Comparative Medicine
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Vol 67, No 1
February 2017
Pages 22–27

Original Research

Age-Associated Differences in Hematopoietic Stem and Progenitor Cells of Mice

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Establishing the appropriate yet minimal number of control mice for experiments is a critical step in experimental design. This decision is particularly important regarding the study of the hematopoietic system over time, given various age-associated changes in murine hematopoietic cell populations. Here we used flow cytometry to serially monitor the frequencies of hematopoietic stem cells, common lymphoid progenitor cells, and common myeloid progenitor cells and RT-PCR assays to study the levels of *Ly6a* (*Sca1*), *Slamf1*, *Ikzf1*, and *Cebpa*—4 genes that control the hematopoietic process—in wildtype male and female mice with a B6SJL genetic background. These analyses revealed many differences, both at the cellular and mRNA levels, between immature and mature mice at various developmental stages. In conclusion, although it is necessary to minimize the number of mice possible insofar as possible to reduce animal use and meet animal welfare requirements, the numerous differences shown by our findings highlight the need to establish controls for every time point selected for the study of the hematopoietic system cells. This need is especially crucial when comparing immature and mature stages of mouse development.

Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell

The establishment of optimal numbers of animals for experiments continues to be an important issue in biomedical research. Differences due to sex-related factors as well as the natural maturity and aging process can arise and thus complicate the selection of the appropriate sex balance and number of the animals for research studies.[4,17,19]

Many of the studies of the hematopoietic system have involved mice. At the top of the hematopoietic hierarchy are hematopoietic stem cells (HSC); these multipotent cells are self-renewing and give rise to every type of blood cell. Their closest progeny are hematopoietic progenitor cells, specifically the common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), which are oligopotent cells with limited self-renewal capacity. CLP and CMP give rise to the lymphoid and myeloid lineages, respectively.[8,22,25] Collectively HSC and hematopoietic progenitor cells are known as hematopoietic stem and progenitor cells (HSPC) and are responsible for hematopoietic homeostasis. HSPC also have an important role in pathologic conditions, by rapidly supplying hematopoietic progenitor cells which can, in turn, give rise to numerous specialized immune cells.[21,22]

Regulation of hematopoiesis is orchestrated through many genes, including *Ly6A* (*Sca1*), *Slamf1*, *Ikzf1*, and *Cebpa*. *Ly6a* plays an essential role in the regulation of the HSC cycle and the commitment of these cells to become hematopoietic progenitors.[6] *Slamf1* is highly expressed in the subpopulation of long-term HSC, which have increased self-renewal potential and are prone to commit to the myeloid lineage.[3] *Ikzf1* expression is necessary for the commitment of multipotent progenitor cells to the lymphoid lineage (that is, it controls lymphopoiesis),[24] whereas *Cebpa* instructs multipotent progenitor cells to differentiate toward the myeloid lineage.[26] In this way, *Ikzf1* and *Cebpa* act as 'switches' for lineage commitment.

Many studies have addressed the variations in the immune system throughout the embryonic development and aging of mice, but little has been studied regarding the progression from postnatal immature stages to adulthood and even less about the steps involved during the maturation of HSPC. For example, T cells are known to be present in low numbers immediately after birth[2] and that their population increases as the thymus develops and increases the output of naïve T cells.[11] After a while, however, T cells numbers begin to diminish in number and function over time, due to the inexorable involution of thymus, which starts at a young age (around sexual maturity or even earlier) in both humans and mice.[13] In addition, as the mice age, HSC show a decrease in their self-renewal capacity but an increase in frequency and bias toward differentiation along the myeloid lineage, to the detriment of lymphoid lineage progenitors and mature lymphocytes.[11,14] In comparison, in both humans and mice, myeloid cells are present at high frequency during the first weeks after birth, due to the migration of these cells to colonize lymphoid and nonlymphoid tissues, particularly the gastrointestinal tract, where they exert a vital role in the establishment of the microbiota.[12,23] Once this process is complete and the animal is close to sexual maturity, myeloid cell frequencies stabilize, and the size of this

Received: 06 Apr 2016. Revision requested: 12 May 2016. Accepted: 31 Jul 2016.
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Pathogens tested	Screening method
Breeding colony	
Klebsiella oxytoca, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Entamoeba muris*, *Tritrichomonas muris*, *Aspiculuris tetraptera*, *Citrobacter rodentium*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Cryptosporidium* spp., *Entamoeba muris*, *Giardia muris*, *Helicobacter bilis*, *Helicobacter ganmani*, *Helicobacter hepaticus*, *Helicobacter mastomyrinus*, *Helicobacter rodentium*, *Helicobacter spp.*, *Helicobacter typhlonius*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Myocoptes* spp., *Pasteurella pneumotropica* biotypes Heyl and Jawetz, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Radfordia* (*Myobia*) spp., *Salmonella* spp., *Spironucleus muris*, *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Syphacia obvelata*, *Tritrichomonas muris*, and β-hemolytic *Streptococcus* spp. groups A, B, C, and G	PCR assay
Clostridium piliforme, *Mycoplasma pulmonis*, cilia-associated respiratory bacillus, ectromelia, enzootic diarrhea of infant mice virus, Hantaan virus, K virus, lymphocytic choriomeningitis virus, lactate dehydrogenase elevating virus, mouse adenovirus types 1 and 2, murine cytomegalovirus virus, mouse hepatitis virus, mouse norovirus, mouse parvovirus, mouse thymic virus, minute virus of mice, polyoma virus, pneumonia virus of mice, reovirus 3, Sendai virus, Theiler murine encephalomyelitis virus, *Encephalitozoon cuniculi*	Serology
Maintenance colony	
Aspiculuris tetraptera, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Cryptosporidium* spp., *Giardia muris*, *Helicobacter bilis*, *Helicobacter ganmani*, *Helicobacter hepaticus*, *Helicobacter mastomyrinus*, *Helicobacter rodentium*, *Helicobacter* spp., *Helicobacter typhlonius*, *Myocoptes* spp., *Pasteurella pneumotropica* biotypes Heyl and Jawetz, *Radfordia* (*Myobia*) spp., *Salmonella* spp., *Spironucleus muris*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Syphacia obvelata*, and β-hemolytic *Streptococcus* spp. groups *A*, *B*, C, and G	PCR assay
Clostridium piliforme, *Mycoplasma pulmonis*, *ectromelia*, enzootic diarrhea of infant mice virus, lymphocytic choriomeningitis virus, mouse adenovirus types 1 and 2, mouse hepatitis virus, mouse norovirus, mouse parvovirus, minute virus of mice, pneumonia virus of mice, reovirus 3, Sendai virus, and Theiler murine encephalomyelitis virus	Serology

Figure 1. Pathogens monitored through annual testing of mouse colonies.

population decreases compared with its size at the immature stage.

The aim of the current study was to investigate the frequencies of HSC, CLP, and CMP under physiologic conditions in immature and mature wild-type mice with a B6SJL background, to assess the need for time-point–specific experimental controls. To this end, we collected serial blood samples for analysis of these cell populations by flow cytometry. In addition, we used RT-PCR assays to evaluate the expression profiles of 4 genes important in the hematopoietic process—*Ly6A* (*Sca1*), *Slamf1*, *Ikzf1*, and *Cebpa*.

Materials and Methods

Animals. Wild-type mice on a B6SJL genetic background (B6SJL-Tg(SOD1-G93A)1Gur/J; The Jackson Laboratory, Bar Harbor, ME) were used for this study. Pelleted food (no. 2914 for maintenance and no. 2918 for breeders, Harlan Laboratories, Indianapolis, IN) and tap water were provided without restriction. Mice were housed in IVC (Tecniplast, Buguggiate, Italy) under a 12:12-h light cycle at the Unidad Mixta de Investigación (University of Zaragoza, Spain). Ambient conditions included a room temperature of 21 to 23 °C and a relative humidity of 55%. Cages contained bedding (Lignocel Select Fine for maintenance and ARBOCEL Performance Small for breeders, Rettenmaier Ibérica, Barcelona, Spain) but no other enrichment items. The mice were routinely screened (IDEXX, Westbrook, ME) for bacterial, viral, and parasitic pathogens according to FELASA guidelines and by using standard procedures (Figure 1). All experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. Animal care and experimentation were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE. The methodology enabled accurate characterization of the cells in small volumes of whole blood, which is in accordance with previous studies.[7] Notwithstanding, we did not test any mice younger than 30 d, to avoid undue stress during the first weeks of life and possible developmental effects.

Blood extraction and flow cytometry. For the flow cytometry study, 30 wildtype B6SJL (15 male and 15 female) were used. Serial blood samples were collected on postnatal days 30, 50, 75, 105, and 120. The selected ages correspond to various development milestones in mice. The tails of the mice were exposed to an infrared light for 5 min to dilate the veins. The tail skin was disinfected with alcohol, and blood was collected by puncturing one of the lateral tail veins by using a 25-gauge needle and collecting the blood into capillary tubes containing EDTA (Microvette CB 300, Sarstedt, Nümbrecht, Germany). The amount of blood collected ranged between 20 and 100 µL. For identification of HSC, samples were stained with antibodies to CD3 (clone 17A2), CD45R (B220), CD11b (m1/70) TER119, and LyG6/Gr1 (RB6-C5) (Mouse Hematopoietic Lineage eFluor 450 Cocktail, eBioscience, San Diego, CA), Ly6A/E (Sca1; PE-conjugated, eBioscience), and CD117 (c-kit) (APC-conjugated, eBioscience). To differentiate CMP from CLP, samples were stained with PE–Cy7-conjugated antiCD127 (IL7RA) antibody (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were lysed, and samples were analyzed by flow cytometry (Gallios Flow Cytometer, Beckman Coulter, Brea, CA); resulting data were analyzed by using Kaluza Flow Analysis Software (Beckman Coulter) and exported to a spreadsheet (Excel, Microsoft, Redmond, WA). Compensation controls were run for each antibody to correct spectral overlap.

Real-time PCR analysis. For real-time PCR analysis, 8 wildtype B6SJL mice (4 male and 4 female) per time point (age, 30, 50, 75, 105, and 120 d) were euthanized by CO_2 anesthesia, and blood obtained by cardiocentesis was collected into 2-mL EDTA tubes. Total RNA was extracted (Purelink Total RNA Blood Purification Kit, Invitrogen, Life Technologies; Waltham, MA) from each sample, treated with DNAse (Turbo DNA-free Kit, Ambion, Life Technologies, Waltham, MA) to eliminate genomic DNA, and reverse-transcribed (SuperScript First-Strand Synthesis System kit,

Invitrogen). Probes for *Ly6a* (*Sca1*) (catalog no. m00726565_s1), *Slamf1* (Mm00443316_m1), *Ikzf1* (Mm01187882_m1), and *Cebpa* (Mm00514283_s1) were obtained from Applied Biosystems (Life Technologies, Foster City, CA). The endogenous gene *Gapdh* (Mm03302249_g1; probe from Applied Biosystems) was used to normalize the data.

Statistics. Data were analyzed by using SPSS Statistics version 19.0 (IBM, Armonk, NY). Changes in gene expression and cell frequencies throughout the animal development were assessed by using repeated-measures ANOVA after checking the normality assumption by using the Kolmogórov–Smirnov test, and the corresponding Bonferroni posthoc test was used to analyze specific differences between the time points. The data are presented as mean ± SEM (error bars). A P value less than 0.05 was used to define statistical significance.

Results

HSPC profiles at various stages of sexual maturity in mice. Our first step was to use flow cytometry to study HSC, CLP, and CMP profiles in serial blood samples obtained from immature (postnatal days 30 and 50) and mature (days 75, 105, and 120) mice; sexual maturity in laboratory mice is considered to between days 45 to 60.[18] Flow cytometry results revealed that most of the differences were set between the immature and the mature stages of mice development (Figure 2). HSC frequencies at days 30 and 50 (which did not differ from each other) both were greater than those at each later time point (Bonferroni $P < 0.001$ for all cases; Figure 2 A). Differences in CLP frequencies were similar in magnitude but showed the opposite relationship, as CLP percentages were lower at 30 and 50 d and dramatically increased at 75 d followed by a gradual decrease over later time points (Bonferroni P between days 30 and 75, $P < 0.05$; between days 30 and 120, 50 and 75, and 50 and 105: $P < 0.001$; Figure 2 B). For CMP, the inflection point occurred at day 75 and was therefore slightly delayed relative to those of HSC and CLP (day 50; Bonferroni P between days 50 and 120, $P < 0.01$; between days 30 and 105, 30 and 120, and 50 and 105: $P < 0.001$; Figure 2 C).

In addition to the differences between immature and mature time points, HSC profiles within each developmental stage differed. The percentages of CLP and CMP decreased slightly (Bonferroni $P < 0.001$) between days 30 and 50 (Figure 2 B and C). Regarding the later time points, mature stages, HSC frequency decreased slightly (Bonferroni $p < 0.05$) from 75 to 105, followed by slight increase (Bonferroni $P < 0.001$) between days 105 and 120 (Figure 2 A). In contrast, both CLP and CMP consistently decreased from day 75 to 120 (Bonferroni $P < 0.001$ for both CLP comparisons; Bonferroni $P < 0.01$ for both CMP comparisons).

mRNA expression profile. We next assessed whether the mRNA expression profiles of genes important in the control of hematopoiesis followed similar trends to those observed at the cellular level. mRNA levels of *Ly6a* (*Sca1*) and *Slamf1* l showed similar patterns to those of HSC and CLP frequency, with expression levels differing mainly between immature and mature stages (Figure 3 A and B). Expression levels of *Ikzf1* and *Cebpa* tended to parallel CLP and CMP frequency trends in immature mice but sometimes differed from population data in mature mice (Figure 3 C and D).

Regarding differences in transcription levels at various time points within a developmental stage, *Ly6a* (*Sca1*) was the only gene to show differences (Bonferroni $P < 0.05$) in immature mice

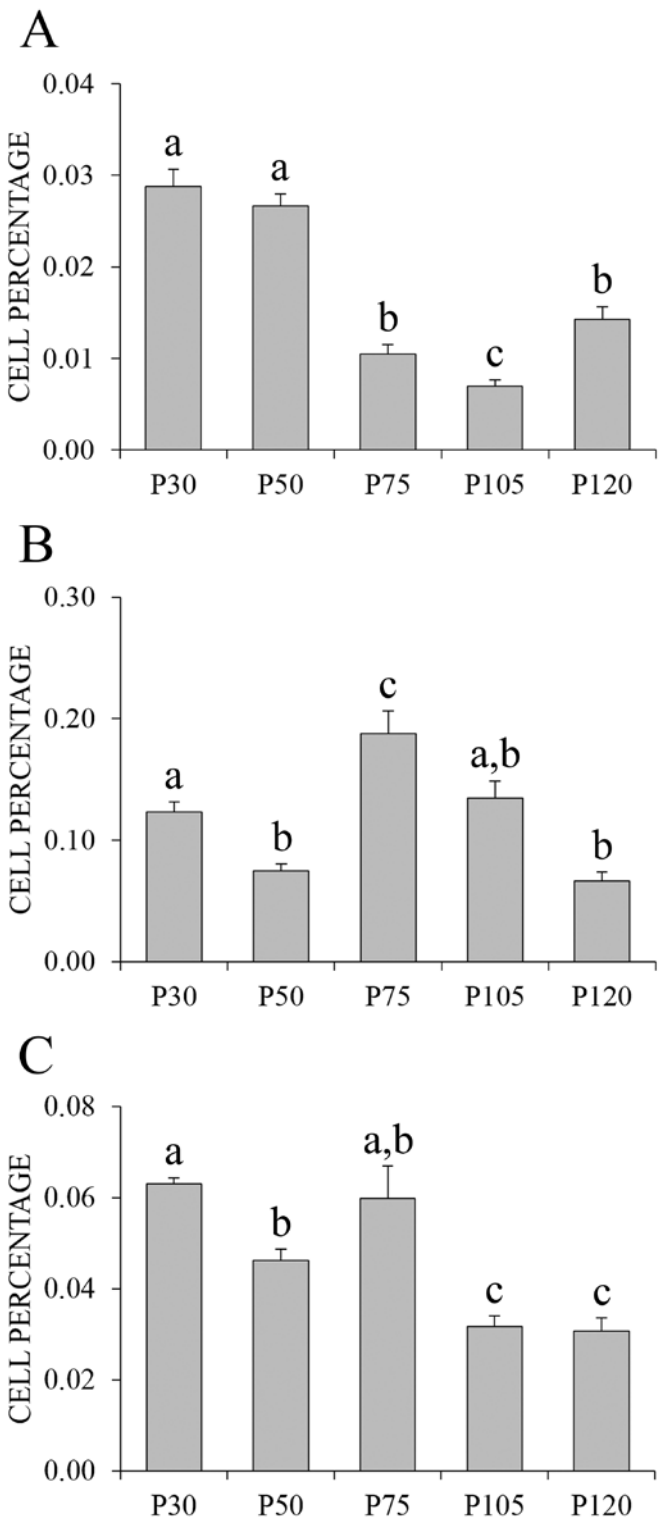

Figure 2. Flow cytometric analysis of HSPC frequencies (%; mean ± SEM) in the peripheral blood of mice at postnatal day (P) 30, 50, 75, 105, and 120 d. HSC were identified as being Lin$^-$Sca1$^+$c-Kit$^+$; CLP were Lin$^-$Sca1^{low}c-Kit^{low}CD127$^+$; and CMP were Lin$^-$Sca1$^-$c-Kit$^+$CD127$^-$. (A) HSC percentages. (B) CLP percentages. (C) CMP percentages. Different lowercase letters indicate significantly ($P < 0.05$) different values; $n = 30$ (15 male and 15 female, monitored serially).

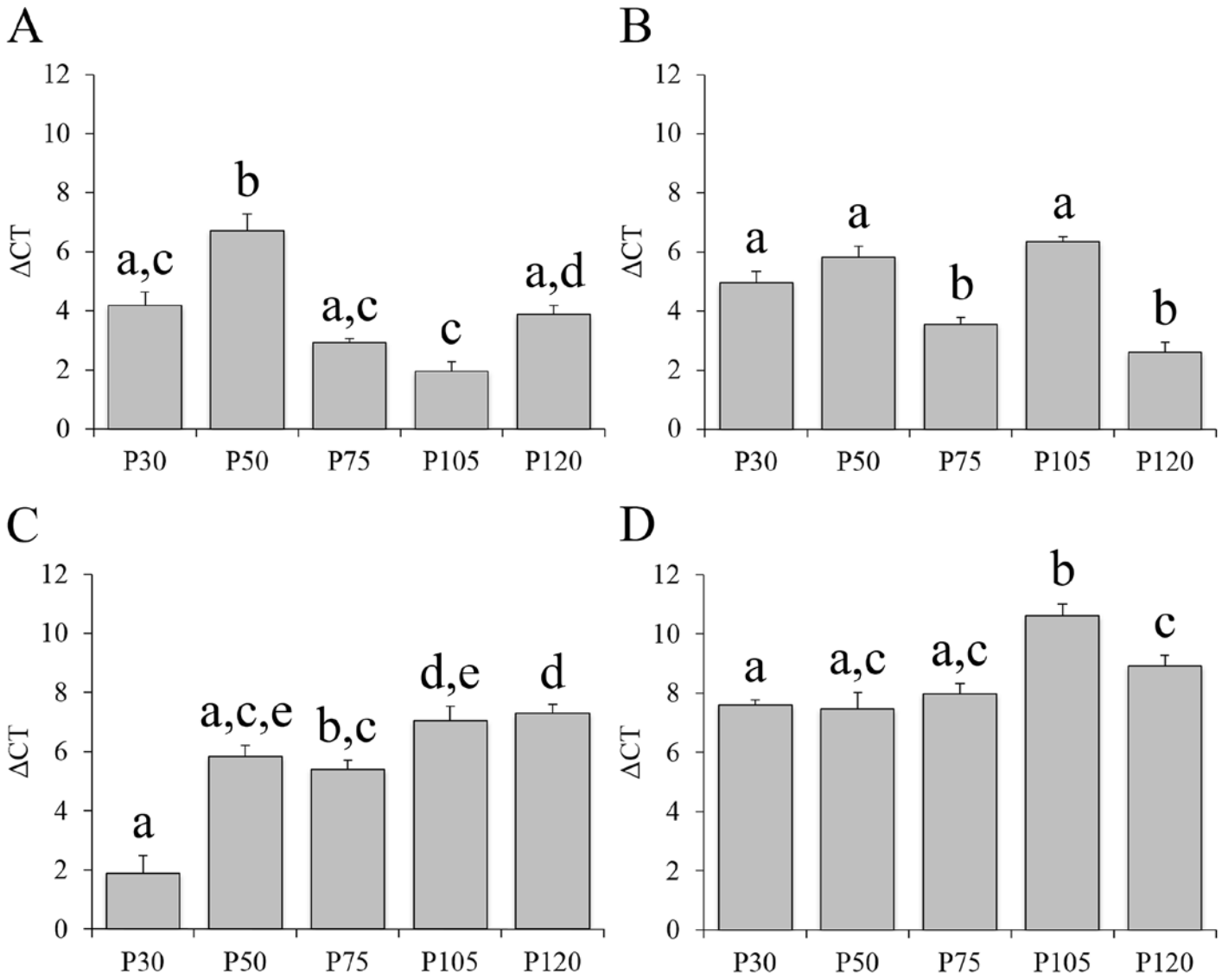

Figure 3. RT-PCR analysis of circulating mRNA transcript levels (cycle threshold [ΔC_t] value; mean ± SEM) of (A) *Ly6a* (*Sca1*), (B) *Slamf1*, (C) *Ikzf1*, and (D) *Cebpa* in mice at postnatal days (P) 30, 50, 75, 105, and 120. Different lowercase letters indicate significantly ($P < 0.05$) different values; $n = 40$ in total, 4 male and 4 female at each time point.

(that is, between days 30 and 50). With regard to mature mice, all 4 genes showed differences in expression between days 75, 105, and 120. *Ly6a* (*Sca1*) transcript levels differed between days 105 and 120 (Bonferroni $P < 0.05$); *Slamf1* between days 75 and 105 and days 105 and 120 (Bonferroni $P < 0.001$ for both comparison); *Ikzf1* between days 75 and 105 (Bonferroni $P < 0.05$) and days 75 and 120 (Bonferroni $P < 0.01$); and *Cebpa* expression differed between days 75 and 105 (Bonferroni $P < 0.01$) and days 105 and 120 (Bonferroni $P < 0.05$).

Discussion

Our flow cytometric results revealed that, overall, the HSC, CLP, and CLM profiles of immature mice differed from those of mature mice (Figure 2). The inflection point of cell frequencies was set between days 50 and 75, with HSC being higher at the immature stage and then decreasing and CLP and CMP percentages acting in the opposite way. Our HSC data are in accordance with studies showing that the frequency of HSC is higher during the immature stages of fast development and declines once the mice reach maturity.[9] Whereas the point of inflection was day 50 for CLP as for HSC, that for CMP was delayed slightly, to day 75. The different inflection times for CLP and CMP might reflect the adjustment of the immune system to the process of developmental maturation, given that members of the myeloid lineage are more prominent and numerous than are those of the lymphoid lineage at the immature stages of hematopoietic development. Myeloid-derived suppressor cells are known to be abundant in young mice and have a prominent role in modulating the early immune response,[16] thus perhaps explaining the delayed decrease in CMP frequency that we observed. In addition, the higher frequency of CMP in immature mice could be due to the continued circulation of these cells in the blood to colonize lymphoid and

nonlymphoid tissues; unlike that in humans, this colonization is not completed before birth in mice.[12] Finally, during the first weeks after birth, the gastrointestinal tract is colonized by the microbiota, thereby providing another possible explanation for the increased presence of circulating CMP, given that their mature progeny are important in the early acquisition of tolerance to the microbiota.[1] In contrast, CLP were decreased in frequency at early experimental time points, perhaps owing to the generally immature state of the lymphoid system at birth and during the first weeks of life.[2] Once mice became mature, however, the lymphoid lineage inverted the CMP–CLP relationship, and CLP reached much higher frequencies before decreasing over time. This decrease was observed previously in 7-mo-old mice, in which the CLP frequency in the bone marrow was decreased, seemingly due to the diminished lymphopoietic potential of the HSC and perhaps also reflecting defects in the survival or proliferative potential of CLP themselves.[20]

Regarding our study of the mRNA expression patterns of 4 genes related to the hematopoietic system function (Figure 3), *Ly6a* (*Sca1*) and *Slamf1* transcript levels were similar in overall pattern to the evolution of HSC and CLP frequencies, with marked differences in immature compared with mature mice. Given that these 2 genes regulate HSC function and their commitment to lineage progenitors, similar trends between their expression and the frequency of these cells was logical.[3,6,10] *Ikzf1* and *Cebpa* expression trends were similar to those of *Ly6a* (*Sca1*) and *Slamf1* in immature but not mature mice. CLP frequency decreased from day 75, but *Ikzf1* expression levels did not precisely follow this pattern. In addition, *Cebpa* levels at days 105 and 120 deviated from the population findings, in that CMP frequency was lower at these 2 time points compared with earlier points. Ikzf1 and Cebpa are essential in the commitment of multipotent progenitor cells to either the lymphoid or myeloid lineage, respectively.[5,15,26] The parallel increases in *Ikzf1* and *Cebpa* expression levels and CLP and CMP populations in immature mice is in accordance with their function and with the frequencies observed for these cells. The similarities between the mRNA profiles and the cell frequencies might reflect the activation of these genes to promote the proliferation of the cells in which they play a prominent role, at least early during development.

The findings obtained from our flow cytometric and RT-PCR analyses of hematopoietic cells and genes reveal the need to establish an appropriate population of control animals at each of the specific age points selected for an experiment. Although minimizing the number of control animals is laudable, using a single control group for an overall developmental stage (for example, immature and mature) is in appropriate for hematopoiesis research, given the rapid variation in the HSPC profile throughout development process (as little as 25 d, in the case of the differences found between the 50- and 75-d time points). Just as the maximal number of animals used as controls should minimized, it is mandatory to establish an appropriate minimal number of controls for every time point selected in the study of the hematopoietic system to ensure the validity of research results.

Acknowledgments

This work was supported by Grant FIS (PI14/00947), Instituto de Salud Carlos III, and FEDER Funds. We thank Miriam de la Torre, Silvia Garcés, and Pilar Lierta for their expert technical assistance.

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