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Effects of Aging on the Common Lymphoid Progenitor to Pro-B Cell Transition

Hyeyoung Min, Encarnacion Montecino-Rodriguez, and Kenneth Dorshkind

The number of common lymphoid progenitors (CLP) and their pre-pro-B and pro-B cell progeny is reduced in old mice, but the age-related changes responsible for these declines have not been fully elucidated. The aim of this study was to provide additional insights into the impact of senescence on early B cell development by analyzing the CLP and pro-B cell compartments under steady-state conditions and after cytoablation with 5-fluorouracil. 5-Fluorouracil subjects the hemopoietic system to acute stress and has the advantage of revealing defects in progenitors that may otherwise be subtle. The data demonstrate significant, age-related defects in the proliferative potential of early B cell precursors and suggest that the ability of CLP to differentiate into pre-pro-B cells is also compromised by senescence. These age-related changes in early B lymphopoiesis do not result from a general defect in HSC or the bone marrow microenvironment that impairs development in all hemopoietic lineages. Instead, data demonstrating that myeloid progenitor number and developmental potential do not decline with age indicate that B lymphopoiesis is particularly sensitive to defects that accumulate during senescence. The Journal of Immunology, 2006, 176: 1007–1012.

B lymphocytes, like myeloid and erythroid cells, are derived from hemopoietic stem cells (HSC) present in the medullary cavity of the bone marrow (1, 2). During their development, B lineage cells progress through a series of distinct developmental stages defined by various phenotypic markers and the rearrangement and expression of Ig genes. Common lymphoid progenitors (CLP), defined as lineage-negative (Lin−) Sca-1low CD117(c-Kit)low CD127(IL-7Rα)+ cells (3), have traditionally been considered to be the intermediate through which both B and T cells are generated, but recently revised models of lymphopoiesis suggest that cells with this phenotype are actually early B-lineage-specified progenitors (4, 5). CLP have initiated Ig H chain gene recombination (6), and rearrangement continues as they mature into pro-B cells. Pro-B cells were originally characterized by the CD45R−CD43+ phenotype, but it is now appreciated that this combination of surface markers defines a heterogeneous population that includes non-B lineage cells in addition to B cell progenitors at different stages of development. The latter precursors include CD45R−CD43+AA4.1+ CD19−Ly6C− pre-pro-B cells and CD45R−CD19−CD43+AA4.1+ pro-B cells (7–10). Upon completion of a functional Ig H chain gene rearrangement, pro-B cells mature into cytoplasmic μ H chain protein expressing pre-B cells. Finally, surface IgM+ B lymphocytes are produced from pre-B cells after productive Ig L chain gene rearrangement (1, 2, 7).

It is well established that the frequency and number of pre-B cells are reduced with age, and numerous studies have demonstrated that this is due in part to inefficient pro-B cell maturation (11–16). However, the CLP to pro-B cell transition has not been studied as extensively. In fact, it has been appreciated only recently that senescence is accompanied by a reduction in CLP, pre-pro-B, and pro-B cells numbers, and that aged CLP exhibit a diminished responsiveness to IL-7 (17).

The aim of this study was to provide additional insights into the impact of senescence on early B cell development. To do so, CLP and pro-B cells in young and old mice were characterized under steady-state conditions and after treatment of animals with 5-fluorouracil (5-FU). 5-FU subjects the hemopoietic system to acute stress and has the advantage of revealing defects in progenitors that may otherwise be subtle (18, 19). The data demonstrate significant, age-related defects in the proliferative potential of CLP and pre-pro-B cells and suggest that the ability of CLP to differentiate into pre-pro-B cells is also compromised by senescence. These age-related changes in early B lymphopoiesis are not the result of a general defect in HSC or the bone marrow microenvironment that impairs development in all hemopoietic lineages. Instead, data demonstrating that myeloid progenitor (MP) growth and differentiation are normal in old mice indicate that B lymphopoiesis is particularly sensitive to defects that accumulate during aging.

Materials and Methods

Mice

Four- to 6-wk-old C57BL/6J (B6) mice were obtained from The Jackson Laboratory. Eighteen- to 20-mo-old B6 mice were purchased from the National Institute of Aging colony. Mice were housed at the Division of Laboratory Animal Medicine vivarium at University of California-Los Angeles. Animals were maintained, and experiments were conducted according to University of California-Los Angeles institutional animal care and use committee guidelines.

Administration of 5-FU and BrdU

Mice were administered a single i.v. dose of PBS or 5-FU (150 mg/kg body weight; Sigma-Aldrich) (18, 19). Bone marrow cells were prepared from 3 to 12 mice at various times after 5-FU treatment.
In those studies in which production rates were calculated, mice received an i.p. injection of BrdU (0.8 mg/mouse; Sigma-Aldrich) on day 7 after 5-FU administration and 12 h thereafter as described previously (11). Groups of mice were killed at various times after the first or second injection, and incorporation of the thymidine analog BrdU by various hematopoietic subpopulations was determined.

**Immunofluorescence and flow cytometry**

Pre-pro-B cells were defined as Lin- (CD11b+ Gr-1- TCRAβ+ TER119+ CD8- TCRγδ+ NK1.1- IgM- CD45R+ AA4.1- CD19- Ly-6C-), and pro-B cells were defined as CD19+ AA4.1+ CD43- cells (17). CLP were defined as Lin- (Lin = CD45R, TER-119, Gr-1, CD8α, and CD3ε) CD127(IL7Rα)+ Sca-1low CD117(c-kit)+ cells (3) and were isolated by labeling bone marrow cells with anti-CD127, anti-Sca-1, and anti-CD117 Abs. MPs were defined as Lin- (Lin = CD3ε, CD8α, CD11b, CD45R, Gr-1, and TER-119), Sca-1+ CD127- CD16/32+ CD117+ cells, and early B1- (CD127- CD172a-) phenotype distinguished them from HSC or CLP. Common MPs (CMP) were identified as Lin Sca-1- CD127- CD117+ CD34+ CD16/32+ and megakaryocyte-erythroid progenitors (MEP) were defined as Lin Sca-1- CD127- CD117- CD54- CD16/32- (20).

FITC-, PE-, Tricolor-, allophycocyanin-, or CyChrome-conjugated or biotinylated Abs to the following cell surface determinants were used for surface staining: Sca-1 (clone 2B8), CD127 (IL-7Rα), CD117 (c-Kit), CD16/32 (FcRII/III), CD19 (clone 1D3), CD34 (clone RAM34), CD43 (clone S7), CD45R (clone RA3-6B2), CD117 (c-Kit, clone 2B8), CD127 (IL-7Rα, clone A7R34), Sca-1 (clone E13-161.7), TER119 (clone Ter-119), TCRαβ (clone H57-597), TCRγδ (clone UC7-13D5), NK1.1 (clone PK136), Ly6C (clone AL-21), AA4.1 (clone 2.4G2), Gr-1 (clone RB6-8C5), and IgMα (clone DS1). The appropriate dilution of each Ab was determined before use. All Abs were obtained from BD Pharmingen, except for goat anti-mouse IgM (Southern Biotechnology Associates), TER119 (eBioscience), and Tricolor- or allophycocyanin-Cy7-conjugated avidin (Caltag Laboratories). Stained cells were analyzed on a FACSScan (BD Biosciences), FACSCalibur (BD Biosciences), or FACS Aria (BD Biosciences) at the Flow Cytometry Core of Jonsson Comprehensive Cancer Center at University of California-Los Angeles.

**Detection of incorporated BrdU and calculation of production rates**

Cells that had incorporated BrdU were detected with the BrdU Flow Kit (BD Pharmingen) according to the manufacturer’s instructions. After cell surface staining, populations were fixed, permeabilized, DNase treated, and stained with anti-BrdU Ab. Flow cytometric analysis was performed using linear regression analysis as previously described (11). The regression line was fitted through at least three time points.

**Detection of proliferating cells**

Bone marrow cells were harvested from PBS- or 5-FU-treated mice on day 8 after treatment. CLP and pro-B cell proliferative status was analyzed by Ki-67 staining. Ki-67 is a nuclear Ag found exclusively in cells in late G1, S, G2, and M phases of the cell cycle (21). After cell surface staining, cells were fixed, permeabilized, and stained with an anti-Ki-67 Ab (BD Pharmingen) or mouse IgG1 isotype control before analysis on a FACSscan or FACS Aria according to the manufacturer’s instructions.

**Statistical evaluation**

Statistical significance of the differences of the means was evaluated by two-tailed Student’s t test. Production rates were compared using analysis of covariance (comparison of slopes). A value of p < 0.05 was considered significant.

**Results**

**CLP, pre-pro-B, and pro-B cell numbers are reduced in old mice**

The age-associated reduction in the frequency and number of CLP, pre-pro-B, and pro-B cells in the bone marrow of old mice has been reported only recently (17). The data in Fig. 1 confirm these observations by demonstrating that the frequency and absolute number of CLP and pre-pro-B cells are significantly reduced in old mice. The frequency and absolute number of pro-B cells were, on the average, also lower in old mice. However, due to the considerable variation between animals, the level of reduction was not significant.

**CLP from old mice exhibit reduced proliferative potential**

To obtain insights into why their number declines with age, CLP proliferation was assessed in young and old mice by labeling Lin- Sca-1low CD117low CD127+ cells with Ki-67. As shown in Fig. 2A, the frequency of Ki-67+ CLP in old mice was marginally reduced compared with that in their counterparts from young animals.

To reveal potential defects that may be subtle under steady-state conditions, the ability of old mice to replenish the CLP compartment at early times after treatment with 5-FU was determined. As shown in Fig. 2B, CLP number was markedly depressed in both young and old mice 3 days after treatment with 5-FU. Recovery in
young animals initiated thereafter, with a transient spike in production observed between days 5 and 8. Cell production at this time correlated with a significant increase in the frequency of CLP in cycle. Although 40% of CLP in PBS-treated young mice were Ki-67$$^+$$, this value had increased to 65% in the young 5-FU-treated animals (Fig. 2A). CLP number in old mice also increased after 5-FU treatment. However, the spike in cell production observed in young mice did not occur, and no compensatory increase in proliferation was detected (Fig. 2).

Pre-pro-B cell production is reduced in old mice

The above data suggest that CLP recovery in 5-FU-treated young mice is more robust than that in old animals. In this case, a prediction is that pro-B cell recovery should also be more vigorous in young animals. Initial experiments demonstrating that the production rate of CD45R$$^+$$CD43$$^+$$ cells in 5-FU-treated young mice was almost 40-fold higher in young than in old mice appeared to confirm this hypothesis (Fig. 3A). However, because the CD45R$$^+$$CD43$$^+$$ compartment is developmentally heterogeneous, subsequent experiments were performed to assess pre-pro-B cell recovery after 5-FU treatment in more detail. As shown in Fig. 3B, by day 4 after 5-FU administration, pre-pro-B cells were barely detectable in mice regardless of age. However, there was a spike in pre-pro-B cell production on day 10 in young mice that occurred subsequent to the peak of CLP production on day 8 (compare Figs. 2B and 3B). In contrast, pre-pro-B cell production in old mice remained low at all time points.

To gain additional insight into these observations, the pre-pro-B cell proliferative status was assessed in young and old mice under steady-state conditions and on day 8 after 5-FU administration. The latter time point was chosen because it immediately preceded the rise in pre-pro-B cell numbers in young mice between days 8 and 10. As shown in Fig. 3C, the frequency of Ki-67$$^+$$ pre-pro-B cells was increased in both young and old mice on day 8 after 5-FU administration. However, although pre-pro-B cells in some old animals were proliferating at levels comparable to those in young mice under both steady-state conditions and after 5-FU treatment, on the average, these levels were lower than those in young animals.

FIGURE 2. Proliferation of CLP in young and old mice. A, Bone marrow cells were harvested from mice 8 days after PBS or 5-FU administration, and CLP were labeled with Abs to Ki-67 to determine their proliferation status. Six to 11 mice/group were individually analyzed to calculate the mean ± SD frequency of Ki-67$$^+$$ cells. FACS plots are from a representative mouse from each group. B, Recovery of CLP number after 5-FU treatment. Four to six mice were individually processed at each time point, and data were pooled to obtain the mean ± SD. Data are based on CLP number per femur and tibia.

FIGURE 3. Effects of age on the development and proliferation of pre-pro-B cells. A, Rates of production of CD45R$$^+$$CD43$$^+$$ cells in young and old mice on days 7–8 after 5-FU treatment were determined by plotting the number of BrdU-labeled pro-B cells over time. The slope of the regression lines was calculated using linear regression analysis and was used to determine production rates. Four to six mice were analyzed at each time point. B, Recovery of Lin$$^-$CD45R$$^+$$AA4.1$$^-$$CD19$$^-$$Ly-6C$$^-$$ pre-pro-B cell numbers after 5-FU administration. Four to six mice were individually processed at each time point, and data were pooled to obtain the mean ± SD number of cells per two femurs. FACS plots of pre-pro-B cells in young and old mice on day 10 after 5-FU administration are shown. C, Bone marrow cells were harvested from mice 8 days after PBS or 5-FU administration, and Lin$$^-$CD45R$$^+$$AA4.1$$^-$$CD19$$^-$$Ly-6C$$^-$$ pre-pro-B cells were labeled with Abs to Ki-67 to determine their proliferation status. Each symbol indicates an individual mouse, with the average indicated by the horizontal line. The mean ± SD for each group is indicated. n.s., Not significant.
A similar pattern was observed in the pro-B cell compartment. Although the frequency of Ki-67<sup>+</sup> pro-B cells had increased in both young and old mice on day 8 after 5-FU administration, the frequency of cycling cells was significantly lower in old mice under steady-state conditions and after 5-FU administration (Fig. 4).

**Recovery of B-lineage cells remains depressed in old mice**

The above experiments focused on the initial wave of cell production after 5-FU treatment, but the recovery of B-lineage cells at more extended times was also assessed. As shown in Fig. 5, the number of CD45R<sup>+</sup>CD43<sup>+</sup> cells in young mice remained higher than that in old animals on day 28 after 5-FU treatment. Even though these findings were based on analysis of CD45R<sup>+</sup>CD43<sup>+</sup> cells, the analysis of a cohort of three young and two old mice confirmed that differences were due to lower numbers of B-lineage cells. In this regard, the frequency of pre-pro-B (young, 0.119 ± 0.005; old, 0.084 ± 0.008; p < 0.01) and pro-B (young, 5.35 ± 1.14; old, 1.76 ± 0.875; p < 0.03) cells on day 28 after 5-FU administration was significantly lower in old mice. The data in Fig. 5 also demonstrate that the number of CD45R<sup>+</sup>CD43<sup>+</sup> cells remained lower in old mice on day 50 after treatment as well.

**Early MP production is not affected by aging**

The above age-related changes in early B lymphopoiesis could have resulted from a general defect in HSC or the bone marrow microenvironment that impairs development in all hemopoietic lineages. To determine whether this was the case, MP cell number and function were assessed. CMP have been identified as Lin<sup>+</sup> CD127<sup>-</sup> CD117<sup>-</sup> CD43<sup>-</sup> CD16/32<sup>low</sup> cells. As these cells mature, they generate GMP progeny (20). As shown in Fig. 6, the frequency of CMP in young and old mice was the same, and GMP numbers were significantly increased in old mice under steady-state conditions. Consistent with these observations, the frequency and absolute number of CD11b<sup>+</sup> bone marrow cells were higher in old (71.3%; 28.3 × 10<sup>6</sup> cells) than in young (57.5%; 15.9 × 10<sup>6</sup> cells) animals. Preliminary data indicated that the frequency of MEP was also not reduced significantly in old mice (Fig. 6A and data not shown).

In contrast to age-related defects in lymphoid progenitor growth and differentiation, no evidence of defective myelopoiesis was observed. The recovery of MPs was comparable in young and old mice after 5-FU treatment (Fig. 6D), and the number of CD11b<sup>+</sup> cells returned to pre-5-FU levels with comparable kinetics in both groups of animals (Fig. 6E). Similar results were obtained even when mice received a second injection of 5-FU 2 wk after the initial administration. Two weeks after this second treatment, there was no significant difference in the frequency of CD11b<sup>+</sup> (young, 87.1 ± 3.4; old, 80.6 ± 6.1) or Gr1<sup>+</sup> (young, 89.7 ± 3.3; old, 85.6 ± 5.6) cells in the bone marrow of young and old animals.

**Discussion**

The decline of primary B cell development with age is a well-recognized phenomenon, and there is an extensive literature describing how aging affects pre-B cells and the pro-B to pre-B cell transition (11, 12, 16). However, it has been appreciated only recently that senescence is also accompanied by a reduction in the number of CLP and pro-B cells (17). To delineate further the effects of aging on early B cell development, cells in the CLP, pre-pro-B, and pro-B cell compartments were characterized under steady-state conditions and during recovery from hemopoietic stress induced by 5-FU administration. It has previously been demonstrated that 5-FU efficiently depletes B-lineage cells within the first week after its administration (18). This effect is due in part to the drug’s propensity to kill cycling cells. However, the number of cells depleted exceeds those in cycle, so additional effects on non-cycling populations must also be occurring (18, 22). This issue aside, the kinetic analyses of early B-lineage cells under steady state conditions and after recovery from 5-FU indicate that early B-lineage cells exhibit age-related defects in growth and differentiation.

Initial studies that focused on CLP suggest that in aged animals these cells exhibit numerous developmental defects. For example, although differences were not dramatic, the frequency of CLP in cycle, estimated by assessing Ki-67 expression, was lower in old animals under steady-state conditions, and over time this could account for reduced numbers of total CLP. The existence of this proliferative defect in CLP was more clearly demonstrated when recovery from 5-FU was measured. CLP number declined in both young and old mice, but different patterns of recovery were observed. A well-defined burst in the production of B-lineage cells is known to occur after suppression of B cell differentiation (23, 24), and this response was observed in young mice. However, CLP in old mice did not exhibit this response. Additional data demonstrating that the frequency of Ki-67<sup>+</sup> CLP in young, but not old, mice had increased above steady-state levels were consistent with these observations. Taken together, these results support a model in which aging affects the CLP proliferative potential, and this effect
may account, at least in part, for their decline in number with age. Additional factors that could include diminished input into this compartment from more immature hemopoietic precursors may be operative and need to be investigated.

Subsequent studies that measured pro-B cell production rates suggested that aged CLP also exhibit differentiation defects. Even though the CD43+/CD45R− population, which includes pre-pro-B, pro-B, and non-B-lineage cells, was assessed in those experiments, the data were, nevertheless, revealing, in that the production of CD43+/CD45R+ cells was ~40-fold less in old animals. These results appear to contradict recent reports, in which the CD43+/CD45R+ population was also analyzed, indicating that pro-B cell production rates in young and old mice are similar (11, 15). However, it is important to note that the latter studies analyzed the CD43+/CD45R+ compartment in unmanipulated animals and not under stress conditions, in which the pro-B cell compartment is ablated and must be almost completely renewed from precursors.

Additional studies that assessed pre-pro-B cell recovery at early times after 5-FU treatment of young and old mice confirmed the diminished input into the pro-pro-B cell compartment. The number of pre-pro-B cells in both young and old mice was markedly depressed by day 5 after 5-FU treatment. Subsequently, however, pre-pro-B cell production ensued in young animals, and by day 10, numbers in young mice exceeded those in old animals. This recovery of pre-pro-B cells occurred subsequent to the rise in CLP numbers on day 8 and provides circumstantial evidence that the Lin−/Sca-1low/CD117low cells identified in mice after 5-FU administration are bona fide CLP. In contrast, pre-pro-B cell recovery in old mice remained depressed.

In addition to reduced input from CLP, the lower number of pre-pro-B cells in old mice after recovery from 5-FU is exacerbated by the fact that the pre-pro-B cells produced proliferate less than their young counterparts. For example, the frequency of young Ki-67+/pre-pro-B cells had increased from 30% under steady-state conditions to 50% on day 8 after 5-FU administration. A compensatory increase in the level of Ki-67 expression by old pre-pro-B cells was also observed. However, regardless of whether measurements were made under steady-state conditions or after recovery from 5-FU, the level of proliferation by old pre-pro-B cells was lower than that in young animals. Similar observations were made upon analysis of pre-B cells.

However, it is important to emphasize that these conclusions only apply on a population basis. In this regard, when the frequency, total number, and Ki-67 labeling of CLP, pre-pro-B, and pro-B cells were assessed, some young mice had characteristics more comparable to their old counterparts, and some old animals exhibited properties that were more generally attributable to young animals. These observations are consistent with data from Witte et al. (11), who previously noted that the decline in B lymphopoiesis in old mice exhibits considerable variability, as determined by assessment of pre-B cell numbers. The finding that not all mice, even those of the same sex and strain, age at a comparable rate indicates that additional factors, perhaps environmental in nature, are also operative and impact upon how ageing affects B-lineage cells.

At least some of the diminished proliferative and differentiative potential of CLP and pre-pro-B cells from old mice is probably due to cell autonomous changes that accumulate with age, and these age-related defects were exacerbated in the 5-FU-treated mice. Although we cannot exclude the possibility that the sensitivity of progenitors to 5-FU is altered in older animals, and that the reduced proliferation and differentiation observed in these animals is a consequence of 5-FU damage, proposing that the existence of
intrinsic defects in early B-lymphoid progenitors exists is consistent with data demonstrating that hematopoietic stem cells from old mice do not efficiently repopulate lymphoid cells in young recipients (25) and down-regulate genes necessary for lymphoid specification (26). However, the present studies do not exclude the possibility that age-related microenvironmental effects could also be occurring (15, 27). Additional studies are needed to distinguish the degree to which intrinsic vs extrinsic influences affect the behavior of early B-lineage cells.

Although the quality of early B-lymphoid progenitors is diminished with age, myelopoiesis remained robust. The frequency and absolute number of CMP and GMP were the same in young and old animals, and the recovery of myelopoiesis after even two rounds of 5-FU treatment was comparable in young and old mice. Preliminary data also indicate that MEP numbers do not decline with age. The up-regulation of genes that promote myelopoiesis may be in part responsible for these observations (26). Even if this is the case, the data suggest that a general defect in HSC or the bone marrow microenvironment that impairs development in all hematopoietic lineages is not a feature of aging and that the B lineage is particularly sensitive to the effects of senescence. Why this is the case is not clear. By the time the CLP stage of development has been reached, progenitors have initiated Ig gene rearrangements (6). In addition to the temporal expression of critical transcription factors (16), DNA repair mechanisms must remain intact in these cells to repair dsDNA breaks. Such defects would affect the function and, ultimately, the survival and number of cells that have initiated the B cell development program.

Disclosures

The authors have no financial conflict of interest.

References