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Cutting Edge: Senescent BALB/c Mice Exhibit Decreased Expression of λ5 Surrogate Light Chains and Reduced Development Within the Pre-B Cell Compartment[1]

Erin M. Sherwood, Bonnie B. Blomberg, Wei Xu, Cynthia A. Warner, and Richard L. Riley[2]

Although senescent BALB/c mice (~2 years old) have reduced numbers of small pre-B cells, early pre-B cells ($CD43^+CD25^+B220^+$) are present in comparable numbers within the bone marrow of both young (3–6-month-old) and senescent BALB/c mice. The transition of $CD43^+$ pre-B cells to the $CD43^-$ pre-B cell compartments is dependent on proliferation and clonal maturation dictated by the pre-B cell receptor (μ/λ5/VpreB). In vivo, senescent $CD43^+B220^+$ pro-B/early pre-B cells demonstrated reduction of λ5 mRNA, by RT-PCR analysis, and of both surface and cytoplasmic λ5 protein. Decreased λ5 protein expression was also seen among pro-B/pre-B cells derived from senescent bone marrow after stimulation in vitro with IL-7. We propose that diminished expression of the λ5 surrogate light chain results in decreased pre-B cell receptor formation and contributes to reduced recruitment of nascent $CD43^+$ pre-B cells into the $CD43^-$ large and small pre-B cell compartments. ***The Journal of Immunology,*** **1998, 161: 4472–4475.**

Senescent mice typically exhibit decreased numbers of pre-B cells in their bone marrow, suggesting that abnormal B lymphopoiesis accompanies the aging process (1–3). Although pre-B cell numbers are reduced in senescent murine bone marrow, it has been reported that the numbers of pro-B cells remain unchanged (2, 3), indicating that the abnormal development of pre-B cells in aged mice occurs at the pro-B cell to pre-B cell transition. Stephan et al. (4) have also suggested that decreased proliferation in response to the growth cytokine IL-7 may contribute to diminished pre-B cell production by limiting expansion of nascent pre-B cells.

Expression of the pre-B cell receptor (μ/λ5/VpreB) is required for optimal clonal expansion and maturation of pre-B cells (5–7). Although critical to the establishment of normal steady state numbers of pre-B cells, there currently is no information regarding expression and/or function of the pre-B cell receptor during senescence. In this report, we demonstrate that early $CD43^+$ pre-B cells are retained in senescent BALB/c mice while later $CD43^-$ pre-B cells are decreased. Importantly, the expression of λ5 surrogate light chain mRNA and protein within the pro-B/early pre-B cell population is significantly reduced in aged BALB/c mice. We suggest that decreased λ5 surrogate light chain expression contributes to the decline in B lymphopoiesis observed in senescence.

Materials and Methods

Mice

Young male and female BALB/c mice 3 to 6 mo of age and senescent BALB/c mice 22 to 29 mo of age were obtained from the National Institute on Aging colony at Charles River Laboratories, Wilmington, MA. Mice bearing visible tumors were eliminated from the study. Comparable results were seen with both males and females.

Fluorescent staining and cell sorting

Bone marrow cells were stained with mAbs specific for CD43 (clone S7), B220 (clone RA3-6B2), CD19 (clone D7), and/or CD25 (clone 7D4) (PharMingen, San Diego, CA) by methods previously reported (2). The LM34 mAb, specific for murine λ5, was produced by Karasuyama et al. (8) and generously provided by Dr. Hans-Martin Jäck, Department Medicine, University of Erlangen-Nurnberg, Erlangen, Germany, with permission of Dr. Fritz Melchers, Basel Institute of Immunology, Basel, Switzerland. LM34 was biotin labeled with a sulfo-NHS-biotinylation kit (Pierce, Rockford, IL). Panning to remove B cells in some experiments was performed as reported (2). Bone marrow $CD43^+B220^+$ and both large and small $CD43^-B220^+$ or $CD25^+B220^+$ cells were separated on a FACStar^{Plus} cell sorter (Becton Dickinson). Purity of the sorted populations was >95% according to reanalysis for surface stains.

In vitro expansion of IL-7-dependent pro-B/pre-B cells

Bone marrow pro-B/pre-B cells were expanded after culture with recombinant murine IL-7 as previously described (2).

Cell cycle analysis

Sorted $CD25^+$ $B220^+$ cells were stained with propidium iodide after ethanol fixation and RNase treatment (2).

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[3] Abbreviation used in this paper: G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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SDS-PAGE and Western blot analysis

In vitro IL-7 expanded populations of bone marrow pro-B/pre-B cells were harvested and lysed in detergent buffers as described (9) and electrophoresed on 4 to 12% SDS-PAGE gels under reducing conditions. Blotted and blocked nitrocellulose membranes were incubated with the hamster anti-λ5 mAb FS1 (10) or with affinity-purified rabbit anti-actin IgG Ab and appropriate horseradish peroxidase-labeled secondary Abs. Membranes were developed by chemiluminescence and exposed to Hyperfilm (Amersham, Arlington Heights, IL).

RNA isolation and RT-PCR analysis

B-lineage cells were sorted from young and aged mice and pelleted, and polyadenylated mRNA were extracted using a Quick Prep Micro mRNA purification kit (Pharmacia Biotech, Piscataway, NJ) or sorted directly into TRIzol reagent (Life Technologies, Gaithersburg, MD) and total RNA extracted. RNA was reverse transcribed to cDNA with murine leukemia virus reverse transcriptase and amplified using the GeneAmp PCR kit with AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). PCR primers are specific for murine λ5 (spanning exons 1 through 3 (11); (amplicon size, 361 bp) or for murine glyceraldehyde-3-phosphate dehydrogenase (G3PDH)[3] cDNA (Clontech Laboratories, Palo Alto, CA; amplicon size, 452 bp). PCR amplification was performed with modified hot start for 30 cycles with 1 cycle consisting of 95°C for 2 min, 60°C for 2 min, and 72°C for 2 min, with 1 additional extension step at 72°C for 3 min. PCR products were Southern blotted, hybridized to gene-specific radiolabeled probes, and densitometrically quantitated.

Statistics

The significance of the results was evaluated by Student's *t* test.

Results and Discussion

The reduced numbers of pre-B cells in senescent mice could result from failure to generate new pre-B cells from the pro-B cell pool, reduced transition of early pre-B cells to later developmental stages, altered kinetics of transit through the pre-B compartments, and/or to limited expansion of these pre-B cell populations. To address this issue, we assessed not only pro-B cell but also both early ($CD43^+$) and later stage ($CD43^-$) pre-B cell populations in aged BALB/c mice. As shown in Figure 1*A*, $CD43^+CD25^-B220^{low}$ pro-B cells were only slightly reduced and early pre-B cells ($CD43^+CD25^+B220^+$) were not significantly reduced in aged bone marrow. However, decreased numbers of both large and small $CD43^-CD25^+B220^{low}$ pre-B/B cells were seen in aged mice. CD43 (S7) has been shown to be expressed on B1 cells in the periphery (12) and aged mice have been reported to have increased numbers of B1 cells (13). To ensure that B cells were not contributing to $CD43^+$ B-lineage populations, bone marrow from four aged mice identified as deficient in small pre-B/B cells and bone marrow from four young mice were depleted of B cells and reanalyzed for CD43, CD25, and B220 expression.

As shown in Figure 1*B*, the proportion of surface $Ig^-CD43^+CD25^+B220^{low}$ early pre-B cells was largely retained although proportions of $Ig^-CD43^-CD25^+B220^{low}$ large and small pre-B cells were extensively reduced, results analogous to those obtained with unfractionated bone marrow cells (Fig. 1*A*). We conclude that pro-B cell and early pre-B cell formation occurs relatively normally in senescent BALB/c mice; however, the transition from nascent $CD43^+$ pre-B cells to the $CD43^-$ pre-B cell compartments is rendered less efficient. Numbers of nucleated viable bone marrow cells were not statistically different between young and senescent BALB/c mice (young, $3.3 \pm 0.9 \times 10^7$ cells; aged, $3.9 \pm 1.2 \times 10^7$ cells per femur/tibia pair); therefore, alterations in the proportions of B-lineage cells seen in senescent BALB/c bone marrow also reflect changes in their absolute numbers.

Since the transition from early ($CD43^+$) to later ($CD43^-$) pre-B cell stages is dependent on expression of the pre-B cell receptor (μ/λ5/VpreB) (5–7), decreased formation of pre-B cells in aged

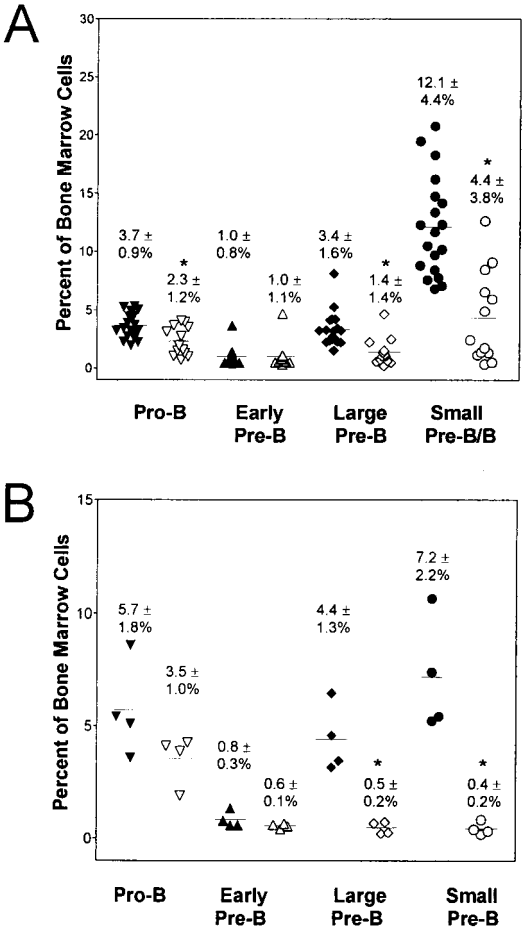

FIGURE 1. Aged BALB/c mice exhibit loss of late stage pre-B cells, but retention of early pre-B cells. *A*, Bone marrow cells from 19 individual BALB/c mice at 3 to 6 mo of age and from 13 BALB/c mice at 24 to 29 mo of age were analyzed for surface CD43 (PE-S7), CD25 (biotin-D7/CyChrome streptavidin), and B220 (FITC-RA3-6B2). The percentage of total nucleated bone marrow cells for each population is shown. $CD43^+CD25^-B220^{low}$ cells are designated as pro-B cells, $CD43^+CD25^+B220^{low}$ cells are early pre-B cells, and $CD43^-CD25^+B220^{low}$ cells are designated as large and small pre-B/B cells. Dark symbols are for young mice; clear symbols for aged mice. In *B*, to eliminate B cells from the pre-B/B cell populations for flow cytometry analysis, bone marrow cells from four individual young (3-mo-old) and aged (22–24-mo-old) BALB/c mice were first panned on anti-IgM/IgG-coated plates and then stained and analyzed as in *A*. These aged mice were previously shown to have significantly decreased proportions of $CD43^-$ $B220^{low}$ bone marrow cells. ∗, data from the aged group are significantly different from the young group at $p < 0.05$.

mice could result from diminished expression or function of the pre-B cell receptor complex. Expression of μ heavy chain in early pre-B cells appears unaffected by the aging process (data not shown); therefore, we have assessed the expression of the λ5 surrogate light chain. As shown in Figure 2, $CD43^+B220^{low}$ pro-B/early pre-B cells from young BALB/c mice exhibited both surface λ5 staining and cytoplasmic λ5 staining by the LM34 mAb, with 73 ± 9% ($n = 10$; range, 58–87%) of these B-lineage precursors expressing levels of cytoplasmic λ5 protein staining above that seen with negative controls. In contrast, $CD43^+B220^{low}$ pro-B/early pre-B cells present in senescent BALB/c mice generally expressed decreased surface and cytoplasmic λ5 protein (55 ± 20% cytoplasmic $\lambda5^+$ (range, 13–85%), $n = 12$, with $p < 0.05$ when

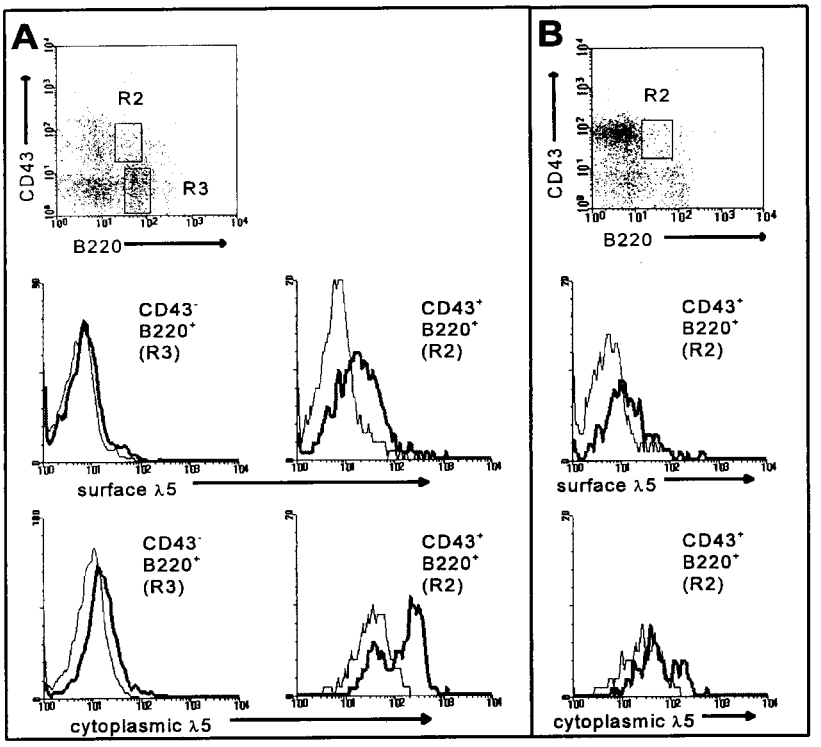

FIGURE 2. Decreased surface and cytoplasmic expression of λ5 protein by aged B-lineage cells. BALB/c bone marrow cells from young 3- to 6-mo old BALB/c mice (*A*) and aged 22- to 29-mo-old BALB/c mice (*B*) were stained for CD43 (PE-S7) and B220 (FITC-RA3-6B2) as well as for λ5 (biotin-LM34/CyChrome streptavidin). Staining for λ5 was performed on intact cells (surface staining) or after fixation and permeabilization (cytoplasmic staining). The λ5 staining for gated $CD43^-B220^+$ pre-B/B cells and $CD43^+B220^+$ pro-B/early pre-B cells is shown. Data are representative of 10 young and 12 aged BALB/c mice examined. Light lines are staining with secondary CyChrome streptavidin reagent alone.

compared with values for young mice). Negligible λ5 protein, either on the surface or within the cytoplasm, was detected among late stage $CD43^-$ $B220^+$ pre-B/B cells.

As shown in Figure 1*A*, some aged mice have proportions of late stage B cell precursors which overlap those seen with young mice, while markedly lower levels of B cell precursors are clearly seen in other aged mice. Bone marrow cells from five aged mice which had "young-like" $CD43^-CD25^+B220^{low}$ small pre-B/B cell levels (4.9–12.6%; average, 7.7 ± 3.0%) exhibited staining for cytoplasmic λ5 protein (62 ± 23%) in their $CD43^+$ $B220^{low}$ pro-B/early pre-B cells which was not significantly different from that seen with young mice. In contrast, pro-B/early pre-B cells from five aged mice identified as having low pre-B/B cell proportions (0.4–1.4%; average, 1.0 ± 0.5%) had reduced staining for cytoplasmic λ5 protein (41 ± 16%). Therefore, even within the aged mouse population, levels of cytoplasmic λ5 protein in pro-B/early pre-B cells generally correlated with proportions of late stage B lineage precursor cells.

The decreased expression of λ5 protein among senescent B lineage precursor cells in vivo was also seen under in vitro culture conditions. In vitro IL-7-expanded pro-B/early pre-B cells from both young and aged BALB/c mice were uniformly $CD43^+$, BP-1^+, and $B220^+$; variably positive for CD25 (10–90%); and ≤25% cytoplasmic μ $chain^+$. Pro-B/pre-B cells from senescent BALB/c mice had λ5 protein levels as determined by Western blot analysis which were generally decreased by two- to fourfold (Fig. 3).

The relative levels of λ5 mRNA were also determined, by semiquantitative RT-PCR, in ex vivo bone marrow cells from young and aged mice. In five aged mice tested, the levels of bone marrow λ5 mRNA were significantly reduced when compared with young bone marrow cells (data not shown). When sorted populations of $CD43^+$ $B220^+$ pro-B/early pre-B cells as well as $CD43^-B220^+$ pre-B/B cells from senescent BALB/c mice were analyzed for λ5 mRNA expression, levels of λ5 mRNA were decreased by ~three- to fourfold (Fig. 4).

The reduced expression of λ5 surrogate light chains among pro-B/early pre-B cells would be anticipated to have substantial ramifications for B lymphopoiesis. Expression of λ5 is not essential for development and maintenance of $CD43^+B220^+$ pro-B cells (14). Therefore, in senescent BALB/c mice, reduced λ5 surrogate light chain expression would not be expected to dramatically alter pro-B/early pre-B cell numbers, consistent with our observations. However, given the importance of the pre-B cell receptor in the progression and expansion of early pre-B cells, population of the $CD43^-$ pre-B cell compartments in aged mice would be compromised if reduced λ5 levels result in decreased assembly or surface expression of pre-B cell receptors.

We have also directly assessed the mitotic activity of the reduced population of large pre-B cells in aged mice. Sorted populations of large $CD25^+B220^+$ pre-B cells from five senescent

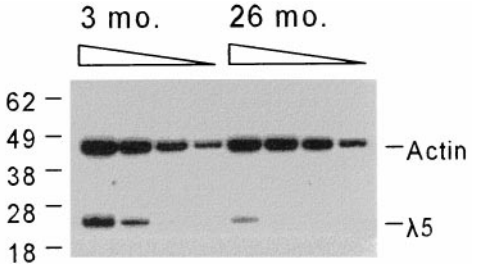

FIGURE 3. IL-7-stimulated pro-B/pre-B cells from aged BALB/c mice express decreased λ5 protein. Bone marrow cells from 3- and 26-mo-old BALB/c mice were stimulated in vitro with IL-7 as described in *Materials and Methods*. Expanded populations of pro-B/pre-B cells were harvested 7 days after initiation of culture, and detergent lysates were prepared. Lysates from equivalent numbers (50, 25, 12.5, 6.25 × 10^4) of harvested pro-B/pre-B cells were separated by SDS-PAGE, Western blotted, and λ5 protein detected with FS1 Ab and actin protein detected with rabbit anti-actin Ab with chemiluminescent development as described in *Materials and Methods*. Results are representative of 12 analogous experiments.

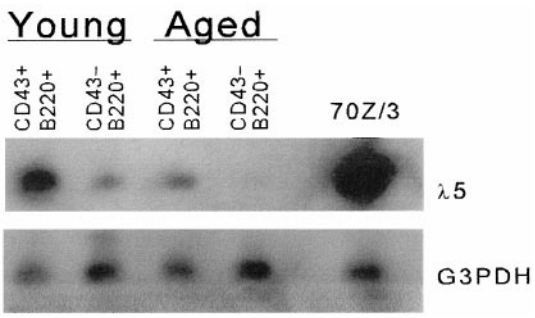

FIGURE 4. Decreased λ5 mRNA expression in aged B-lineage cells. Polyadenylated RNA was isolated from sorted bone marrow B lineage precursors as described in *Materials and Methods*. Polyadenylated RNA was prepared from 8.9×10^4 to 1.4×10^5 $CD43^+B220^+$ cells and from 3.3×10^5 to 1.3×10^6 $CD43^-B220^+$ cells from young and aged mice. One-fifth of the RNA sample was used for reverse transcription and one-fourth of the reverse transcriptase preparation was used for PCR with either λ5 or G3PDH primers. *Lane 1*, Young (3 mo) $CD43^+B220^+$ pro-B/early pre-B cells; *Lane 2*, Young (3 mo) $CD43^-B220^+$ late stage pre-B/immature B cells; *Lane 3*, old (24 mo) $CD43^+B220^+$ pro-B/early pre-B cells; *Lane 4*, old (24 mo) $CD43^-B220^+$ late stage pre-B/immature B cells; *Lane 5*, 70Z/3 pre-B cells as positive control. Data are representative of sorted cell populations from one of two experiments. Densitometric scans of λ5 bands were normalized to those of G3PDH.

BALB/c mice had 61 ± 7% of cells in S + G_2-M, comparable with results obtained with sorted large $CD25^+B220^+$ pre-B cells from young mice (61 ± 4%, $n = 4$) (data not shown). Therefore, although decreased in number, the large pre-B cells in aged mice retain their characteristic high mitotic activity. We postulate that these pre-B cells probably express levels of pre-B cell receptors appropriate to provide the requisite signals for proliferation. Therefore, the decline in surrogate light chain expression may affect the numbers of nascent pre-B cells recruited into the highly mitotically active compartment, but not their capacity to undergo division.

While our hypothesis suggests that decreased λ5 expression may ultimately affect the production of large and small $CD43^-$ pre-B cells in senescence, it is also possible that the decline in late stage B cell precursors seen in aged mice results from altered transit through the pre-B cell compartments or to enhanced emigration from the bone marrow to the periphery. However, recent kinetic studies suggest that the loss of late stage pre-B cells in aged mice reflects neither more rapid progression through the pre-B cell stage nor altered turnover rates among pro-B cells (15, 16). Previous studies have shown that surface λ5 can be up-regulated on pro-B cells by culture with the supportive bone marrow stromal cell line, FLST2, together with IL-7 (10). Stromal cells isolated from senescent BALB/c mice have been shown to be poorer in their capacity to support the growth of pro-B cells in vitro (4), implying functional differences in stromal cells concomitant with the aging process. Alternatively, reduced responsiveness of B cell precursors from aged mice to stromal cell-derived signals may also contribute to poor surrogate light chain expression.

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