once the mice reach maturity (Copley and Eaves, 2013). The sudden increase of CLPs once maturity is reached could be probably related to the general immature state of the lymphoid system at birth and in the first weeks of life. Once maturity is reached, however, the lymphoid lineage reaches higher frequencies which tend to normalize over time (Adkins et al., 2004). Reversely, CMPs showed a higher frequency at the immature stages, slowly decreasing their percentage over time. This could be due to CMPs presenting a relevant role in the early immune response and the colonization of lymphoid and non- lymphoid tissues (De Kleer et al., 2014; Gantt et al., 2014).

After sexual maturity, however, CLPs take the leading role of the immune system. The lack of differences in the frequency of HSCs, CLPs, and CMPs between sexes is in concordance with the results of Nakada et al. (2014) in bone marrow, as their works showed that there was not a frequency difference in HSPCs, but rather a different asymmetrical differentiation pattern, with female HSPCs being more active than male HSPCs. Interestingly, our study considered for the first time very early stages, namely P30 and P50, thus reinforcing and complementing the results of Nakada et al. (2014).

In spite of the absence of differences in the frequency of HSCs, CLPs and CMPs between sexes by flow cytometry along the time, our next step was to test if mRNA expression profiles followed a similar trend to that observed at the transcriptional level. To study the role of Ly6a (Sca-1), Slamf1, Gata2, Id1, Ikzf1, and Cebpa in the hematopoietic system in male and female WT mice, gene expression profiles were monitored by real-time PCR analysis in peripheral blood from mice starting at P30 and up to P120. The reason behind the selection of these genes was thoroughly described above and corresponded with the genes better suited to assess the activity and state of the HSPCs studied. Ly6a (Sca-1) expression levels seemed to be lower in females than in males at P30, P50, P75, and P105, though there were no significant differences at any stage (Figure 2A). Slamf1 expression levels were statistically lower in females than males only at P30 ($*P < 0.05$) and P120 ($*P < 0.05$) (Figure 2B). Also, although Gata2 expression levels were apparently lower in females than in males, significant differences were only found at P50 ($*P < 0.05$) (Figure 2C). Interestingly, females presented lower expression levels for both Slamf1 and Gata2 at P30 and P50, respectively. As Slamf1 is a gene related to the more undifferentiated LT-HSCs (Beerman et al., 2010), it seems that this type of HSCs could be less active at the mRNA level in females than in males at P30 and P120. The differences in Gata2, together with the changes observed in Slamf1 could mean that female HSCs are more actively entering the cell cycle and differentiating than their male counterparts at P30, P50, and P120 (Rodrigues et al., 2012; Hirabayashi et al., 2017), suggesting different transcriptional processes for these genes at different time points. However, despite these slight differences in mRNA expression levels, no reper- cussions were found at the protein level. These results are in accordance with the asymmetrical differentiation pattern along time demonstrated by Nakada et al. (2014), and the flow cytometry results shown above. Finally, Id1 expression levels were higher in females than in males at P30, P105, and P120, but no significant differences were found. At P50 and P75, female Id1 expression levels tended to be lower than those found in males, although no significant differences between sexes were detected (Figure 2D). Similarly, Ikzf1 and Cebpa expression levels remained unchanged between sexes at every stage (Figures 2E and 2F).

Conclusions

In conclusion, our findings revealed that both male and female mice can be used to perform descriptive studies in the peripheral hematopoietic system of WT mice. Although minor differences at specific time points were detected in the case of Slamf1 and Gata2 mRNA levels between sexes, the lack of differences in the frequency of HSPCs at the protein level along time suggests that HSPC pool remains constant in both sexes along their develop- ment process. Therefore, we open the door to future studies using the B6SJL WT mice for the analysis of the hematopoietic system in peripheral blood using both sexes equally without fearing sex-related differences and thus avoiding sex biased results. This lack of differences would allow to diminish the number of animals needed for the experiments, as any of the sexes could be used indistinctly for descriptive studies of the murine hematopoietic system in peripheral blood. In addition, the use of serial extractions in the same animals drastically decreases the number of animals needed, which in our case supposed a fivefold decrease in the number of animals used.

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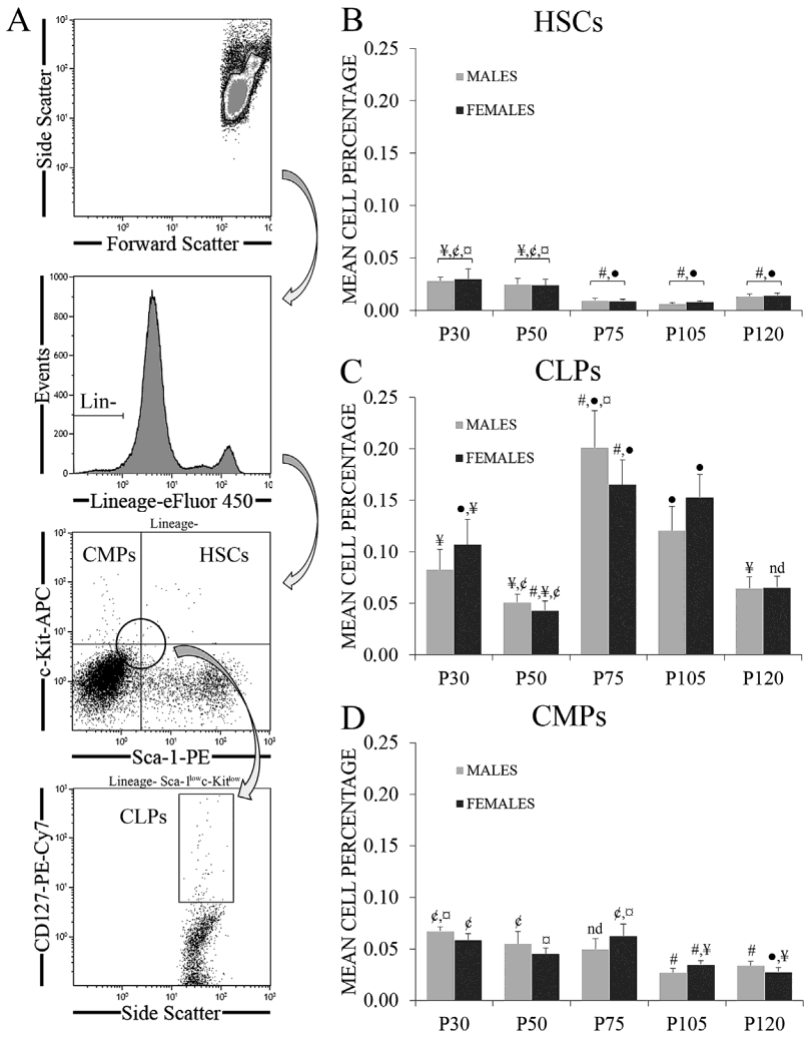

Figure 1 HSC, CLP, and CMP quantification in peripheral blood using flow cytometry at ages P30, P50, P75, P105 and P120. (A) Flow cytometry analysis followed for the quantification of HSPCs. Quantification was made using cell percentages. HSCs were identified as Lin-Sca-1 + c-Kit+, CLPs as Lin-Sca-1lowc-KitlowCD127+ and CMPs as Lin-Sca-1-c-Kit + CD127-. Males are represented with light gray bars, while females are represented with dark gray bars. (B) HSC, (C) CLP, and (D) CMP mean percentages at each age stage. Data shown as mean cell percentages of the mice at each stage SEM. No differences were found between sexes but some longitudinal differences were found for each sex and are highlighted with symbols, P-value < 0.05. #, significant difference from P30; *, significant difference from P50; ¥, significant difference from P75; ¢, significant difference from P105; ¤, significant difference from P120; nd, no significant differences. n = 30 mice (serial blood extractions were performed using the same animals each time).

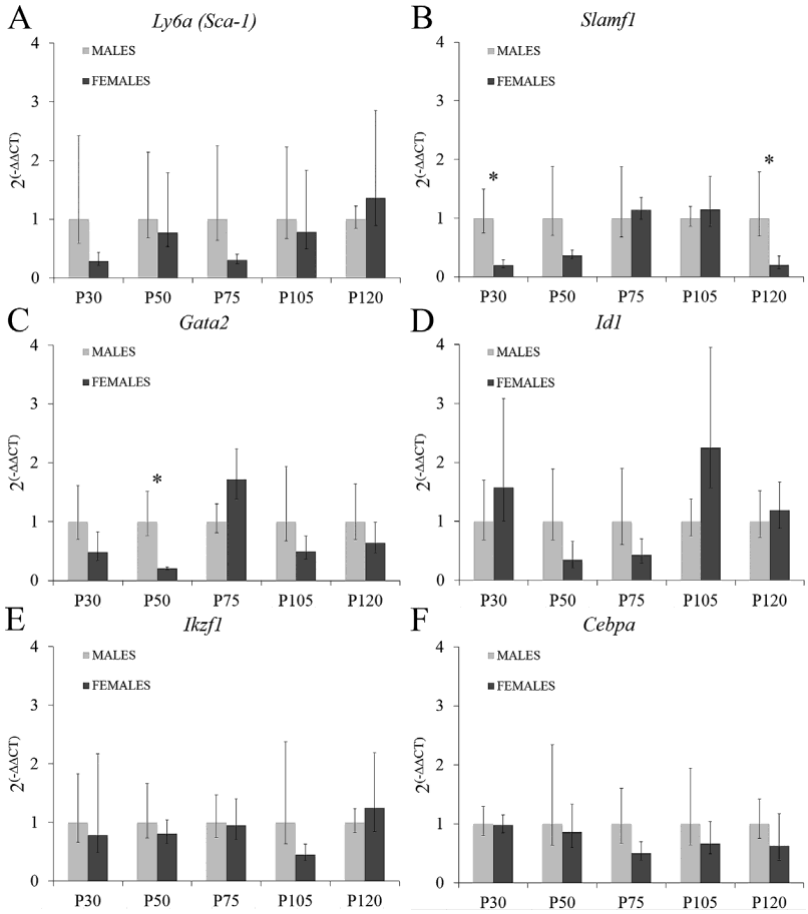

Figure 2 Relative expression values of Ly6a (Sca-1), Slamf1, Gata2, Id1, Ikzf1, and Cebpa mRNA expression levels in peripheral blood analyzed by RT-PCR. Males are represented using light gray bars, while females are represented with dark gray bars. (A) Ly6a, (B) Slamf1, (C) Gata2, (D) Id1, (E) Ikzf1, and (F) Cebpa expression levels for males and females at P30, P50, P75, P105, and P120 are shown. Fold-change represents the relative expression of the target genes in females compared with that of males at each age point. Each data point represents the mean of the mice at each stage. Error bars were calculated as described by Livak and Schmittgen (2001). Asterisks represent an independent t-test P-value < 0.05 (*); n = 4 for each time-point and sex group.