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Lymphoid-Biased Hematopoietic Stem Cells Are Maintained with Age and Efficiently Generate Lymphoid Progeny

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SUMMARY

Current models propose that reductions in the number of lymphoid-biased hematopoietic stem cells (Ly-HSCs) underlie age-related declines in lymphopoiesis. We show that Ly-HSCs do not decline in number with age. Old Ly-HSCs exhibit changes in gene expression and a myeloid-biased genetic profile, but we demonstrate that they retain normal lymphoid potential when removed from the old *in vivo* environment. Additional studies showing that interleukin-1 inhibits Ly-HSC lymphoid potential provide support for the hypothesis that increased production of inflammatory cytokines during aging underlies declines in lymphocyte production. These results indicate that current models proposing that lymphopoiesis declines with age due to loss of Ly-HSCs require revision and provide an additional perspective on why lymphocyte development in the elderly is attenuated.

INTRODUCTION

Aging alters the pattern of hematopoiesis in mice (Geiger et al., 2013; Montecino-Rodriguez et al., 2013) and humans (Pang et al., 2011; Rundberg Nilsson et al., 2016), with declines in lymphocyte production being a prominent feature. The significant reduction in the number of B lineage cells produced (Johnson et al., 2002; Miller and Allman, 2003; Min et al., 2006; Riley et al., 2005) results in reduced replenishment of naive B cells in secondary lymphoid tissues. Intrinsic and/or extrinsic perturbations may underlie the decline in lymphopoiesis (Florian et al., 2012; Labrie et al., 2004; Sudo et al., 2000), which may in turn contribute to an increased susceptibility of the elderly to infection and a reduced vaccination efficacy (McElhaney, 2005). In contrast, myelopoiesis predominates in the marrow of old individuals, although age-related deficiencies have been reported (Dykstra et al., 2011; Signer et al., 2007).

Multiple processes such as proliferation, DNA repair, and cell polarity are altered with age in HSCs (Geiger et al., 2013, 2014; King and Goodell, 2011). To provide a molecular basis for these observations, several laboratories compared the transcriptomes of young and old stem cells (Chambers et al., 2007; Grover et al., 2016; Kirschner et al., 2017; Kowalczyk et al., 2015; Rundberg Nilsson et al., 2016; Sun et al., 2014). Consistent with their altered functions, old HSCs exhibit changes in expression of genes that regulate processes that include self-renewal and differentiation (Kowalczyk et al., 2015; Sun et al., 2014). In addition, an increase in the expression of myeloid lineage genes, including those specifying platelet/megakaryocyte differentiation (Grover et al., 2016), and a downregulation of genes that specify lymphoid production (Rossi et al., 2005), were observed. Whether these age-related changes in gene expression irreversibly compromise lymphoid differentiation is unclear.

The HSC compartment includes lymphoid-biased (Ly-HSCs) and myeloid-biased (My-HSCs) subpopulations (Dykstra et al., 2007; Muller-Sieburg et al., 2004), and Muller-Sieburg and colleagues reported that the age-associated decline in lymphopoiesis is due to reductions in the number of Ly-HSCs (Muller-Sieburg and Sieburg, 2008). Ly-HSCs and My-HSCs express low and high levels, respectively of the CD150 (*Slamf1*) cell surface determinant (Beerman et al., 2010; Challen et al., 2010), and phenotypic analyses demonstrated that the proportion of Ly-HSCs is reduced in old mice while that of My-HSCs is increased (Beerman et al., 2010; Challen et al., 2010). Based on these results, the current model is that the loss of Ly-HSCs contributes to declines in lymphocyte production with age (Elias et al., 2016; Montecino-Rodriguez et al., 2013; Muller-Sieburg et al., 2004).

This view of HSC aging does not take into account the increase in total HSCs with age (de Haan et al., 1997; Dykstra et al., 2011). We now show that when this is considered, the number of Ly-HSCs does not decrease in old mice. We also demonstrate that, upon removal from the old *in vivo* environment, the potential of old Ly-HSCs to produce lymphoid progenitors is comparable with that of their young counterparts. This is the case even though old Ly-HSCs acquire a myeloid-biased pattern of gene expression. Instead, our data support the view that the increased production of inflammatory cytokines in the old environment is responsible for age-related declines in lymphopoiesis. These observations indicate that current models proposing

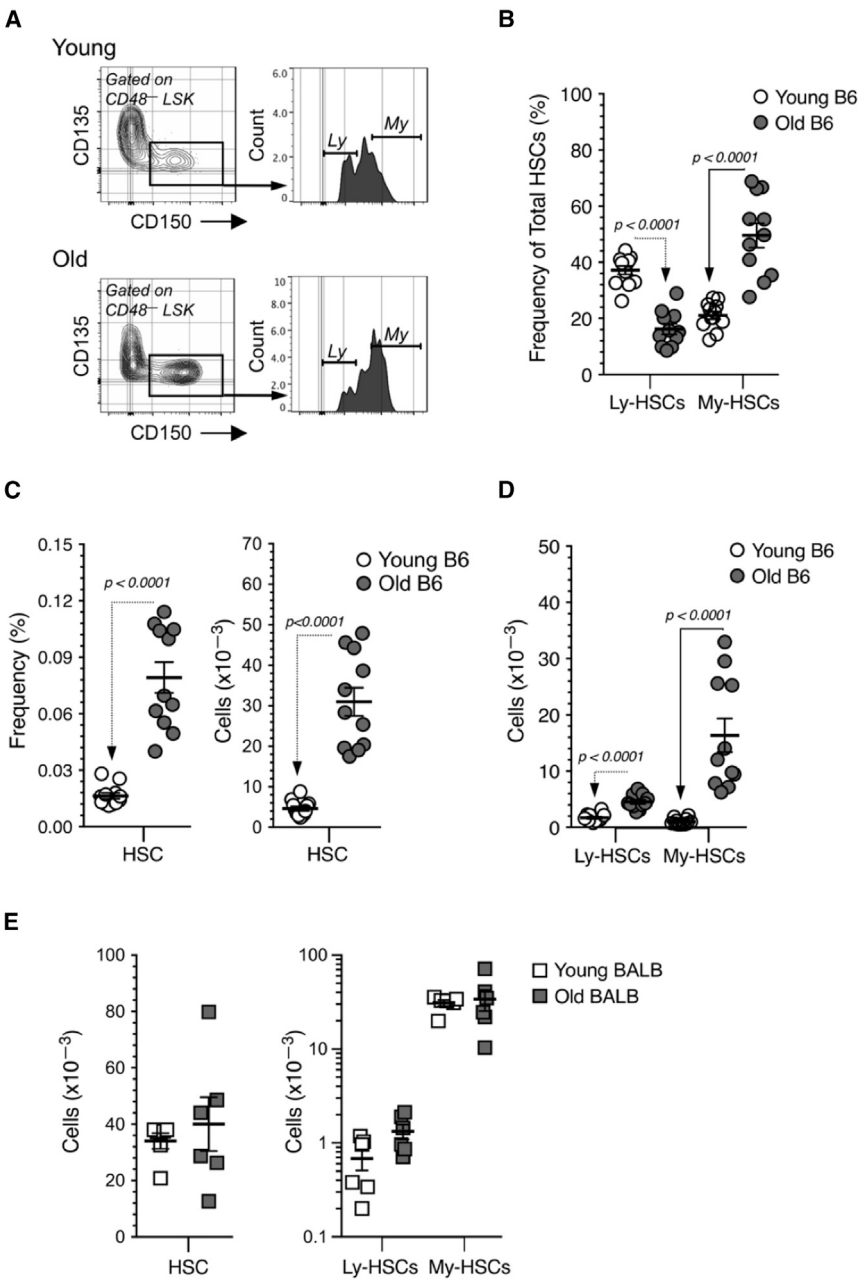

Figure 1. Quantification of Ly-HSCs and My-HSCs in the Marrow of Young and Old Mice

(A) Fluorescence-activated cell sorting (FACS) plots showing the strategy used for the resolution of CD135$^-$ CD150^{low} Ly-HSCs and CD135$^-$ CD150^{high} My-HSCs within the lineage-negative, Sca-1$^+$ CD117 (c-Kit)$^+$ (LSK) CD48$^-$ population. Figure S1A shows the gating strategy.

(B) Relative frequency of Ly-HSCs and My-HSCs within the total HSCs in the bone marrow of young and old B6 mice.

(C) Frequency and total number of HSCs in the bone marrow of young and old B6 mice.

(D) Total number of Ly-HSCs and My-HSCs in the bone marrow of young and old B6 mice.

(E) Number of total HSCs (left panel) and Ly-HSCs and My-HSCs (right panel) in the bone marrow of young and old BALB/c mice. (B–E) Each symbol represents a mouse (young, 8–12 weeks; old, 17–18 months); levels of significance for differences between populations are indicated. Error bars indicate means ± SEM.

that lymphopoiesis declines with age due to loss of Ly-HSCs need revision.

RESULTS

The Number of Ly-HSCs Does Not Decline with Age

Current models propose that the number of Ly-HSCs declines and that the number of My-HSCs increases with age (Elias et al., 2016; Montecino-Rodriguez et al., 2013; Muller-Sieburg et al., 2004). We quantified the frequency of lineage-negative, Sca-1$^+$ CD117(c-Kit)$^+$ (LSK) CD48$^-$ CD135$^-$ CD150^{low} Ly-HSCs and LSK CD48$^-$ CD135$^-$ CD150^{high} My-HSCs (Figure S1A), in young and old C57BL/6 (B6) mice and found that, consistent with previous reports (Beerman et al., 2010; Challen et al., 2010), the proportion of Ly-HSCs significantly declines with age while that of My-HSCs significantly increases (Figures 1A and 1B). In agreement with other reports (Dykstra and de Haan, 2008; Geiger and Van Zant, 2002), the number of HSCs increases with age in B6 mice (Figure 1C). When this is considered, it is clear that there is a significant

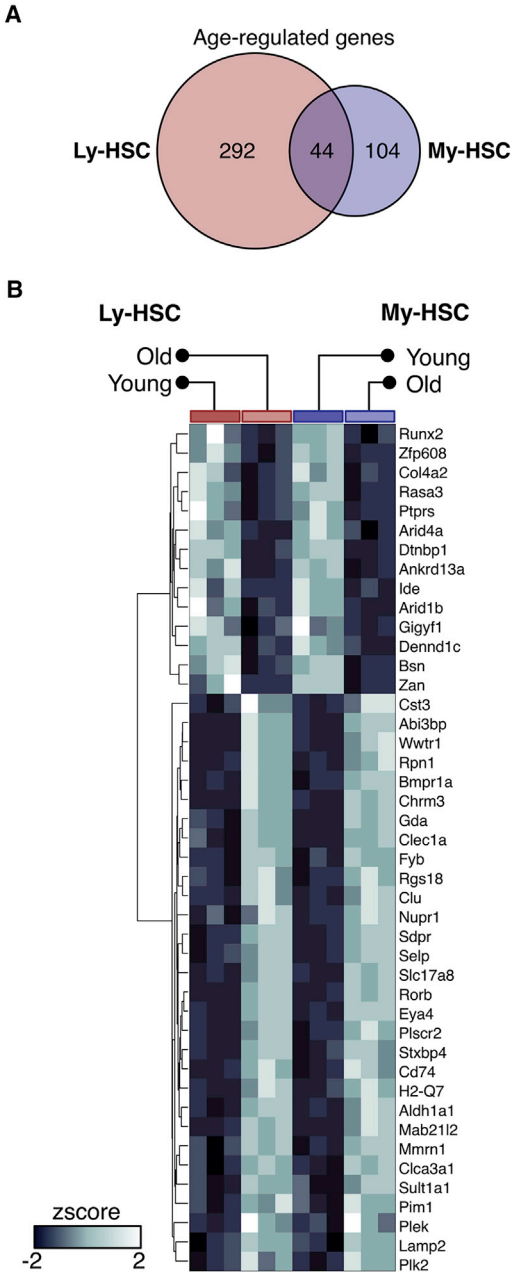

Figure 2. Ly-HSCs and My-HSCs Exhibit Specific Changes in Gene Expression with Age

(A) Venn diagram showing the number of genes whose expression changed significantly (adjusted p value <0.05) between young and old Ly-HSCs and young and old My-HSCs and which changed in common in both subsets.

(B) Hierarchical clustering for the 44 genes classified as age regulated in both Ly-HSCs and My-HSCs for each individual biological replicate.

increase in My-HSC number and that they are the predominant stem cell population in old mice. It is also evident that the total number of Ly-HSCs does not decline and is significantly higher in old compared with young bone marrow (Figure 1D). A similar result was obtained with BALB/c mice (Figure S1B). Total HSC number did not significantly change with age in that strain but the total number of Ly-HSCs did not decline with age (Figure 1E).

Ly-HSCs Exhibit Changes in Gene Expression with Age

Aging affects gene expression in total HSCs (Chambers et al., 2007; Grover et al., 2016; Kirschner et al., 2017; Kowalczyk et al., 2015; Rundberg Nilsson et al., 2016; Sun et al., 2014). To determine if such age-related genetic alterations occurred in Ly-HSCs and My-HSCs, we performed RNA sequencing (RNA-seq) on these populations isolated from three independent groups of young and old mice. All profiled samples were at least 98% pure, and those that did not achieve this level in the initial sort were resorted resulting in 100% purity (Figure S1C). We compared our sequencing data with two separate sets of gene expression signatures (ImmGen and The Mouse Body Atlas), and all samples showed maximal and consistent enrichment for HSC-specific signatures (Figures S2A and S2B; Table S1).

Expression of 336 genes changed significantly between young and old Ly-HSCs, and this was over 2-fold greater than the 148 genes that changed between young and old My-HSCs (Figure 2A). The magnitude of gene expression changes in Ly-HSCs was often markedly higher and skewed toward extreme values (two-sample Kolmogorov-Smirnov test p value < 0.05, Table S2 and data not shown) compared with those in My-HSCs. This trend was particularly significant for genes annotated in intracellular signaling pathways (Rho GTPases, MAPK), response to stress, DNA damage, and cell-cycle control (Table S2). Only 44 genes changed in common in both stem cell subsets (Wald test adjusted p value < 0.05; Figure 2B) and included *Plk2*, *Lamp2*, *Pim1*, *Mmrn1*, *Aldh1a1*, *Cd74*, *Rorb*, *Clu*, and *Selp*. The latter genes have been reported as markers of aging in total HSCs (Chambers et al., 2007; Grover et al., 2016; Kirschner et al., 2017; Kowalczyk et al., 2015; Rundberg Nilsson et al., 2016; Sun et al., 2014).

Analysis of the data by multidimensional scaling revealed that old Ly-HSCs acquired a pattern of gene expression that overlapped with My-HSCs, and that this was even more pronounced when age-responsive genes specific to Ly-HSCs were considered (Figure 3A). We identified the genes whose change in expression contributed to the myeloid-biased signature of old Ly-HSCs by grouping old Ly-HSCs with young and old My-HSCs and comparing their combined pattern of gene expression to that in young Ly-HSCs. The rationale for combining old Ly-HSCs together with young and old My-HSCs was based on the multidimensional scaling showing that these three groups of stem cells clustered together (Figure 3A). This analysis identified 507 differentially expressed genes (Wald test

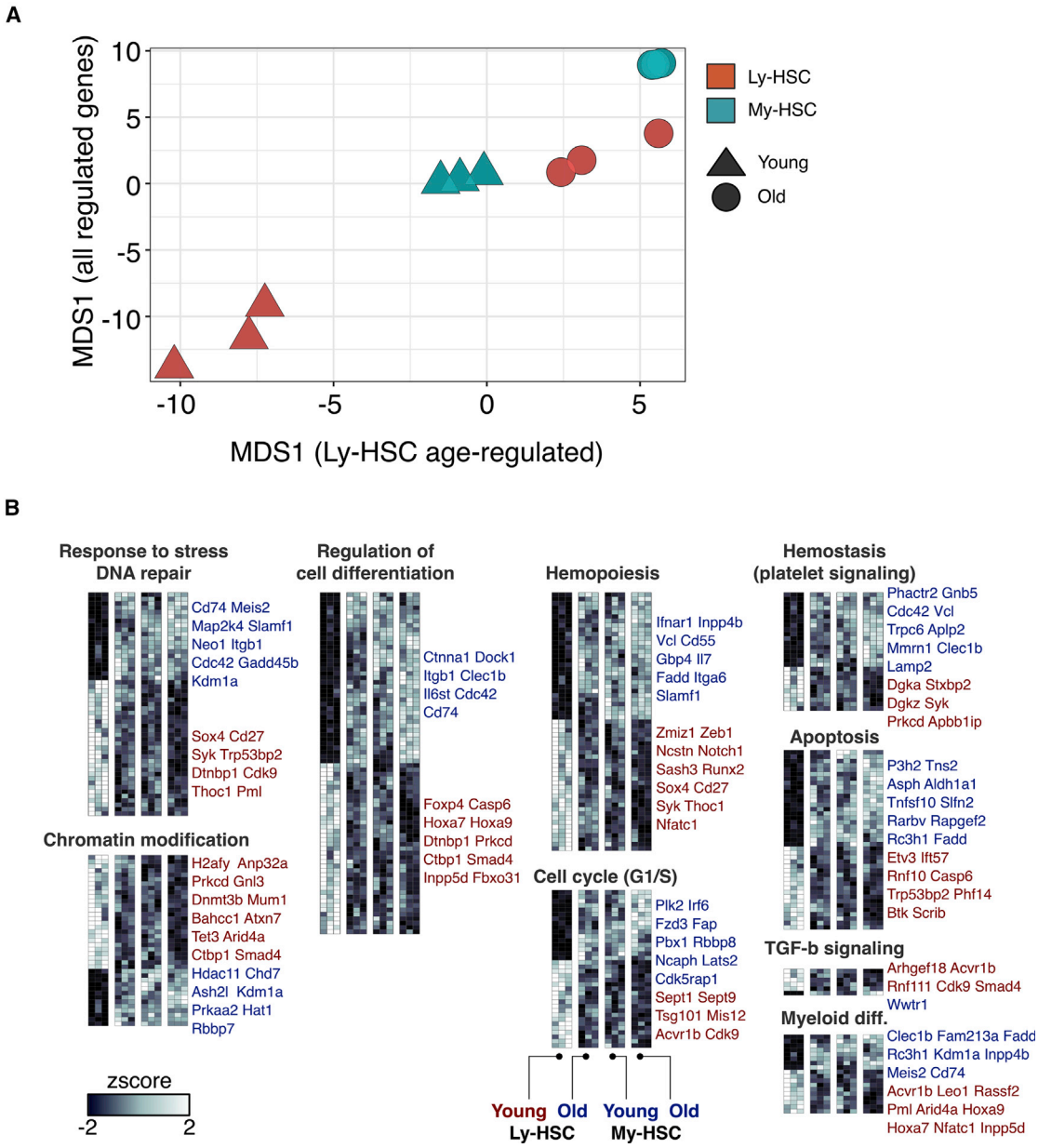

Figure 3. Ly-HSCs Acquire a Myeloid-Biased Pattern of Gene Expression with Age

(A) Multidimensional scaling analysis of young and old Ly-HSCs and My-HSCs. Each symbol represents a distinct cohort of Ly-HSCs or My-HSCs isolated from 6 to 8 B6 mice. The scatterplot shows first multidimensional scales, obtained from all variable (age and lineage bias) genes (1,062 genes, y axis) or from those identified as regulated between young and old Ly-HSCs (337 genes, x axis).

(B) Hierarchical clustering for genes classified as significant (adjusted p value <0.05) in a pairwise test between young Ly-HSCs and the rest of the samples. Genes are grouped by functional categories (only significant categories are shown, $p < 0.01$). Representative gene names are shown (blue, lower expression; red, higher expression in young Ly-HSCs). A list of the specific genes in each plot can be found in Table S2.

adjusted p value < 0.05) that were associated with response to stress/DNA damage, chromatin/histone modification, regulation of cell differentiation, hemopoiesis, cell cycle, hemostasis (platelet signaling), apoptosis, transforming growth factor β signaling, and myeloid differentiation (Figure 3B; Table S2). We also performed a supervised analysis in which we compared the gene expression changes observed in old Ly-HSCs to a myeloid signature list defined

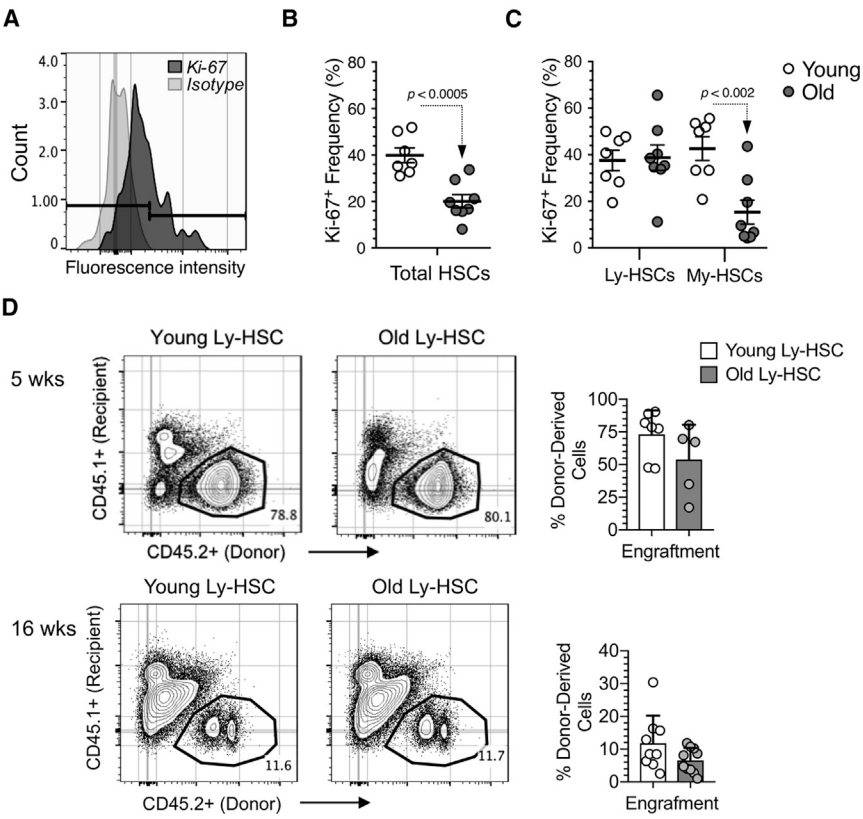

Figure 4. Young and Old Ly-HSCs Exhibit Similar Levels of Proliferation and Reconstitution Potential

(A) Representative FACS plot showing Ki-67 staining of total young HSCs with Ki-67 antibody and isotype control.

(B) Frequency of Ki-67 expressing young and old total HSCs.

(C) Frequency of Ki-67 expressing young and old Ly-HSCs and young and old My-HSCs. Levels of significance are indicated. Each symbol in (B and C) represents an individual mouse. Error bars indicate means ± SEM.

(D) Left panels: representative FACS plot showing resolution of CD45.1 recipient and CD45.2 donor cells in the bone marrow of Busulfan-conditioned CD45.1 B6 mice 5 and 16 weeks after transplantation. Right panels: percent donor chimerism in the marrow of Busulfan-conditioned CD45.1 mice at 5 and 16 weeks post-transplantation. No difference in total bone marrow cellularity was observed in any of the recipients at either time point. Error bars indicate means ± SD. Each symbol represents an individual recipient mouse.

in a previous study (Chambers et al., 2007). This revealed that some myeloid-specific genes were overexpressed in old Ly-HSCs at levels similar to those in old My-HSCs (Figure S3A).

The Lymphoid Potential of Young and Old Ly-HSCs Is Equivalent

To determine if the above gene changes affected Ly-HSC function, we first compared proliferation in young and old Ly-HSCs by Ki-67 labeling (Figures 4A–4C). Total HSC proliferation declines with age (Dykstra et al., 2011; Geiger et al., 2014), and our data are in agreement with this finding (Figure 4B). We found that there was no difference in Ki-67 levels between young and old Ly-HSCs. In contrast, we observed that old My-HSCs exhibited a significant decrease in Ki-67 positivity compared with their young counterparts (Figure 4C). These results suggest that the age-related reductions in the proliferation of total HSCs (Dykstra et al., 2011; Geiger et al., 2014) largely reflect changes in My-HSCs. We also found that proliferation of young Ly-HSCs and young My-HSCs was similar. This result is not in agreement with a previous study (Challen et al., 2010), but the precise reasons for this discrepancy remain to be determined.

We then determined if the above gene changes affected HSC differentiation. Previous studies reported that old Ly-HSCs do not efficiently engraft in irradiated recipients (Beerman et al., 2010), making it difficult to compare their hematopoietic potential to that of young Ly-HSCs *in vivo*. We searched for an alternative conditioning regimen that would obviate this complication and chose to use Busulfan based on studies showing that robust hematopoietic reconstitution was observed in mice treated with doses of 20 mg/kg or higher (Hsieh et al., 2007; Yeager et al., 1991). We transplanted 200 young or old Ly-HSCs isolated from CD45.2$^+$ donors into Busulfan-conditioned CD45.1 SJL recipients and examined reconstitution 5 and 16 weeks later.

Recipients of young and old Ly-HSCs exhibited similarly high levels of donor cell engraftment in the bone marrow at 5 weeks post-reconstitution. No differences in levels of bone marrow engraftment by young and old Ly-HSCs were observed at 16 weeks post-transplantation as well, although levels of donor cell chimerism were markedly lower (Figure 4D). This result is consistent with a previous study demonstrating that Ly-HSCs are bona fide stem cells but do not exhibit robust long-term reconstitution potential (Challen et al., 2010). There were no significant differences in the proportion of donor HSCs in recipients of young or old Ly-HSCs at either time point (Figure 5A). Because of the higher level of donor chimerism at the 5-week time point, we were able to determine that the

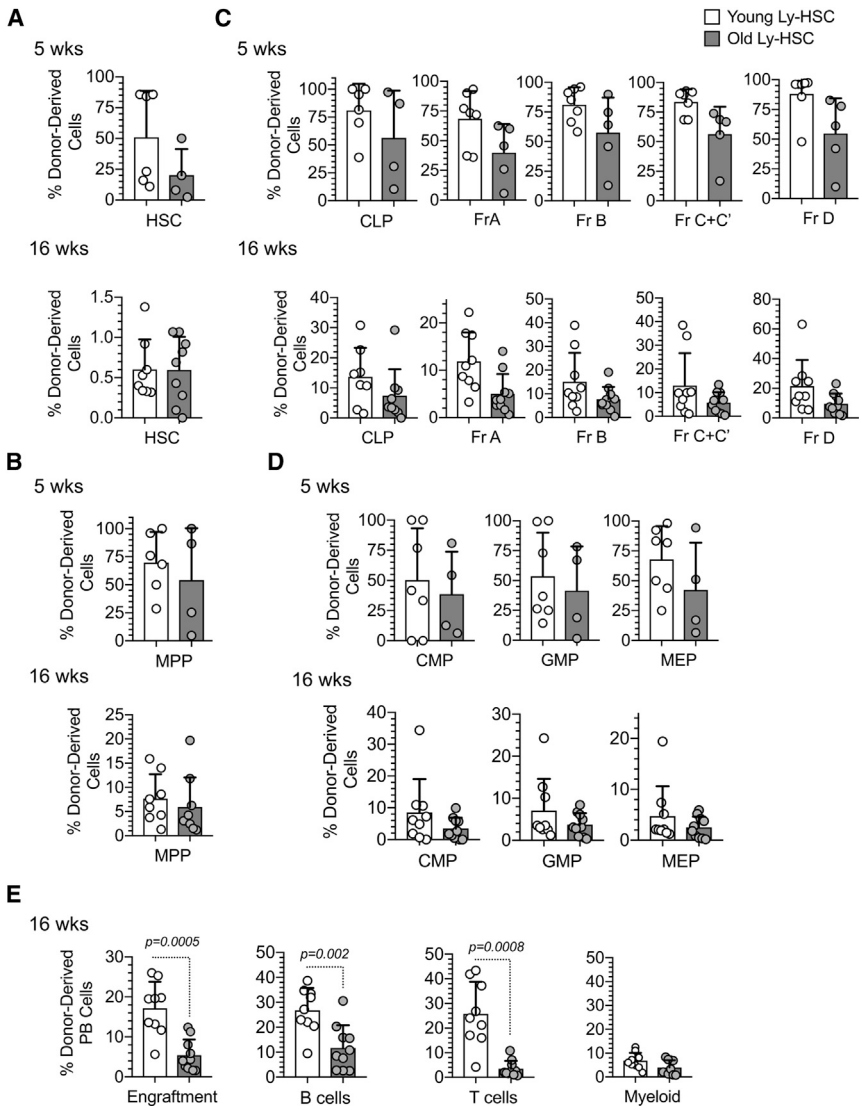

Figure 5. The Developmental Potential of Young and Old Ly-HSCs Is Similar

(A and B) Relative frequency of donor (A) HSCs and (B) MPPs in recipients of 200 young and 200 old Ly-HSCs at 5 and 16 weeks post-reconstitution.

(C) Relative frequency of donor CLPs, Fraction A pre-pro-B, Fraction B early pro-B, Fraction C + C′ late pro-B, and Fraction D pre-B cells in recipients of 200 young and 200 old Ly-HSCs at 5 and 16 weeks post-reconstitution.

(D) Relative frequency of donor CMPs, GMPs, and MEPs in recipients of 200 young and 200 old Ly-HSCs at 5 and 16 weeks post-reconstitution.

(E) Donor cell engraftment and relative frequency of surface IgM^+ B cells, combined $CD4^+$ and $CD8^+$ T cells, and $CD11b^+$ myeloid cells in the peripheral blood of recipients of young or old Ly-HSCs 16 weeks post-reconstitution. The mice analyzed are the same as those described in Figure 4. Each symbol is an individual recipient mouse. Error bars indicate means ± SD.

donor-derived HSCs were $CD150^{low}$ Ly-HSCs (Figure S4). This result is consistent with previous observations indicating that the lineage bias of Ly-HSCs is maintained following transplantation (Beerman et al., 2010; Challen et al., 2010). Multipotential progenitor (MPP) levels in recipients of young and old Ly-HSCs were also equivalent at 5 and 16 weeks post-reconstitution (Figure 5B).

B cell development proceeds through common lymphoid progenitor (CLP), pre-pro-B (Fraction A), early pro-B (Fraction B), late pro-B (Fraction C + C′), pre-B (Fraction D), and surface immunoglobulin M-positive (IgM^+) B cell stages of development (Fractions E) (Hardy et al., 1991, 2007) resolved as described previously (Montecino-Rodriquez et al., 2016). We found no significant differences in the level at which young and old Ly-HSCs reconstituted each of these stages of development at either time point (Figure 5C). Despite the acquisition of a myeloid gene signature by old Ly-HSCs, they did not generate elevated numbers of common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs) (Figure 5D).

Following their production in the bone marrow, immature B cells and myeloid cells traffic to the periphery and can be found in the circulation. Similarly, newly produced T cells exit from the thymus. Despite the fact that young and old Ly-HSCs reconstituted B lymphopoiesis and myelopoiesis to similar levels, overall donor cell chimerism in peripheral blood was significantly lower in recipients of old Ly-HSCs at 16 weeks post-transplantation. This was due to differences in levels of circulating B and T cells, because the frequency of mature $CD11b^+$ myeloid cells in recipients of young and old Ly-HSCs was the same (Figure 5E).

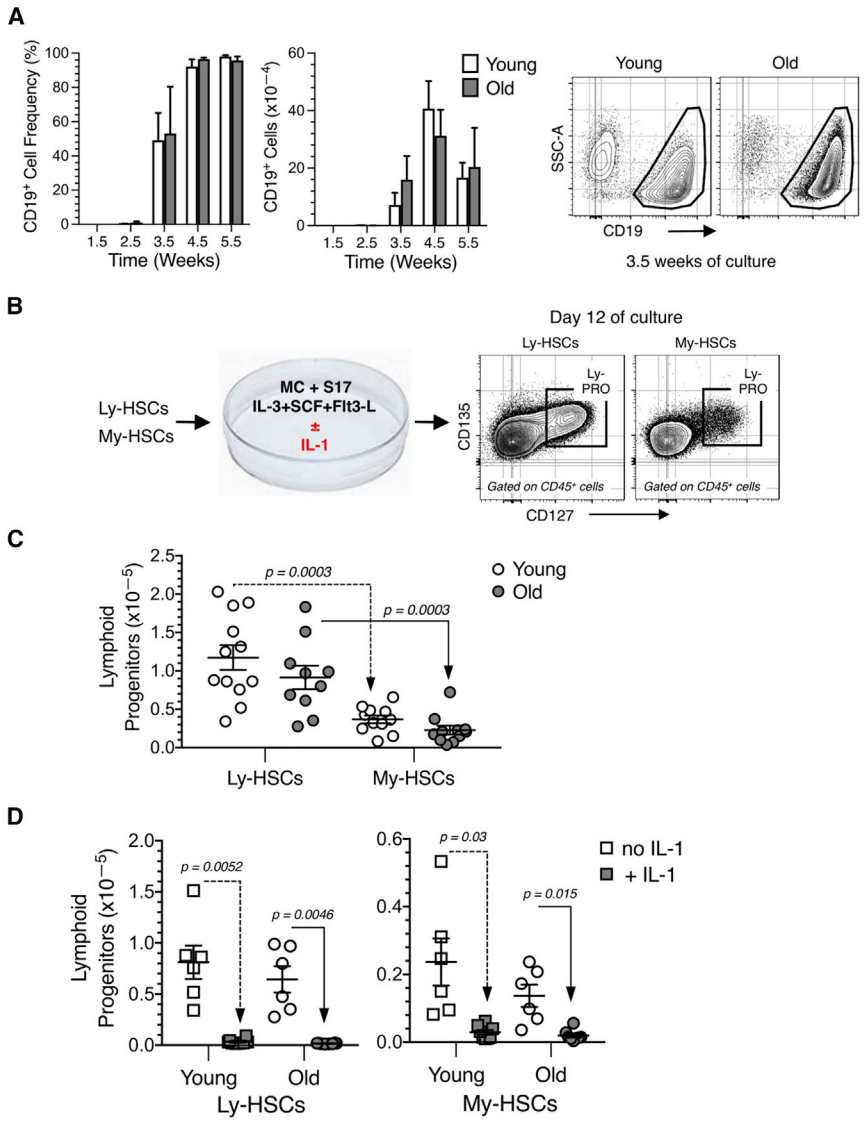

Figure 6. Old Ly-HSCs Generate Lymphoid Progenitors *In Vitro*

(A) Frequency and number of CD19⁺ B lineage cells produced in culture from 150 Ly-HSCs purified from young and old mice seeded on OP9 stroma *in vitro*. Representative FACS plots show CD19⁺ cell production at 3.5 weeks of culture. Data are representative of 3 experiments.

(B) Diagram of the *in vitro* assay used to generate lymphoid progenitors from Ly-HSCs and My-HSCs. Representative FACS plots showing CD127⁺ CD135⁺ lymphoid progenitors generated from young Ly-HSCs and My-HSCs. MC, methylcellulose supplemented medium.

(C) Total number of CD127⁺ CD135⁺ lymphoid progenitors generated by young and old Ly-HSCs and My-HSCs.

(D) Total number of CD127⁺ CD135⁺ lymphoid progenitors generated by young and old Ly-HSCs and My-HSCs in the presence or absence of 1 ng/mL of IL-1. Each symbol in (C) and (D) represents production per culture dish. Cultures were initiated with Ly-HSCs and My-HSCs purified from 3 to 2 independent cohorts of 6 young (8–12 weeks) and 4 old (18 months) B6 mice, respectively. Levels of significance for the differences between populations are indicated.

IL-1 Blocks Lymphocyte Development from Ly-HSCs

The above results indicate that old Ly-HSCs exhibit normal lymphoid developmental potential when removed from the old hematopoietic environment. We confirmed this observation using an *in vitro* system in which Ly-HSCs were seeded on OP9 stroma under long-term culture conditions. Consistent with the transplantation data, young and old Ly-HSCs generated a similar number of CD19⁺ B lineage cells over the course of 5 weeks (Figure 6A).

The production of inflammatory cytokines increases with age in the medullary cavity (Henry et al., 2015; Kennedy and Knight, 2017) and this has been linked to age-related declines in lymphocyte production. For example, interleukin-1 (IL-1) can inhibit B cell development from total HSCs (Kennedy and Knight, 2015). To test the effects of inflammation on lymphocyte development from Ly-HSCs and My-HSCs, we developed an *in vitro* system in which the combination of stromal cell signals and lymphopoietic factors promoted the formation of CD127⁺ CD135⁺ CD19⁻ early lymphoid progenitors (Igarashi et al., 2001) and CLPs (Hardy et al., 2007) following 12 days of culture (Figure 6B). Young and old Ly-HSCs generated a similar number of lymphoid progenitors in these cultures (Figure 6C). This result was consistent with the above *in vivo* studies showing that Ly-HSCs have normal lymphoid potential when removed from the old environment. Young and old My-HSCs also generated lymphoid progenitors but, regardless of age, at lower levels compared with Ly-HSCs (Figure 6C), indicating that the lineage-biased potential of Ly-HSCs and My-HSCs is maintained with age *in vitro* as well as *in vivo* as described (Challen et al., 2010). The addition of IL-1 (1 ng/mL) to the lymphoid progenitor cultures

at their initiation almost completely blocked the emergence of $CD127^+$ $CD135^+$ $CD19^-$ lymphoid progenitors from Ly-HSCs and My-HSCs (Figure 6D), implicating inflammatory factors in aging bone marrow as suppressors of B lymphopoiesis.

DISCUSSION

We demonstrate that age-related declines in lymphopoiesis do not result from a loss of Ly-HSCs. Instead, the data show that, when total cell number and not just frequency is taken into account, the number of Ly-HSCs is significantly increased in 18-month-old B6 mice. This strain exhibits declines in B cell development as early as 6 months of age (Miller and Allman, 2003). Ly-HSC number was also maintained in old BALB/c mice, which exhibit age-related declines in B lymphopoiesis after 1 year of age (Riley et al., 1991). These data indicate that current models of HSC aging presented in various reviews (Cho et al., 2008; Elias et al., 2016; Montecino-Rodriguez et al., 2013) proposing that the age-related reduction in lymphocyte development is due to a loss of Ly-HSCs need to be revised. The results further indicate that, while changes in gene expression occur in old Ly-HSCs, these do not irreversibly block lymphopoiesis.

Our whole-transcriptome analysis of young and old Ly-HSCs and My-HSCs complement a previously described microarray-based genomic study of these cells (Challen et al., 2010) and allowed us to assess the effects of aging on these populations. The age-related changes in gene expression in Ly-HSCs and My-HSCs are for the most part distinct. The gene expression data also demonstrate that old Ly-HSCs acquire a myeloid-biased pattern of gene expression. Rossi et al. (2005) reported an increased expression of myeloid lineage genes in total old HSCs. Our results indicate that, in addition to the increased number of My-HSCs in the bone marrow of old mice, gene expression changes in Ly-HSCs also contribute to the myeloid-biased signature of old HSCs.

Despite these changes in gene expression, lymphocyte production from young and old Ly-HSCs was equally efficient and the latter cells did not exhibit enhanced myelopoietic potential. These observations indicate that, if the altered pattern of gene expression in old Ly-HSCs inhibits their potential to generate lymphocytes and stimulates their production of myeloid cells, this only occurs as long as cells are exposed to the old milieu. In this case, the normal developmental potential of old Ly-HSCs observed following their transplantation into young recipients suggests that the effects of altered gene expression are reversible/erasable. This possibility is consistent with findings that old HSCs acquire epigenetic modifications that can affect differentiation (Sun et al., 2014).

The possibility that reconstituting mice with 200 young and old Ly-HSCs may have saturated stem cell niches, thereby masking deficits in lymphoid reconstitution by the old stem cells that were too subtle to be revealed in our analyses, cannot be excluded. However, if old Ly-HSCs had acquired permanent, cell-intrinsic changes that compromised their lymphoid potential, some deficit in one or more stages of primary B cell development regardless of whether or not a saturating number of cells had been transplanted should have been observed. This was not the case, and we also observed no defect in the ability of old Ly-HSCs to generate lymphoid progenitors *in vitro*. These observations are consistent with the fact that old Ly-HSCs did not express $p16^{Ink4a}$, a biomarker and effector of aging (He and Sharpless, 2017; Martin et al., 2014) (Table S1), in agreement with a previous analysis of old total HSCs (Sun et al., 2014).

While we could not identify differences in primary lymphopoiesis between young and old Ly-HSCs, the number of circulating B and T cells in peripheral blood was significantly lower in recipients of old donor cells. This deficiency may be due to the impaired migratory potential of the newly produced cells. Following their generation in the bone marrow, naive B cells migrate to the spleen where they progress through distinct transitional stages before becoming mature effector cells (Allman and Pillai, 2008). The ability of these immature B cells to exit the bone marrow and enter the circulation is significantly compromised in old mice (Johnson et al., 2002; Wols et al., 2010). Newly generated T lymphocytes in old mice share this defect. The thymus involutes with age, but this can be reversed by administration of keratinocyte growth factor (KGF) (Min et al., 2007; Rossi et al., 2002). Nevertheless, the T cells produced in the thymus of KGF-treated old mice inefficiently exit that organ to enter the peripheral blood (Thompson et al., 2018). These migratory defects likely contribute to the decreased number of mature cells in the circulation. It is tempting to speculate that this deficiency is due to the gene changes that occur in old Ly-HSCs. This hypothesis is in agreement with the suggestion that the effects of altered transcription of genes in HSCs may be manifest in downstream progeny (Rossi et al., 2005). The ability to distinguish effects of aging on primary lymphocyte development from those acting on newly produced B and T cells was possible because the Busulfan conditioning of recipients allowed comparable engraftment of young and old donor Ly-HSCs.

With the exception of reports from Labrie et al. (2005, 2004), our transplantation results are not in accord with studies concluding that old HSCs generate fewer lymphoid progeny compared with young stem cells following

transplantation into irradiated recipients (Rossi et al., 2005; Sudo et al., 2000). However, this result may reflect the impaired ability of the old stem cells to home to the bone marrow of irradiated recipients (Liang et al., 2006) and not a lymphoid differentiation defect. In this case, if fewer old Ly-HSCs engrafted in recipients compared with young Ly-HSCs, lower levels of lymphopoiesis would be observed. Such reconstitution deficits by old Ly-HSCs were not observed in Busulfan-conditioned recipients, as the number of young and old donor Ly-HSCs was similar at 5 weeks post-reconstitution. In view of these observations, our data indicate that, in contrast to irradiation, efficient engraftment of old stem cells occurs in Busulfan-conditioned recipients and suggest that, if the goal is to measure lymphocyte development, this provides a more physiologic means to condition recipients.

The production of inflammatory factors increases with age systemically and in the bone marrow (Henry et al., 2015; Kennedy and Knight, 2017), and previous reports and data presented herein demonstrate that such cytokines can inhibit lymphopoiesis (Dorshkind, 1988, 1991; Kennedy and Knight, 2015, 2017; Ueda et al., 2005). Short-term inflammation may provide a means to produce increased numbers of mature myeloid cells that provide an initial response against infection (Ueda et al., 2005). While this response is likely transient in young individuals, and hematopoiesis returns to steady-state levels after the infectious stimulus subsides, the chronic inflammation associated with aging may result in a continual stimulation of myeloid cell production and inhibition of lymphopoiesis. Such increased inflammation may have effects at the stem and progenitor cell levels (Nagai et al., 2006). In particular, IL-1 blocks lymphocyte development from total HSCs *in vitro* (Kennedy and Knight, 2015, 2017), and our data demonstrate that this is the case for both Ly-HSCs and My-HSCs.

This sensitivity of lymphopoiesis to inflammatory cytokines may explain why, in contrast to what has been observed in irradiated recipients, old Ly-HSCs exhibited efficient lymphoid potential in Busulfan-treated mice. In this regard, IL-1 levels are significantly higher in mice conditioned with irradiation compared with Busulfan (Wilkinson et al., 2013; Xun et al., 1994). Additional inflammatory cytokines such as IL-6 (Maeda et al., 2005) and tumor necrosis factor alpha (TNF-α) (Ueda et al., 2004) can inhibit bone marrow B cell development, and these factors are also significantly elevated in irradiated compared with Busulfan-treated mice (Henry et al., 2015; Xun et al., 1994). Based on these observations, we propose that transplantation of stem cells from old mice into irradiated recipients transfers them from one environment rich in inflammatory cytokines known to inhibit B lymphopoiesis to another with the same characteristics. In contrast, the environment in Busulfan-conditioned young mice may be more permissive for lymphopoiesis from old Ly-HSCs, because levels of inflammation are low. Further studies are needed to assess these possibilities. In addition, the precise effects of inflammation on Ly-HSCs need to be determined. On the one hand, factors such as IL-1, IL-6, and/or TNF-α may trigger cell intrinsic changes that inhibit the ability of Ly-HSCs to generate lymphoid progenitors; these might be reversed following transfer of the cells to a less inflammatory milieu, thus "rescuing" their lymphoid potential. Alternatively, Ly-HSCs in old mice may retain normal lymphoid potential, which is blocked due to residence in an inflammatory environment. In this case, their transfer to a less inflammatory milieu would allow an activity (i.e., lymphoid potential) that was never lost to be revealed.

In summary, the data in this report demonstrate that the number of Ly-HSCs does not decline with age, necessitating a revision of current models of stem cell aging proposing that reduced Ly-HSC number accounts for the age-related diminution of lymphopoiesis (Elias et al., 2016; Montecino-Rodriguez et al., 2013; Muller-Sieburg et al., 2004). The results further show that the lymphoid output of Ly-HSCs is influenced by conditions in their environment. Finally, the data indicate that not all reconstitution models are equivalent, and, if the goal is to measure lymphocyte development from old stem cells, the use of irradiation to condition recipients may not be optimal.

EXPERIMENTAL PROCEDURES

Mice

Young 8–12-week-old C57BL/6J (B6), BALB/cJ (BALB), and B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1 B6) mice were obtained from The Jackson Laboratory or the UCLA Division of Laboratory Animal Medicine. Seventeen- to 18-month-old B6 and BALB mice were obtained from the National Institute on Aging colony. All animals were housed in the UCLA Division of Laboratory Animal Medicine vivarium. Animal care and use were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Flow Cytometry

Peripheral blood was obtained by cardiac puncture following euthanasia. Bone marrow cell suspensions were prepared as described previously (Min et al., 2006; Montecino-Rodriquez et al., 2016). HSCs, CMPs, GMPs, MEPs, CLPs, and Fractions A-F B lineage cells were resolved using specific combinations of FITC-, PE–, PerCP/Cy5.5-, PE/Cy7-, APC-efluor780-, Pacific Blue-, and efluor-605NC-conjugated antibodies as previously described (Montecino-Rodriquez et al., 2016). The lineage cocktail included antibodies to CD3ε, CD8α, CD45R(B220), CD48, Gr-1(Ly-6G), IgM, NK1.1, TCRβ, TCRγδ, and TER-119. Ly- and

My-HSCs were resolved within the lineage-negative $CD48^-$ population using antibodies to CD117(c-Kit), Sca-1, CD150, and CD135, as shown in Figure S1A. For Ki-67 staining, cells were fixed, permeabilized, and stained with PE/Cy7-conjugated anti-Ki-67 antibody or rat IgG2a,κ isotype control, as per the manufacturer's instructions (Life Technologies). Cells produced in long-term cultures were analyzed for expression of CD11b and CD19. The antibody clones used and their sources are listed in Table S3. HSCs were purified using Aria Cell Sorters (BD Biosciences) located in the Jonsson Comprehensive Cancer Center flow cytometry core and analyses were performed on an LSRII (BD Biosciences) located in the Broad Stem Cell Research Center flow cytometry core, both at UCLA.

Bone Marrow Transplantation

Eight- to 12-week-old CD45.1 B6 female mice were obtained from the Jackson Laboratory and underwent myeloablative conditioning via two intraperitoneal injections of Busulfan (Sigma) at 48 and 24 h prior to transplantation with young or old Ly-HSCs purified from CD45.2 B6 donors. The donor cells were derived from two independent cohorts of young and old mice. The mice processed at 5 weeks post-reconstitution were conditioned with 25 mg/kg of drug per injection and the mice analyzed at 16 weeks received 10 mg/kg of drug per injection. Previous studies have shown that mice conditioned with these two doses of Busulfan exhibit similar levels of hematopoietic reconstitution (Hsieh et al., 2007; Yeager et al., 1991). Mice were killed 5 or 16 weeks later and the frequency of donor CD45.2 HSCs, MPPs, lymphoid and myeloid progenitors, and mature lymphoid and/or myeloid cells in the bone marrow, and peripheral blood were determined by flow cytometry.

In Vitro Lymphoid Assays

The lymphoid potential of Ly-HSCs was assessed by seeding 150 purified stem cells per well of 12 well plates on confluent layers of OP9 stroma cells (Nakaono et al., 1994) in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol (2ME) (Sigma), 2 mM L-glutamine, 50 μg/mL gentamicin, 100 U/mL streptomycin, 100 μg/mL penicillin, 0.1 mM minimum essential medium (MEM) vitamins, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (all from Gibco). The contents of the wells were harvested weekly by trypsinization. Cells were counted and one-tenth was used for phenotypic analysis and the remaining cells were seeded on newly established OP9 stromal cells. This cycle of harvest and re-seeding was repeated for approximately 5 weeks.

Lymphoid progenitor production from Ly-HSCs and My-HSCs isolated from young and old B6 bone marrow was measured by mixing 200 purified stem cells with 5.0×10^4 S17 stromal cells (Collins and Dorshkind, 1987) in 1.5 mL of methylcellulose (MC) medium. MC medium was prepared by supplementing α-MEM with 30% heat-inactivated FCS, 1% methylcellulose (STEMCELL Technologies), 5×10^{-5} M 2ME, 2 mM L-glutamine, 50 μg/mL gentamicin, 100 U/mL streptomycin, 100 μg/mL penicillin, 0.1 mM MEM vitamins, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 20 ng/mL stem cell factor, 20 ng/mL Flt-3L ligand, and 50 ng/mL IL-7 (all from Biosource). In some experiments the cultures were additionally supplemented with 1 ng/mL of IL-1 (Biosource). The mixture was plated in non-tissue-culture-treated 3.5-cm^2 dishes (Becton Dickinson). Following 12 days of culture, the contents of the plates were harvested, cells were enumerated, and examined for production of $CD45^+$ $CD127^+$ $CD135^+$ $CD19^-$ lymphoid progenitors by flow cytometry. All cultures were placed at 37°C, 5% CO_2 humidified incubators until processing.

RNA-Seq and Data Analysis

Whole-transcriptome profiling of Ly-HSCs and My-HSCs purified from three independent groups of young and old mice was performed. RNA was extracted using the Zymo Direct-Zol RNA MiniPrep Plus (cat. no. R2070, Irvine, CA, USA) as per the manufacturer's instructions. Total RNA was quantified and 260/280 ratios determined using NanoDrop. RNA-seq and library preparation were performed in the Jonsson Comprehensive Cancer Center Genomics Shared Resource using the KAPA Stranded mRNA-seq kit (Roche Sequencing, cat. no. KK8421, Pleasanton, CA, USA), according to the manufacturer's instructions. The work-flow consisted of mRNA enrichment, cDNA generation, end repair to generate blunt ends, A-tailing, adaptor ligation, and PCR amplification. Different adaptors were used for multiplexing samples in one lane. Sequencing was performed on the HiSeq3000 System for a paired-ended 150-bp run. All samples (12 total, 2 cell types, 2 ages, 3 biological replicates each) were pooled and sequenced in two different lanes of the flow cell.

The STAR ultrafast universal RNA-seq aligner v.2.5.2b (Dobin et al., 2013) was used to generate the genome index and perform paired-end alignments. Reads were aligned to a genome index that includes both the genome sequence (GRCm38 primary assembly) and the exon/intron structure of known gene models (Gencode M12 genome annotation). Alignment files were used to generate strand-specific, gene-level count summaries with STAR's built-in gene counter. Technical replicates showed high reproducibility and were pooled. Only protein-coding genes in the Gencode M12 annotation were considered (85% of total counts on average). Independent filtering was applied as follows: genes with no counts in any sample, count outliers or low mappability were filtered out for downstream analysis (Casero et al., 2015). Counts were normalized per sample in units of fragments per kilobase of transcripts per million mapped reads (FPKM) after correcting for gene-mappable length and sample total counts. The table of expression estimates (FPKM) was used as input for SaVanT (Lopez et al., 2017) to compute enrichment scores on two different databases of mouse gene expression signatures: ImmGen (http://immgen.org) and the Mouse MOE430 Gene Atlas (http://biogps.org/). Non-default parameters for SaVanT were "Convert matrix values to ranks" and "Compute null distribution with 10,000 iterations." The most significant enrichment scores are shown in Figure S1, and unabridged output from SaVanT is provided in Table S2.

Differential expression analysis was performed with *DESeq2* (Love et al., 2014). Count data were fitted to additive models using *Age* (young/old) and *Bias* (lymphoid/myeloid) as explanatory factors. The individual effect of each factor on the expression of each gene was tested using a contrast with reduced models (likelihood ratio test). Pairwise differential expression (young versus old

Ly-HSCs and My-HSCs, young and old Ly-HSCs versus My-HSCs) was performed to classify genes as differentially expressed between any two conditions. The set of most variable genes was generated after pooling all genes that were classified as significant (Wald or likelihood ratio tests, adjusted p value < 0.05) by additive of pairwise tests. An additional pairwise test was performed (young Ly-HSCs versus the rest of the samples) to identify genes similarly expressed in old Ly-HSCs and My-HSCs (507 genes, Figures 4B; Table S1).

Functional enrichment was performed with Metascape (http://metascape.org) using gene ontology biological processes annotations in mouse. Hierarchical clustering (Figures 3, 4A, and 5B) was performed and visualized in MATLAB using *Z* scores from variance-stabilized data as input for each group of genes. Multidimensional scaling was performed with the function *cmdscale* in R (https://www.R-project.org/) using variance-stabilized data as input.

Statistical Analysis

Data are expressed as a means ± SD or SEM as indicated in the figure legends. Differences between groups were tested by a two-tailed, unpaired Student's t test ($\alpha = 0.05$).

ACCESSION NUMBERS

Raw sequence files are available at NCBI's GEO (GEO: GSE112769).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and three tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2019.01.016.

AUTHOR CONTRIBUTIONS

E.M.-R., Y.K., K.D., A.R., and P.D.P. conceived, designed the experiments, conducted the experiments, and/or analyzed the data. D.C. performed the bioinformatics analysis. All authors participated in the writing of the manuscript.

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