UNIVERSITY OF CALIFORNIA

Los Angeles

Effects of Aging on the Lymphoid Potential of Hematopoietic Stem Cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular, Cellular & Integrative Physiology

by

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2018

ABSTRACT OF THE DISSERTATION

Effects of Aging on the Lymphoid Potential of Hematopoietic Stem Cells

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Doctor of Philosophy in Molecular, Cellular, & Integrative Physiology

University of California, Los Angeles, 2018

Professor Kenneth A Dorshkind, Chair

Lymphocyte development declines with age, which in turn contributes to the reduced replenishment of naïve lymphocytes in secondary lymphoid organs. An impaired ability of old hematopoietic stem cells (HSCs) to generate lymphoid progeny is thought to contribute to this attenuation of lymphopoiesis. It is now recognized that the HSC compartment is heterogeneous and includes lymphoid biased (Ly-HSCs), myeloid biased (My-HSCs) and balanced (Bal-HSCs) hematopoietic stem cells. Previous studies have demonstrated that the frequency of Ly-HSCs is reduced with age, and this observation has resulted in the formulation of a model of stem cell aging which proposes that the age-related decline in lymphocyte development is due to a reduction in the number of these lymphoid biased precursors. However, findings from this thesis show that this model needs to be revised. In particular, Ly-HSCs do no decline and increase

significantly during aging. The lymphoid potential of old Ly-HSCs is similar to young Ly-HSCs ex vivo shown by the number and aging marker expression of produced lymphoid cells. But the genomic pattern of old Ly-HSCs turns into a myeloid biased pattern indicated by RNA-Sequencing results, which correlates with altered myeloid production from old Ly-HSCs. In terms of reduced lymphoid development in vivo during aging, the fact that elevated level of pro inflammatory cytokines, one character of senescence-associated secretory phenotype, was detected to significantly and irreversibly prohibit the lymphoid differentiation from Ly-HSCs. Based on the above results, we propose the revised aging model of hematopoietic stem cells: Ly-HSCs do not decline during aging; their lymphoid potential remains similar to young Ly-HSCs ex vivo, but when being under the pro inflammatory cytokines, their differentiation is irreversible blocked; Whereas, their myeloid production is altered even removing from proinflammatory condition, which correlates with the fact that old Ly-HSCs show myeloid-biased transcriptional pattern. Throughout the projects, the methods utilized include cells staining, flow cytometry, cell sorting, lymphoid and myeloid assays, HSC-OP9 co-culture, cell transplantation, qPCR and RNA-Sequencing. Two supplementary files were also included separately: Table 2-11 “Ly-HSC and My-HSC gene expression estimates and differential expression results” and Table 2-12 “Ly-HSC and My-HSC gene signature analysis and functional enrichment results”.

The dissertation of Ying Kong is approved.

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Dedication

This dissertation is dedicated to my loving parents Linghong Kong and Linlin Ma. I thank for their endless and unconditional love and support. I will never forget all their support, guidance, encouragement and sacrifices to sustain me in growth, dream and career. They always place my well-being first over their own. None of my accomplishments would have been possible without them. Thank you for your constant love and support.

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I would like to thank all the wonderful people that I have worked with. I would like to thank all the former and current members of the Dorshkind lab, particularly Michael Fice and Dr. Pete D. Pioli for their help and valuable suggestions; Dr. David Casero for supporting all bioinformatics analysis; the MCIP program for great assistance in career development; the flow cytometry core laboratory at the Jonsson Comprehensive Cancer Center.

Chapter 2 is a version of article in submission to Stem Cells Report: Ying Kong*, Encarnacion Montecino-Rodriguez*, David Casero*, Kenneth Dorshkind, and Peter D. Pioli, Lymphoid Biased Hematopoietic Stem Cells Are Maintained with Age and Acquire a Myeloid Gene Expression Signature,

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Chapter 1:

Introduction

Human life span has significantly increased over the course of the past 150 years due to advances in medicine and improved public health practices. As a result, it is not surprising that the total number of elderly people has risen as well. It has been estimated that the number of octogenarians will rise from 125 million in 2015 to 434 million in 2050 (WHO, Aging and Health). In fact, the number of individuals aged 65 and older is expected to increase significantly in multiple countries between now and 2050 (Figure 1-1).

Although many individuals who reach advanced age are healthy, aging often negatively impacts the function of one or more organs and processes, which can in turn compromise health. For example, a loss of muscle stem cells can lead to sarcopenia, and multiple deficiencies that affect various aspects of nervous system function have been described. It is also well recognized that age-related defects in the immune system that in turn compromise immune function can occur. The result is that the elderly are particularly susceptible to infections and vaccination efficacy is significantly reduced.

There are two major effects of aging on the immune system (Figure 1-2). First, the function of mature immune cells in secondary lymphoid tissues such as the spleen and lymph nodes declines. The composition of the $CD4^+$ T cells compartment shifts from naïve T cells to memory cells and the number and proportion of regulatory $CD4^+$ T cells increases in both mice and human (1-3). The reduced number of naïve T cells is primarily due to the involution of the

thymus, which shrinks in size and function with age. Aging can also skew the repertoire of $CD8^+$ T cells. For example, $CD8^+$ T cells with reactivity towards previously encountered antigens such as cytomegalovirus accumulate in secondary lymphoid tissues (4). This in turn may compromise the response to new antigens (5). Age-related changes in patterns of gene expression can also affect expression of various cell surface receptors and cell signaling cascades. For example, the expression of CD27 and CD28 is lost, and there is up-regulation of the dual-specificity phosphatases that in turn affects MAP kinase signaling (6).

Mature B cell function is also compromised by aging. Although total serum immunoglobulin (Ig) levels remain intact (7), fewer high-avidity antibodies are produced and adaptive immune reactivation to newly encountered antigens is diminished. Furthermore, instead of a diverse response to new antigen, humoral immunity shifts from production of polyclonal to monoclonal immunoglobulin (8). Some of these age-related changes in mature B cells may result from cell intrinsic effects. In this regard, the expression of E2a and AID (9) is decreased in mature B cells leading to decreased Ig class switching and somatic hypermutation (10). However, it is also possible that secondary effects are also operative. Mature B cell function requires T cell help, and age-related defects in mature T cells could in turn compromise humoral immunity.

One way to circumvent these age-related deficiencies would be to replace old, defective B and T cells with newly produced, naïve lymphocytes. However, the

second major effect of aging on the immune system is diminished lymphocyte development. The thymus is involuted in old individuals, and B cell development in the bone marrow is suppressed (Figure 1-3). As a result, few naive lymphocytes populate peripheral lymphoid tissues in old individuals.

If the production of naïve B and T cells in the bone marrow and thymus could be stimulated in the elderly, then it may be possible to replace old, defective B and T cells with newly produced populations. However, achieving this goal will be dependent on a detailed understanding of why lymphocyte development declines with age. This is the focus of the research described in this thesis.

In order to provide the background for this work, we first provide an overview of normal hematopoiesis and the hematopoietic microenvironment. We then describe the effects of aging on blood cell production and the ability of the environment to support that process. As our focus will be on hematopoietic stem cells (HSCs), we will place particular emphasis on these cells and highlight how the current research addresses gaps in our understanding of how aging affects them.

Hematopoiesis

Blood cell development is generally depicted as a hierarchical process in which HSCs mature into intermediates that are lymphoid or myeloid specified. These cells then generate progenitor populations that ultimately become committed to

generating a specific type of blood cell.

i. Hematopoietic stem cells

The majority of blood cells are derived from HSCs that are generally depicted as being at the head of the hematopoietic hierarchy. HSCs can both self-renew and generate differentiated progeny that ultimately become specified to a particular hematopoietic lineage.

HSCs develop in the fetus from hemogenic endothelium (11). In mice, this process occurs at around embryonic day 10.5. HSCs then seed other fetal tissues such as the placenta and liver where they undergo expansion and then differentiation (12-14). However, by late gestation HSCs seed the bone marrow and after birth that tissue is the primary site of hematopoiesis.

HSCs are enriched in fraction of Lineage negative (Lin^-) $CD117(c\text{-}Kit)^{high}$ $Sca\text{-}1^+$ cells (LSKs). Lineage negative indicates that the cells do not express a variety of lineage markers that include $CD3\varepsilon$, $CD8\alpha$, $TCR\beta$, $TCR\gamma\delta$, NK1.1, TER-119, Gr-1, B220 (CD45R) and IgM. However, most groups now use a more sophisticated isolation strategy that employs the SLAM marker CD150 (15-17), which is expressed on HSCs, and exclusion of additional cell surface determinants such as CD48 and CD135 (18-20). Additional markers and strategies for HSC purification have been described. For example, in addition to cell surface markers,

the Goodell laboratory resolves HSCs based on their ability to efflux the Hoechst dye (21). In humans, HSCs are enriched in the population of Lineage$^-$ CD34$^+$ CD38$^-$ CD90$^+$ CD45RA$^-$ cells (22).

Most schemes of hematopoiesis depict HSCs as a single, homogeneous population. However, studies from the Muller-Sieburg laboratory revolutionized our thinking by showing that the HSC compartment is heterogeneous and includes subpopulations with biased developmental potential. These investigators sorted individual HSCs that were allowed to expand in culture for a few days, and this population was then transplanted into individual mice. As a result of this experimental design, the donor cells repopulating each recipient are derived from a single stem cell clone. They observed that some recipients showed equivalent lymphoid and myeloid reconstitution while others exhibited reconstitution biased towards lymphopoiesis or myelopoiesis. These results led to the concept that the HSC compartment includes lymphoid biased HSCs (Ly-HSCs), myeloid biased HSCs (My-HSCs), and balanced HSCs (Bal-HSCs) (23-26). Subsequent reports from other labs have also defined megakaryocyte/platelet-biased HSCs (27, 28). The developmental relationship between these stem cell subsets remains to be defined. They could all be derived from a single precursor stem cell or they could be autonomously generated.

As noted above, HSCs express the CD150 cell surface determinant, and the level at which it is expressed allows Ly-HSCs, My-HSCs, and Bal-HSCs to be resolved. Ly-HSCs and My-HSCs can be identified based on their expression of

low and high levels of CD150, respectively, while Bal-HSCs express intermediate levels (18, 20). Additional markers such as CD229 may be useful for isolation of these cells as well (29). Our strategy for resolution of these HSC subpopulations, based on these reports, is shown in Figure 1-4.

ii. Multipotential Progenitors

As HSCs differentiate, they mature into multi potent progenitors (MPPs), which are defined by their Lin^- $Sca\text{-}1^{high}$ $CD117(c\text{-}Kit)^{high}$ $Flt3^+$ $CD34^+$ phenotype. As with HSCs, MPPs may be a heterogeneous population containing subsets with distinct lineage potential (30). MPPs can reconstitute all lineages following transplantation into irradiated mice. However, in contrast to HSCs, they cannot stably do so for the lifetime of the recipient, indicating that their self-renewal potential is limited.

iii. Myeloid Progenitors

One developmental option for MPPs is to generate myeloid cells via the production of common myeloid progenitors (CMPs) that can be identified by their Lin^- $Sca\text{-}1^-$ $CD117(c\text{-}Kit)^{high}$ $CD34^+$ $CD16/32^{lo}$ phenotype. CMPs can then differentiate into lineage biased progenitor populations that include Lin^- $Sca\text{-}1^-$ $CD117(c\text{-}Kit)^{high}$ $CD34^+$ $CD16/32^+$ granulocyte-macrophage progenitors (GMPs) or Lin^- $Sca\text{-}1^-$ $CD117(c\text{-}Kit)^{high}$ $CD34^-$ $CD16/32^-$ megakaryocyte/erythrocyte

progenitors (MEPs). GMPs give rise to granulocytes and monocytes while MEPs differentiate into megakaryocytes and erythrocytes (31).

iv. Lymphoid Progenitors

MPPs also have the option of maturing into Lin^- Sca-1^{high} CD117(c-Kit)^{high} $Flt3^{high}$ lymphoid-primed multipotent progenitors (LMPPs). Although LMPPs are lymphoid primed, they retain granulocyte-macrophage (32) and limited megakaryocyte-erythrocyte potential (33).

A subset of LMPPs expresses the recombinase activating (Rag) protein, which is required for the rearrangement of immunoglobulin (Ig) and T cell receptor genes. These Rag expressing LMPPs are referred to as early lymphocyte progenitors (ELPs) (34).

If LMPPs remain in the bone marrow, they differentiate into Lin^- Sca-1^{low} CD117(c-Kit)^{low} $Flt3^+$ $CD127^+$ common lymphoid progenitors (CLP). Although CLPs may exit the bone marrow and migrate to the thymus, they are not generally considered to be the main intermediate from which T cells are produced as discussed below. Instead, CLPs can be considered as B lineage specified progenitors. CLPs mature through pre-pro B (Lin^- $CD45R^+$ $CD43^+$ $CD93^+$ $CD19^-$ $Ly6C^-$), pro-B (Lin^- $CD45R^+$ $CD43^+$ $CD93^+$ $CD19^+$ $Ly6C^-$), and pre-B (Lin^- $CD45R^+$ $CD43^-$ $CD93^+$ $CD19^+$ $Ly6C^-$) intermediates before maturing into B

cells that express IgM on their surface. The use of these marker combinations to define the various stages of B cell development was first described by Hardy and colleagues (35, 36). For simplicity, these stages are often designated as Fractions A-F as shown in Figure 1-5.

Newly produced B cells in the bone marrow are functionally immature, and they migrate to the spleen where they progress through various transitional stages before generating either follicular or marginal zone B cells. The precise pathway through which transitional cells mature depends on their exposure to B cell activating factor (BAFF), engagement of the Notch2 ligand, and antigen. As shown in Figure 1-5, transitional cells that engage Notch2 move into the marginal zone compartment. Marginal zone and follicular B cells have distinct function. Most mature B cells home to B cell follicles in secondary lymphoid organs and are referred to as follicular B cells. They participate in T cell-dependent immune responses. Marginal zone B cells reside in the outer white pulp of spleen between the marginal sinus and the red pulp. They express high levels of CD21 and CD1d and respond vigorously to blood borne pathogens (37, 38).

LMPPs may also exit the bone marrow and migrate to the thymus, which is the site of T cell development. The thymus does not contain self-renewing stem cells and must be replenished by precursors from the bone marrow. Upon entering the thymus, LMPPs are thought to rapidly differentiate into early thymocyte progenitors (ETPs), which are the most immature intrathymic progenitors. In

response to signals from the thymic microenvironment, ETPs differentiate through double negative (DN) 2, 3, and 4 stages of development before becoming $CD4^+CD8^+$ double positive thymocytes. These cells then mature into CD4 helper or CD8 single positive T cells then exit the thymus and populate secondary lymphoid tissues, shown in Figure 1-6.

The Hematopoietic Microenvironment

Hematopoiesis in the bone marrow occurs in association with a supporting network of stromal cells that provide the environment that supports blood cell production. Figure 1-7 shows basic elements of the medullary circulation. A series of venous sinusoids originate at the endosteal surface of the bone and then traverse the medullary cavity before ending in a central sinus. The spaces between these sinusoids are occupied by a three-dimensional network of bone marrow stromal cells. Developing hematopoietic cells associate with the stroma, which regulate blood cell development through cell to cell interactions and by the secretion of various salutary cytokines (39-41).

The localization of hematopoietic cells in the bone marrow is not random. It is thought that HSCs are preferentially found at the endosteal surface of the bone or in association with the sinuses (42). Developing B cells move between various niches as they mature. For example, IL-7 is an obligate B lymphopoietic factor, and as pro-B cells mature into pre-B cells, they are thought to migrate from IL-7

producing to IL-7 non-producing niches (43).

Effects of Aging on Lymphocyte Development

The effects of aging on hematopoiesis have been studied in multiple laboratories, and it is clear that the properties of HSCs as well as lymphoid progenitors are affected.

i. Effects of Aging on HSCs

Multiple stem cell properties are compromised by aging (Table 1-8). For example, the number of HSCs is increased with age (20, 44), their proliferative/self-renewal capacity is decreased (45-47) compared to their young compartments, and homing and engraftment potential is compromised (45, 48). As a result of these deficiencies, old HSCs do not effectively compete with young HSCs in competitive reconstitution assays (20, 45, 49, 50). The other major functional change is that old HSCs do not efficiently generate lymphoid progeny following transplantation and instead exhibit a myeloid biased pattern of differentiation (20, 47) in both mice (20) and humans (22, 51).

The analysis of young and old bone marrow has shown that the frequency of Ly-HSCs is reduced and that of My-HSCs is increased with age. This has resulted in the formulation of the current model of HSC aging, shown in Figure 1-9, in which

age-related declines in lymphocyte development are due to a loss of Ly-HSCs. In this case, there are fewer stem cells capable of generating lymphocytes (25) and an abundance of those with myeloid potential. This shift in the Ly-HSC/My-HSC balance has been proposed by Rossi and co-workers (52) to explain why myeloid lineage genes were upregulated and lymphoid genes were down regulated in HSCs that were profiled by microarray analysis.

Several studies have compared the transcriptomes of young versus old total HSCs, including at the single cell level, using microarray analysis and whole transcriptome profiling (RNA-Seq) (18, 28, 53, 54). Gene Ontology analysis of the data indicates that categories related to Cell Adhesion, Cell Proliferation and the Ribosome are up regulated while DNA Base Excision Repair, DNA replication and Cell Cycle are down regulated (53). Epigenetic changes also characterize aging HSCs as demonstrated by studies from the Goodell and Rossi laboratories. Expression of key epigenetic regulators such as DNA methyltransferases *Tet1* and *Tet3* and repetitive element, decreases with age and there are age dependent DNA methylation changes and histone modifications. The altered DNA methylation may interfere with the transcriptional network. Beerman et al (55) found that proliferation triggered DNA hypermethylation, and this contributed to the functional decline of HSCs during aging. However, no studies have transcriptionally profiled young versus old Ly-HSCs in order to obtain insights in to the genetic changes that may occur in them.

ii. B cell progenitors

B cell development is particularly impacted by aging as demonstrated by a reduction in the frequency and total number of CLPs, pre-pro-B cells, pro-B cells, and pre-B cells (56-58) These progenitors exhibit multiple, age-related defects that include decreased proliferative potential, increased rates of apoptosis, and diminished ability to differentiate (59).

Multiple molecular defects in developing B lineage cells have been described. For example, they exhibit impaired V-D-J heavy chain gene recombination (60), possibly because of decreased expression of the *E2A* encoded transcription factors E12 and E47 (10). *P16^{Ink4}a* and *Arf*, are preferentially expressed in aged lymphoid progenitors and contribute significantly to their decline in proliferative potential and survival (61, 62).

iii. T cell progenitors

The thymus in old mice and humans is severely involuted (63, 64), and the frequency and total number of ETPs is significantly reduced compared to young mice. ETPs from the old thymus exhibit attenuated proliferation and increased apoptosis (65, 66). Similar to B cell progenitors, they also express high levels of *p16^{ink4a}* (61). As a result of these changes, few mature cells are generated in the old thymus.

iv. Myelopoiesis

In contrast to these drastic changes in lymphocyte development, myelopoiesis is intact in old mice and humans. The number of CMPs, GMPs and mature myeloid cells is higher in old compared to young mice (59). However, subtle changes in myeloid progenitors have been reported (47, 67).

In summary, current models of hematopoietic aging propose that the number of My-HSCs increases with age and the number of Ly-HSCs declines. This leads to the production of normal to elevated numbers of myeloid progenitors and a significant decline in the number of lymphoid progenitors. In addition, the quality of those B and T cell progenitors that are produced is severely attenuated by aging. These changes are summarized in Figure 1-10.

Effects of Aging on the Hematopoietic Environment

There are two hypotheses to explain why patterns of hematopoiesis change during aging. One is that cell intrinsic changes develop in hematopoietic stem and progenitor cells and this leads to alterations in their number and/or developmental potential. The other possibility is that changes in stem and progenitor cells are triggered as a result of their residence in an aging environment (Figure 1-11).

These are not mutually exclusive alternatives and in fact, it is possible that age-related changes in the environment trigger changes in stem and progenitor cells that compromise their number and/or differentiation. For example, interleukin 7 (IL-7) is expressed in bone marrow stromal cells and it is required for normal B and T cell development. It has been proposed that aging impairs IL-7 secretion by stromal cells (68), which could then account for the decreased proliferation and impaired differentiation of B cell progenitors. In addition to variations in bone marrow stromal cells, the microenvironment that supports the survival and proliferation of peripheral B cells is altered during age as well. For example, survival factors such as B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are significantly reduced in the blood plasma of elderly people (69). There is also an extensive literature, beyond the scope of the current discussion, demonstrating that age-related changes in the thymic microenvironment underlie declines in thymopoiesis (70, 71).

A major environmental change that occurs during aging is an increased production of inflammatory cytokines and this has been referred to as “inflammaging” (72). Cytokines such as IL-6, TNF-α, IL-1Rα, C-reactive protein, and IFN-γ have been reported to be increased in elderly individuals (73-77). The precise source of these inflammatory factors is not known, but their increased production has significant ramifications for hematopoiesis. In this regard, HSCs and progenitor cells express a broad spectrum of inflammatory cytokine/chemokine receptors and can thus directly respond to pro-inflammatory

factors in their milieu (78-80).

Myeloid skewing may be one major consequence of increased inflammation. Inflammatory factors can stimulate myeloipoiesis and a diversion of CD19 expressing cells from the B lineage to dendritic cells (81). Factors such as IFN-γ can impair HSC proliferative capacity, and thus dysregulate HSC maintenance (82). Inflammatory factors may also increase the number of $CD150^{hi}$ HSCs (18); as noted above, high levels of CD150 are a characteristic of My-HSCs which increase in number with age.

Summary

It is evident from the above review that a significant amount of information about normal hematopoieisis and how aging affects that process has been acquired. However, significant gaps in our understanding of why lymphocyte development is so severely affected by aging remain. My research has addressed two main questions.

I. How Does Aging Affect Ly-HSCs?

As noted, current models of stem cell aging as shown in Figure 1-9 propose that lymphocyte development is reduced with age because the number of Ly-HSCs declines. However, as we discuss in Chapter 2, while the frequency of Ly-HSCs

is reduced with age, their total number remains stable. This indicates that a reduction in their developmental potential, and not their number, underlies age related declines in lymphopoiesis. In order to obtain a better understanding of this, we compared the transcriptomes of young and old Ly-HSCs and compared them to young and old My-HSCs.

II. What are the effects of inflammation on Ly-HSCs and My-HSCs?

While HSCs respond to inflammatory factors and this may skew their development, we propose that inflammation triggers changes in Ly-HSCs that block their development. As described in Chapter 2 and 3, we have compared the effects of inflammation on Ly-HSCs and My-HSCs.

F

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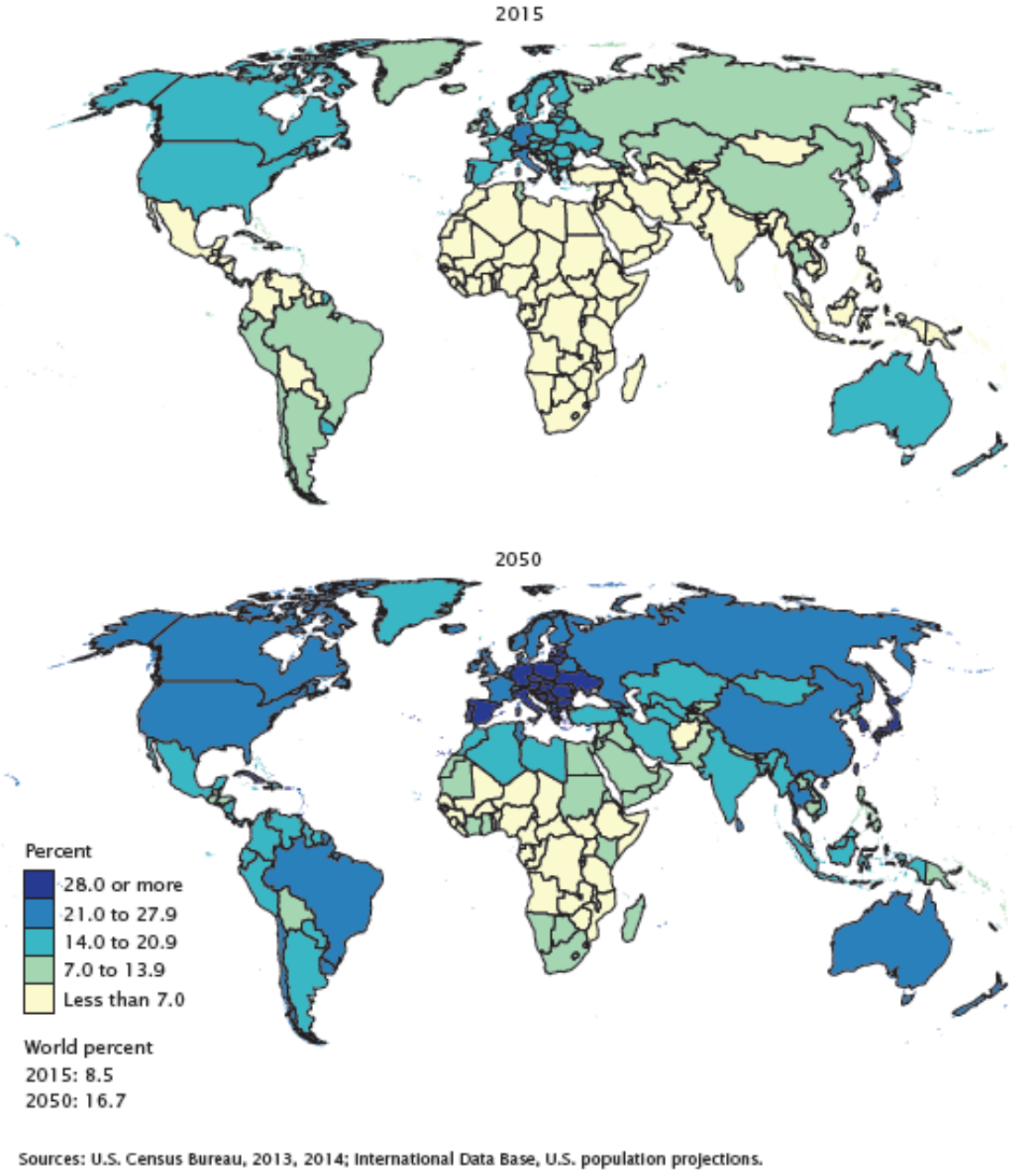

Figure 1-1. Percentage of population aged 65 and over worldwide between 2015 and 2050. Data from the US Census Bureau.

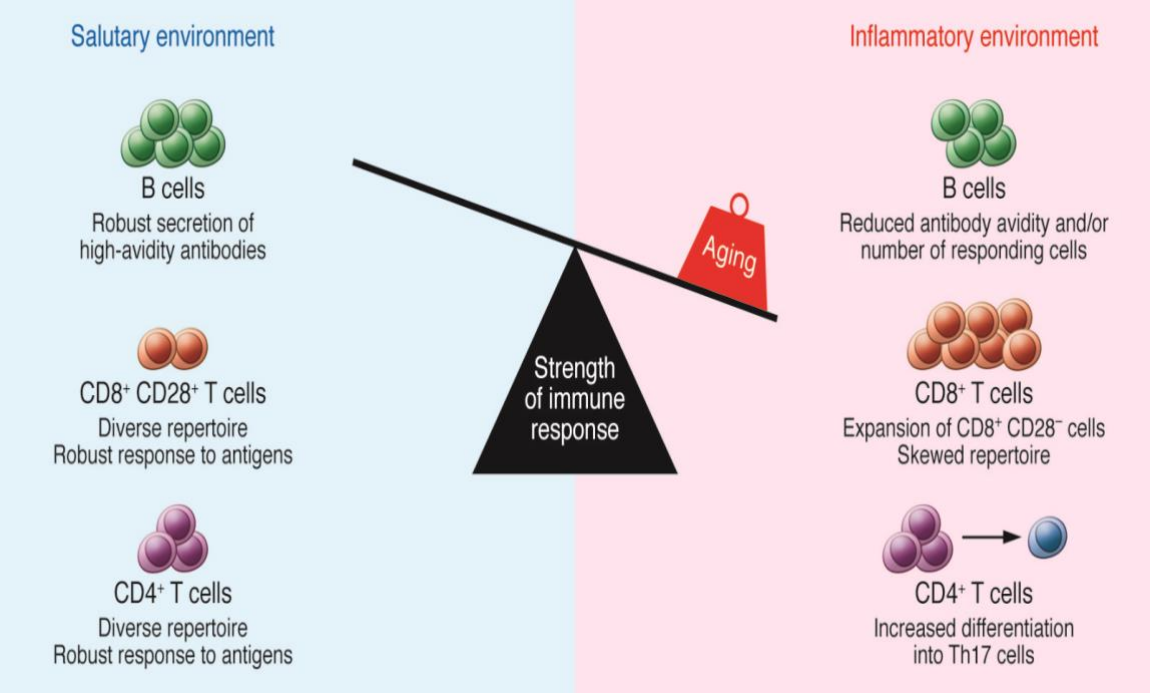

Figure 1-2. Changes in the function of mature B and T cells during aging. Figure from Montecino-Rodriguez E, Berent-Maoz B, Dorshkind K. *J Clin Invest.* 2013 *Mar;123(3):958-65.*

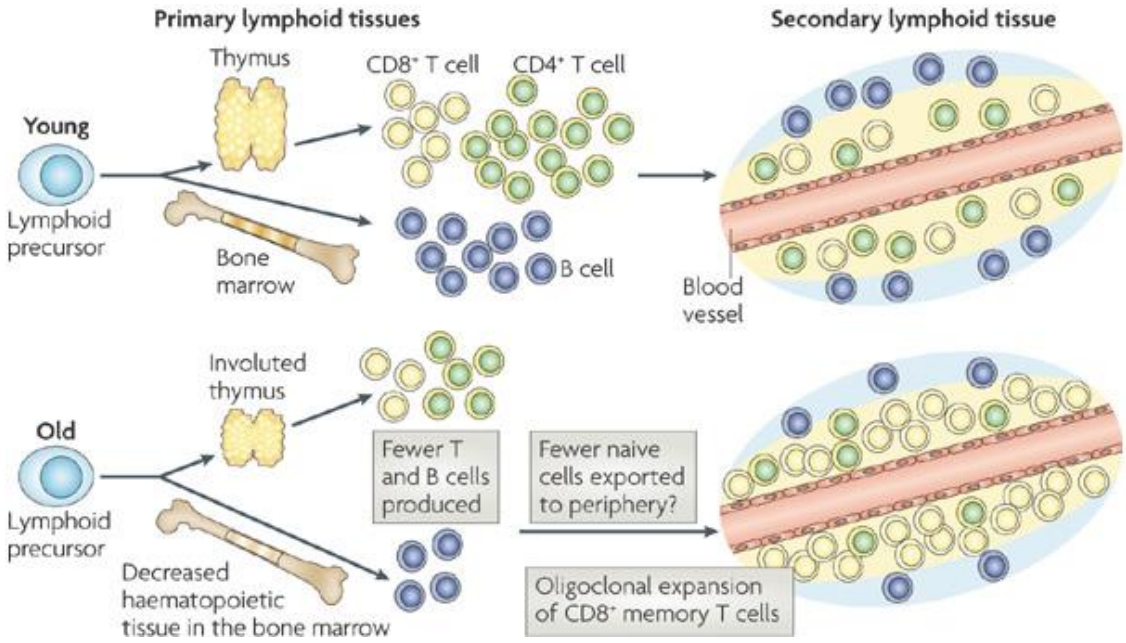

Figure 1-3. Declines in B and T cell development are a feature of aging. Figure from Dorshkind K, Montecino-Rodriguez E, Signer RA, *Nat Rev* Immunol *2009 Jan;9(1):57-62.*

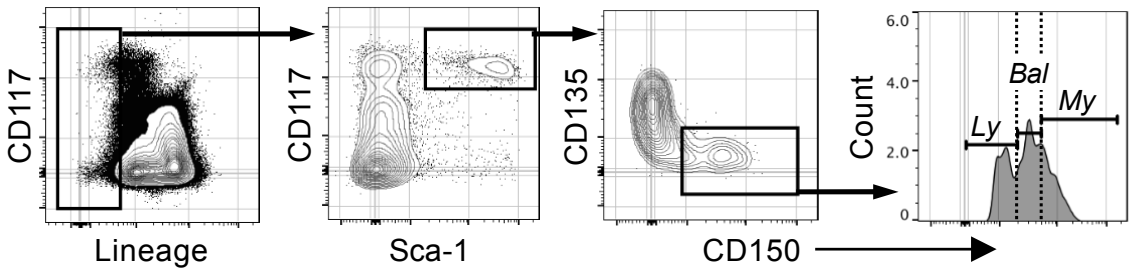

Figure 1-4. The resolution of lymphoid biased, myeloid biased, and balanced hematopoietic stem cells by flow cytometry.

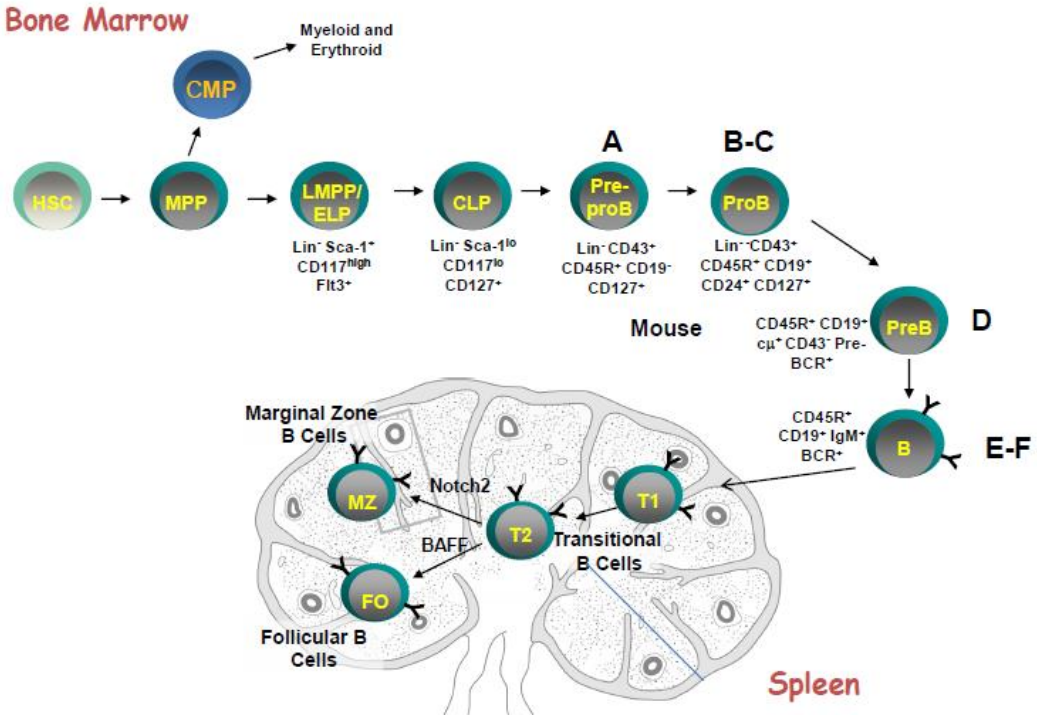

Figure 1-5. B cell development. All stages of B cell development in the bone marrow can be distinguished by their unique cell surface profile. A = pre-pro-B cells; B-C = pro-B cells; D = pre-B cells; E-F = surface Ig expressing B cells. Newly produced B cells exit the bone marrow and mature in the spleen through transitional B cell stages before maturing into marginal zone or follicular B cells.

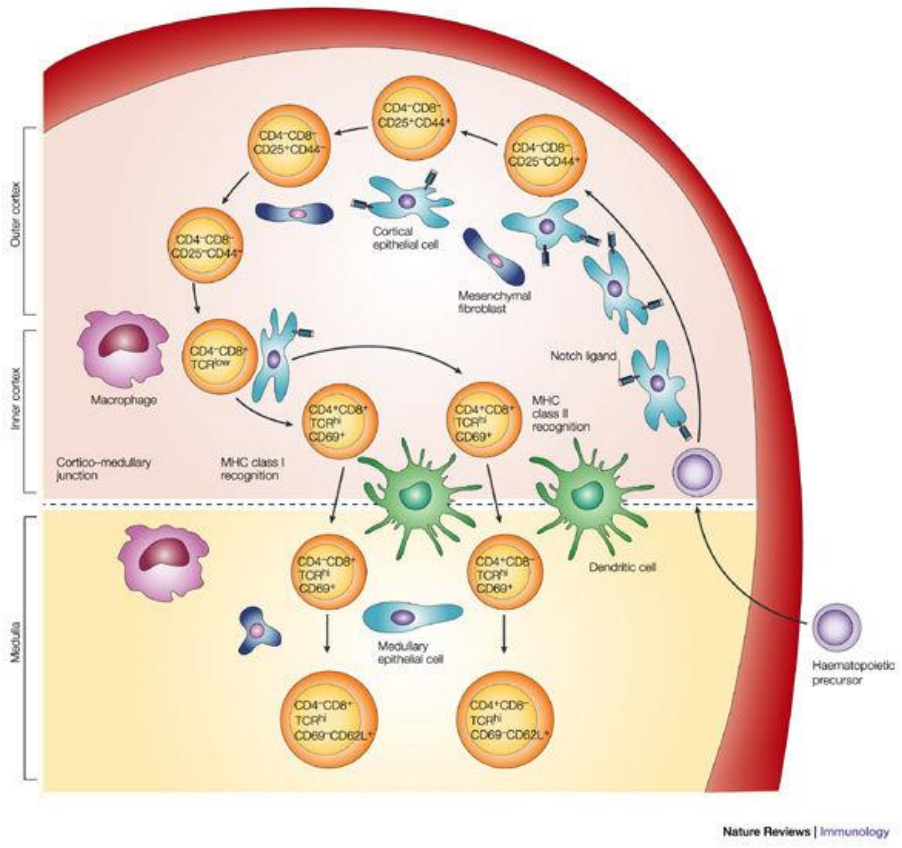

Figure 1-6. T cell development. Cells at distinct stage of development can be identified based on the expression of various cell surface determinants. Figure from Zúñiga-Pflücker J, *Nat Rev Immunol 2004 4: 67–72.*

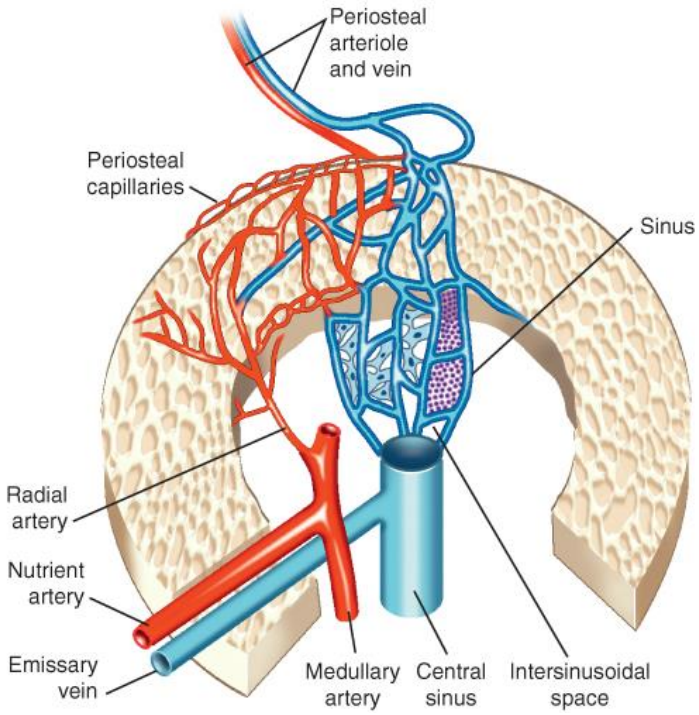

Figure 1-7. The hematopoietic microenvironment. Cross-section of bone showing elements of the medullary circulation, the marrow sinusoids, and the location of stromal cells. Figure from Dorshkind K, *Annu Rev Immunol*. 1990;8:111-37.

Hallmarks of HSC aging	Mouse	Human
Number/Frequency	Increased	Increased
Self-renewal	Reduced	?
Heterogeneity	Altered, increase in CD41+ cell number (myeloerythroid and megakaryocytic primed) clonality increased	Altered
Differentiation	Myeloid-biased	ncreased myeloid contribution decreased myeloid contributior
Localization	More distant from endosteum	?
Homing	Reduced	?
Mobiliaztion efficiency	Enhanced	?
Stem cell polarity	Apolar	?
Stem cell niche	RANTES elevated Alters clonality in HSCs upon aging	?

Table 1-8. Effects of aging on hematopoietic stem cells number and function. Table modified from Geiger H, Denkinger M, Schirmbeck R, *Current Opinion in Immunology 2014, 29:86-92*

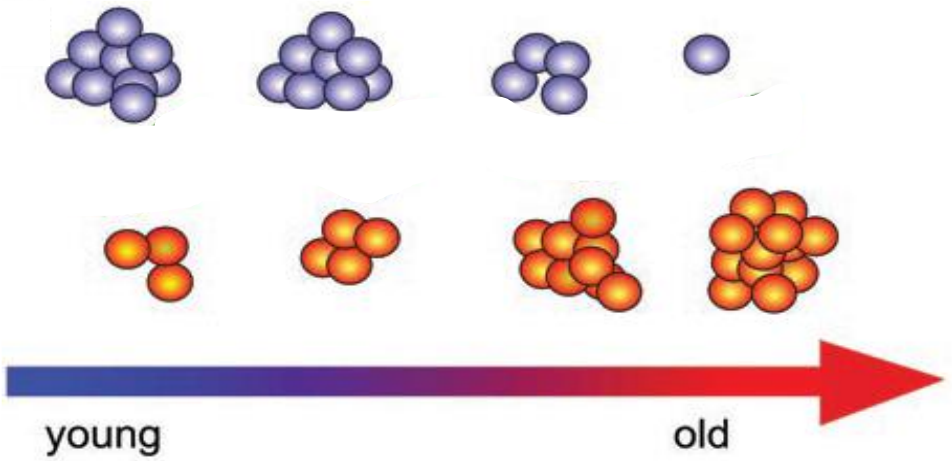

Figure 1-9. Current model of HSC aging. During aging, Ly-HSCs decrease in number whereas My-HSCs increase significantly. As a result, My-HSCs predominate in the bone marrow of old individuals. Figure from Muller-Sieburg C, Sieburg HB. *Cell Cycle. 2008 Dec 15;7(24):3798-804.*

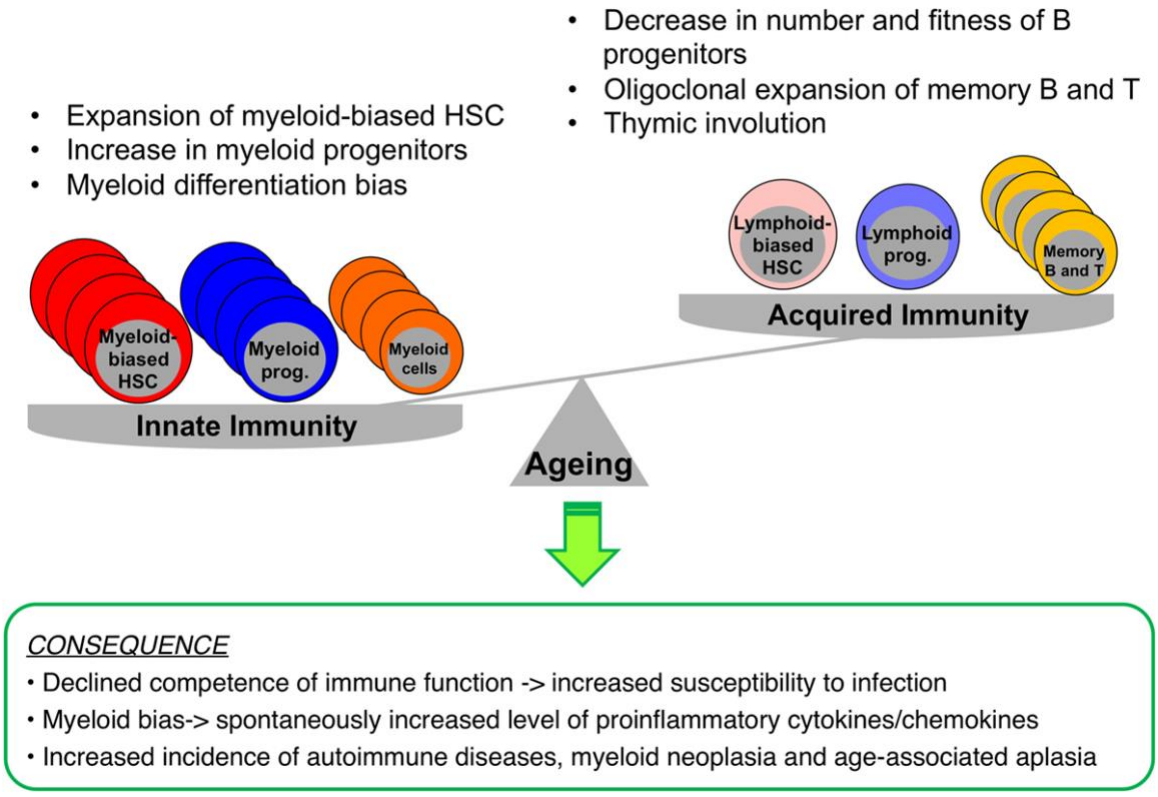

Figure 1-10. Summary of aging effects on hematopoiesis. Figure from Kovtonyuk LV, Fritsch K, Feng X, Manz MG, Takizawa H. *Front Immunol. 2016 Nov 14;7:502*

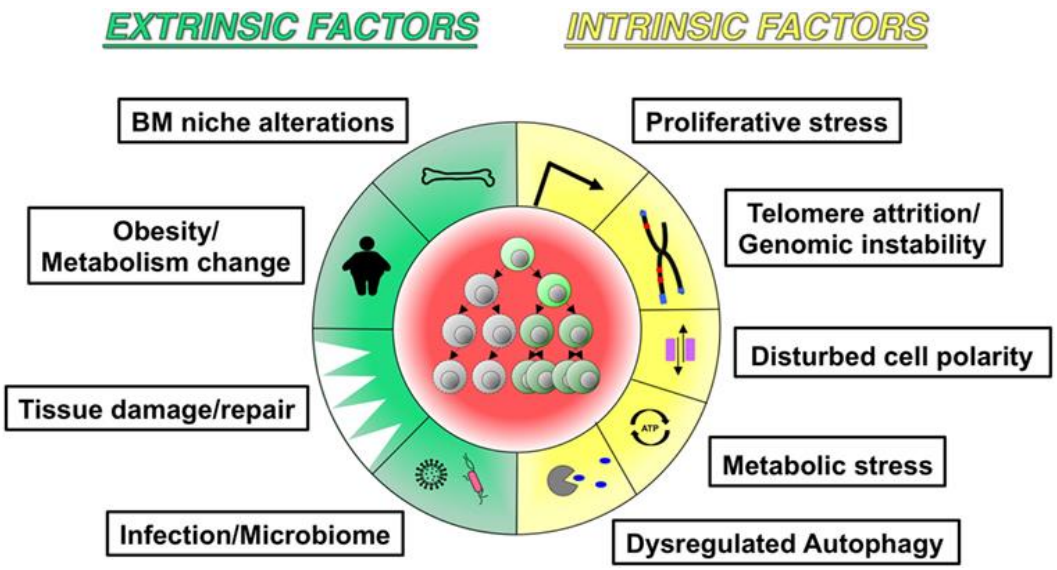

Figure 1-11. Effects of extrinsic and intrinsic factors on age related changes in hematopoiesis. Figure from Kovtonyuk LV, Fritsch K, Feng X, Manz MG, Takizawa H, *Front Immunol. 2016 Nov 14;7:502.*

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Chapter 2:

Lymphoid Biased Hematopoietic Stem Cells Are Maintained with Age and Acquire a Myeloid Gene Expression Signature

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Summary

Current models propose that reductions in the number of lymphoid biased hematopoietic stem cells (Ly-HSC) underlie age-related declines in lymphopoiesis. We show that Ly-HSCs do not decline in number with age and retain normal lymphoid potential when removed from the old in vivo environment. We compared gene expression in young and old Ly-HSCs as well as young and old myeloid biased HSCs (My-HSCs) and found that aging differentially affects the transcriptomes of these stem cell subsets. While the genetic changes that occur in old Ly-HSCs do not affect their potential to generate lymphoid progenitors, they acquire an imprinted, myeloid biased pattern of gene expression that correlates with changes in the types of myeloid cells generated. 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 the genomic changes that occur in HSCs during aging.

Introduction

Aging significantly alters the pattern of blood cell production in the bone marrow of mice (1, 2) and humans (3, 4) with declines in lymphocyte production being a prominent feature. The significant reduction in the frequency and total number of B lineage cells produced (5-8) results in reduced replenishment of naïve B cells in secondary lymphoid tissues. Intrinsic hematopoietic stem cell (HSC) perturbations are thought to contribute to the decline in lymphopoiesis (9-12), which may in turn contribute to an increased susceptibility of the elderly to infection and a reduced efficacy of vaccination (13). In contrast, myelopoiesis predominates in the bone marrow of old individuals, although age-related deficiencies have been reported (14, 15).

Multiple processes that include proliferation, DNA repair, and cell polarity are altered with age in HSCs (1, 16, 17). In order to provide a molecular basis for these observations, several laboratories compared the transcriptomes of young and old stem cells (4, 18-22). Consistent with their altered functions, old HSCs exhibited changes in expression of genes that regulate multiple processes that include self-renewal and differentiation (18, 20). In addition, alterations consistent with their myeloid bias were also evident. In particular, an increase in the expression of myeloid lineage genes, including those specifying platelet/megakaryocyte differentiation (21), and a downregulation of genes that specify lymphoid production were observed (23). Whether or not these age-

related changes in gene expression compromise lymphoid differentiation has not been determined.

The realization that the HSC compartment is heterogeneous and includes lymphoid biased (Ly-HSCs) and myeloid biased (My-HSCs) subpopulations (24, 25) provided an additional perspective on why age-related declines in lymphopoiesis occur. In this regard, Muller-Sieburg and colleagues reported that the age-associated attenuation of lymphopoiesis is due to reductions in the number of Ly-HSCs (26). Ly-HSCs and My-HSCs express low and high levels, respectively of the CD150 (*Slamf1*) cell surface determinant (11, 27), and phenotypic analyses demonstrated that the proportion of Ly-HSCs is reduced in old mice while that of My-HSCs is increased (11, 27). Based on these results, the currently accepted model is that the loss of Ly-HSCs contributes to declines in lymphocyte production in aging individuals (2, 24, 28).

However, this view of hematopoietic stem cell aging does not take the fact that the total number of HSCs increases with age into account (14, 29). We now show that when this point is considered, the number of Ly-HSCs does not decrease in old mice, and they retain a normal capacity to produce lymphoid progenitors upon removal from the in vivo, inflammatory milieu. This is the case even though transcriptome changes occur in old Ly-HSCs as determined by whole transcriptome sequencing. In particular, analysis of the gene expression data revealed that old Ly-HSCs acquire a myeloid biased pattern of gene expression

that correlates with a change in the types of myeloid cells they generate. These observations provide an additional perspective on the genomic changes that occur in HSCs over time and indicate that current models proposing that lymphopoiesis declines with age because of a loss in Ly-HSCs are in need of revision.

Results

The number of Ly-HSCs does not decline with age

Current models propose that reductions in the number of Ly-HSCs contribute to age-related declines in lymphopoiesis and that the number of My-HSCs and myelopoiesis increases (2, 24, 28). We quantified the frequency of Ly-HSCs and My-HSCs, based on their lineage negative, Sca-1^+ CD117(c-Kit)$^+$ (LSK) CD48$^-$ CD135$^-$ CD150^{low} and LSK CD48$^-$ CD135$^-$ CD150^{high} phenotypes, respectively (Figure 2-8A), in young and old C57BL/6 (B6) mice and found that, consistent with previous reports (11, 27), the proportion of Ly-HSCs significantly declines with age while that of My-HSCs significantly increases (Figures 2-1 A and B). However, in agreement with published observations (30, 31), both the frequency and number of HSCs increases with age in B6 mice (Figure 2-1C). When this is considered, it is clear that there is a significant 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 with age and is significantly

higher in old compared to young bone marrow (Figure 2-1D). A similar result was observed when we quantified HSC numbers in young and old BALB/c mice which do not exhibit an increase in HSCs number with age (30, 31). We found that the total number of Ly-HSCs also did not decline with age in the BALB/c strain (Figure 2-1E).

Old Ly-HSCs efficiently generate immature lymphoid progenitors in vitro

We seeded young and old Ly-HSCs on OP9 stromal cells in vitro to assess how aging affects Ly-HSC developmental potential. The results showed that they generated similar numbers of $CD19^+$ B lineage cells over the course of several weeks in culture (Figure 2-2A). In order to more accurately quantify the number of lymphoid progenitors generated, we developed an in vitro system in which the combination of stromal cell signals and lymphopoietic factors promote the formation of $CD127^+$ $CD135(FLT3)^+$ $CD19^-$ progenitors from HSCs following 12 days of culture (Figure 2-2B). The $CD127^+$ $CD135(FLT3)^+$ $CD19^-$ phenotype identifies a subset of lymphoid primed multipotential progenitors (LMPPs), referred to as early lymphoid progenitors (ELPs) (32), as well as common lymphoid progenitors (CLPs), both of which are early intermediates in the B cell differentiation pathway (33). We found that old Ly-HSCs generated a similar number of $CD127^+$ $CD135(FLT\text{-}3)^+$ progenitors as young Ly-HSCs (Figure 2-2C). Young and old My-HSCs also generated $CD127^+$ $CD135(FLT\text{-}3)^+$ cells but, regardless of age, the numbers were consistently lower compared to Ly-HSCs

(Figure 2-2C), indicating that the lineage bias of Ly-HSCs and My-HSCs is maintained in these cultures.

Ly-HSCs acquire specific changes in gene expression with age

The observation that old Ly-HSCs had normal lymphoid developmental potential was surprising in view of several reports showing that aging affects gene expression in total HSCs (4, 18-22). This raised the possibility that transcriptome changes do not occur in Ly-HSCs or that the changes that occur do not affect their lymphoid developmental potential. We performed RNA sequencing (RNA-Seq) on Ly-HSCs and My-HSCs isolated from three independent groups of young and old mice to distinguish between these possibilities. We compared our data to two separate sets of gene expression signatures (ImmGen and The Mouse Body Atlas, see Materials and Methods). For both sets, all our samples showed maximal and consistent enrichment for HSC-specific signatures (Figure 2-9A and B, and Table 2-11).

We identified 1062 genes whose expression was significantly altered by age and/or differed between the two HSC lineages (Table 2-11). Gene ontology analysis of these genes resulted in the formulation of a functional network that included cell homeostasis (including myeloid cell homeostasis), myeloid cell differentiation, cytokine production and the regulation of lymphocyte cell adhesion and differentiation as key nodes (Figure 2-10, Table 2-12).

Clear differences between Ly-HSCs and My-HSCs irrespective of age were apparent for genes in most functional categories (Figure 2-3). For example, we found that genes encoding GTPase and G-protein coupled receptor pathway elements were more frequently expressed in a lineage-specific manner (hypergeometric test p-value < 0.001, Table 2-12). Since they function in the transduction of external signals, these results suggest that the response of Ly-HSCs and My-HSCs to environmental signals is distinct.

Nevertheless, age induced variations were also evident (Figure 2-3) and were overall more prevalent than differences between Ly-HSCs and My-HSCs (Wald test adjusted p-value <0.05, Table 2-11). Genes significantly regulated by age regardless of lineage bias were over-represented in both myeloid differentiation and regulation of cell adhesion (hypergeometric test p-value < 0.001, Table 2-12). We also found that age-induced variations in gene expression occurring in Ly-HSCs largely mirrored those in My-HSCs (Pearson correlation = 0.47, Student's t test p-value <10 e-58 for all variable genes). In particular, some genes changed in common in both stem cell subsets (Wald test adjusted p-value <0.05, Figures 2-3 and 2-4A) and included *Plk2, Lamp2, Pim1, Mmrn1, Aldh1a1, Cd74, Rorb, Clu and Selp,* that have been reported by others as markers of aging in total HSCs (4, 18-22)

However, we also found that gene expression changes in Ly-HSCs were often markedly higher and skewed toward extreme values (two-sample Kolmogorov-Smirnov test p-value < 0.05, Table 2-12) compared to those which occurred in My-HSCs; this was particularly significant for genes annotated in intracellular signaling pathways (Rho GTPases, MAPK), response to stress, DNA damage, and cell cycle control (Table 2-12). As a result, at any given significance threshold, the number of genes classified as age-regulated in Ly-HSCs was higher than in My-HSCs (Figure 2-4B, 292 and 104, respectively for Wald test adjusted p-value < 0.05). These observations emphasize the importance in separately profiling Ly-HSCs and My-HSCs in order to understand how aging affects patterns of gene expression in the stem cell compartment. Because the HSC population predominantly includes My-HSCs (Figure 2-1D), gene expression analysis of young and old total HSCs would be dominated by age-induced changes in My-HSCs. We found this to be the case (Figure 2-4C), for many genes previously identified as makers of aging in total HSCs (22).

The fact that age-related gene changes in Ly-HSCs and My-HSCs were often distinct suggested that similar biological processes might be altered by aging in both subsets but in a reflection of the transcriptional heterogeneity between them, not in an identical manner. As shown in Figure 2-3 and Table 2-12, the expression of genes involved in cell cycle regulation changed in Ly-HSCs and My-HSCs with age but with a distinctly different pattern. We therefore measured Ki-67 expression in young and old total HSCs (Figure 2-4D), and in agreement

with previous reports (14, 16), we found that stem cell proliferation/cell cycle entry is reduced with age (Figure 2-4E). However, analysis of Ly-HSCs versus My-HSCs showed that while My-HSCs exhibited a significant decrease in Ki-67 positivity compared to their young counterparts, the frequency of young and old Ly-HSCs that were Ki-67^{+} was equivalent (Figure 2-4F). These results indicate that the reduced proliferation in total HSCs from old mice (14, 16) primarily reflects changes in My-HSCs.

Taken together, the above analyses demonstrated that age-related changes in gene expression occur in Ly-HSCs and revealed that aging has distinct effects on the two HSC subpopulations.

IL-1 blocks lymphocyte development from Ly-HSCs

The production of inflammatory cytokines such as IL-1 increases in the bone marrow with age (34, 35), and these factors can inhibit lymphopoiesis and stimulate myelopoiesis (36). We determined how IL-1 affects lymphocyte development from Ly-HSCs and My-HSCs in view of the RNA-Seq data showing that Ly-HSCs expressed genes encoding the IL-1 receptor as well as various RhoGTPases such as *Cdc42.* RhoGTPases play multiple roles within cells including the transduction of signals from the IL-1 receptor (37, 38). We found that the addition of 1 ng/ml of IL-1 to the lymphoid progenitor assay almost completely blocked the emergence of CD127^{+} CD135(FLT3)$^{+}$ CD19^{-} cells from

Ly-HSCs, regardless of age. In addition, IL-1 treatment also abrogated the limited lymphopoietic potential of My-HSCs (Figure 2-2D).

Ly-HSCs acquire a myeloid biased pattern of gene expression with age

Further analysis of our RNA-Seq data by multidimensional scaling revealed that old Ly-HSCs acquired a pattern of gene expression that overlapped with My-HSCs, and this was even more pronounced when age-responsive genes specific to Ly-HSCs were considered (Figure 2-5A).

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 2-5A). This analysis identified 507 genes (Wald test 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, TGF-□ signaling, and myeloid differentiation (Figure 2-5B and Table 2-12). We also performed a supervised analysis in which we compared the gene changes observed in old Ly-HSCs to a myeloid signature list defined in a previous study (19). This analysis revealed that some of these myeloid specific

genes were overexpressed in old Ly-HSCs at levels similar to those in old My-HSCs (Figure 2-5C).

Young and old Ly-HSCs generate phenotypically distinct myeloid progeny

These above observations raised the possibility that the myeloid potential of old Ly-HSCs is affected by aging. We tested this by culturing young and old Ly-HSCs and My-HSCs under myeloid conditions and examining the phenotype of the myeloid cells produced 11 days later. The majority of myeloid cells produced by young Ly-HSCs were Gr-1^{+} CD11b^{high} while old Ly-HSCs primarily generated Gr-1^{+} CD11b^{low} cells. The cell populations produced by old Ly-HSCs were similar to those generated from young My-HSCs and, in agreement with the multidimensional scaling data, even more so to those produced by old My-HSCs (Figure 2-6A). Median fluorescence intensity (MFI) analyses of CD11b and Gr-1 expression levels confirmed a specific decrease in CD11b expression by the myeloid progeny of old Ly-HSCs (Figures 2-6B and 2-6C). We also observed that Ly-6C expression levels on the myeloid progeny of old Ly-HSCs was lower when compared to myeloid cells generated from young Ly-HSCs and more similar to that of My-HSC derived myeloid cells (Figure 2-6D).

Discussion

The central conclusion of this study is that age-related declines in lymphopoiesis do not result from a loss of Ly-HSCs in the bone marrow. As a result, current models of HSC aging presented in various reviews, including from our own laboratory (2, 12, 28), need to be revised to indicate that the number of Ly-HSCs is maintained with age and they develop a myeloid biased pattern of gene expression (Figure 2-7). In addition, our results indicate that the various age-related defects identified in total HSCs (16) are not necessarily associated with all HSC subpopulations. For example, while old HSCs exhibit declines in proliferation, our data indicate this is primarily a property of My-HSCs. These observations demonstrate that a full understanding of how aging affects HSCs will be dependent upon assessing the various stem cell populations.

Our data also demonstrate that the Ly-HSCs present in old mice have normal lymphoid developmental potential ex vivo. We used two in vitro assays to show this and purposely avoided the use of transplantation assays for two reasons. First, the irradiation used to condition recipients can cause numerous systemic changes, such as increased inflammation (39), that could differentially affect the young and old stem cells. In addition, old HSCs exhibit bone marrow homing defects (16, 40). In this case, reports that old Ly-HSCs exhibit diminished lymphoid reconstituting potential (24, 27) may reflect the fact that fewer of these

cells seeded the marrow rather than a defect in their ability to generate downstream progeny.

Ly-HSCs did not decline in number with age and retained normal lymphoid potential ex vivo, indicating that the gene changes in them do not disable their lymphoid developmental program. These results raised the question of why few immature lymphoid progenitors, such as CLPs (6, 7) are present in the bone marrow of old mice. We demonstrate that exposure of Ly-HSCs to inflammatory cytokines whose production is known to increase with age in the bone marrow (34, 35) blocked their ability to produce lymphoid progenitors (Figure 2-7). We focused on IL-1 because it is a key effector of the senescence associated secretory phenotype (41), and it can stimulate myelopoiesis (42) and inhibit lymphocyte development (43, 44). In addition, Ly-HSCs expressed high levels of various RhoGtpases such as *Cdc42*, which are important for IL-1 signal transduction (37, 38). We also found that IL-1 blocked lymphocyte development from My-HSCs. Thus, age-related declines in lymphopoiesis may reflect the reduced production of lymphoid progeny from both stem cell subsets. Taken together, these results suggest that residence in the aging environment underlies the reduced production of B lineage cells from Ly-HSCs and My-HSCs. Inflammatory cytokines have differential effects on Ly-HSCs and My-HSCs (11, 45), and the data provide additional information regarding their effects on lymphoid development from them in the context of aging.

This report provides the first sequencing-based resource of gene expression data for young and old murine Ly-HSCs and My-HSCs that will be of value to future studies of these populations. They also complement a previously described microarray based genomic analysis that compared young Ly-HSCs and young My-HSCs (11). We found that some differences in gene expression between Ly-HSCs and My-HSCs occurred irrespective of age and reflected distinctions between these two stem cell populations. However, the majority of differences in gene expression between Ly-HSCs and My-HSCs were age induced, and the number of genes whose expression changed with age was almost three fold higher in Ly-HSCs compared to My-HSCs. In addition, Ly-HSCs exhibited more extreme changes in gene expression with age, particularly with regard to genes involved in the response to external cues. Together, these results indicate that Ly-HSCs are subject to stronger transcriptional regulation than My-HSCs. Such differences between young and old Ly-HSCs could not be elucidated in the multiple studies that compared the transcriptomes of young and old total HSCs.

An unexpected observation was that old Ly-HSCs had acquired a myeloid biased pattern of gene expression. This result is consistent with previous observations that total HSCs acquire a platelet/megakaryocyte biased pattern of gene expression (21). Rossi and colleagues reported an increased expression of myeloid lineage genes in total HSCs during aging (23). Our results indicate that, in addition to the increased number of My-HSCs, gene expression changes in Ly-HSCs also contribute to this myeloid biased signature HSCs from old mice. The

gene changes that occurred in old Ly-HSCs, which are likely to be epigenetically induced, had an enduring effect on myelopoiesis even upon removal of the cells from the old environment. In this regard, young Ly-HSCs produced a high proportion of $CD11b^{high}$ myeloid cells while old Ly-HSCs, along with young and old My-HSCs, primarily produced $CD11b^{low}$ progeny ex vivo (Figure 2-7). The functional ramifications of this shift in cell production remain to be determined. In this regard, it is known that the $CD11b^{+}$ $Gr\text{-}1^{+}$ phenotype identifies a population of myeloid derived suppressor cells (46, 47) that have been implicated in the age-related declines in lymphocyte development (43).

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 (2, 24, 28) proposing that reduced Ly-HSC number accounts for the age-related diminution of lymphopoiesis. The results further show that despite changes in their transcriptome, the inhibition of lymphoid development from old Ly-HSCs is dependent on their continued exposure to external signals, because they had normal lymphoid potential when removed from the old environment (Figure 2-7). The latter result is in accord with genomic analyses showing that Ly-HSCs exhibit significant changes in the expression of genes involved in the response to external cues. The normal developmental potential of old Ly-HSCs is also consistent with the fact that *p16*^{Ink4a}, a biomarker and effector of aging (48, 49) that is expressed in old pro-B cells and compromises their proliferation and differentiation (50), was not detected in old Ly-HSCs (Table 2-11). A previous

study also detected no change in *p16^{Ink4a}* expression between young and old total HSCs (18). These results suggest that targeting changes that occur in the aging environment may be fruitful in rejuvenating lymphocyte production in old individuals.

Experimental Procedures

Mice

Young 8-12 week-old C57BL/6 (B6) and BALB/c (BALB) mice were obtained from The Jackson Laboratory or the UCLA Division of Laboratory Animal Medicine. Old B6 and BALB mice were obtained from the National Institute on Aging colony and were used at 18 months of age. All experiments were conducted according to UCLA Institutional Animal Care and Use Committee guidelines. 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

Bone marrow cell suspensions were prepared as previously described (6, 51). HSCs 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 (51). The lineage cocktail included antibodies to CD3ε, CD8α, TCRβ, TCRγδ, NK1.1, TER-119, Gr-1, B220(CD45R) and IgM. 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 2-8. For Ki-67 staining, cells were fixed, permeabilized, and stained with PE/Cy7 labeled anti-Ki-67 antibody or rat IgG2a, isotype, as per manufacturer instructions (Life Technologies). Cells produced in long term-cultures and colony assays were analyzed for expression of CD45, CD135, CD127, CD19, CD11b, and Ly-6C and/or Gr-1 as indicated in the figure legends. All the antibody clones used and their sources are listed in Figure 2-8B. 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.

In vitro lymphoid and myeloid assays

The lymphoid potential of Ly-HSCs was initially assessed by seeding 150 purified stem cells per well of 12 well plates on confluent layers of OP9 stroma cells (52) in RPMI 1640 supplemented with 10% heat inactivated FCS, $5x10^{-5}$ M 2ME (SIGMA), 2 mM L-glutamine, 100 U/ml streptomycin, 50 µg/ml gentamycin, 100 U/ml streptomycin, 100 µg/ml penicillin, 0.1 mM 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 five 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 x 10^4 S17 stromal cells (53) in 1.5 ml of methylcellulose (MC) medium. MC medium was prepared by supplementing α-MEM with 30% heat inactivated FCS, 1% methylcellulose (Stem Cell Technologies), 5x10^{-5} M 2ME, 2 mM L-glutamine, 50 μg/ml gentamycin, 100 U/ml streptomycin, 100 μg/ml penicillin, 0.1 mM MEM vitamins, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (all from GIBCO) and 20 ng/ml SCF, 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.

Myeloid assays were initiated by resuspending 200 purified stem cells in 1.5 ml α-MEM supplemented with 30% FCS, 1% methylcellulose, 5x10^{-5} M 2ME, 2 mM L-glutamine, 50 μg/ml gentamycin, 100 U/ml streptomycin, 100 μg/ml penicillin,

0.1 mM MEM vitamins, 0.1 mM nonessential amino acids,1 mM sodium pyruvate (all from GIBCO) and 20 ng/ml IL-3, and 50 ng/ml SCF and 10 ng/ml IL-6 in 3.5 cm^2 petri dishes in triplicate. Following 11 days of culture the contents of the plates were harvested and tested for production of CD11b, Gr-1 and Ly-6C expressing myeloid lineage cells by flow cytometry.

All cultures were placed at 37^oC, 5% CO_2 humidified incubators until processing.

RNA-Sequencing 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# R2070, Irvine, CA) as per the manufacturer's instructions. Total RNA was quantified and 260/280 ratios determined using Nanodrop. RNA sequencing and library preparation were performed in the Jonsson Comprehensive Cancer Center Genomics Shared Resource using the KAPA Stranded mRNA-seq kit (Roche Sequencing, cat#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 150bp 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. Raw sequence files are available at NCBI's Gene Expression Omnibus (GSE112769).

The STAR ultrafast universal RNA-seq aligner v2.5.2b (54) 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 (55). Counts were normalized per-sample in units of FPKMs after correcting for gene mappable length and sample total counts. The table of expression estimates (FPKM) was used as input for SaVanT (56) 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 10000 iterations*". The most significant enrichment scores are shown in Figure 2-9, and unabridged output from SaVanT is provided in Table 2-12.

Differential expression analysis was performed with *DESeq2* (57). 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 vs. old Ly-HSCs and My-HSCs, young and old Ly-HSCs vs. 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 pair-wise tests. An additional pair-wise test was performed (young Ly-HSCs vs. the rest of the samples) to identify genes similarly expressed in old Ly-HSCs and My-HSCs (507 genes, Figure 2-5B and Table 2-11).

Functional enrichment was performed with Metascape (http://metascape.org) using Gene Ontology (GO) biological processes annotations in mouse. The network of ontology terms in was computed in-house and visualized with Cytoscape (58) (Figure 2-10). Nodes with the same color are specific ontologies in the same GO generic class, and are labeled using a representative member (Table 2-12). Node size is proportional to statistical significance (hypergeometric p-value as provided by Metascape). Edge thickness is proportional to between-node similarity and was computed in-house in Matlab (Release 2017a, The MathWorks, Inc) using Kappa statistics, and reflects the overlap between the gene sets annotated in both ontology terms. Hierarchical clustering (Figure 2-3,

2-4A and 2-5B) was performed and visualized in Matlab using z-scores from variance-stabilized data as input for each group of genes. Multi-dimensional scaling (MDS) 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 mean ± SEM as indicated in the figure legends. Differences between groups were tested by a two-tailed, unpaired Student's t test ($\alpha = 0.05$).

Figures

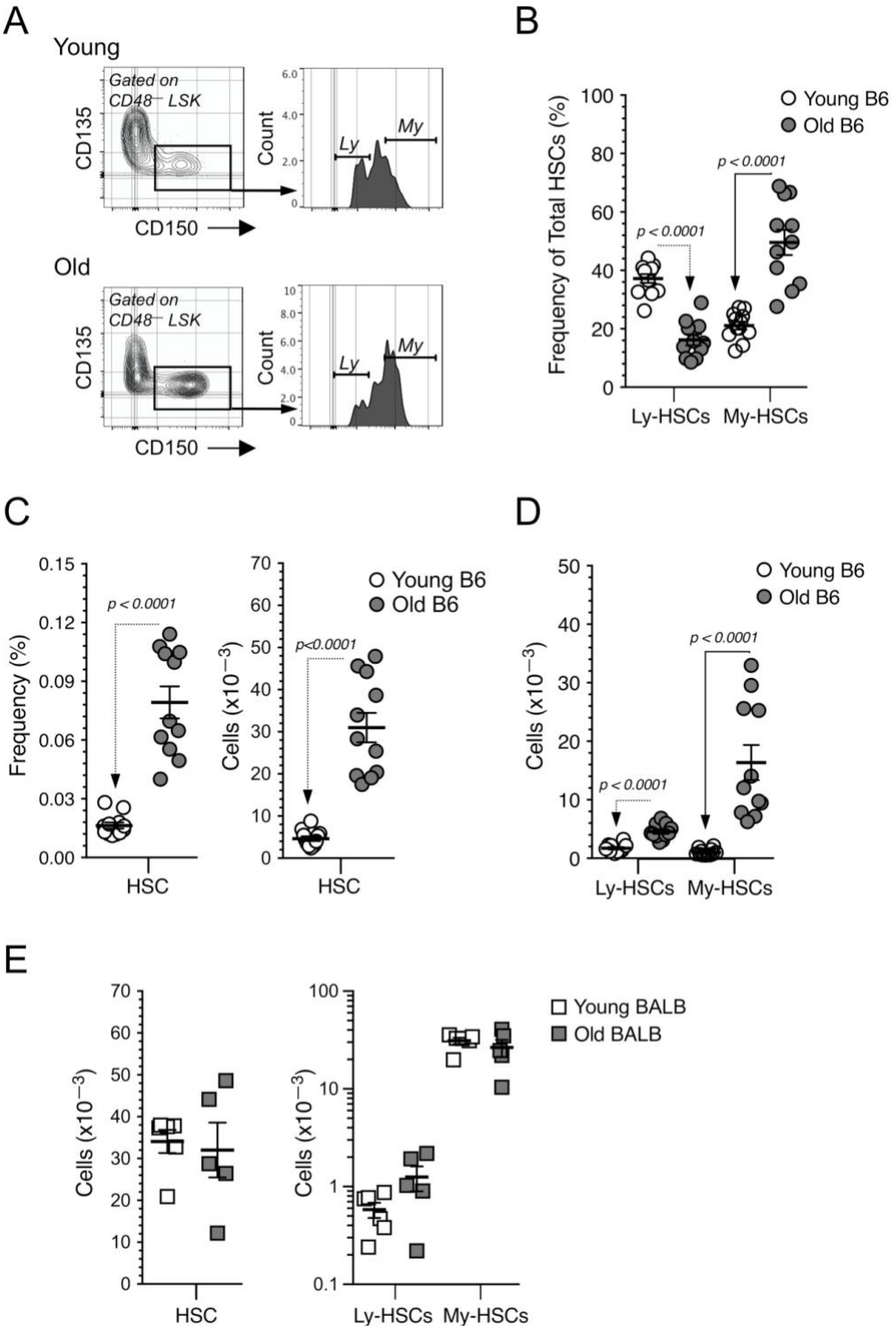


Figure 2-1. Quantification of Ly-HSCs and My-HSCs in the bone marrow of young and old mice. (A) FACS plots showing the strategy used for the resolution of $CD150^{low}$ $CD135^{-}$ Ly-HSCs and $CD150^{high}$ $CD135^{-}$ My-HSCs within the

lineage negative, Sca-1^{+} CD117(c-Kit)$^{+}$ (CD48^{-} LSK) population. (A complete gating strategy is shown in Figure 2-8A). (B) Relative frequency of Ly-HSCs and My-HSCs within the total HSC population 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 of Ly-HSCs and My-HSCs (right panel) in the bone marrow of young and old BALB/c mice. Panels A-E: Each symbol represents an individual young (8 to 12 week) or old (18 months) mouse; levels of significance for the differences between populations are indicated.

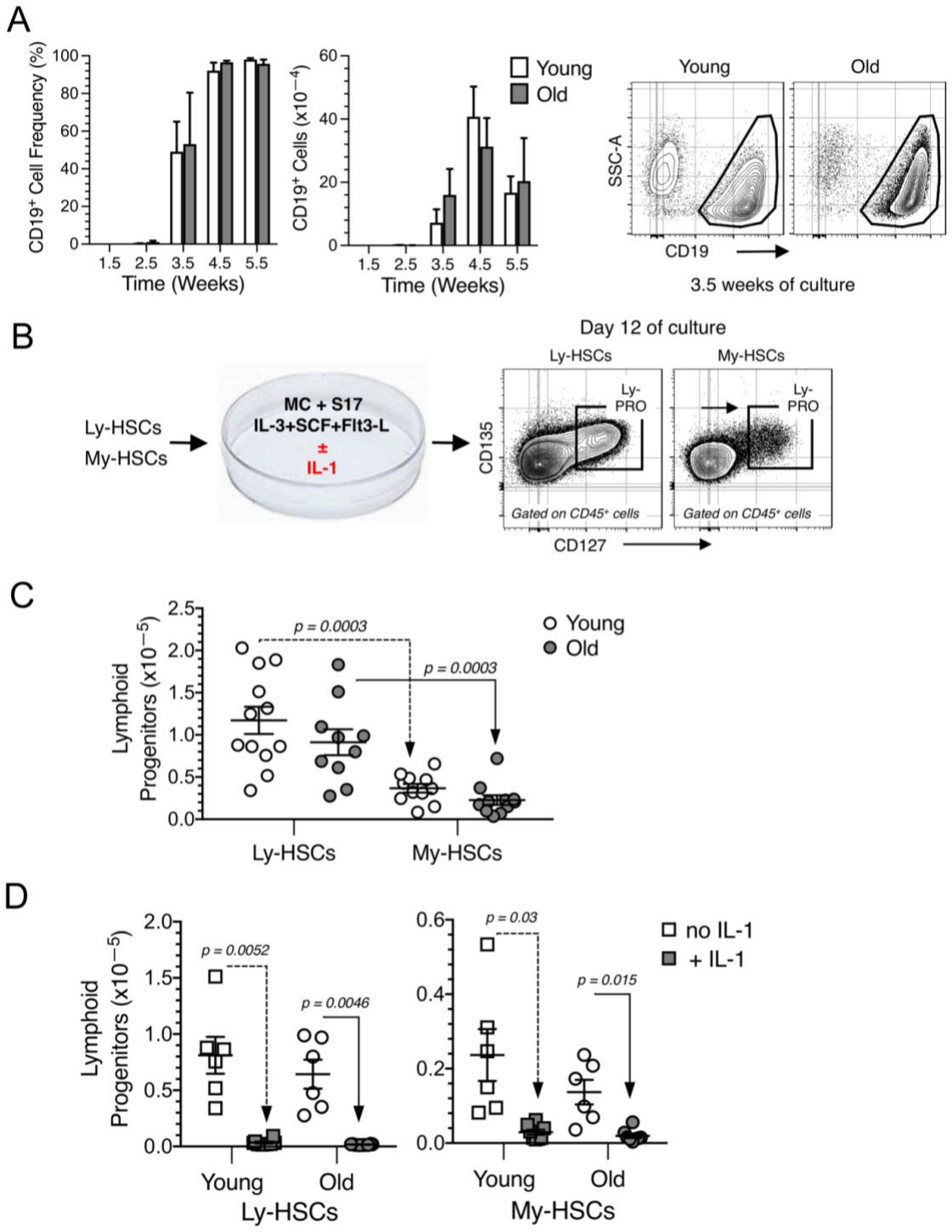


Figure 2-2. Old Ly-HSCs generate lymphoid progenitors in vitro. (A) Frequency and number of CD19⁺ B lineage cells produced in culture from 150 Ly-HSCs purified from young and old mice seeded on OP9 stroma in vitro. Representative FACS plots show CD19⁺ cell production at 3.5 weeks of culture. Data are representative of 3 experiments. (B) Diagram of the in vitro assay used to

generate lymphoid progenitors from Ly-HSCs and My-HSCs. (A detailed protocol is described in the methods section). 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 panels C and D represents production per culture dish. Cultures were initiated with Ly-HSCs and My-HSCs purified from 3 and 2 independent cohorts of 6 young (8 to 12 weeks) and 4 old (18 months) B6 mice, respectively. Levels of significance for the differences between populations are indicated.

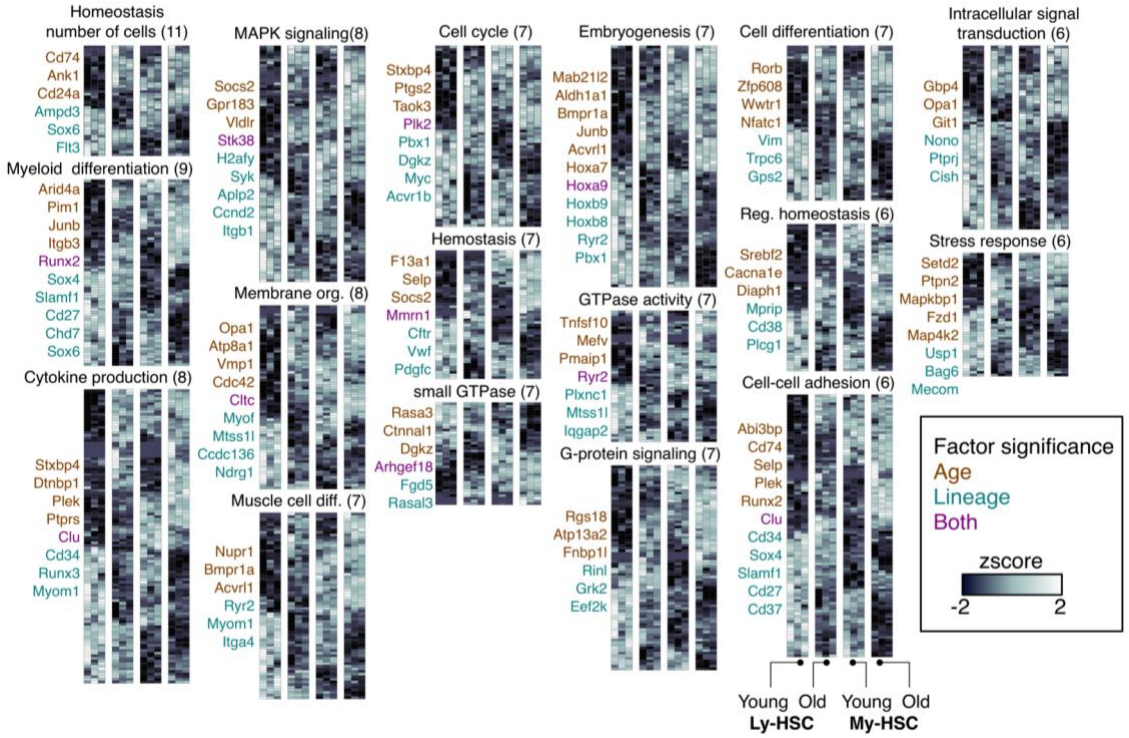


Figure 2-3. Gene ontology analysis of age and/or lineage biased gene expression in young and old Ly-HSCs and My-HSCs. Hierarchical clustering showing the expression levels for genes in selected ontology categories. The results for each biological replicate are shown. Categories are sorted by overall statistical significance from top left to bottom right with levels of significance shown in parentheses (e.g. 11 stands for p-value = 10e–11). The gene font colors distinguish genes that are age regulated (orange), lineage regulated (green), or both age and lineage regulated (purple), following results from additive models for both factors (Table 2-11). Only a few genes in each category are shown to provide representative examples of lineage and/or age biased genes. Full results are provided in Table 2-12. Genes annotated in several categories are only shown for the most significant function, with the exception of genes highlighted for cell-cell adhesion (See also Figure 2-9 and 2-10).

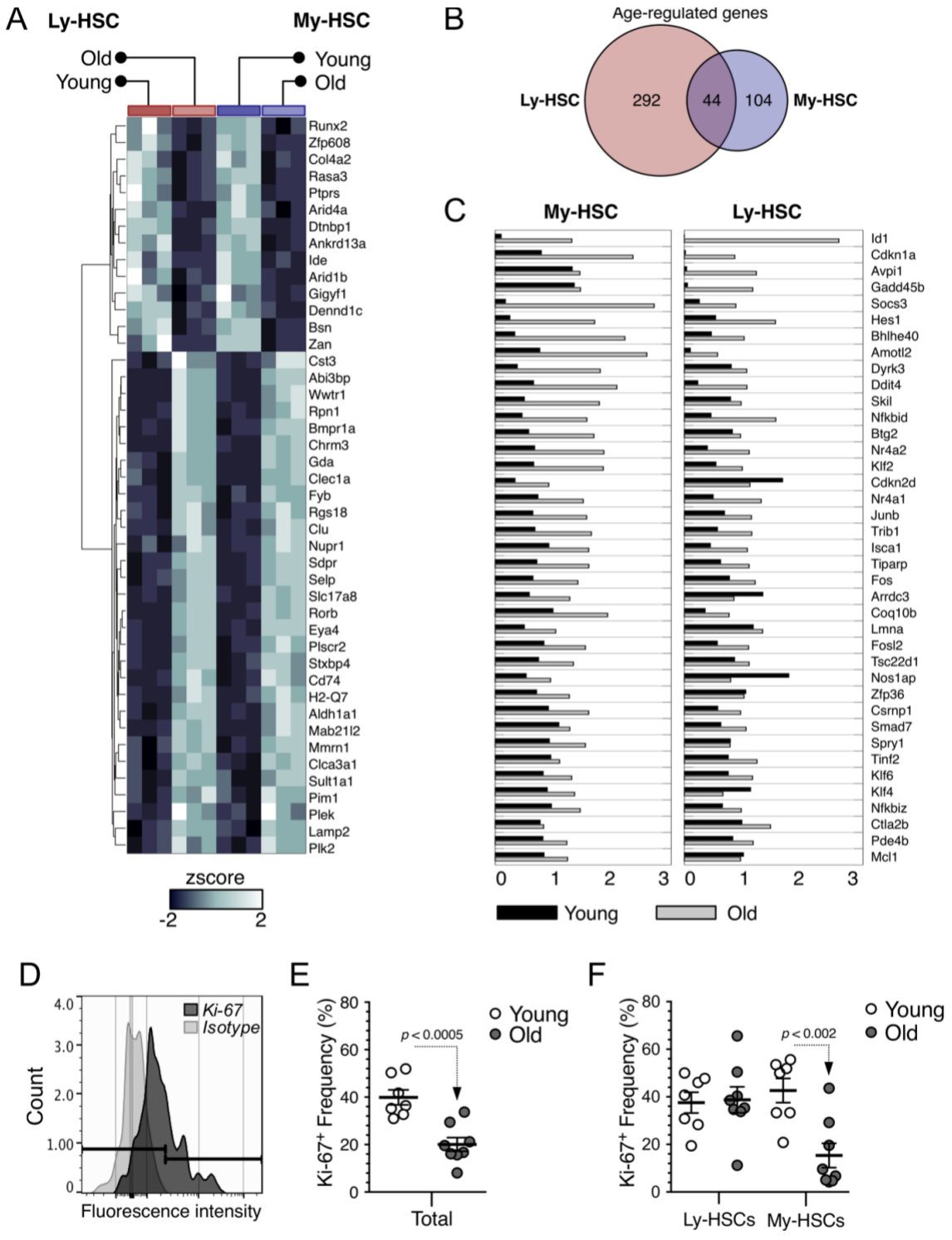


Figure 2-4. Ly-HSCs and My-HSCs exhibit specific changes in gene expression with age. (A) Hierarchical clustering for the 44 genes classified as age-regulated in both Ly-HSCs and My-HSCs for each individual biological replicate. (B) Venn

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 stem cell subsets. (C) Barplots of average expression levels (relative to the mean across all samples) for genes classified as markers of an “old specific cluster” by single-cell RNA-Seq analysis of total HSCs (22). Each bar represents the average of three biological replicates. (D) FACS plot showing Ki-67 staining of total HSCs with Ki-67 antibody and isotype control. (E) Frequency of Ki-67 expressing young and old total HSCs. (F) Frequency of Ki-67 expressing young and old Ly-HSCs and young and old My-HSCs. Each symbol in panels C and D represents cells analyzed from an individual mouse.

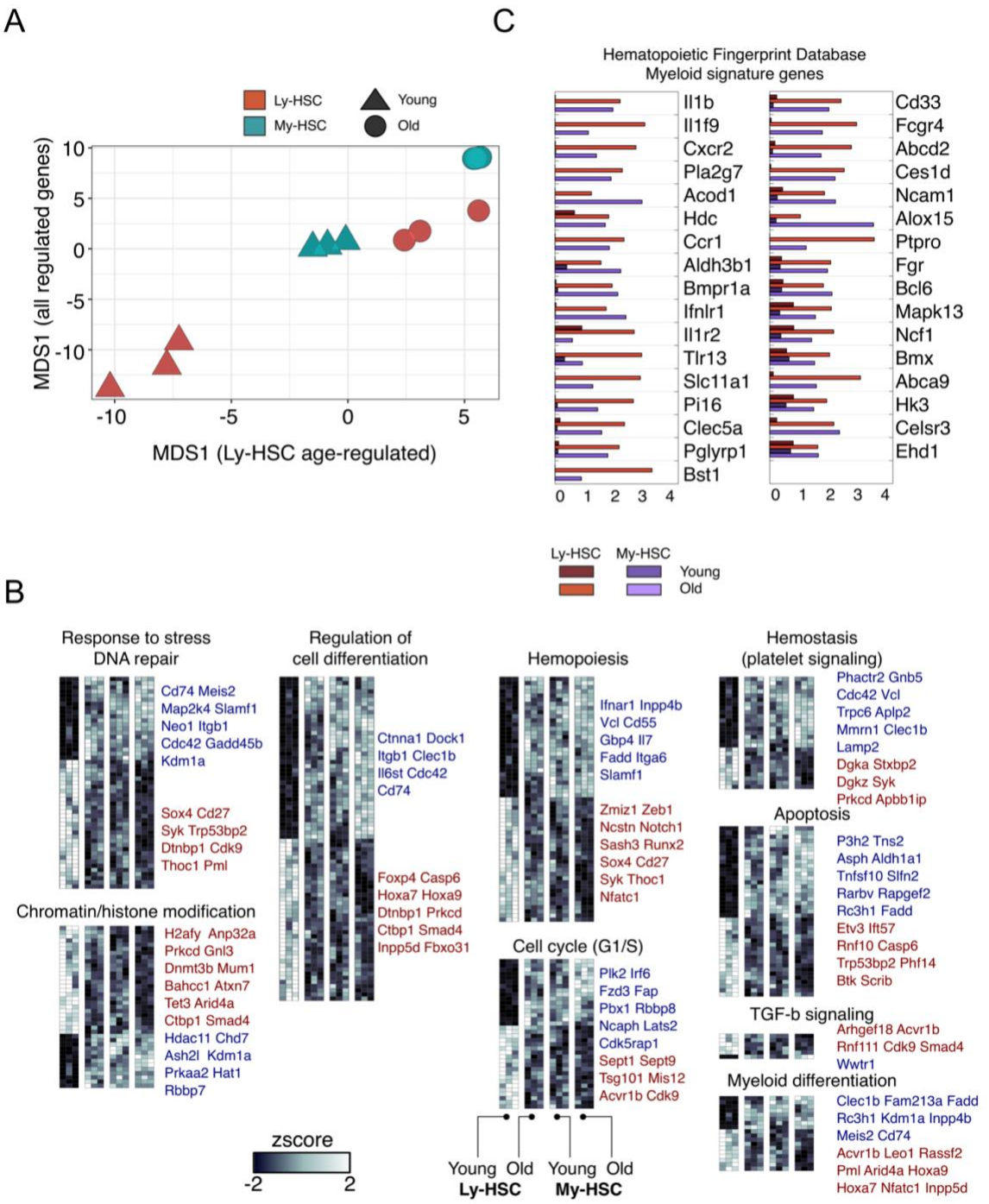


Figure 2-5. Ly-HSCs acquire a myeloid bias with age. (A) Multidimensional scaling analysis of young and old Ly-HSCs and My-HSCs. Each symbol represents a distinct cohort of Ly-HSCs or My-HSCs isolated from 6-8 B6 mice. Shown is a scatterplot of the first multidimensional scales obtained from all variable genes (1062 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 pair-wise test

between young Ly-HSCs and the rest of the samples, thus showing similar expression levels in old Ly-HSCs and My-HSCs. Genes are grouped by functional categories (only significant categories are shown, p-value<0.01). Representative gene names are shown (blue: lower expression and red: higher expression in young Ly-HSCs). A list of the specific genes in each plot can be found in Table 2-12. (C) Barplots of average expression levels (relative to the mean across all samples) for selected genes classified as “myeloid genes” in the Hematopoietic Fingerprints database (19). Each bar represents the average of three biological replicates.

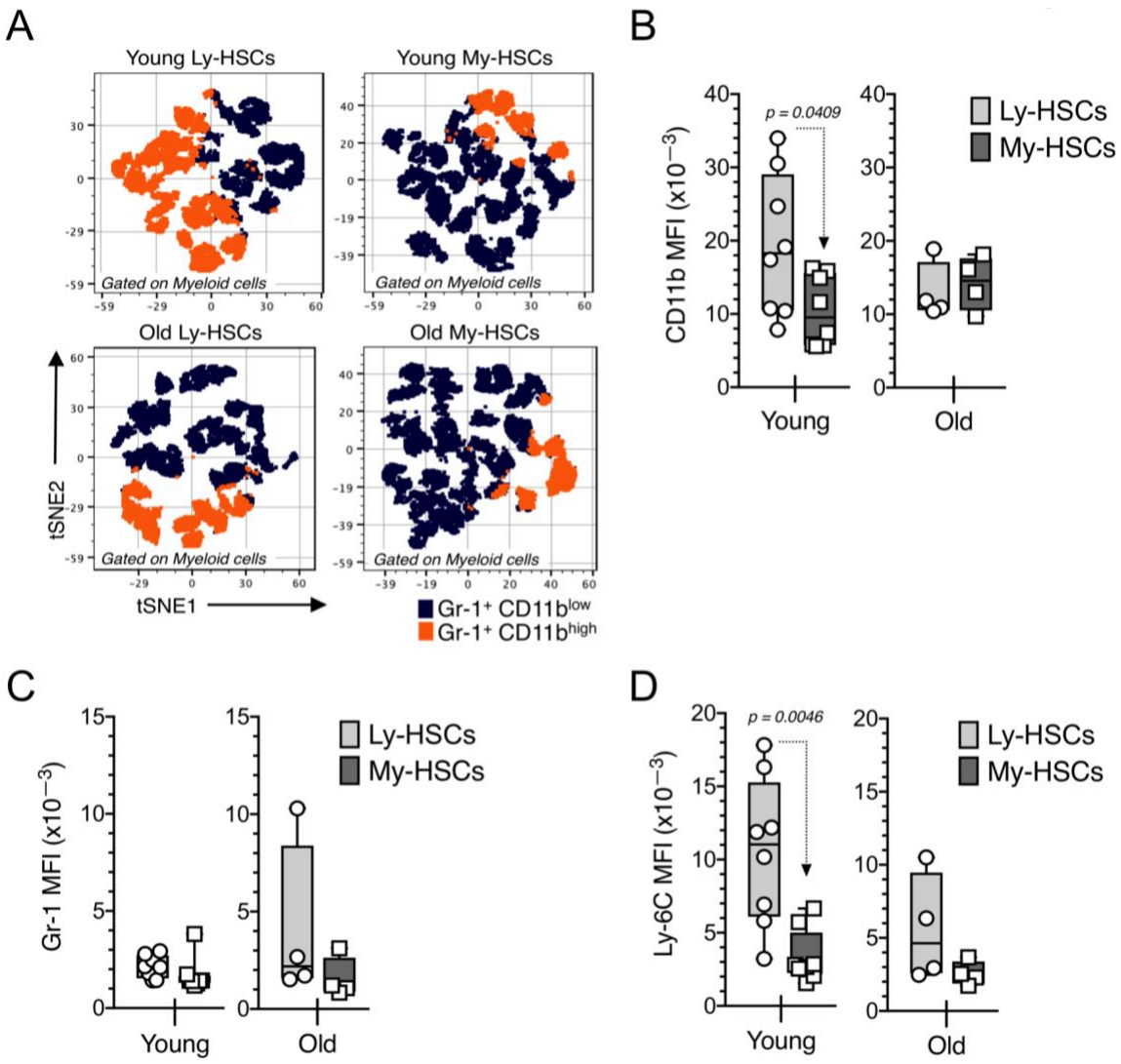


Figure 2-6. Aging affects myeloid cell production from Ly-HSCs. (A) Representative t-SNE plots showing the distribution of Gr-1^{+} CD11b^{high} and Gr-1^{+} CD11b^{low} myeloid cells generated from young and old Ly-HSCs and My-HSCs in culture for 11 days. Mean fluorescence intensity (MFI) of (B) CD11b, (C) Gr-1, and (D) Ly-6C expression by myeloid cells generated by young and old Ly-HSCs and My-HSCs. Each symbol represents cells produced per culture dish from 200 HSCs. Cultures were initiated with Ly-HSCs and My-HSCs purified from 3 and 2 independent cohorts of 6 young and 4 old B6 mice, respectively. Levels of significance for the differences between populations are indicated.

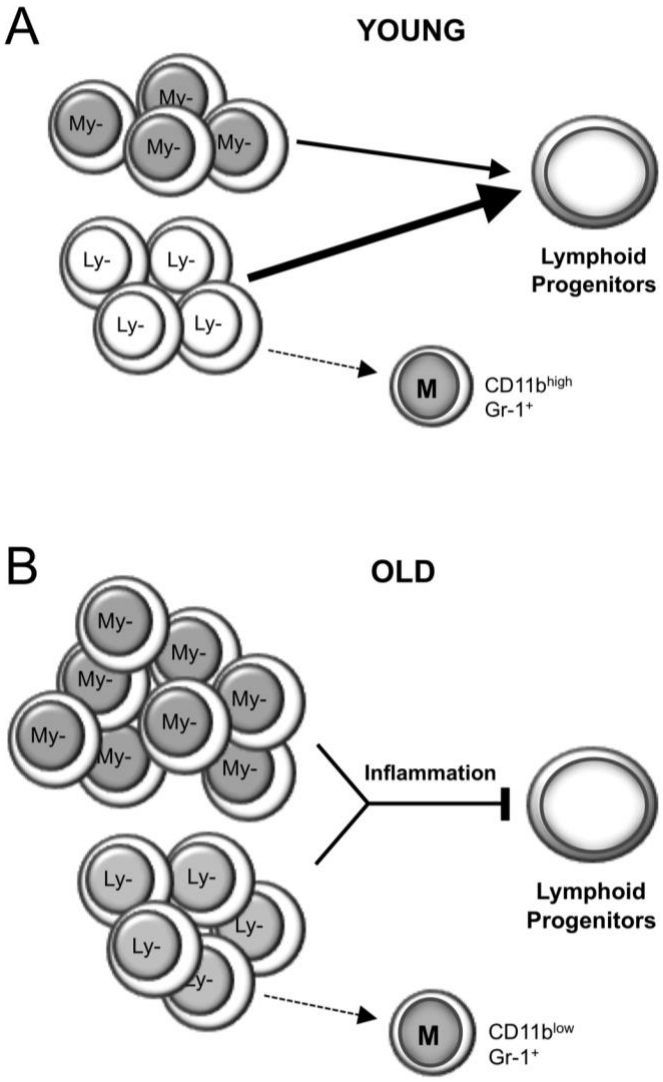
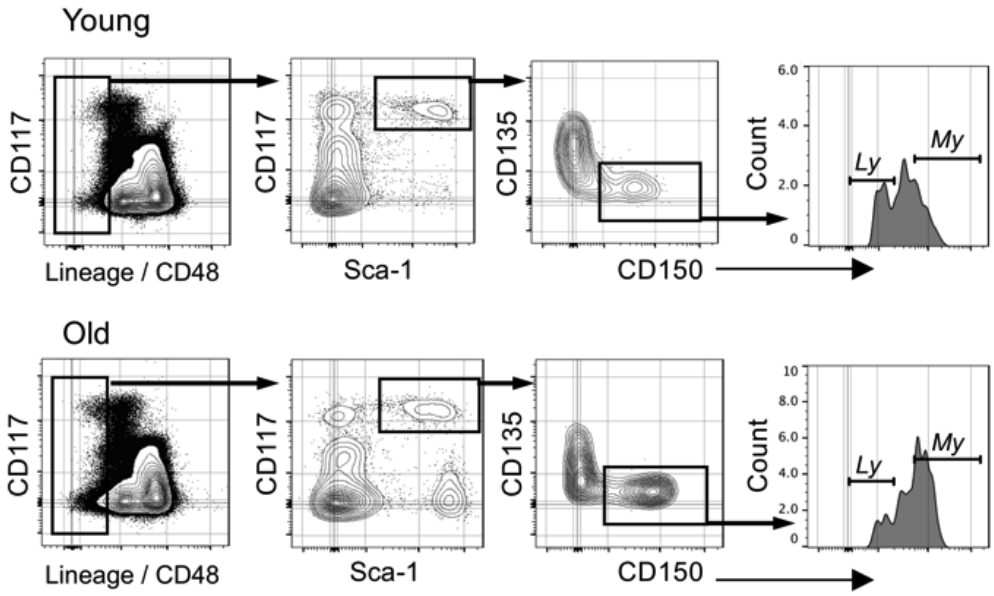


Figure 2-7. Model showing the effects of aging on Ly-HSCs. In young individuals both Ly-HSCs and My-HSCs generate lymphoid progeny, although Ly-HSCs do so more efficiently as indicated by the thicker arrow. The myeloid cells (M) produced by young Ly-HSCs are mostly CD11b^{high} Gr-1^{+}. In contrast to current models of HSC aging, while there is an expansion of My-HSCs in old individuals the data herein indicate that the number of Ly-HSCs does not decline with age. The figure further indicates that the lymphoid developmental potential of old Ly-HSCs and My-HSCs is blocked due to residence in the old, inflammatory environment. Finally, old Ly-HSCs acquire a myeloid biased pattern of gene expression (as indicated by the darker nuclear shading) that is more similar to that of My-HSCs. This in turn correlates with a change in the types of myeloid cells produced; specifically, myeloid cells produced form old Ly-HSCs have a CD11b^{low} Gr-1^{+} phenotype that is more similar to the myeloid cells generated from My-HSCs.

Supplemental Information

A



B

	Antibody	*Clone*	*Source*
Fcγ Blocking	CD16/32	FcγRII-III; 93	Life Technologies
Lineage cocktail			
	CD3ε	145-2C11	Life Technologies
	CD8α	53-6.1	Life Technologies
	Gr-1(Ly-6G)	RB6-8C5	Life Technologies
	IgM	—	Southern Biotechnology
	NK1.1	PK136	Life Technologies
	TCRβ	H57-597	Life Technologies
	TCRγδ	UC7-13D5	Life Technologies
	TER-119	TER-119	Life Technologies
	CD48	HM48-1	Life Technologies
HSC			
	CD117	2B8	Life Technologies
	CD150	TC15-12F12.2	Biolegend
	Sca-1	D7	Life Technologies
	CD135	A2F10	Biolegend
Ki-67 staining			
	Ki-67-PE/Cy7	SolA15	Life Technologies
	PE/Cy7 Rat IgG2a, κ (isotype control)	eBR2a	Life Technologies
Others			
	CD19	1D3	Life Technologies
	CD11b	M1/70	Life Technologies
	CD127	eBio5B1/199	Life Technologies
	CD45	30-F11	Life Technologies
	Ly-6C	AL-21	BD Biosciences

Optimal working dilutions of all antibodies were determined before use. *HSC*, Hematopoietic stem cell

Figure 2-8. Reagents and strategy used to resolve bone marrow HSC subsets. (A) Detailed strategy for resolution of Ly-HSCs and My-HSCs from the bone

marrow of 8-12 weeks old young and 18 months old B6 mice. (B) List of antibody clones used in this study and their sources.

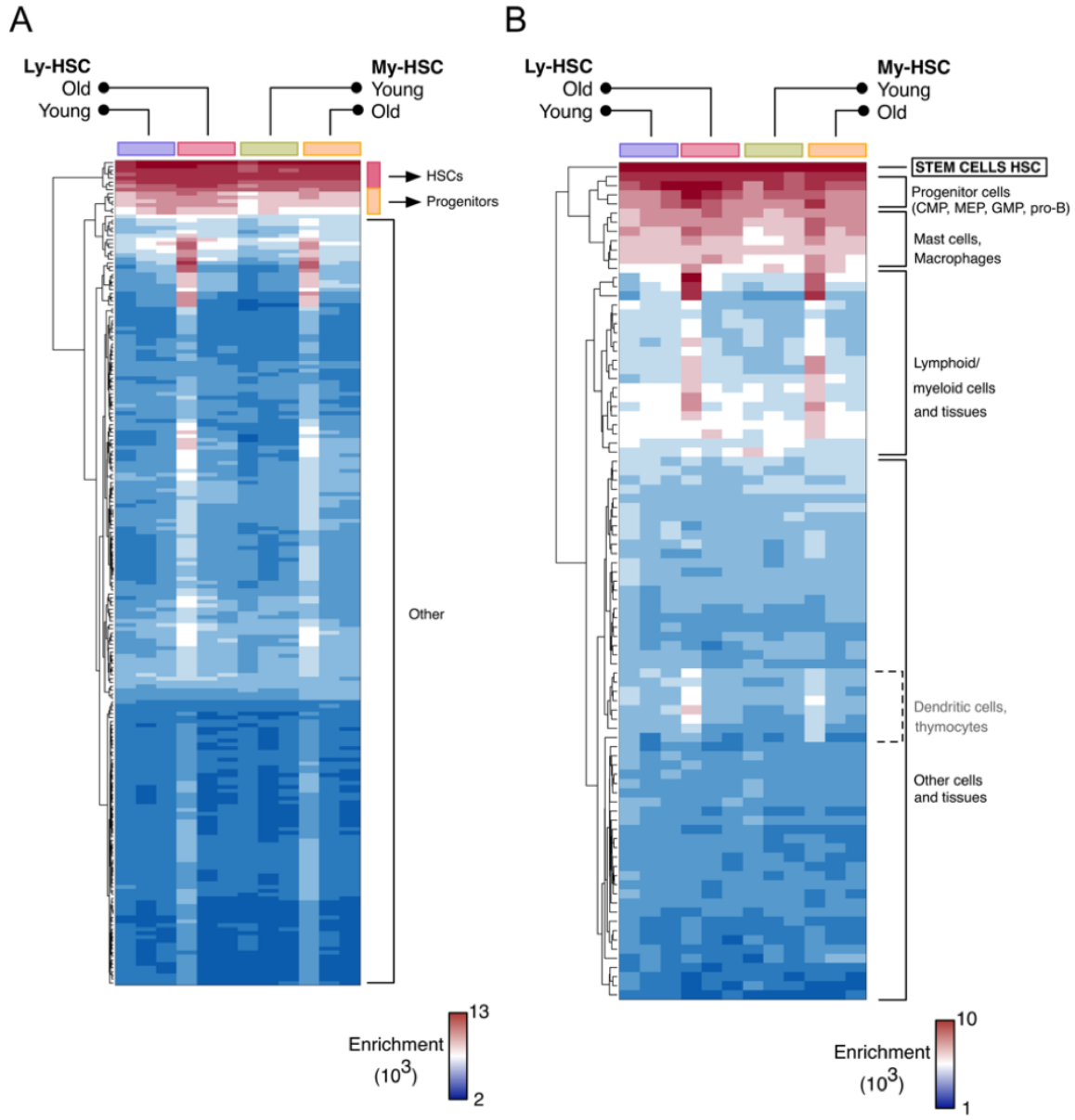


Figure 2-9. SaVanT enrichment scores for the RNA-Seq samples in this study using all the expression signatures from the (A) Immunological Genome Project (ImmGen) and (B) Mouse MOE430 Gene Atlas (BioGPS). Highlighted ImmGen signatures in panel (A) are: HSCs: HSC signatures (LTSL BM, STSL BM, ST34F BM, LT34F BM, LTSL FL, MPP34F BM, CMP BM and STSL FL); Progenitors: MLP, ETP, pre-T; Other: all other expression signatures.

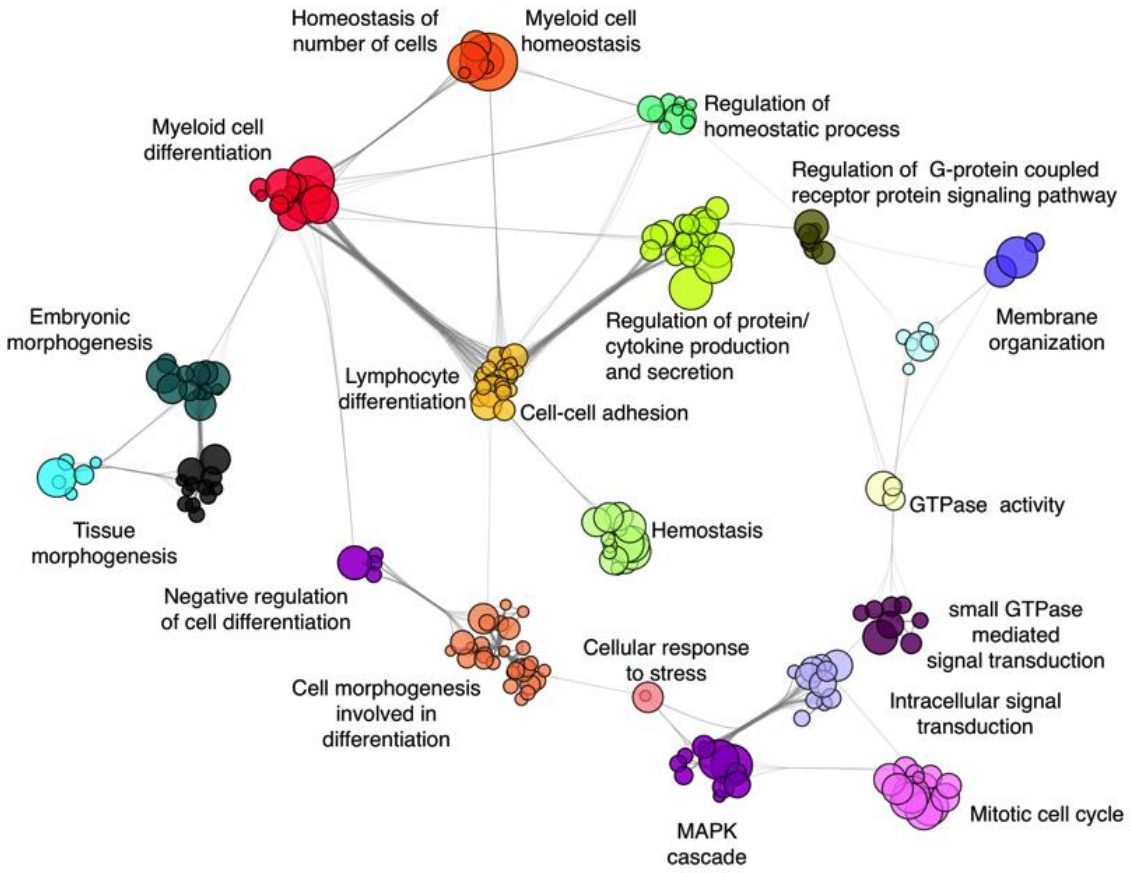


Figure 2-10. Functional enrichment network for all genes differentially expressed in Ly-HSCs and My-HSCs. Individual gene ontology terms with similar gene members are grouped by categories (node color), and labeled using a representative member. Node size is proportional to statistical significance. Edge thickness is proportional to between-node similarity and reflects the overlap between the gene sets annotated in both ontology terms. Only edges representing a Kappa similarity score greater than 0.2 are shown. Only significant ontology terms are shown (hypergeometric p-value $p<0.01$).

Table 2-11. Ly-HSC and My-HSC gene expression estimates and differential expression results.

Table 2-12. Ly-HSC and My-HSC gene signature analysis and functional enrichment results.

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Chapter 3:

The Effects of Inflammation on Hematopoietic Stem Cells Subpopulations

Introduction

The effects of aging on stem and progenitor cells can be manifest at several levels. For example, intrinsic changes may occur within these populations as a result of programmed events. However, stem and progenitor cells are also subject to local, tissue, and systemic influences. As a result, many of the intrinsic changes in these populations may be induced via their interactions with environmental components throughout life. One prominent feature of aging in this regard is chronic inflammation. The term, “inflammaging”, describes this low-grade, chronic condition that correlates with increasing rates of morbidity and mortality in the elderly (1, 2).

Multiple factors contribute to inflammaging including debris from damaged cells and free radicals from oxidative stress (3). Various microbial constituents may also do so. In particular, the ability of the gut to sequester microbes and/or their products declines with age, and the leakage of these elements into the circulation can lead to chronic low-grade inflammation in tissues (4). Even without such leakage, the composition of the intestinal microbiota might change leading to the production of inflammatory cytokines in the gastrointestinal tract. Finally, many cells that become senescent acquire a senescence-associated secretory phenotype (SASP) (5). Such populations then secrete one or more inflammatory factors. Some cells with a SASP may express *p16*^{Ink4}, and their removal has been shown to reverse or delay many aging effects (6). Thus, there is evidence

that targeting inflammatory factors may provide a way to mitigate some effects of aging.

Both animal and human epidemiological studies have identified many of the inflammatory factors produced during aging, and these include interleukin-6 (IL-6) (7), tumor necrosis factor-α (TNFα), interleukin-1 α (IL-1α), and interferon γ (IFNγ) (8). It has been proposed based on a cardiovascular health study that levels of IL-6 and soluble TNFα receptor 1 are predictors of 10-year mortality rates (9).

Hematopoiesis was traditionally thought to be a programmed process not affected by local or systemic events such as infection. However, it is now recognized that this view is not correct and that hematopoietic stem cells (HSCs) and progenitors express receptors for various cytokines and bacterial products. For example, HSCs express Toll-Like Receptor 4, which allows them to respond to bacterial lipopolysaccharide. When these agents bind to stem and progenitor cells, this results in proliferation and skewing to myeloid differentiation (10-12). Inflammatory cytokines, such as IL-6 and IFNs, appear to stimulate HSCs to proliferate in the short term but little is known about the long-term effects of sustained inflammation (13, 14). For example, sustained exposure of HSCs to IFNγ impairs their proliferation and maintenance (15), and chronic TNFα signaling has been associated with myelodysplastic syndrome and bone marrow failure (16).

While the effects of inflammatory cytokines on hematopoiesis are increasingly well defined, how these factors affect the developmental potential of specific HSC subsets has not been fully explored. Using a long-term culture system in which the lineage bias of Ly-HSCs and My-HSCs is maintained in vitro, we confirm and extend the conclusions in Chapter 2 showing that inflammatory factors can inhibit lymphocyte development from HSCs. We show that chronic exposure of HSCs to inflammation blocks lymphocyte development and we identify bone marrow adipocytes, which increase in number with age (17), as a source of inflammatory cytokines.

Results:

Old Ly-HSCs have diminished lymphoid potential in vivo

It has been reported that Ly-HSCs from old mice exhibit a diminished capacity to produce lymphoid progeny based on their potential to reconstitute donor cells in the peripheral blood of lethally irradiated recipients (20). However, this study did not examine the status of B cell development in the bone marrow of recipients.

We repeated these experiments to address this issue. We harvested bone marrow Ly-HSCs from 8-week old and 18-month old B6 mice and transplanted 300-400 cells into sublethally irradiated (550R) young recipients (Figure 3-1). We (21, 22) and others have shown that the effects of aging on lymphocyte

development are clearly evident in 18-month old mice (20, 23, 24). The donor cells were from mice that expressed the CD45.2 allele and the recipients expressed the CD45.1 allele, which allowed us to identify donor cells in the recipients (Figure 3-1).

The status of B cell development in the bone marrow of recipients was determined six weeks later by measuring the frequency and number of donor (CD45.2) cells that expressed the CD45R (B220) cell surface determinant that is a characteristic of B lineage cells (25). The results showed that the total number of donor B lineage cells was significantly lower in recipients of old compared to young Ly-HSCs (Figure 3-2).

Ly-HSCs retain normal developmental potential in vitro

The in vivo reconstitution data are not in accord with the conclusions in Chapter 2 that Ly-HSCs exhibit normal developmental potential when removed from the in vivo environment and cultured in vitro. We therefore developed a second in vitro system in which stem cells are seeded on OP9 stroma to confirm that the developmental potential of old Ly-HSCs is normal when they are removed from the old milieu. The protocol for this system, which is described in the Methods section, is shown in Figure 3-3A.

Cobblestone areas of hematopoiesis emerge around 5 to 6 days after seeding Ly-HSCs while these develop from My-HSCs at around day 9 to 10 following initiation of the cultures. At weekly intervals the frequency and number of $CD19^+$ B lineage and $CD11b^+$ myeloid cells were determined in cultures initiated with Ly-HSCs and My-HSCs. Although B lineage cells develop from Ly-HSCs a week earlier than from My-HSCs, it is clear that the developmental bias of these stem cell subsets is maintained in culture. While Ly-HSCs produce some myeloid cells, most cell production is lymphoid biased (Figure 3-3B). In contrast, My-HSCs produce few lymphoid cells while myelopoiesis predominates (Figure 3-3C).

Old Ly-HSCs efficiently generate lymphocytes

We used this culture system to compare the lymphoid developmental potential of young and old Ly-HSCs by seeding 150 Ly-HSCs per well from young (8-12 weeks) or old (18 months) mice on OP9-GFP stromal cells. The production of CD19 and CD11b expressing cells was compared at weekly intervals. The results show that when Ly-HSCs are removed from old mice and cultured in vitro, they can generate lymphoid progeny as efficiently as young Ly-HSCs (Figure 3-4). These data confirm the conclusions in Chapter 2 that upon removal from the in vivo environment, old Ly-HSCs can produce lymphoid progeny as well as their young counterparts.

The expression of *p16^{Ink4a}*, which is both a biomarker and effector of aging, increases in B lineage cells with age (21). *P16^{Ink4a}* is not expressed in young cells due to high expression of *Bmi1* (26). We examined *Bmi1* expression in the B lineage cells generated in the cultures from young or old Ly-HSCs by quantitative RT-PCR. CD19^{+} cells were harvested from the cultures between days 36-41 days after initiation. As shown in Figure 3-5, no significant difference in *Bmi1* expression was detected between the B lineage cells generated from young and old Ly-HSCs.

Exposure to pro-inflammatory cytokines inhibits lymphocyte development from Ly-HSCs

The production of inflammatory cytokines increases with age and these factors are thought to affect patterns of hematopoiesis (27). The data in Chapter 2 demonstrate that these cytokines can inhibit lymphocyte development from Ly-HSCs. We used the in vitro system described above to provide additional insights into the effects of inflammation on Ly-HSCs.

First, young Ly-HSCs were pre-incubated with a pro-inflammatory cytokine cocktail that contained IL-1α, IFNγ and TNFα for 15 hours before seeding on OP9-GFP stromal cells. The pre-incubation with inflammatory cytokines did not affect the kinetics with which cobblestone areas emerged in the cultures (data

not shown) or the production of B lineage cells. In addition, no significant effects on myeloid cell production were observed (Figure 3-6).

These data indicated that short-term exposure to inflammatory cytokines had no major effect on hematopoietic development from Ly-HSCs. We next tested the effect of extended exposure of hematopoietic cells to inflammatory cytokines. Ly-HSCs were seeded on OP9 stromal cells in medium supplemented with or without inflammatory cytokines; these factors were left in the cultures up until the time cobblestone areas emerged. The inflammatory factors were then removed and the cultures were maintained in medium without them.

The presence of inflammatory cytokines did not affect the time at which cobblestone areas emerged in cultures seeded with Ly-HSCs (day 6 after seeding). However, as shown in Figure 3-7, B lineage cells did not emerge in the cultures. Nevertheless, myeloid cells were produced in the inflammatory cytokine supplemented cultures.

HSCs are not maintained in vitro, and long-term cell production in the above cultures almost certainly reflects the activity of progenitor cells. To understand the effects of pro-inflammatory effects on cells at these stages of development, we added pro-inflammatory cytokines to cultures after 3 weeks after their initiation with Ly-HSCs (Figure 3-8). The results clearly showed that the inflammatory mediators induced a suppression of lymphopoiesis. We then

removed the inflammatory cytokines to determine if B cell production would re-initiate and found that this was the case. This result is in agreement with previous study (28), which reported that IL-1 inhibition of B lymphopoiesis by IL-1 is reversible.

Adipocytes inhibit lymphocyte development from Ly-HSCs

Adipocytes have been shown to be a source of various cytokines, including inflammatory factors, and their number increases in the bone marrow with age (29). In view of this, we tested the hypothesis that fat cells would affect lymphocyte production from Ly-HSCs. We induced fat cell accumulation in our cultures in order to do so.

The development of bone marrow fat from mesenchymal stem cells is dependent on expression of the peroxisome proliferator-activated receptor gamma (PPAR-γ) transcription factor (30) and it can be induced by Rosiglitazone (RGZ) (31). We treated OP9 stromal cells, which share properties with mesenchymal stem cells (32), with 10 μM RGZ for 1 week (33). This protocol resulted in induction of PPAR-γ expression (Figure 3-9A) and adipocyte formation (Figure 3-9B) in the cultures.

RGZ was washed from the cultures, which were then seeded with young Ly-HSCs. Adipocyte numbers remain constant in the cultures for at least a week

following drug removal (data not shown). The production of $CD19^+$ cells was lower in the RGZ treated compared to control cultures (Figure 3-9C). In order to correlate these results with the production of inflammatory cytokines, we quantified *IL-6* expression in control cultures and those treated with RGZ. As shown in Figure 3-10, there was a significant increase in *IL-6* expression by the RGZ treated stromal cells.

Discussion:

A key conclusion from this study is that HSCs exhibit relatively normal lymphoid developmental potential when they are removed from the old in vivo environment. These observations are consistent with the conclusions in Chapter 2 in which we measured the potential of young and old Ly-HSCs to generate $CD127^+$ $CD19^-$ early lymphoid progenitors (ELPs) and common lymphoid progenitors (CLPs). The studies in Chapter 2 as well as the current chapter also addressed the question of why lymphocyte development is blocked in vivo but not in vitro. Our working hypothesis in this regard was that HSCs in the old environment are exposed to inflammatory mediators that block their differentiation while in vitro they are removed from this deleterious milieu. Previous studies have shown that inflammatory factors can inhibit lymphocyte development from total young stem cells (13), but an analysis of their effects on young and old Ly-HSCs has not been reported.

In order to address this issue, we developed an in vitro culture system in which stromal cells supported the differentiation of Ly-HSCs or My-HSCs into lymphoid or myeloid cells. A distinguishing feature of this culture system is that the lymphoid and myeloid biased pattern of Ly-HSC and My-HSC differentiation observed using in vivo transplantation studies is reproduced in vitro. We chose to monitor the emergence of B lineage cells in these cultures by labeling them with antibodies to CD19, in contrast to CD45R(B220), which is commonly used to identify B lineage cells. One reason for this is that harvest of cells from the cultures at weekly intervals was dependent on trypsinization. This process can remove CD45R(B220) from the cell surface but it does not affect CD19 expression (data not shown). Second, CD45R(B220) can be expressed on many non-B lineage cells (34) while CD19 is a B lineage restricted cell surface determinant (25).

We observed that old Ly-HSCs retain the ability to generate $CD19^+$ B lineage cells in the in vitro cultures. This result is in agreement with data in Chapter 2 showing that conditions in the old in vivo environment were responsible for the inhibition of B cell development. In view of the considerable literature implicating the increased production of inflammatory cytokines during aging on patterns of hematopoiesis (27), we investigated their effects in the in vitro cultures.

We found that short-term exposure of Ly-HSCs to a cocktail of inflammatory factors did not affect their lymphoid potential. However, when cytokines were

maintained in the cultures for the first week after their initiation, B cell development was suppressed. Cobblestones areas of hematopoiesis formed and hematopoietic cells formed in these cultures, but cell production in these cultures was limited to $CD11b^+$ myeloid cells. These results are consistent with previous studies indicating that lymphoid and myeloid development are differentially sensitive to inflammatory factors (35). The addition of inflammatory cytokines to cultures that had established allowed us to test their effects on progenitors, and here too we found that B cell development was suppressed. These results, which extend the observations made in Chapter 2, indicate that chronic exposure of HSCs to inflammatory factors is required to affect their developmental potential. In addition, we found that when the inflammatory cytokines were removed from the cultures, B cell development re-initiated. The latter result is also consistent with data showing that old Ly-HSCs can generate B lineage cells in vitro when removed from the old in vivo milieu.

Our results further suggest that adipocytes may be the source of inflammatory factors that inhibit B lymphopoiesis. Consistent with previous studies that indicate fat has a negative effect on hematopoiesis (29, 36), we found that the production of lymphoid cells was suppressed by fat containing stroma, which produced IL-6. Additional studies are needed to determine if adipocytes are the key stromal cell element responsible for the age-dependent declines in B cell development or other microenvironmental components also play a role.

The results from our in vitro studies showing that Ly-HSCs have normal lymphoid developmental potential conflicts with studies showing that old Ly-HSCs do not reconstitute donor cells as efficiently as their young counterparts when injected into lethally irradiated recipients (20). That study only examined total donor cells in peripheral blood, and we have extended it to demonstrate that old Ly-HSCs do not efficiently reconstitute B cell development in the bone marrow of the recipients. There are several possible reasons for why the in vitro and in vivo differentiation data are not in agreement. HSCs are thought to develop homing defects with age (37), but these are not operative in the in vitro system. Thus, when old and young Ly-HSCs are injected in vivo, the old cells may home to the bone marrow less efficiently. Another consideration is that the irradiation used to condition the recipients may differentially affect the young and old Ly-HSCs. Irradiation induces inflammation (38), and as a result donor cells are being injected into a systemic inflammatory environment. Thus, the old cells are removed from one inflammatory environment (39) and transferred into another one (Figure 3-11). The inflammation in the recipients may also affect the young donor cells but not to the same degree, because they are derived from a young, non-inflammatory environment. These hypotheses are consistent with the well-known effects of inflammation on suppression of lymphoid production and effects on HSCs (15, 35).

In summary, the results from this study provide a better understanding of why lymphocyte production declines with age. The fact that B cell development can

initiate when HSCs are removed from the inflammatory environment is also a key finding, because it raises the possibility that interventions that reduce inflammation may result in the rejuvenation of B lymphopoiesis.

Methods:

Mice

8-12 weeks old C57BL/6J mice were purchased form the Jackson laboratory or the UCLA Division of Laboratory Animal Medicine; 18 months old C57BL/6J mice were obtained from the National Institute on Aging colony. For Ly-HSC transplantation, recipients were 6-week old female CD45.2 RAG-2/SJL mice (strain TAC 000461-M) purchased from Taconic Laboratories. All animals were housed in the vivarium of the Division of Laboratory Animal Medicine, University of California at Los Angeles. Animal care and use were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

Flow Cytometry

Bone marrow cell suspensions were prepared as previously described (18). HSCs and B lineage cells were resolved with specific combinations of FITC, PE, PerCP Cy5.5, APC, Pacific Blue and Biotin conjugated antibodies as described in chapter 2. All sorting experiments used Aria I Cell sorters (BD Biosciences)

located in the Jonsson 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 at UCLA.

Ly-HSC Transplantation

Ly-HSCs were pooled from either 9-week old (n=6, 3 female and 3 male) or 18-month old (n=6, 3 female and 3 male) CD45.2 B6 bone marrow. Cells were transplanted into 6-week old CD45.1 recombinase activation gene deficient RAG-2/SJL female recipients (n=3, 300-500 Ly-HSCs per recipient) that had been conditioned 24 hours prior to transplantation with 550 R of sublethal irradiation form a ^{137}Cs irradiator. Reconstitution of B lineage cells was examined 6 weeks later.

In vitro cultures

5x10^4 OP9-GFP stromal cells were seeded per well in 12-well plates in α-Minimal Essential Medium supplemented with 5% Fetal Calf Serum, 1 mM L-glutamine, 100 U/ml streptomycin and 100 µg/ml penicillin. One day later various numbers of Ly-HSCs or My-HSCs were seeded into these wells in triplicate in 1.5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1 mM L-glutamine, 100 U/ml streptomycin, 100 µg/ml penicillin, 50 µM 2-ME, 50 µg/ml gentamicin, 0.1mM non-essential amino acid, 0.1mM vitamin and 1mM pyruvate.

Cells were fed every two days thereafter by removing 0.5 ml of medium and adding 0.5 ml medium to the wells.

The contents of the wells were harvested by trypsinization ten days later. One tenth of the cells was used for phenotypic analysis and the remaining cells, depleted of any residual OP9 stroma, were seeded on newly established OP9-GFP stromal cells as just described. OP9 stroma was depleted by seeding cells in a 10 cm-diameter tissue culture dish for 45 minutes at 37℃, 5% CO_2 air humidified incubator with gentle swirling of the plates every 15 minutes at 3 times totally. This process was repeated at weekly intervals for up to six weeks post-initiation of the cultures.

Addition of inflammatory cytokines to cultures

In some experiments Ly-HSCs or My-HSCs in 100 µL were incubated in wells of 96-well U bottom culture plates in SCF (20 ng/mL), IL-3 (20 ng/mL), TPO (10 ng/mL) and Flt-3L (10 ng/mL) supplemented culture medium (19) at 37°C in a 5% CO_2 air humidified incubator. Some wells were maintained under these conditions while others were additionally supplemented with a pro-inflammatory cytokine cocktail that contained IL-1α (15 U/ml), IFNγ (15 U/ml) and TNF α (10 ng/ml). Following a 15-hour incubation, the cells were washed 5 times in PBS and seeded on OP9-GFP confluent stromal cells prepared as described above.

In other experiments, 150 Ly-HSCs or My-HSCs were seeded on OP9 stromal cells in medium supplemented with or without IL-1α (15 U/ml), IFNγ (15 U/ml) and TNFα (10 ng/ml). Once cobble stone areas had formed, the inflammatory cytokine supplemented medium was removed from the cultures and replaced with fresh medium.

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was extracted with the RNeasy Plus microkit from flash-frozen aliquots of purified cells and used to synthesize cDNA with the RT First Strand kit (both from Qiagen) by the manufacturer's instructions. Reactions were run in 20 µl volumes with Taqman PCR master mix (Bio-Rad) as recommended by the manufacturer. Amplification efficiencies were routinely found to be between 95 and 105%, and all reactions were run in duplicate. All primers were purchased from Thermo Fisher Scientific and included *mBmi1* (Mm03053308_g1) and *mAcb* (Mm00607939_s1). The presence of PCR products was confirmed by melt curve analysis. Data were analyzed with Bio-Rad IQ5 software using the Pfaffl method. All reactions were run 2 times using 3 independent biological samples.

Statistical Analysis

Data are expressed as a mean ± SEM as indicated in the figure legends.

Differences between groups were tested by a two-tailed, unpaired Student's t test ($\alpha = 0.05$).

Figures:

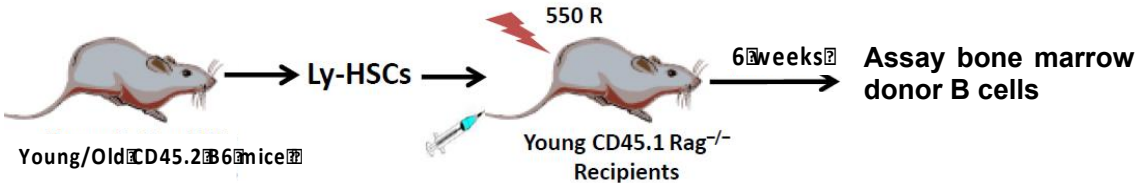

Figure 3-1. In vivo transplantation protocol. Ly-HSCs purified from CD45.2 mice were injected intravenously into young (10-12 weeks old) CD45.1 recombinase activation gene deficient RAG-2/SJL recipients. This strain is immunodeficient due to its inability to recombine immunoglobulin and T cell receptor genes. The mice are pre-conditioned with sublethal irradiation (550R) to facilitate lymphoid reconstitution and do not require transplantation of carrier bone marrow for survival. All recipients received an injection with the same number of young and old donor CD45.2 cells. Six weeks after transplantation, the frequency and number of donor CD45.2 B lineage cells in the recipients' bone marrow were enumerated.

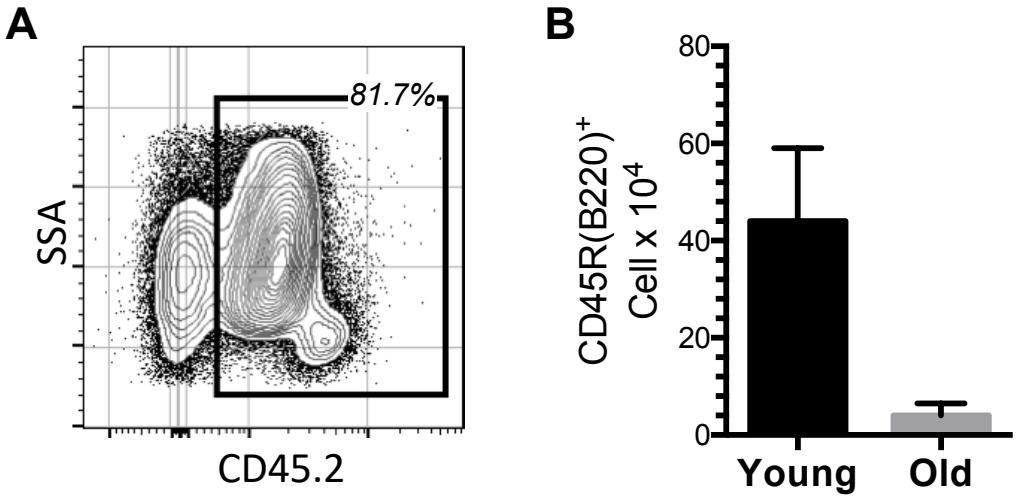

Figure 3-2. Old Ly-HSCs have diminished lymphoid potential. Two out of 6 Rag recipients of old Ly-HSCs had significant levels of CD45.2 donor cells in their lymphoid tissues compared to 5 out of 6 Rag mice injected with young Ly-HSCs. (A) Representative FACS plot showing frequency of donor CD45.2$^+$ cells in recipient bone marrow. (B) Number of donor B lineage CD45R(B220)$^+$ cells in the bone marrow of recipients.

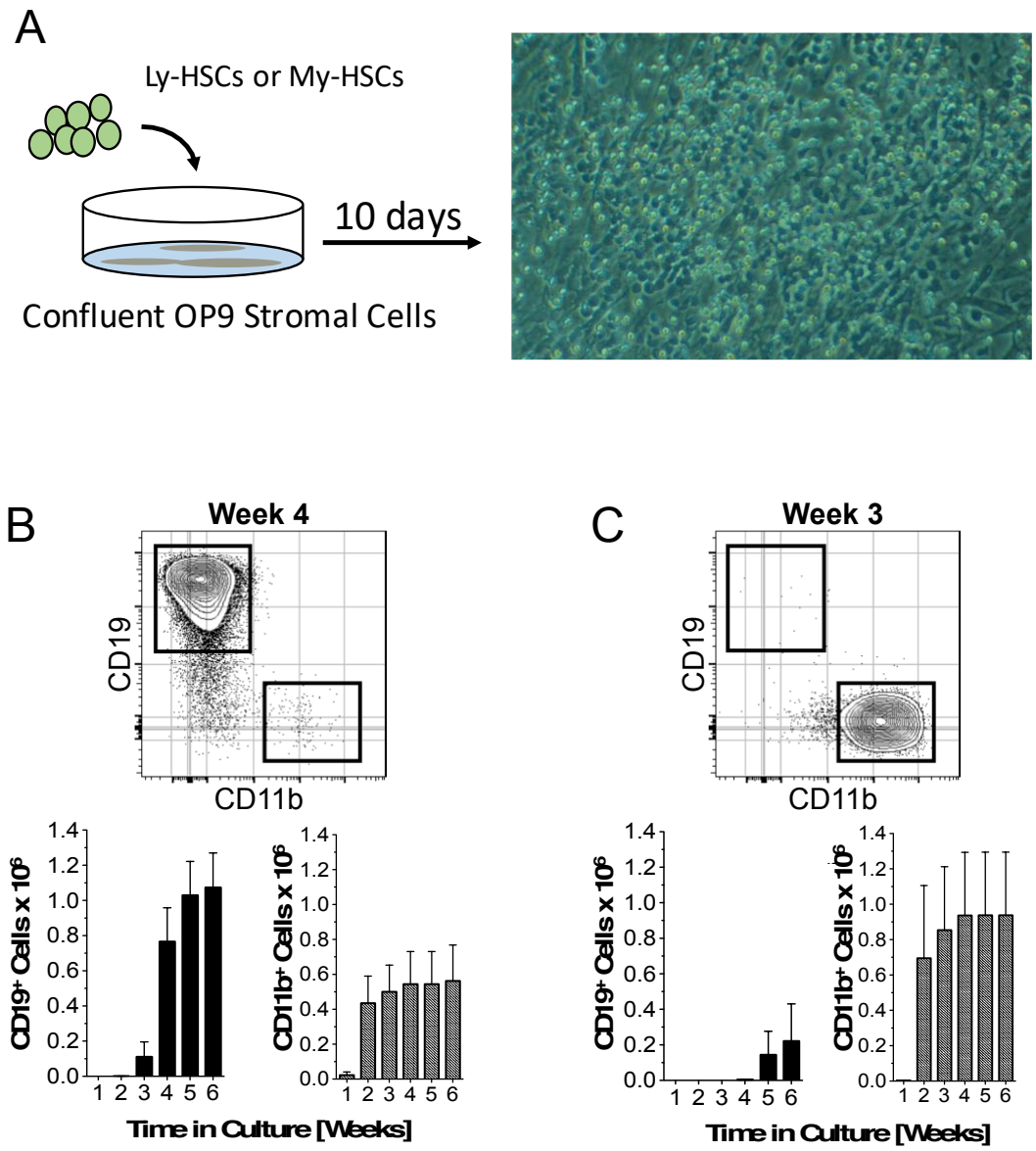

Figure 3-3. Ly-HSCs and My-HSCs retain their developmental bias in vitro. (A) Protocol for initiation of cultures (left panel) and appearance of cultures 10 days after seeding 150 HSCs on OP9 stroma. The round refractile cells are on top of the stroma while the round dull cells are beneath the stroma. OP9 stromal cells were seeded with (B) 150 Ly-HSCs or (C) 150 My-HSCs. Cells were harvested from cultures weekly, counted, and analyzed for CD19 and CD11b expression. The FACS plots show resolution of $CD19^+$ and $CD11b^+$ cells in cultures seeded with Ly-HSCs (week 4 after seeding) and My-HSCs (week 3 after seeding).

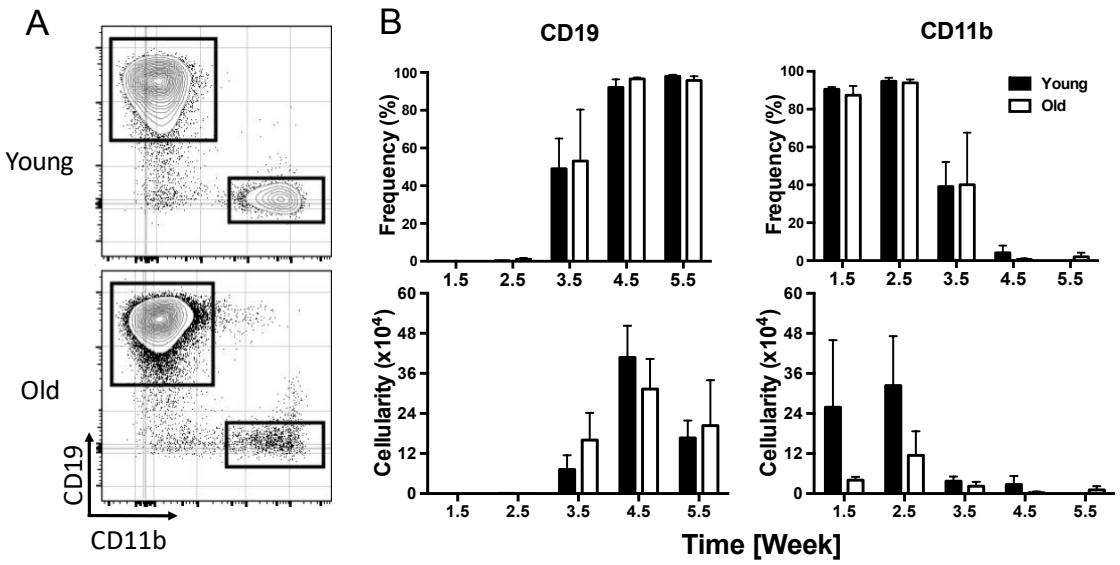

Figure 3-4. The comparison of lymphoid and myeloid cell production from young and old Ly-HSCs in vitro. (A) FACS plots show resolution of $CD19^+$ and $CD11b^+$ cells in cultures seeded with young and old Ly-HSCs at week 3.5 after culture initiation. (B) Frequency and number of $CD19^+$ and $CD11b^+$ cells in cultures established with young and old Ly-HSCs. The data are based on cell production in 3 independent sets of cultures.

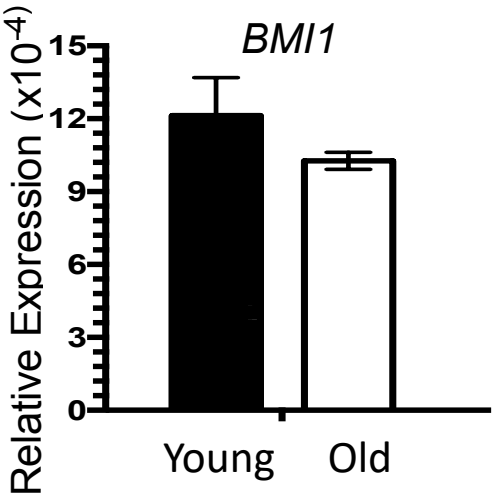

Figure 3-5. The relative level of *Bmi1* expression is similar in CD19^{+} cells generated from young and Ly-HSCs old HSCs. Relative expression of Bmi1 in CD19^{+} cells generated from young and old Ly-HSCs at 36-41 days after initiation. The data are based on 3 biologic replicates.

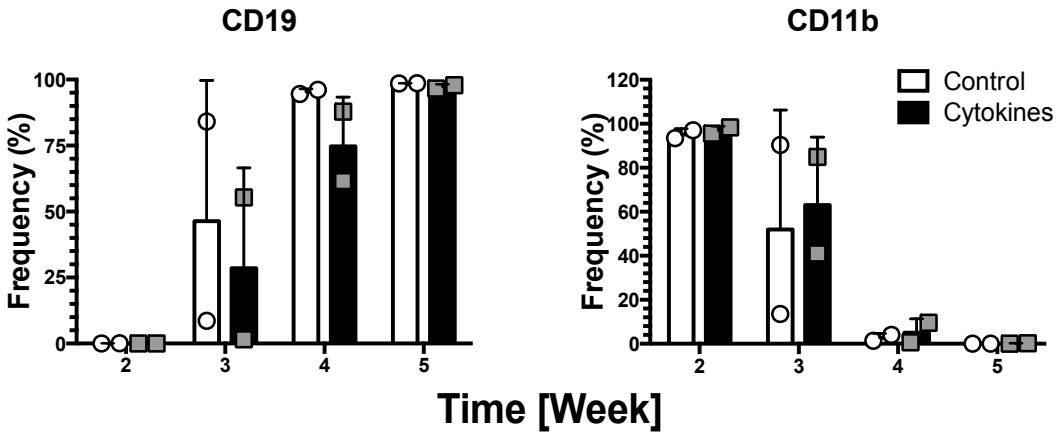

Figure 3-6. Lymphoid and myeloid cell production in cultures initiated with young Ly-HSCs pre-treated with inflammatory cytokines (IL-1α, IFNγ and TNFα) for 15 hours before seeding on OP9 stromal cells. Each time point is based on two biological replicates

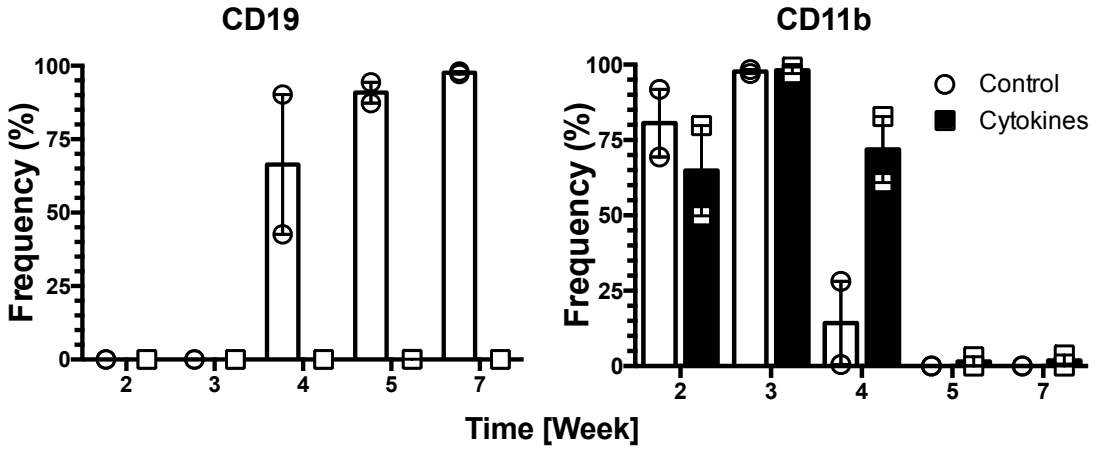

Figure 3-7. Effect of inflammatory cytokines (IL-1α, IFNγ and TNFα) on the production of lymphoid and myeloid cells from young Ly-HSCs. The cytokines were present in the cultures up until the time that cobble stone areas emerged. Cytokines were then washed from the cultures which were then maintained for several weeks thereafter. Each time point is based on two independent biologic experiments.

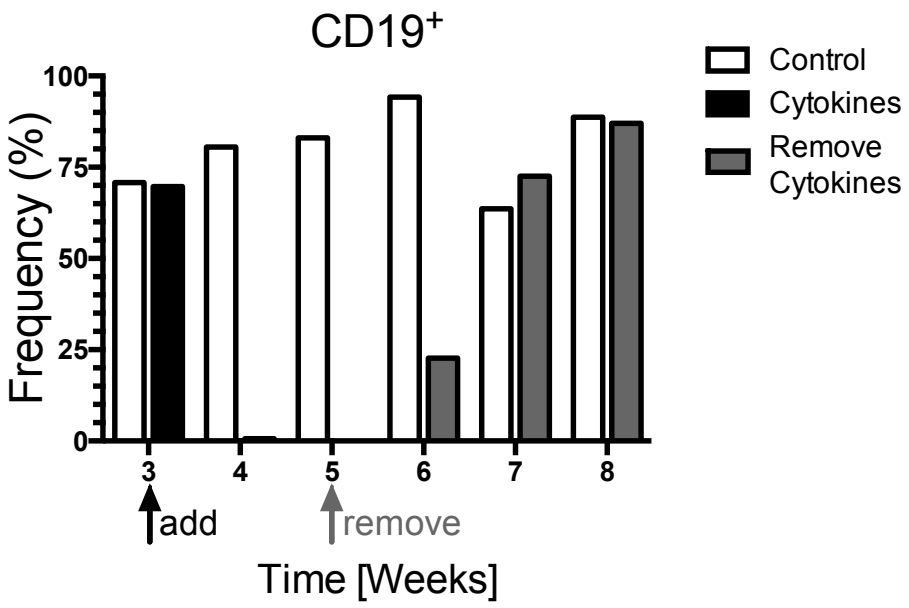

Figure 3-8. Effects of inflammatory cytokines on the production of lymphoid cells from progenitors in cultures initiated with Ly-HSCs. Number of $CD19^+$ expressing cells in cultures at weekly intervals in the presence of cytokines (IL-1α, IFNγ and TNFα) and following their removal. The times at which cytokines were added and removed from the cultures is indicated by the arrows.

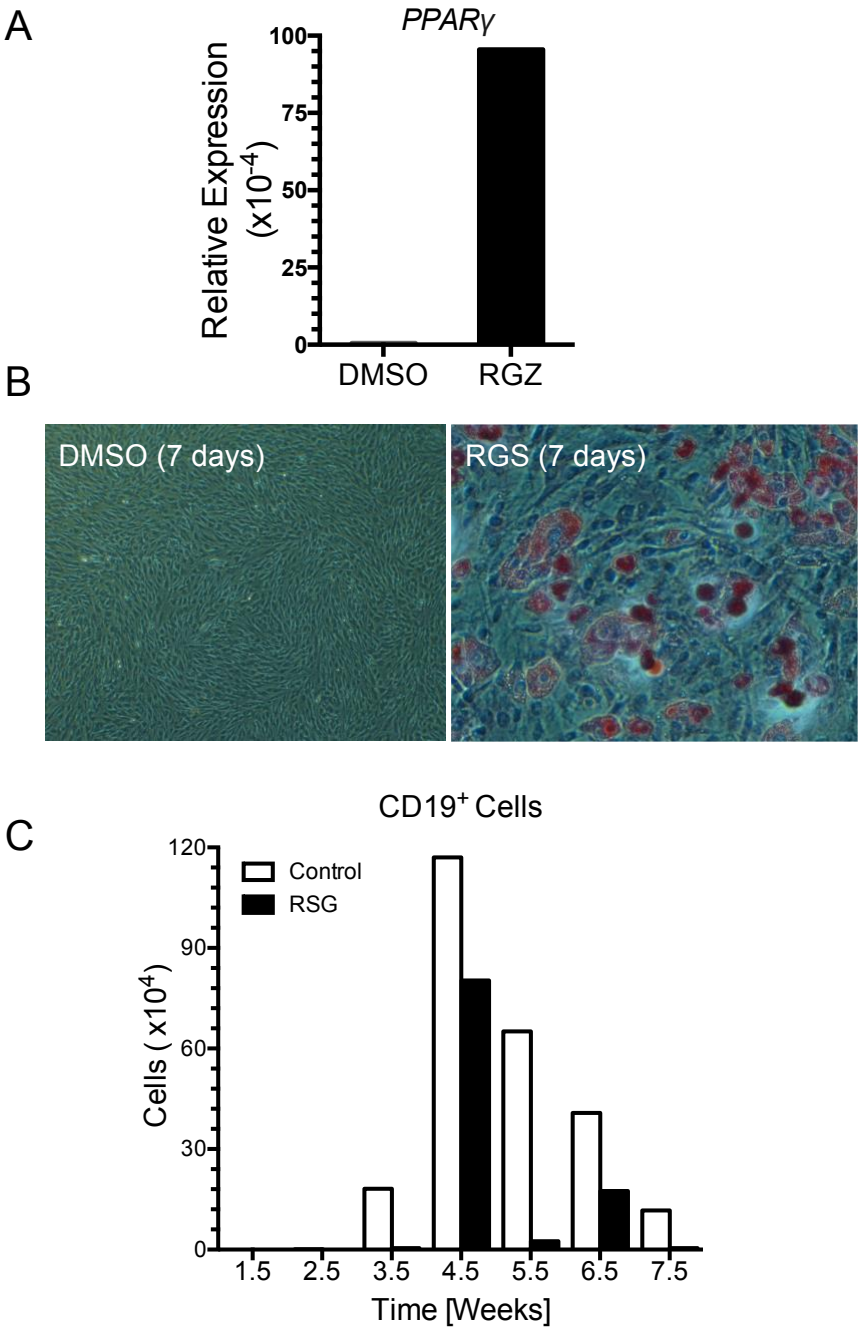

Figure 3-9. Adipocytes inhibit B lymphopoiesis in vitro. (A) Induction of *PPARgamma* in stromal cell cultures treated with Rosiglitazone (RGZ). *PPARgamma* expression measured by qRT-PCR. (B) Appearance of cultures after treatment with RGZ for one week. Fat cells were identified based on Oil Red O straining. (C). Production of $CD19^+$ lymphoid cells in control and adipocyte containing cultures seeded with Ly-HSCs.

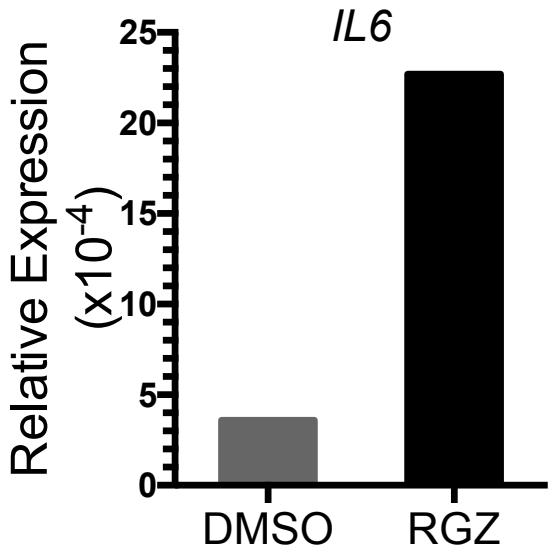

Figure 3-10. Adipocytes produce IL-6. Induction of *il6* in stromal cell cultures treated with Rosiglitazone (RGZ). Levels of *il6* measured by qRT-PCR. Cultures were treated with RGZ for 7 days. Drug was then removed, and cultures were maintained for an additional 7 days at which time the stromal cell layer was harvested for analysis of gene expression.

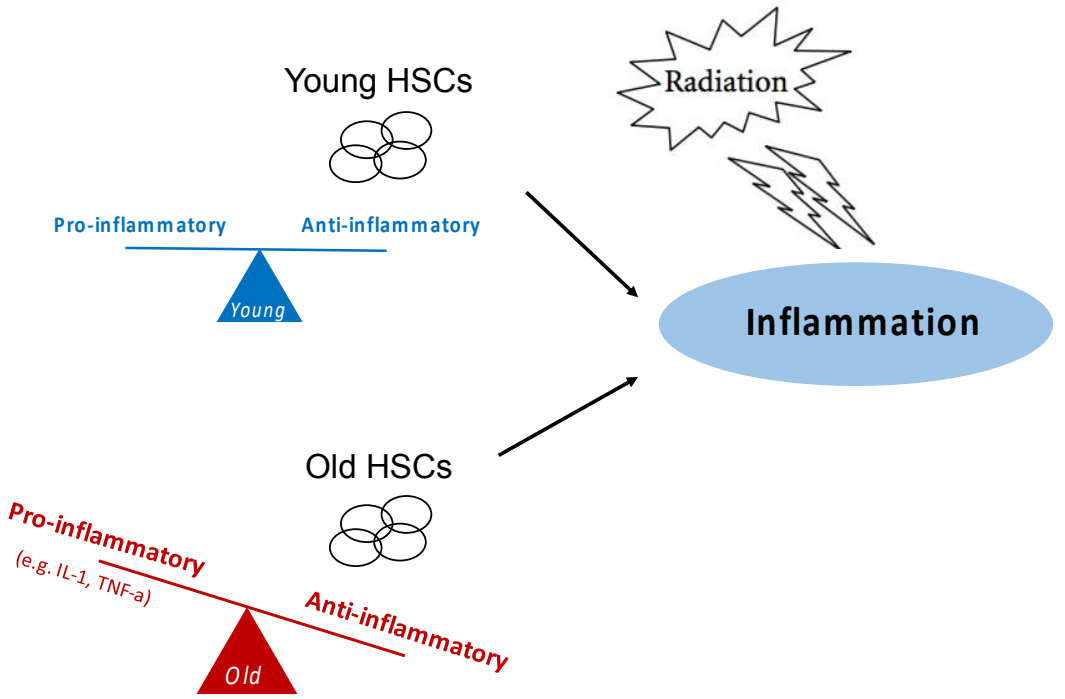

Figure 3-11. Irradiation induces inflammation. Old Ly-HSCs were transferred from the inflammatory environment into another inflammatory environment induced by irradiation. In contrast, young Ly-HSCs had not been exposed previously to inflammation in the young environment.

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Chapter 4:

Conclusions

One of most prominent changes that occurs during aging is the decline in immune function. As reviewed in Chapter 1, the number of naïve B and T cells in secondary lymphoid tissues is reduced and functional changes in mature lymphocytes result in attenuated responses to new antigens. One way to address these issues would be to replace old lymphoid cells with newly produced naïve B and T cells. However, as we have emphasized throughout this thesis, lymphocyte production in the bone marrow and thymus is depressed in old individuals. The aim of our work has been to understand why this occurs as a basis for developing strategies for rejuvenation of lymphocyte production in elderly individuals. Our focus was on hematopoietic stem cells (HSCs), as age-related defects are thought to underlie their reduced ability to produce lymphoid progeny. The results we have obtained have provided the following new insights:

I. The number of lymphoid biased HSCs does not decline with age

It is now accepted that the HSC compartment is heterogeneous and includes lymphoid biased (Ly-HSCs) and myeloid biased (My-HSCs) stem cells (1). Current models of HSC aging propose that lymphocyte development declines because the number of Ly-HSCs is reduced. However, this conclusion is based on frequency data alone and does not incorporate changes in cell number. We show in chapter 2 that Ly-HSCs do not decline in number in the elderly and instead significantly increase. This finding necessitates a revision of current models of HSC aging.

II. Ly-HSCs acquire changes in gene expression that correlate with defects in stem cell processes

The data in Chapter 2 further demonstrate that Ly-HSCs exhibit changes in gene expression that are associated with alterations in various stem cell processes such as cell cycle progression, apoptosis, and DNA damage repair. These changes are in accord with various studies that have shown that HSCs acquire various functional deficits as they age (2). The transcriptomes of young and old total HSCs were compared, and the data we present in Chapter 2 are the first to show the genetic changes that occur in Ly-HSCs and My-HSCs with age. The genetic databases we have generated will be of value to future researchers as they investigate how aging affects HSCs subsets.

III. Old Ly-HSCs retain normal developmental potential

We unexpectedly found that the genetic changes that occur in old Ly-HSCs do not block their lymphoid potential, as they efficiently produced lymphoid progenitors in vitro. This result raised the question of why lymphocyte development declines with age.

IV. Exposure of Ly-HSCs to inflammation inhibits lymphocyte development

It is recognized that the production of inflammatory factors increases with age, so we examined their effect on lymphocyte development from Ly-HSCs and report in Chapter 2 that they almost completely inhibited that process. Thus, our view is that cell extrinsic signals are largely responsible for the age-related decline in lymphocyte production. Chapter 3 extended these conclusions by examining the effects of specific inflammatory cytokines on HSCs and by demonstrating that chronic exposure of stem cells to inflammatory factors is required to block lymphocyte development. In this regard, we incubated Ly-HSCs with IL-1α, TNFα and IFNγ for 15 hours before seeding them in our in vitro differentiation system and found that this did not block lymphocyte development. However, if IL-1α, TNFα and IFNγ were maintained throughout the culture period, lymphocyte development was inhibited. It is known that adipocyte derived factors can inhibit lymphocyte development, and Chapter 3 provides evidence that these cells interfere with lymphocyte development from lymphoid biased stem cells. We also demonstrate that adipocyte derived factors include IL-6. Finally, we showed that upon removal of the cytokines from the cultures, lymphocyte development re-initiated. This is an important observation, because it raises the possibility that interventions that reduce inflammation may result in the rejuvenation of B lymphopoiesis.

V. Ly-HSCs produce myeloid cells in response to inflammation

Although exposure of Ly-HSCs to inflammatory factors blocked lymphocyte development, they produced myeloid cells. This result correlates with a myeloid biased pattern of gene expression in old Ly-HSCs.

VI. In vivo systems may not accurately measure stem cell developmental potential

We transplanted young and old Ly-HSCs into young recipient mice and found that old Ly-HSCs do not reconstitute B lineage cells as well as their young compartments. This result is in agreement with a report from Rossi et al. who found that the reconstitution efficiency of old Ly-HSCs is compromised (3). However, the results in Chapters 2 and 3 show that old Ly-HSCs have normal lymphoid developmental potential when removed from the aging bone marrow and cultured in vitro. Our view is that in vivo reconstitution is not an appropriate model to compare the developmental potential of young and old stem cells because the latter populations acquire homing defects with age. As a result, differences in developmental potential between young and old stem cells in vivo may reflect differences in bone marrow homing potential rather that differences in developmental potential. Furthermore, irradiation used to condition the recipients may differentially affect young and old Ly-HSCs.

VII. **A model of lymphocyte development in young and old mice**

We have proposed a revised aging model of stem cell aging (Figure 4-1), based on our results, which proposes that Ly-HSCs do not decline during aging but their differentiation is blocked due to residence in an inflammatory environment.

Future perspectives:

The work in this thesis also raises a number of interesting questions.

I. What bone marrow cells are affected by inflammatory cytokines?

Inflammatory factors may be acting at the stem cell level as well as on downstream intermediates in the lymphoid developmental pathway. In addition to these direct effects, indirect effects are also possible. For example, inflammatory factors might affect stromal cell function. Further studies are needed to define direct and indirect effects of inflammatory cytokines on lymphocyte development and to identify the precise stage(s) at which they act.

II. Do HSC subsets exist in humans?

A fundamental question is whether HSC subsets exist in humans. If so, they might express different cell surface determinants that allow their isolation. Thus,

it will be important to resolve human HSCs and then determine if they can be further divided based on expression of additional cell surface determinants. If so, these human HSC subpopulations can be isolated and their developmental potential tested using in vivo or in vitro systems.

III. What genetic changes occur in HSCs during aging?

Our studies have compared overall patterns of gene expression in young and old stem cells. However, the expression and/or function of additional regulatory elements such as long non-coding RNAs (lncRNAs) may also be affected by aging. In this regard, lymphoid commitment can be characterized by lncRNA expression patterns, which are highly stage specific and more lineages specific than protein-coding genes (4). It would be of interest to compare lncRNA patterns in young versus old HSCs subsets to determine if there is a change of lineage specificity of each subset during aging. lncRNAs may also play a role in epigenetic regulation of HSC function (5), and this is also in need of further study.

IV. What is the relationship between Ly-HSCs and My-HSCs?

The origin of Ly-HSCs and My-HSCs is not clear. It has been suggested that CD150 high HSCs can generate CD150 low populations (6), raising a precursor progeny relationship between stem cell subsets. This has generally been tested in vivo and further studies are needed to validate or refute this model. Another

possibility is that Ly-HSCs and My-HSCs develop independently from distinct precursors (7).

Figure:

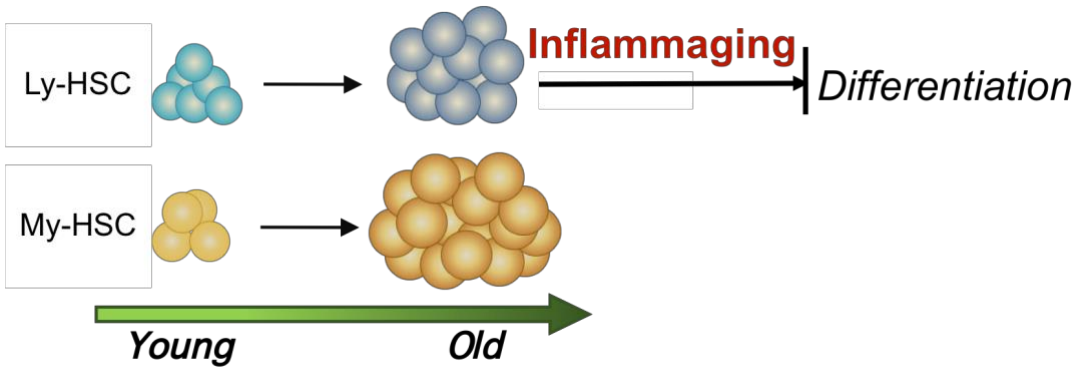

Figure 4-1. Revised model of Ly-HSCs and My-HSC aging. The model proposes that Ly-HSCs are maintained at normal levels with age but their differentiation is blocked as a result of residence in an inflammatory environment.

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