Multiple prethymic defects underlie age-related loss of T progenitor competence

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Running Title: Loss of T potential in Aged BM Progenitors

Word Count (Text): 4,118
Word Count (Abstract): 197

Scientific category: Hematopoiesis

Keywords: hematopoietic stem cells, aging, T cell progenitors, bone marrow transplantation.
Abstract
Aging in mice and humans is characterized by declining T lymphocyte production in the thymus, yet it is unclear whether aging impacts the T lineage potential of hematopoietic progenitors. Although alterations in the lymphoid progenitor content of aged mouse bone marrow (BM) have been described, irradiation-reconstitution experiments have failed to reveal defects in T lineage potential of BM hematopoietic progenitors or purified hematopoietic stem cells (HSC) from aged mice. Here, we assessed T progenitor potential in unmanipulated recipient mice without conditioning irradiation. T progenitor potential was reduced in aged BM compared to young BM, and this reduction was apparent at the earliest stages of intrathymic differentiation. Further, enriched populations of aged HSC or multipotent progenitors (MPP) gave rise to fewer T lineage cells than their young counterparts. Whereas the T precursor frequency within the MPP pool was unchanged, there was a fourfold decline in T precursor frequency within the HSC pool. In addition, among the T-competent HSC clones, there were fewer highly proliferative clones in the aged HSC pool than in the young HSC pool. These results identify T-compromised aged HSC, and define the nature and cellular sites of prethymic, age-related defects in T lineage differentiation potential.
Introduction

Aging in mice and humans is characterized by declining T lymphocyte production by the thymus. This is associated with impaired immune function in the elderly. Further, age-related declines in thymic function is a barrier to effective recovery of the peripheral T cell compartment after lymphocyte depleting events, such as chemotherapy or HIV infection. Despite its clinical importance, cellular mechanisms governing age-related loss of thymic function are not well defined. Alterations in thymic stromal cells do occur, but whether aging impacts BM-derived hematopoietic progenitors is unclear.

Previously, the earliest progenitor within the mouse thymus was thought to be in the double negative 1 (DN1) (CD4^- CD8^- CD44^+ CD25^-) population. Because this population is intact in aged thymi, an intrathymic block in the development of T cell progenitors after the DN1 stage was predicted. However, it is now established that the earliest progenitors in the thymus are a population of CD4^lo Lineage-marker^- CD25^- c-Kit^hi cells termed early T lineage progenitors (ETP) that only partially overlap with the DN1 population. ETP in aged thymi are reduced compared to young thymi, thus raising the possibility that prethymic progenitors upstream of ETP in the BM are defective in producing T cells.

Several studies have shown alterations in lymphoid progenitor content in aged BM, including a lower frequency of multipotent progenitors (MPP). Subsets of MPP are thought to settle within the thymus, so reduced MPP frequency in aged BM predicts that T progenitor potential will also be reduced. However, whether there are functional defects in aged BM progenitors remains unclear. Aged BM administered intravenously (i.v.) reconstitutes the thymus of irradiated mice with slower kinetics than young BM, but eventually produces similar numbers of thymocytes. Studies using fetal thymic organ culture showed that aged BM is less efficient at reconstituting thymic lobes compared to young BM in competitive conditions. It is possible that these data reflect reduced MPP frequency in aged BM, since MPP can rapidly develop into thymocytes. However, because these studies did not assess purified populations, they did not distinguish between defects within individual hematopoietic progenitor cells versus age-related changes in frequencies of hematopoietic progenitor populations in the BM preparations.
Reduced MPP frequency in aged BM suggests that there may be upstream functional defects in hematopoietic stem cells (HSC). Multiple studies using long-term irradiation-reconstitution models to assess aged HSC in competitive conditions suggested that lymphoid differentiation may be impaired, but these results are difficult to collectively interpret. Reconstitution of lethally irradiated mice with mixtures of old and young BM cells did not reveal any competitive defects in the ability of aged cells to reconstitute peripheral T and B cells, suggesting intact T potential of aged HSC.\textsuperscript{19-21} Experiments using small numbers of purified aged HSC to competitively reconstitute irradiated mice have shown defects in B lineage but not T lineage reconstitution.\textsuperscript{13} A similar study reported reduced B and T lymphoid reconstitution relative to myeloid by aged HSC, but this was never compared to young HSC.\textsuperscript{20} Thus, whether aged HSC are intrinsically defective at giving rise to T lymphocytes in vivo remains an open question.

In this study, we establish that aged BM progenitors are less efficient at giving rise to T lineage cells by using experimental conditions that do not involve irradiation. We show that aged unfractionated BM, purified MPP and purified HSC produce fewer T lineage cells than their young counterparts when assessed in vitro and in vivo in young host animals. Limit dilution analysis of purified HSC in vitro revealed that a lower fraction of aged HSC possessed T potential. Additionally, single cell analysis showed that aged T-competent HSC gave rise to fewer T lineage cells. In contrast, aged MPP did not have a lower T precursor frequency even though they produced fewer T lineage cells in bulk assays. These data suggest that T-compromised HSC do not transition to the downstream MPP pool, and that the smaller burst size of T-competent HSC is also reflected in their MPP progeny. This work therefore demonstrates that multiple age-related defects in T potential occur within the HSC population.

**Materials and Methods**

**Mice.** 2 mo and 18-24 mo C57BL/6 female mice were obtained from the National Institute on Aging. No differences in progenitors were observed between 18 mo and 24 mo of age, so data from these groups were combined. C57BL/6 Ly5\textsuperscript{SJL} mice from the National Cancer Institute were used as host mice in all irradiation transplant experiments. To overcome minor histoincompatibilities between NIA mice and congenic C57BL/6
Ly5SJL mice during non-irradiated transplant experiments, the two lines were crossed to yield F1 C57BL/6 Ly5B6xLy5SJL progeny that served as host mice. For irradiation-reconstitution experiments, recipient mice were lethally irradiated (900 rads) and i.v. injected with 1:1 mixtures of whole BM (3x10^5 cells each). Experiments were performed with IACUC approval.

**Cell preparation.** Thymi were harvested, dissociated into single cell suspensions, and total cellularity was calculated after obtaining cell counts with a hemacytometer. An aliquot was set aside, and the remaining cells were depleted of CD4^{hi} and CD8^{hi} cells using anti-CD4 (GK1.5) and anti-CD8 (53-6.7) and anti-Rat IgG conjugated magnetic beads (Qiagen, Hilden, Germany). BM was flushed from the hindlimbs and RBC were lysed using ACK lysing buffer (Cambrex, Walkersville, MD, USA). For non-irradiated transplant experiments, BM was depleted of T lymphocytes using anti-CD4 and anti-CD8 antibodies to prevent graft-versus-host disease, and also depleted of B220^{+} B lymphocyte progenitors for accurate assessment of donor Pro B cell (B220^{+} CD43^{+} CD19^{+} AA4.1^{+}) production after transplant. Peripheral blood was collected retro-orbitally.

**Flow cytometry.** All antibodies were from BD Pharmingen (San Jose, CA, USA) or Ebiosciences (San Diego, CA, USA). For phenotypic analysis of cells, the following antibodies were used: FITC-conjugated CD45.1 (A20); PE-conjugated CD43 (S7), Gr-1 (8C5), CD4 (GK1.5), Thy-1.2 (53-2.1); Biotinylated CD19 (1D3), CD25 (PC61), Flt3 (A2F10); APC-conjugated B220 (RA3-6B2), CD11b (M1/70), CD8 (53-6.7), c-Kit (2B8); PECy5.5-conjugated CD45.2 (104), B220 (RA3-6B2), Sca-1; PECy7-conjugated AA4.1, c-Kit (2B8); and APC-Alexa 750-conjugated CD25 (PC61). Biotinylated antibody was visualized with Streptavidin-APC. To exclude mature cells, a Lineage cocktail of the following FITC- or PE-conjugated antibodies was used: B220 (RA3-6B2), CD19 (1D3), CD11b (M1/70), Gr1 (8C5), CD11c (HL3), NK1.1 (PK136), Ter119, CD8α (53-6.7), CD8β (53-5.8), TCRγδ (GL-3), TCRβ (H57) and CD3ε (2C11). Dead cells were excluded with 4,6-diamidino-2-phenylindole (DAPI). Cells were analyzed on a four laser LSR II (Becton Dickinson, San Jose, CA, USA) and data were analyzed using FlowJo (Tree Star, Ashland, OR, USA). Cells were sorted on a FACSaria (Becton Dickinson) and sort purity was routinely checked.
Real-time quantitative PCR. mRNA from sorted populations was isolated using the RNeasy kit (Qiagen) and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA, USA). Resultant cDNAs were amplified and detected using pre-made FAM-labeled probes purchased from Applied Biosystems. Amplification and analysis were carried out on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

OP9-DL1 culture. OP9-DL1 cells were kindly provided by J.C. Zuniga-Pflucker, and cultures were carried out as described. OP9-DL1 cells were kindly provided by J.C. Zuniga-Pflucker, and cultures were carried out as described. For bulk assays, 200-250 purified HSC or MPP were plated per well of a 24-well plate. For limit dilution and single cell experiments, 20, 10, 5, or 1 HSC or MPP were plated per well in 96-well plates. In some burst size experiments, 5 HSC were plated per well to increase colony formation. The probability of monoclonality of T colonies from such wells is at least 0.84. For all cultures, Flt3 Ligand was added at a final concentration of 5 ng/ml and IL-7 at 1 ng/ml. Hematopoietic cells were transferred to fresh stromal layers with fresh cytokines at day 8-10 of culture. HSC cultures were harvested for analysis at day 18-20, and MPP at day 8-10. Numbers of cells produced per well were obtained by resuspending each well in a constant volume and counting on a flow cytometer at a constant flow rate for a defined period of time. Absolute cell numbers were calculated from the cytometry data using a standard curve generated with known numbers of cells.

Intrathymic injections. Two thousand purified progenitors or 2.5x10^5 – 5x10^5 whole BM cells were injected into the thymi of non-irradiated anesthetized F1 mice.

Statistics. Poisson statistics were calculated using L-Calc software (Stem Cell Technologies, Vancouver, BC, Canada).

Results

Reduced T potential of aged progenitors

Previous studies have not detected defects in T lineage potential of aged progenitors using BM chimeras, but these earlier studies examined peripheral T cell reconstitution rather than thymus reconstitution. Thus it was possible that reduced T lineage differentiation by aged progenitors was masked by homeostatic proliferation of aged progenitor-derived T cells in the periphery. To address this, we reconstituted lethally irradiated mice with mixtures of equal numbers of 2 mo Ly5SJL and 18-24 mo
Ly5\textsuperscript{B6} whole BM. As controls, we mixed 2 mo Ly5\textsuperscript{B6} with 2 mo Ly5\textsuperscript{SJL} BM. Mice were analyzed at least 10 wk later for granulocyte and CD4\textsuperscript{+} CD8\textsuperscript{+} double positive (DP) thymocyte chimerism. Thymocyte production by aged progenitors in radiation chimeras was similar to that by young progenitors (Figure 1A), consistent with previous work.\textsuperscript{19,20}

Irradiation is associated with increased production of cytokines and chemokines, and transplanted BM progenitors undergo rapid proliferation in irradiated recipients.\textsuperscript{24,25} Thus, it was possible that changes in aged progenitor function were masked after exposure to the irradiated environment. To address this possibility, we transplanted non-irradiated Ly5\textsuperscript{B6} x Ly5\textsuperscript{SJL} F1 recipient mice with 20 x 10\textsuperscript{6} T-depleted BM cells from 2 mo or 18-24 mo mice and analyzed them 13-16 wk later for production of granulocytes and committed T precursors in the thymus. At this late time point, production of granulocytes and committed T precursors reflects the activity of HSC in the donor inoculum.\textsuperscript{11,14,26} Analysis of blood granulocytes showed that aged and young BM engrafted at a comparable level, with ~3\% chimerism, as expected in non-irradiated recipients.\textsuperscript{14,27} Interestingly, aged BM gave rise to ~10-fold fewer DP thymocytes than young BM (Figure 1B, 1C). There was a slight drop in myeloid chimerism, but this was not statistically significant. Hence, defects in T lineage potential are present in aged HSC, and can be revealed in non-irradiated hosts.

Altered progenitor content of aged BM

Progenitors introduced i.v. must undergo two distinct processes to give rise to thymocytes: first migrate into the thymus and then respond to intrathymic inductive signals to differentiate along the T pathway.\textsuperscript{11} We have shown recently that HSC do not directly settle in the thymus of non-irradiated recipients, but instead the thymus is likely settled at least in part by Ccr9-expressing cells contained within the MPP population.\textsuperscript{14} Therefore, the mechanism responsible for the apparent loss of T potential in vivo could be either a decreased generation of thymus settling progenitors, or decreased intrathymic expansion of progenitors. To determine if thymus settling progenitors were reduced in aged BM, we examined the frequencies of Lin\textsuperscript{lo} Sca-1\textsuperscript{+} c-Kit\textsuperscript{hi} (LSK) cells. These LSK cells consist of a Flt3\textsuperscript{−} subset highly enriched in HSC, and a Flt3\textsuperscript{+} subset which are downstream non-renewing MPP.\textsuperscript{26} There was a reduced frequency of Flt3\textsuperscript{+} cells within
the LSK population at 12 mo of age, and this decreased further by 18 mo (Figure 2A). This correlated with reduced expression of Flt3 transcripts in LSK cells of aged mice (Figure 2C). Absolute numbers of total LSK cells were stable, but numbers of HSC were increased and numbers of MPP were decreased with age (Figure 2B), consistent with previous work. We also examined Ccr9 expression on the MPP population by flow cytometry and quantitative PCR to ask if thymus settling cells were further reduced within this subset. We did not find significant differences in the expression of Ccr9 in young and aged MPP (data not shown). These results show that frequencies and numbers of HSC and MPP are consistently altered in aging BM. As expected from previous work, we also found a reduction in downstream early B-lineage progenitors (Lin⁻ c-Kit⁺ IL-7Rα⁺ AA4.1⁺ Flt3⁺) (data not shown).

Reduced T potential of aged thymus settling progenitors

The above data indicate that the frequency of MPP is reduced in aged BM. As these subsets contains progenitors able to directly migrate into the thymus and initiate T lymphopoiesis, these data predict that unfractionated aged BM progenitors delivered i.v. would be defective in short-term T lymphopoietic assays. We therefore assessed the ability of aged BM to give rise to thymocytes in short-term transplant experiments. Twenty million CD4, CD8, and B220-depleted BM cells from 2 mo or 18-24 mo mice were injected i.v. Because generation of large numbers of DP thymocytes takes approximately 3 wk in this system, recipient mice were analyzed 3 wk later for the presence of donor-derived thymocytes and myeloid cells (Figure 3A). Blood granulocyte chimerism was similar in recipients of aged or young BM, but analysis of thymi revealed that donor-derived thymocyte chimerism was ~4-fold lower in recipients of aged BM than young BM (Figure 3B). This trend was also apparent when absolute numbers of donor-derived DP thymocytes were compared (young: 1.1x10⁶±1.8x10⁵; old: 0.28x10⁶±7.2x10⁴; p<0.001). Further, the reduction in thymocyte chimerism in mice that had received aged BM was apparent at the ETP stage and persisted to the DP stage (Figure 3B).

This T lymphopoietic defect could reflect reduced migration ability and/or reduced T differentiation/expansion ability of aged progenitors. To distinguish between
these, we injected BM cells directly into the thymus of non-irradiated mice, thus bypassing the migration requirement (Figure 3C). Aged BM cells maintained a defect in DP thymocyte production, indicating that they were deficient in T lineage differentiation. Although a migration defect could not be ruled out, it was not sufficient to explain reduced thymocyte production by aged BM. Consistently, B lineage production, which does not require thymus migration, was also decreased (Figure 3B).

**Aged MPP produce fewer T lineage progeny**

The experiments described above establish reduced T potential in aged BM progenitors. We next asked if there were functional defects on a per progenitor basis. We injected equal numbers of purified MPP into the thymus of non-irradiated recipients and analyzed DP thymocyte production 3 wk later (Figure 4A). Aged MPP gave rise to 2.4-fold fewer thymocytes than young MPP. To confirm this result in vitro, we used OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1) to generate T lineage cells. In this system, adult BM progenitors efficiently differentiate into DN2/DN3-stage CD25+Thy-1+ cells, although further differentiation to the DP stage is inefficient. We cultured purified MPP on OP9-DL1 layers, and subsequently analyzed cells for expression of Mac-1, B220, CD25, and Thy-1 (Figure 4B). Although no B220+ cells were detected, substantial numbers of Mac-1+ cells were present. Production of myeloid lineage cells from young and aged MPP was equivalent, but production of Thy-1+ CD25+ T lineage cells by 18-24 mo MPP was decreased 3.3-fold compared to 2 mo MPP. Together, these data show that reduced T lineage output is an intrinsic feature of aged MPP.

To ask whether the defect in T lineage output by aged MPP could be accounted for by a lower T precursor frequency within this population, we performed limit dilution analysis using the OP9-DL1 system (Figure 4C). We detected no significant difference in precursor frequencies of T or myeloid cells between aged and young MPP (T precursors: 1 in 21 aged versus 1 in 19 young, p=0.6; Myeloid precursors: 1 in 10 aged versus 1 in 13 young, p=0.3). These results indicate that although a similar fraction of MPP respond to T inductive signals, aged MPP produce fewer T lineage progeny on a per
progenitor basis. However, producing fewer progeny is not a general feature of aging progenitors, since myeloid production is intact.

**Multiple defects in the aged HSC pool**

The data above establish that there are fewer MPP in aged BM and they are impaired in T lineage differentiation. We wished to determine if defective MPP generation and function emanated from defects in upstream HSC. To directly test the T potential of aged HSC, we cultured purified HSC on OP9-DL1, and analyzed numbers of T and myeloid progeny (Figure 5A). Production of Thy-1^+ CD25^+ T lineage cells from 18-24 mo HSC was decreased compared to production from 2 mo HSC. In contrast, myeloid production by aged HSC was increased. Thus, the T lineage differentiation defect observed in long-term non-irradiated transplant recipients (Figure 1C) is reproducible in vitro, consistent with the defect being intrinsic to HSC. Intrathymic injection assays also showed reduced thymocyte production by aged HSC (data not shown). Together, these results indicate that a loss of T potential occurs in the HSC compartment and this change is cell intrinsic, as it is evident ex vivo (Figure 5A) and in vivo in young hosts (Figure 1B).

We reasoned that production of fewer T lineage progeny by aged HSC could be a result of a reduced proliferative burst of committed T lineage cells and/or a reduction in T precursor frequency within the HSC pool. To test these possibilities, we performed a limit dilution analysis of 2 mo and 18-24 mo HSC using OP9-DL1 (Figure 5B). Poisson statistical analysis indicated that whereas 1 in 15 young HSC produced T lineage cells in this assay, only 1 in 57 aged HSC did so ($p=0.0001$). For myeloid cell production, no significant difference was observed between aged and young HSC (1 in 14 aged versus 1 in 10 young, $p=0.2$).

To assess burst size of young and aged HSC during differentiation, single cells were plated onto OP9-DL1, and the number of T and myeloid lineage cells produced per well were counted (Figure 5C). For both young and aged HSC, there was large variation in T and myeloid output between individual clones. Aged HSC had more highly proliferative myeloid-competent cells than young HSC. However, among T-competent clones, a smaller fraction of aged clones were highly proliferative compared to young
clones (Figure 5C). Together, these data show that production of fewer T lineage cells by aged HSC is the result of both a lower fraction of T-competent cells within the aged HSC pool, as well as a smaller T proliferative burst by T-competent HSC.

**Discussion**

In this study, we have shown that BM progenitors from aged mice have defects in T lineage differentiation. These defects are maintained when aged progenitors are placed in a young host for extended periods of time, and are also evident when single aged HSC are assessed in vitro, indicating they are cell-intrinsic. Multiple cellular mechanisms account for the observed defective T differentiation by aged progenitors. In the HSC pool, there are two distinct factors: 1) There is an accumulation of T-compromised cells within the HSC pool, and 2) The HSC that are T-competent make on average fewer T lineage cells. In contrast, the MPP pool does not exhibit an age-related accumulation of T-compromised cells, however, aged MPP give rise to fewer T progeny. The simplest interpretation of these data is that T-compromised HSC do not transition to the downstream MPP pool, and the smaller burst size of T-competent HSC is also reflected in their MPP progeny. Importantly, we did not detect any reduction in myeloid production by aged progenitors. Hence, the mechanism underlying reduced T output by aged MPP and the T-competent subset of aged HSC is not a global defect, but instead is linked to defective T or lymphoid lineage differentiation in aged cells.

The loss of T lineage potential in aged HSC has historically been difficult to detect, mainly because of the reliance on irradiation models to assay HSC function. It is unclear why age-related changes in T potential are not readily revealed in irradiated mice, but one possible explanation lies in the observation that hematopoiesis in irradiated mice is driven by a small number of stem cell clones; and other clones, while present, remain quiescent.\(^3^1,3^2\) It is possible that T-compromised HSC are more likely to be forced into quiescence in the irradiated host, while the competent HSC drive hematopoiesis. Alternatively, aged T-compromised clones may not engraft in irradiated hosts. Consistently, the engraftment ability of aged HSC is lower than young HSC in irradiated hosts.\(^1^9,3^3\) Another possibility is that transplant into an irradiated host affects both aged and young HSC function by stimulating rapid proliferation and self-renewal. One could
envision a situation where epigenetic control of lymphoid or myeloid gene loci is altered by rapid proliferation. Thus, aged:young mixed marrow radiation chimeras may read out competition between two similarly altered populations. A related possibility is that the irradiated environment may produce some limiting lymphopoietic cytokine or chemokine in excess, reactivating aged cells to make them competitive with young cells. Importantly, our results here indicate that irradiated mice are not always an appropriate system for assessing hematopoietic function.

It is unclear whether the T-compromised cells that accumulate within the aged HSC pool are true stem cells, i.e., have long term self-renewal ability. Other groups have provided evidence for myeloid-biased or myeloid-restricted HSC in young mice. It is possible that the T-compromised cells observed here result from an expansion of such populations with age. Alternatively, the T-compromised cells in the aged HSC pool may not be stem cells at all, and instead may be non-renewing myeloid progenitors. Discrimination between these possibilities will require ways of physically separating the T-competent and T-compromised populations within the “HSC” compartment of aged mice, so that these two subsets can be assessed for long-term self-renewal ability in vivo.

Although we clearly demonstrated age-related defects in T cell production by BM progenitors, it is unknown whether these defects contribute to or initiate thymic involution. Thymic involution is a complex process involving thymic stromal cells and thymocytes, and crosstalk between these two populations is required for normal thymus development. Therefore, defects in one compartment can potentially affect the other. On the progenitor side, it is unknown if reductions in MPP number and function described here are limiting for thymopoiesis. The thymus is thought to be periodically settled by progenitors released from the BM, so it is possible that decreased MPP function may be compensated for by increased progenitor emigration from the BM. Further, though the MPP pathway is likely important for thymopoiesis, additional pathways may exist. Several populations, including the CLP-2 in BM and the CTP in BM and blood, also have T potential and may migrate to the thymus. As these populations have not been assessed in aged mice, it is possible that waning of the MPP pathway is accompanied by an increased role for other pathways. Additionally, these studies focus on the long-lived C57BL/6 mouse strain. However, HSC number and rates
of thymus involution vary between mouse strains.\textsuperscript{45,46} Thus, additional work is needed to determine the contribution of progenitor defects to thymic involution, and to establish commonalities of aging mechanisms among different mouse strains and human populations.

Molecular mechanisms underlying defective T differentiation by aged progenitors remain obscure. However, parallels in age-related changes in T lymphopoiesis and B lymphopoiesis, and relative absence of myelopoietic defects, hint that the mechanism may be common to both lymphoid lineages. Like MPP and ETP, early B lineage progenitors are reduced in frequency and number in aged mice.\textsuperscript{12} Additionally, they are less efficient at producing B lineage progeny in vitro, which may be a result of reduced responsiveness to IL-7.\textsuperscript{12,47} Diminished response to IL-7 is an attractive possible mechanism for progenitor aging, as it would be predicted to impact B and T lineage differentiation early in development, without affecting myelopoiesis.\textsuperscript{48}

The data presented here resolve the question of whether T lineage potential of BM progenitors is impacted by aging. We identified T-compromised progenitors within the HSC compartment of aged mice. Further, we showed that aged clones competent for T lineage differentiation produce fewer T lineage progeny. Our observations may have important implications for patients who experience profound lymphocyte depletion, usually as a result of chemotherapy or HIV infection. Recovery of T cell numbers by autologous BM transplant is impaired with age, and this is correlated with generation of fewer T cells by the thymus.\textsuperscript{3,4} Although age-related changes in the thymic stroma may play a role in reduced thymic output, our data suggest that age-related changes in BM progenitors are an additional obstacle for efficient production of naïve T cells. Additionally, there have recently been efforts to develop effective ex vivo strategies to generate T lineage cells for patients with impaired thymic function.\textsuperscript{49-51} Our data point out that the efficacy of these methods may be reduced when using hematopoietic progenitors from aging patients.
Acknowledgements

Author contributions: V.P.Z. designed research, performed research, analyzed data, wrote paper; I.M. performed research and wrote paper; A.B. designed research and wrote paper. This work was supported by NIH grant AI059621. Individual support was provided by NIH training grant T32-CA09140 for V.P.Z., a Damon Runyon Cancer Research Foundation to I. M. (DRG-102-05), and a Scholar Award from the Leukemia and Lymphoma Society to A. B. The authors have no competing financial interests. We thank Michael Cancro, David Allman, Warren Pear, Arivazhaghan Sambandam and Benjamin Schwarz for critically reading the manuscript.
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Figure Legends

Figure 1. Aged BM is inefficient at producing T lineage cells in vivo in non-irradiated recipients. (A) Lethally irradiated (900 rads) Ly5SJL recipients were i.v. injected with 1:1 mixtures (3x10^5 cells each) of 18-24 mo Ly5B6 BM and 2 mo competitor Ly5SJL BM (dark gray bars). Control chimeras (light gray bars) were generated by injecting mixtures of 2 mo Ly5B6 and 2 mo competitor Ly5SJL BM. Thymocyte and granulocyte populations were analyzed for chimerism at least 10 wk later. Bars represent percentages of Ly5B6 cells within each population. Absolute numbers of thymocytes produced by Ly5B6 BM were also similar (Young: 77.1 x 10^6 ± 19.3 x 10^6; Aged: 64.7 x 10^6 ± 12.8 x 10^6; p=0.3). Data represent 11 individual 2 mo:2 mo chimeras and 22 individual 2 mo:18-24 mo chimeras, and differences between young and old donors in the DP compartment were not significant. Error bars represent 1 SEM. (B) 20x10^6 T-depleted BM cells from 2 mo or 18-24 mo Ly5B6 donors were injected into non-irradiated 5 wk old Ly5B6xLy5SJL recipient mice. Mice were analyzed 13-16 wk later for donor-derived chimerism in the DP thymocyte and blood granulocyte compartments. (C) Summary of data shown in B, representing 4-6 mice per group. Error bars represent 1 SEM. *p<0.01. Absolute numbers of donor-derived thymocytes were also significantly different (Young: 1.58 x 10^6 ± 0.17 x 10^6; Aged: 0.32 x 10^6 ± 0.054 x 10^6; p<0.0001).

Figure 2. Altered frequencies of HSC and MPP in aged BM. (A) BM was harvested from 2 mo, 12 mo, or 18-24 mo mice and LSK cells were analyzed for Flt3 expression by flow cytometry. (B) Numbers of HSC (defined as LSKFlt3-) and MPP (defined as LSKFlt3+) in young and aged BM, calculated from flow cytometric plots in panel A. Numbers are expressed as total cells (counted from 2 femurs plus 2 tibiae plus 2 humeri from each mouse) per gram of body weight. Data represent at least six mice per group. (C) cDNA from total LSK cells was amplified in the presence of gene-specific FAM-labeled Taqman probes. Data is shown as expression relative to Hprt amplification (*p<0.0001).

Figure 3. Reduced T potential of aged BM in short term assays. (A) Ly5B6 donor BM from 2 mo and 18-24 mo mice was depleted of CD4+, CD8+, and B220+ cells, and 20x10^6 of the remaining cells were injected intravenously into non-irradiated mice.
Recipients were analyzed at 3 wk for chimerism in ETP (Linlo c-Kithi CD25−), DN2 (Linlo c-Kithi CD25+), DN3 (Linlo c-Kit− CD25+), and DP (CD4+ CD8+) populations in the thymus, granulocytes in the blood (Mac-1+ Gr-1+), and Pro B (B220+ CD43+ CD19+ AA4.1+) cells in the BM by flow cytometry. (B) Summary of experiments shown in A. Data represent 7 mice per group across 3 independent experiments. Error bars represent 1 SEM. *p≤0.01. Absolute numbers of donor-derived thymocytes were also significantly different (Young: 1.1 x 10⁶ ± 0.17 x 10⁶; Aged: 0.28 x 10⁶ ± 0.072 x 10⁶; p<0.001). (C) BM cells (5x10⁵ – 1x10⁶) were injected into the thymi of non-irradiated mice. Thymi were harvested and analyzed for donor-derived DP thymocytes 21d later. Data are combined from 3 independent experiments each, with 18-20 total mice per group. *p≤0.001.

**Figure 4. Aged MPP are inefficient at producing T lineage cells in vivo and in vitro.**
(A) 2000 sorted MPP from 2 mo or 18-24 mo donors were injected into the thymi of non-irradiated recipients and donor-derived DP progeny were analyzed 21d later. Error bars represent 1 SEM and *p=0.05. (B) 200-250 purified MPP were plated onto OP9-DL1 stromal layers supplemented with IL-7 and FL, and numbers of T lineage (Thy1+ CD25+) and myeloid lineage (Mac1+) progeny were assessed 8-10 d later. For bar graphs, error bars represent 1 SEM and *p<0.01. (C) Limit dilution analysis was performed in vitro by culture of purified 2 mo and 18-24 mo MPP on OP9-DL1 stromal layers. Poisson statistics determined frequencies of lineage competent cells: T lineage: Young 1 in 19 (95% confidence interval 1 in 13 - 26), Aged 1 in 21 (1 in 15 - 29) p=0.6; Myeloid lineage: Young 1 in 13 (1 in 9 - 17), Aged 1 in 10 (1 in 8 - 14); p=0.3. Twenty-eight wells were analyzed for each cell dose.

**Figure 5. The aged HSC pool has a lower frequency of T-competent cells and fewer highly proliferative T-competent clones.** (A) 250 sorted 2 mo or 18-24 mo HSC were plated on OP9-DL1 stromal layers and analyzed on day 17-19. Data shown represents 5 independent experiments (*p<0.001). (B) Limit dilution analysis was performed on purified HSC in vitro by OP9-DL1 co-culture. Poisson statistics determined frequencies of lineage competent cells: T lineage: Young 1 in 15 (95% confidence interval 1 in 11-22), Aged 1 in 57 (1 in 33 – 99) p=0.0001; Myeloid lineage: Young 1 in 10 (1 in 7 – 14), Aged 1 in 14 (1 in 10 – 19); p=0.2. Twenty-four wells were analyzed for each cell dose.
(C) Single HSC were plated on OP9-DL1 layers with cytokines and analyzed by flow cytometry 18d later. Data shown are restricted to those clones that generated detectable T lineage progeny, and are thus T-competent. Data were analyzed using the binomial distribution probability mass function. Colonies with >10,000 progeny were considered highly proliferative (denoted by dashed line), and the frequency of highly proliferative T-competent clones was greater in the young HSC pool than aged (Young: 25 highly proliferative colonies out of 50 total T colonies; Aged: 10 out of 32; p=0.015). Differences were also significant when other arbitrary cutoff values from 5,000 to 100,000 were used. For the myeloid lineage, aged HSC had a higher frequency of highly proliferative myeloid producing clones compared to young HSC (Young: 47 highly proliferative colonies out of 72 total myeloid colonies; Aged: 39 out of 52 p=0.04). Average colony sizes are indicated by horizontal bars.
Figure 1.
Figure 2.
Figure 3.

A

B Intravenous Injection

C Intrathymic Injection

Percent chimerism

Donor-Derived DP (x10^6)

* indicates significant difference between 2mo and 18-24mo groups.
Figure 4.
Figure 5.
Multiple prethymic defects underlie age-related loss of T progenitor competence

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