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_J Immunol_ 2003; 171:2326-2330; doi: 10.4049/jimmunol.171.5.2326

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The Decline in B Lymphopoiesis in Aged Mice Reflects Loss of Very Early B-Lineage Precursors

Juli P. Miller and David Allman

The primary age-related loss in B cell progenitors is thought to be at the pro- to pre-B cell transition. However, we show that the frequencies and absolute numbers of all progenitor populations for the B cell lineage, including B-lineage-committed pro-B cells and multipotent B-lymphoid progenitors, decline in aged C57BL/6 mice. Moreover, when derived from aged mice, lymphoid progenitors within every population examined exhibited suboptimal IL-7 responsiveness, demonstrating that age-associated suboptimal IL-7R signaling is a general property of all early B-lineage precursors. Collectively, these data indicate that aging results in a previously unappreciated decline in the earliest stages of B cell development. The Journal of Immunology, 2003, 171: 2326–2330.

In adults, B-lineage progenitors derive ultimately from hematopoietic stem cells (HSCs) in the bone marrow (BM). After B lineage commitment, pro-B cells with productive Ig H chain (IgH) rearrangements undergo pre-BCR-mediated selection and, as a result, initiate Ig L chain (IgL) rearrangements and differentiate into pre-B cells. After successful IgL recombination, pre-B cells yield sIgM+ B cells that subsequently migrate into peripheral lymphoid tissues (reviewed by Refs. 1 and 2).

It is well established that frequencies of pre-B cells are markedly diminished in senescent mice (3–10). Many of these studies suggest that attrition within the pre-B cell pool stems primarily from diminished pro-B cell differentiation due to either suboptimal pre-BCR signaling and/or diminished synthesis and responsiveness to IL-7 (5–7). Age-related defects in the responsiveness of progenitors for the B cell lineage has not been rigorously evaluated. Thus, it is also conceivable that the age-related loss of pre-B cells reflects an inability to sustain earlier phases of B lymphopoiesis. Early IL-7-responsive B-lineage precursors can be subdivided into several discrete precursor pools characterized by their differential capacities to generate non-B cell progeny. Cells enriched within the earliest of these populations were originally termed common lymphoid progenitors (CLPs) due to their capacity to yield clonally related B and T cells with little or no contribution to nonlymphoid lineages (11, 12). Recent data, however, suggest that CLPs may not contribute significantly to the colonization of the adult thymus (13), suggesting that multipotent lymphoid progenitors within the CLP population are instead early B-lineage precursors (EBPs) characterized by a latent T-lineage potential. In this study, we show that the frequency and absolute number of all progenitor pools for the B cell lineage including EBPs/CLPs are markedly reduced in aged C57BL/6 mice. In addition, we find that IL-7 responsiveness decreases with age in both EBPs/CLPs and pro-B cells. We therefore propose that the previously reported loss of pro-B cells in senescent mice (3–10) is due largely from an inability to generate and/or maintain sufficient numbers of very early B lineage precursors.

Materials and Methods

Mice

Six- to ten-week-old C57BL/6 mice were purchased from the National Cancer Institute animal facility (Frederick, MD). C57BL/6 mice greater than 8 mo of age were aged in our colony or purchased through the National Institute on Aging. NG-BAC transgenic mice (14) were kindly provided by J. Monroe (University of Pennsylvania).

Abs, analytical flow cytometry, and cell sorting

BM suspensions were prepared and stained with optimal dilutions of directly conjugated fluorescent Abs as previously described (12), then analyzed on an 11-parameter MoFlo cell sorter (Cytometry, Fort Collins, CO) or a FACSCalibur (BD Biosciences, San Jose, CA) as recently described (15). All Abs and secondary staining reagents were recently described (15). For cell sorting, stained cell suspensions were applied to our MoFlo at a sheath pressure of 60 p.s.i. and a drop delay frequency of ~98,000 drops/s. This resulted in sorting rates of 25–30,000 cells/s with abort rates of 10–12%.

Cell cultures

One thousand EBPs/CLPs or pro-B cells were cultured in round-bottom 96-well plates in 100 μl complete medium (Opti-MEM with 5% FCS (Irvine, Santa Ana, CA)) containing 10 mM glutamine, 10 mM HEPES, 0.5 mg/ml gentamicin, and 5 × 10⁻⁵ 2-ME. This medium was supplemented with stem cell factor (SCF) at 10 ng/ml and/or IL-7 at the indicated concentrations (R&D Systems, Minneapolis, MN). Stromal cultures were established by sorting defined cell populations onto pre-established S17 stromal cells in flat-bottom 96-well plates and cultured in complete Opti-MEM supplemented with 10 ng/ml IL-7.
marker AA4 (15). In further validation of this strategy, we examined IL-7 responsiveness and RAG2 expression among subsets of CD19<sup>+</sup> B220<sup>+</sup> CD43<sup>+</sup> BM cells. As shown, B220<sup>+</sup> CD43<sup>+</sup> BM cells can be divided into both CD19<sup>+</sup> AA4<sup>+</sup> and CD19<sup>+</sup> AA4<sup>-</sup> cells (Fig. 1A). When equivalent numbers of cells from each population were sorted into S17 stromal cultures supplemented with IL-7, only cells within the AA4<sup>+</sup> subset readily expanded (Fig. 1B). To assess RAG2 expression, we determined green fluorescence protein (GFP) expression among each subset using cells derived from the NG-BAC transgenic mouse. This mouse carries a bacterial artificial chromosome (BAC) transgene in which GFP coding sequences were inserted into the RAG2 coding region (14). As shown, the AA4<sup>+</sup> CD19<sup>+</sup> cells in the B220<sup>+</sup> CD43<sup>+</sup> compartment were GFP<sup>+</sup>, but we could not detect GFP/RAG2 expression among B220<sup>+</sup> CD43<sup>-</sup> AA4<sup>-</sup> CD19<sup>+</sup> BM cells (Fig. 1C).

**AA4<sup>+</sup> pro-B cells decline with age**

We next assessed frequencies and absolute numbers of B220<sup>+</sup> CD43<sup>-</sup> AA4<sup>+</sup> CD24/HSA<sup>-</sup> CD19<sup>+</sup> pro-B cells in C57BL/6 females ranging between 2 and 24 mo of age. As shown, CD19<sup>+</sup> AA4<sup>+</sup> pro-B cells were markedly reduced beginning at 14 mo of age (Fig. 2 and Table I). In addition, whereas all of these data derive from females, we observed a similar decline in aged C57BL/6 males (Table II). Thus, the pro-B cell compartment is markedly diminished in aged mice, suggesting that the primary age-associated defect in B cell development is not due to defective differentiation at the pro-B cell to pre-B cell transition.

**Very early B-lineage precursors decline with age**

The age-associated decline in IL-7-responsive pro-B cells might reflect either defective maintenance of the pro-B cell compartment or defective differentiation and/or maintenance of less mature progenitor pools. To distinguish these possibilities, we first assessed whether the age-associated loss of pro-B cells coincided with a similar loss of earlier B-lineage progenitors. To focus on very early B-lineage progenitors, we exploited our recent findings that multipotent lymphoid progenitors (EBPs/CLPs) are readily identified among Lin<sup>-</sup> IL-7R<alpha><sup>-</sup> AA4<sup>+</sup> Sca-1<sup>low</sup> BM cells (12, 15). Accordingly, we compared frequencies and absolute numbers of this BM population in female C57BL/6 mice at multiple ages. As shown in Fig. 3A, a marked decline in the frequency of EBPs/CLPs was...
observed in mice that were 21 mo of age. Absolute numbers of these cells were also clearly and reproducibly reduced, and this trend began as early as 7 mo of age (Fig. 3B and Table I). Again, whereas all of these data derive from female C57BL/6 mice, we observed a similar decline in aged C57BL/6 males (Table II). Frequencies and absolute numbers of downstream pre-pro-B cells, defined by the surface phenotype Ly-6C$^-$CD24$^+$HSA$^-$B220$^+$AA4$^+$, also declined significantly beginning at 10 mo of age (Tables I and II). We conclude that age-related defects in B lymphopoiesis are due primarily to an inability to generate or maintain numbers of very early B-lineage progenitors, including EBPs/CLPs. Accordingly, Lin$^-$IL-7Ra$^+$AA4$^+$ Sca-1$^{low}$ EBPs/CLPs and B220$^+$CD43$^+$AA4$^+$ CD19$^+$ pro-B cells from 2.5- and 23-mo-old C57BL/6 mice were sorted and cultured with graded doses of IL-7 without the inclusion of stromal cells. A constant dose of SCF was added to parallel cultures to enhance overall cell recoveries. This latter strategy follows from the previous observation that IL-7, but not SCF responsiveness is reduced in aged pro-B cells (5), and our recent observation that SCF enhances cell recoveries of cultured EBPs/CLPs when used in combination with IL-7 (15). Numbers of viable B220$^+$CD19$^+$AA4$^+$ cells in each culture were determined 4 days later. As shown, although B220$^+$CD19$^+$ cells were detected in cultures initiated with aged EBPs/CLPs (Fig. 4C), numbers of B220$^+$CD19$^+$ cells recovered from cultures initiated with either EBPs/CLPs or pro-B cells from 23-mo-old mice were both markedly reduced compared with corresponding control populations from 2.5-mo-old mice (Fig. 4, A and B), indicating that IL-7 responsiveness is diminished in EBPs/CLPs derived from aged mice. These data were not due to lower surface levels of the IL-7R, because EBPs/CLPs from 2.5- and 23-mo-old mice expressed

### Table I. Age-associated loss of early B-lineage progenitor populations in female C57BL/6 mice

<table>
<thead>
<tr>
<th>Age (mo.)</th>
<th>EBP/CLP</th>
<th>Pre-Pro-B</th>
<th>Pro-B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$%$ $^b$</td>
<td>No. $^c$</td>
<td>$p^d$</td>
</tr>
<tr>
<td>2</td>
<td>0.058</td>
<td>1.43</td>
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<td></td>
<td>(0.02)</td>
<td>(0.55)</td>
<td>(13)</td>
</tr>
<tr>
<td>7</td>
<td>0.044</td>
<td>0.67</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.14)</td>
<td>(5)</td>
</tr>
<tr>
<td>10</td>
<td>0.027</td>
<td>0.17</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.11)</td>
<td>(4)</td>
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<tr>
<td>14</td>
<td>0.015</td>
<td>0.10</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.05)</td>
<td>(3)</td>
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<td>20</td>
<td>0.008</td>
<td>0.06</td>
<td>0.0001</td>
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<tr>
<td></td>
<td>(0.01)</td>
<td>(0.06)</td>
<td>(13)</td>
</tr>
<tr>
<td>24</td>
<td>0.001</td>
<td>0.05</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

$^a$ EBPs/CLPs, Lin$^-$IL-7Ra$^+$ AA4$^+$ Sca-1$^{low}$; pre-pro-B, Ly6C$^-$CD24$^+$HSA$^-$B220$^+$ AA4$^+$; pro-B, B220$^+$CD43$^+$CD19$^+$ AA4$^+$.  
$^b$ Mean percentages of the indicated populations are shown with SDs in parentheses.  
$^c$ Mean absolute numbers of the indicated populations were calculated by multiplying the frequencies of cells by the number of cells harvested from two tibia and two femurs from each mouse. SDs are shown in parentheses.  
$^d$ Values of $P$ were calculated for absolute numbers using an unpaired Student’s $t$ test using data from 2-mo-old mice as the control population. Sample sizes are shown in parentheses.

### Table II. Age-associated loss of early B-lineage progenitor populations in male C57BL/6 mice

<table>
<thead>
<tr>
<th>Age (mo.)</th>
<th>EBP/CLP</th>
<th>Pre-Pro-B</th>
<th>Pro-B</th>
</tr>
</thead>
<tbody>
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<td>No. $^c$</td>
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<td>(0.55)</td>
<td>(4)</td>
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<td>0.001</td>
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<td></td>
<td>(0.03)</td>
<td>(0.04)</td>
<td>(3)</td>
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<td>0.006</td>
<td>0.15</td>
<td>0.0002</td>
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<tr>
<td></td>
<td>(0.02)</td>
<td>(0.06)</td>
<td>(3)</td>
</tr>
<tr>
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<td>0.007</td>
<td>0.16</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.06)</td>
<td>(3)</td>
</tr>
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</table>
comparable surface levels of IL-7Rα (Fig. 4D). We therefore conclude that age-associated suboptimal IL-7 responsiveness occurs in both EBPs/CLPs and pro-B cells.

Discussion
Our data suggest that the primary age-associated defect in B lymphopoiesis does not result from suboptimal pro-B cell differentiation. Instead, we find that decreased B lymphopoiesis in aged mice is largely due to loss of very early B-lineage precursor pools. Supporting this conclusion, the frequency and absolute number of AA4+.CD19+ pro-B cells declined beginning as early as 12 mo of age (Fig. 2; Tables I and II). Likewise, earlier B-lineage precursor populations, including Lin−.IL-7Rα+.AA4+.Sca-1low BM cells from one 2- and one 21-mo female C57BL/6 mouse. Two hundred thousand total events were collected from cells stained for expression of the indicated surface proteins, as previously described (15). Numbers in plots indicate percentages of all events collected. Data are representative of six separate experiments. B, Absolute numbers of EBPs/CLPs in each mouse of the indicated age were calculated by multiplying cell recoveries from two legs by the percentage of Lin−.IL-7Rα+.AA4+.Sca-1low BM cells. Each symbol represents data from an individual mouse of the indicated age. The solid lines indicate means for each age group. Means, SDs, and p values relative to 2-mo-old mice are shown in Table I.

FIGURE 3. Decline in very early B-lineage progenitors (EBPs/CLPs) with age. A, Flow cytometric assessment of Lin−.IL-7Rα+.AA4+.Sca-1low BM cells from one 2- and one 21-mo female C57BL/6 mouse. Two hundred thousand total events were collected from cells stained for expression of the indicated surface proteins, as previously described (15). Numbers in plots indicate percentages of all events collected. Data are representative of six separate experiments. B, Absolute numbers of EBPs/CLPs in each mouse of the indicated age were calculated by multiplying cell recoveries from two legs by the percentage of Lin−.IL-7Rα+.AA4+.Sca-1low BM cells. Each symbol represents data from an individual mouse of the indicated age. The solid lines indicate means for each age group. Means, SDs, and p values relative to 2-mo-old mice are shown in Table I.

FIGURE 4. Diminished IL-7 responsiveness of both EBPs/CLPs and AA4+.pro-B cells. A and B, One thousand sorted Lin−.IL-7Rα+.AA4+.Sca-1low (EBPs/CLPs) (A) or B220+.CD43+.CD19+.AA4+.pro-B (B) BM cells sorted from 2.5- or 23-mo-old female C57BL/6 mice were cultured in stromal-free cultures (see Materials and Methods) containing IL-7 at the indicated concentrations. Parallel cultures without (top panels) or with (bottom panels) 10 ng/ml SCF were established, and the total number of B220+.CD19+ cells recovered after 4 days was determined by flow cytometry. Pro-B cells for each group were cultured in triplicate, whereas, due to limited cell numbers in aged mice, certain cultures containing EBPs/CLPs were performed in duplicate. C, Indicate individual recoveries from cultures initiated with limiting cell numbers. Data are representative of three separate experiments. Mean cell recoveries for each condition are shown with SDs indicated by error bars. C, EBPs/CLPs were cultured for 4 days in 2.0 ng/ml IL-7, then stained for surface expression of B220 and CD19 before addition of propidium iodide (PI) to exclude dead cells. PI− cells are shown. D, BM cells from a 2- and 21-mo-old C57BL/6 female were stained to resolve EBPs/CLPs (see Fig. 3). Overlay histograms showing IL-7Rα expression were generated by backgating on Lin−.IL-7Rα+.AA4+.Sca-1low cells. A total of 400,000 events was collected for this analysis. The filled histogram is derived from the 2 mo old; the solid line histogram from the 21 mo old. Data are representative of analyses of at least 10 separate experiments.

It has been suggested that aging negatively impacts the efficiency with which transplanted HSCs engraft irradiated recipient BM (19) and the ability of such cells to differentiate into peripheral blood B cells (20). Whereas it is difficult to determine whether the latter observation reflects defects within the HSC compartment vs later stages of B cell differentiation such as the pro- to pre-B cell transition, our data indicate that diminished B cell production from
transplanted HSCs is most likely due to an inability to initiate and/or sustain the development of very early lymphoid and B-lineage precursor pools.

Would these age-related defects also hamper early T cell development? In considering this question, we must emphasize that the BM progenitor pool responsible for colonization of the postnatal thymus has not been identified. Indeed, we recently provided evidence that T cells can develop via a EBP/CLP-independent pathway, and propose that cells referred to in this work and elsewhere as CLPs are B-lineage-specified precursors defined by their receptivity to signals that can redirect them into the T cell lineage (13). Thus, loss of very early BM lymphoid progenitors need not correspond to diminished frequencies of early thymic T-lineage progenitor pool. Indeed, whether aging impacts negatively on early T-lineage progenitors is currently unclear (21, 22), and our recent data illustrate that CD44+/CD25− thymocytes are heterogeneous, with bona fide early T cell precursors defined as c-kit−high IL-7Rαlow (13). Thus, an assessment of early T cell precursor frequencies in aged mice may resolve the current controversy over the potential effect of aging on very early T cell progenitors in the thymus (21, 22).

Whereas our data support the notion that suboptimal IL-7 responsiveness is a general property among B-lineage progenitors in aged mice (Fig. 4), the inability of old mice to sustain B lymphopoiesis is most likely due to additional factors. Several studies examining the negative impact of increased steroid levels on B lymphopoiesis may provide important insights into this issue. First, two studies from Erben et al. (23, 24) illustrate that B lymphopoiesis in rats can be augmented upon removal of sources of serum levels in aged mice. Indeed, we recently provided evidence that T cells can develop via a EBP/CLP-independent pathway, and propose that cells referred to in this work and elsewhere as CLPs are B-lineage-specified precursors defined by their receptivity to signals that can redirect them into the T cell lineage (13). Thus, an assessment of early T cell precursor frequencies in aged mice may resolve the current controversy over the potential effect of aging on very early T cell progenitors in the thymus (21, 22).

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Acknowledgments
We thank Drs. Jennifer Punt, Avinash Bhandoola, and Michael Cancro for helpful discussions and for critically reviewing this manuscript. We also thank William DeMuth for skilled cell sorting, and Mathew Karnell and Kristina Rudd for expert technical assistance.

References