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In Old BALB/c Mice, Bone Marrow Pre-B Cell and Surrogate Light Chain Reduction Is Associated With Increased B Cell Reactivity to Phosphorylcholine, But Reduced T15 Idiotypic Dominance

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Abstract

In young adult BALB/c mice, antibodies to phosphorylcholine (PC) bearing the T15 (TEPC 15) idiotype confer protection against pneumococcal infections. In old age, even though PC reactive B cells are often increased, the proportion of T15⁺ antibodies declines. We hypothesize that limited surrogate light chain (SLC) and compromise of the pre-B cell receptor checkpoint in old mice contribute to both reduced new B cell generation and changes in the anti-PC antibodies seen in old age. In old mice: 1) early pre-B cell loss is most pronounced at the preBCR checkpoint; however, the reduced pool of early pre-B cells continues to proliferate consistent with preBCR signaling; 2) increased PC reactivity is seen in bone marrow immature B cells; 3) deficient SLC promotes increased B cell PC reactivity and diminished T15 idiotype within a subset of young adult mice; and 4) in old mice, as pre-B cell losses and reduced SLC become progressively more severe, increased T15 negative PC reactive B cells occur. These results associate a reduction in pre-B cells, imposed at the preBCR checkpoint, with increased reactivity to PC, but more limited expression of the protective T15 idiotype among PC reactive antibodies in old age.

Keywords

Aging; immature B cells; pre-B cell receptor; surrogate light chain; T15 idiotype; phosphorylcholine

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AUTHOR CONTRIBUTIONS

Kelly Khomtchouk, Sarah Alter, and Michelle Ratliff performed the experiments, analyzed data, and assisted in writing the paper. Bonnie B. Blomberg was involved in experimental design, data analysis, and assisted in writing the paper. Richard L. Riley oversaw the project, assisted in data analysis and experimental design, and assisted in writing the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest

INTRODUCTION

B cell production is reduced in the bone marrow of mice with advanced age (~ 2 years old)^{1,2} and, while controversial^{3,4}, similar changes may occur in elderly humans^{5,6}. While reduced development of B cell precursors within the bone marrow in old age has been extensively studied in the mouse model, far less is known about the generation of new B cells in old age and how this affects the repertoire of antibody specificities available for immune responses. In aging humans, the primary B cell repertoire is less diverse and this coincides with increased morbidity and mortality⁷. Therefore, understanding the mechanisms that regulate the B cell repertoire, and the alterations that occur in old age, may be of considerable importance not only in mice, but also in humans as well.

Alterations in the immune responses to the phosphorylcholine (PC) epitope in the mouse model, first described almost three decades ago, still remains a well-characterized example of an antigen-specific B cell repertoire change accompanying old age^{8–10}. PC is an immunodominant epitope on pneumococci and, in young adult mice, antibodies reactive with the PC epitope, and particularly those antibodies that express the high affinity T15 (TEPC 15) idiotype, are necessary for protective immunity^{11,12}. In old BALB/c mice, while T15⁺ anti-PC antibodies continue to be produced, an upsurge in production of lower affinity T15^{neg} anti-PC antibodies dilutes the protective response^{8–10} and, consequently, old BALB/c mice exhibit higher morbidity and mortality to pneumococcal infections⁸.

Notably, PC functions not only as a bacterial epitope, but also as a self-antigen expressed on apoptotic cells and anti-PC antibodies likely are involved in their clearance^{13–15}. Moreover, T15⁺ anti-PC antibodies bind to oxidized phospholipids found in atherosclerotic lesions¹³. In humans, increased levels of IgM anti-PC antibodies are associated with diminished risk for cardiovascular disease and stroke^{16,17}. Therefore, humoral immunity to PC functions in normal tissue homeostasis as well as in combating infection. Consequently, understanding the mechanisms responsible for altering the expression of PC-reactive B cells in old age has considerable importance.

Previous studies by Zharhary and Klinman⁹ and Riley, et al.¹⁰, demonstrated that alterations in the PC antibody response seen in primary (naïve) splenic B cells in old mice were also seen among newly formed B cells in the bone marrow. The mechanisms responsible for these changes in B cell specificity with old age have not been determined. We^{18–22} and others (reviewed in [1, 2]) have shown that old mice of several inbred strains have impaired B lymphopoiesis and, in particular, substantially reduced pre-B cells in the bone marrow. We have extensively studied the reduced expression of the surrogate light chain (SLC) in B cell precursors in old age^{18–21}. The SLC proteins $\lambda 5$ and VpreB pair with μ Ig heavy chain to form the preBCR^{23–28}. Low SLC expression likely impairs the functions of the pre-B cell receptor (preBCR) that are critical for both Ig heavy chain variable region selection and pre-B cell clonal expansion^{23–28}.

In this report, we show that the increased reactivity of B cells to PC, and the diminished representation of the T15 idiotype, among anti-PC antibodies in old BALB/c mice coincides

with reduced pre-B cell generation in old age. These findings provide a mechanism which may contribute to the poor humoral immunity to PC-expressing pathogens in old mice.

MATERIALS AND METHODS

Mice

Young (2–5 months old) and aged (24–27 months old) BALB/c female mice were obtained from the National Institute on Aging. Aged animals with obvious malignancies or abdominal tumors were not included in these studies. Young (2–6 months old) C57BL/6 mice were obtained from the Jackson Laboratories, Bar Harbor, ME. Young (2–6 months old) B6.129S2-Igll1^{bm1Cgn}/J (λ 5 gene knockout) mice were originally obtained from the Jackson Laboratories and bred in-house. Animal protocols were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

Surface staining and isolation of B cells

Bone marrow cells were stained for IgM, CD23, CD43/S7, CD19, CD45R (B220) and CD93 (AA4.1) as described in Ratliff et al.^{20,29}. In some experiments, bone marrow cells were stained for determination of Hardy fractions using fluorescent antibodies for surface BP-1, CD24 (heat stable antigen, HSA), CD43/S7, and B220 (CD45R) as described previously³⁰. This allowed discrimination of Hardy Fraction subsets based on staining of B220⁺ cells: CD43⁺ CD24⁻ BP-1⁻ pre-pro-B cells (Fr. A); CD43⁺ CD24⁺ BP-1⁻ pro-B cells (Fr. B); CD43⁺ CD24⁺ BP-1⁺ pro-B cells (Fr. C); and CD43⁺ BP-1⁺ CD24^{high} early pre-B cells (Fr. C'). Late pre-B cells (Fr. D) were identified as surface IgM⁻ CD43⁻ B220⁺ cells. Monoclonal antibodies, as well as isotype controls, labeled with appropriate fluorochromes or biotin and fluorochrome labeled streptavidin, were purchased from BD Biosciences. Appropriate isotype controls labeled with fluorochromes were also obtained from BD Biosciences. Cells were analyzed on either an LSR-Fortessa or a FACS Canto fluorescence flow cytometer (BD Biosciences). Bone marrow immature B cells (surface IgM⁺ AA4.1⁺ CD19⁺) were separated into CD23⁻ and CD23⁺ subsets by fluorescence activated cell sorting using a FACS Aria II (BD Bioscience). Cells were sorted to a purity of 97–99%. Splenic follicular (FO) B cells were isolated either by fluorescence activated cell sorting or by magnetic bead sorting as in Ratliff et al.^{20,29}

Mitotic activity in Hardy Fractions was assessed upon staining with Hoescht 33342 dye (Molecular Probes, Eugene, OR) to label DNA. Cells stained for Hardy Fractions were washed in PBS containing 0.1% glucose, resuspended in 5pg/ml Hoescht dye in PBS and then incubated for 30 minutes at 37°C prior to analysis. Cells were identified as in G0/G1 (2N) vs. S/G2/M (>2N) stages of cell cycle via fluorescence flow cytometry analysis.

Identification of phosphorylcholine reactive B cells by ELISpot

Subsets of CD23⁻ and CD23⁺ immature bone marrow B cells or splenic follicular (FO) B cells were cultured at 1–2×10⁶ cells/ml in GlutaMax RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (low endotoxin; Sigma-Aldrich, St. Louis, MO) plus 1% penicillin-streptomycin and 5 × 10⁻⁵ M 2-mercaptoethanol. Cultures were stimulated with LPS (*E. coli* 055:B5; 10µg/ml) for 4–5 days. Collected cells were then assayed via ELISpot

on phosphorylcholine (PC₂)-BSA (Biosearch, Petaluma, CA) coated plastic plates as described in Ratliff et al.²⁰. Total IgM secreting cells were also assessed on anti-IgM coated plates by ELISpot as in Ratliff et al.²⁰. Serial dilutions of cells were plated and antibody secreting cells (ASC) detected to determine the frequency of ASC by linear regression analysis²⁰. In LPS stimulated cultures, >90% of ASC reactive to PC secreted IgM. LPS stimulation showed experimental variation as measured by IgM ASC per 10³ isolated B cells. The average and standard deviation (SD) of IgM ASC/10³ cells for B cell subsets in young mice were: FO splenic B cells, 57 ± 51; CD23⁻ immature bone marrow B cells 35 ± 34; and CD23⁺ immature bone marrow B cells 25 ± 19. From old mice, IgM ASC/10³ were: FO splenic B cells 109 ± 73; CD23⁻ immature bone marrow B cells 58 ± 62; and CD23⁺ immature bone marrow B cells 36 ± 39. There were no statistically significant differences in the IgM ASC responses obtained for any B cell subset between young adult and aged mice. In the absence of LPS, IgM responses were only ~10% of that seen upon LPS stimulation.

Given the variability in IgM ASC derived per 10³ cells after culture with LPS, PC-reactive ASC were normalized to the number of total IgM antibody secreting cells.

Identification of the T15 idotype

T15 idotype expression was detected on PC reactive antibodies from LPS stimulated B cells in ELISpot assays. LPS stimulated B cells were cultured on PC₂-BSA coated plates. After washing, plates were developed with the anti-T15 IgG1 mAb HB33/AB1-2 (BioXCell, W. Lebanon, NH) followed by HRP-anti-IgG1 specific antibody (Jackson Immunoresearch, West Grove, PA) and colorimetric ELISpot analysis to identify PC-reactive, T15 idotype positive ASC. AB1-2 recognizes a conformational epitope generated by both the Ig heavy chain and κ light chain of T15³¹. Anti-PC antibodies recognized by AB1-2 bear close similarity, albeit not necessarily complete variable region identity, to the prototypic germ-line T15 antibody³². The incidence of PC reactive antibody secreting cells expressing the T15 idotype as detected via AB1-2 binding was compared to the total incidence of PC reactive antibody secreting cells (see above) and percentages of T15⁺ PC reactive ASC were estimated.

In some experiments, ELISA assays were used to assess T15 idotype expression on anti-PC antibodies in supernatants from LPS stimulated B cells after 4–5 days in culture. T15 expressing antibodies were captured plates coated with the anti-T15 idotype antibody AB1-2. Development of these plates was with anti-IgM-alkaline phosphatase (AP) and used an ELISA Amplification System (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Binding of supernatant IgM to AB1-2 (anti-T15) coated plates was compared to the binding of supernatant IgM to PC₂-BSA plates to estimate proportions of T15⁺ anti-PC IgM antibodies. The results of this ELISA assay for T15 were compared, in several experiments, to a competitive assay based on T15 idotype assays described previously^{9,10}. In the competitive ELISA, AB1-2 (10µg/ml) was coated onto MaxiSorb plates (Thermo Scientific, Waltham, MA). Supernatants from LPS stimulated B cells (25µl neat or appropriately diluted) were added to the AB1-2 coated plates followed by addition of 2ng of AP labeled-T15 antibody (TEPC 15; Sigma Aldrich). The extent of competition for

labeled T15 was compared to that for graded concentrations of T15 protein. The amounts of T15 antibody estimated by the solid phase ELISA methods were comparable to those obtained by competitive ELISA.

Labeling of immature bone marrow and splenic transitional B cells with BrdU

Mice were administered 5-bromo-2'-deoxyuridine (BrdU) in drinking water (1mg/ml) supplemented with 5% glucose for a period of 3 days. Cells were stained for surface antigens for immature B cells as described above and for BrdU incorporation with a BD BrdU Flow Kit as previously reported³³.

Statistical Analysis

Groups were compared by unpaired two-tailed Student's t-test or Mann-Whitney U-test with p values shown. P values <0.050 were considered significant

RESULTS

In old mice, loss of pre-B cells coincides with reduced $\lambda 5$ SLC, but proliferation is retained among residual pre-B cells

Our previous studies have shown that a decline in B cell precursors is variable in individual old mice²². Hardy, et al.³⁰, divided B cell precursors into Hardy Fractions A (prepro-B), B and C (pro-B), C' (early pre-B), and D (late pre-B/immature B). In young adult $\lambda 5$ gene knockout mice, which are incapable of preBCR expression, Hardy Fraction C' is highly reduced³⁴, indicating that Fraction C' is at the preBCR checkpoint. In the old BALB/c mice shown in Figure 1A, Fraction D is extensively reduced (~10% of young controls), the pro-B cell Fractions B and C are partially reduced (~40% of young controls) and Fraction C' pre-B cells are highly reduced (~15% of young controls). We have called old BALB/c mice with such a substantial deficit in pre-B cells (>70% loss), "severely pre-B cell depleted"²². As shown in Figure 1B, the late stage pre-B cell losses seen in individual old BALB/c mice are strongly associated with the reductions seen in $\lambda 5$ SLC protein at the pro-B cell stage. This would be anticipated if reduced capacity to assemble the preBCR, and reduced signaling for early pre-B cell expansion, contributes to loss of later stage pre-B cells in old age.

Decline in SLC expression in old B cell precursors may impact the expression of the preBCR in early pre-B cells and reduce proliferation driven by preBCR signals^{23,24,28}. As previously reported³⁰, and shown in Figure 1C, B cell precursors at Hardy Fraction C', e.g., at the preBCR checkpoint, from young adult mice undergo extensive mitosis (~80% in S/G2/M stages of cell cycle). While early pre-B cells (Hardy Fraction C') transiting the preBCR checkpoint are highly reduced in old mice (Fig 1A), these residual Hardy Fraction C' pre-B cells continue to exhibit high levels of proliferation comparable to that seen in young adults. Therefore, early pre-B cells in old mice, albeit fewer in number, appear to undergo proliferation consistent with preBCR signaling. The effects of deficient SLC expression and reduced pre-B generation on the development of immature B cells were next examined.

Immature B cells are heterogeneous based on expression of CD23 and CD43/S7 in young and old BALB/c mice

Increased reactivity to PC has been shown previously among newly derived bone marrow immature B cells in old BALB/c mice^{9,10}. However, it has become apparent that immature B cells are themselves a heterogeneous population. In particular, Lindsley, et al.³⁵, have shown that most immature B cells in young adult bone marrow lack CD23. CD23 serves as a “low affinity 201D; IgE receptor, an activation antigen, and a C-type lectin involved in cell-cell interactions³⁶. CD23 is typically expressed after migration of new B cells from bone marrow to the spleen (e.g., at the transitional T1 [CD23⁻] → T2 [CD23⁺] transition). However, a minor subset of newly generated B cells develops early expression of CD23 while still within the bone marrow³⁵. This early expression of CD23 by bone marrow immature B cells is a likely consequence of activation, possibly driven via the BCR and antigen selection, and is dependent upon Bruton’s tyrosine kinase (Btk)³⁵. We have also described subsets of immature B cells based on differential expression of CD43/S7, which is also associated with activation and Btk expression, and described changes in this population in old age^{19,33}.

As a consequence of this heterogeneity, any changes in antibody repertoires seen among immature B cells in old age may reflect changes in the early expression of new B cell specificities or, alternatively, changes in the representation of particular B cell subsets within the immature B cell pool. In order to assess this, we determined the representation of immature B cells of varying phenotypes in the bone marrow of old BALB/c mice and their reactivity to PC antigens.

As shown in Figure 2A,B, in old BALB/c mice (severely pre-B depleted), total immature B cells (IgM⁺ AA4.1⁺ CD19⁺) were reduced; however, this was particularly evident for those immature B cells that did not express CD23. While CD23⁻ immature B cell numbers in old bone marrow were reduced by ~4-fold; CD23⁺ immature B cells were reduced to a lesser extent (~2-fold). This resulted in an increase in the proportion of CD23⁺ immature B cells in the bone marrow from ~20% in young adults to ~30% in old BALB/c mice (Fig. 2B).

We also noted heterogeneity of expression of CD43/S7 on immature B cells within the bone marrow of old mice. Optimal CD43/S7 expression on immature B cells, like CD23, requires Btk, and consequently BCR signaling³⁸. We have previously reported that CD43/S7 is more prevalent on immature B cells from old mice and that new B cells with CD43/S7 expression are generated more readily from B cell precursors in old mice^{19,33}. Together with the finding that CD23 expression is more prevalent on immature B cells from old mice, increased CD43/S7 expression on new B cells in old mice likely represents increased activation of these B cells^{19,33}.

As shown in Figure 3A,B, the expression of CD43/S7 was very low (~8%) among CD23⁻ immature B cells in young mice, but higher on CD23⁺ immature B cells (~25%). In old BALB/c mice, CD43/S7 expression was surprisingly observed on a larger fraction of CD23⁻ immature B cells, e.g., ~30–40% on average (Fig. 3A,B). Approximately 70% of CD23⁺ immature B cells in old mice expressed CD43/S7. The co-expression of CD23 and CD43/S7 on a subset of immature B cells in young and old mice is consistent with both serving as activation induced surface antigens.

As shown in Figure 3C, while CD23⁻ CD43/S7⁻ and CD23⁺ CD43/S7⁻ immature B cells were reduced by ~80% in old BALB/c bone marrow, both CD43/S7⁺ CD23⁻ and CD43/S7⁺ CD23⁺ immature B cell subsets declined little in senescence. Consequently, CD43/S7 expression defines subsets of immature B cells within both the CD23⁻ and CD23⁺ immature B cell pools which are better maintained in old BALB/c mice, while CD43/S7⁻ CD23⁻ and CD43/S7⁻ CD23⁺ immature B cells decrease substantially within the bone marrow.

We note that CD43/S7⁺ immature B cells continue to be expressed within the bone marrow of old mice. As previously described, these also show increased usage of VhS107.1³³, a commonly used Vh gene for encoding anti-PC antibodies, and have, not surprisingly, increased reactivity with PC antigen as discussed below. This suggests that even as pre-B and immature B cells in general are diminished in old bone marrow, the capacity to produce new B cells which undergo activation, and express CD43/S7 and also CD23, remains relatively intact.

In old mice, do bone marrow immature B cells continue to populate the splenic mature B cell pool

We analyzed 5 old BALB/c mice where the numbers of CD23⁻ immature bone marrow B cells were ~60% reduced and CD23⁺ immature bone marrow B cells were reduced by ~40% (Fig. 4A) compared to young adult mice. In the spleens of these aged mice, T1 (IgM^{hi} CD23⁻ AA4.1⁺ CD19⁺) transitional splenic B cells, representing new emigrants from the bone marrow³⁷, were reduced by ~70% compared to young adults (Fig. 4A) while T2 (IgM^{hi} CD23⁺ AA4.1⁺ CD19⁺) transitional splenic B cells were reduced by ~45%. Given the paradigm that T2 transitional B cells derive from the T1 transitional B cell pool³⁷, the numbers of T2 splenic B cells in old mice appear disproportionately high.

We also fed BrdU in the drinking water of these mice as a means to label newly formed B cells within the bone marrow and track their progress to the spleen. Bone marrow immature and splenic transitional B cells in both young and old mice are not mitotically active^{33,37}. In our study, <7% of immature bone marrow B cells and T1 and T2 transitional splenic B cells from young and old mice were in S/G2/M stages of cell cycle (data not shown). Bone marrow immature B cells and splenic transitional B cells derive from proliferating bone marrow B cell precursors which readily incorporate BrdU into DNA³⁷.

Approximately 95% of CD23⁻ and ~76% of CD23⁺ immature bone marrow B cells in young adults were BrdU labeled at day 3 (Fig. 4B), consistent with their relatively rapid turnover and passage from the bone marrow to the spleen³⁷. Substantially fewer (~62% and ~44% respectively) CD23⁻ and CD23⁺ immature B cells in old bone marrow were BrdU labeled over the 3 day period (Fig. 4B). This suggests that some immature B cells are relatively “stagnant” within the bone marrow of old mice and turnover more slowly, comparable with previous results^{39,40}. Consistent with diminished production of new B cells, the numbers of CD23⁻ immature bone marrow B cells which were labeled with BrdU over 3 days was ~4-fold reduced in old mice (Fig. 4B). However, the reduction seen in BrdU labeled CD23⁺ immature B cells in old bone marrow was only ~2-fold.

In the spleens of old BALB/c mice, after 3 days of BrdU administration, both T1 (CD23⁻) and T2 (CD23⁺) transitional B cells were poorly labeled compared to young controls (~75% labeled in young T1 vs. ~23% in old T1 and ~51% labeled in young T2 vs. ~17% labeled in old T2 B cells; Fig. 4B). Moreover, in numbers, BrdU labeled T1 splenic B cells were diminished ~17-fold compared to young controls (Fig. 4B). In contrast, BrdU labeled T2 splenic transitional B cells in old spleen were reduced by a sizeable amount, ~7-fold less than seen in young controls, but not to the extent expected given the very low numbers of T1 transitional B cells (Fig. 4B).

Immature B cells in old BALB/c bone marrow are enriched for PC reactivity

Immature B cells from the bone marrow of BALB/c young and aged mice were polyclonally activated with LPS in vitro and the reactivity of the resultant antibody secreting cells (ASC) to PC was measured in ELISpot assays. As shown in Figure 5A, CD23⁺ immature B cells from young adult BALB/c mice had ~7-fold greater reactivity to PC (~100 PC reactive ASC/10³ IgM ASC) than was exhibited by CD23⁻ immature B cells (~15 PC reactive ASC/10³ IgM ASC).

While in old BALB/c mice the incidences of PC reactive ASC derived from CD23⁺ immature B cells were comparable to those seen in young adults, the anti-PC reactivity from CD23⁻ immature B cells from aged mice was significantly higher (~4-fold) than was seen with analogous young adult B cells (Fig. 5A). Most of the increases (~70%) seen in the PC reactivity of all immature bone marrow B cells in old mice resulted from the increased PC reactivity seen in the CD23⁻ immature B cell subset. We next addressed whether deficiencies in SLC expression, resulting in poor pre-B and immature B cell generation, would be sufficient to alter immature B cell phenotypes and PC reactivity even in young adults.

SLC λ 5 deficiency in young adults alters immature B cell phenotypes and PC reactivity to resemble old age

As shown in Figure 6A, young adult λ 5 deficient mice had reduced numbers of immature B cells (as expected given their low pre-B cell numbers^{34,41}). Both CD23⁻ and CD23⁺ immature B cell subsets were reduced in the λ 5 knockout and λ 5 heterozygous young adult mice (Fig. 6A) and, as seen in old mice, the decline in CD23⁻ immature B cells in λ 5 knockout mice was somewhat greater than was seen in CD23⁺ immature B cells (e.g., CD23⁻, ~90% loss; CD23⁺, ~80% loss). Like old mice, young adult mice fully deficient or partially deficient in λ 5 expression had immature bone marrow B cells that were enriched for CD43/S7 expression (Fig. 6B).

As shown in Figure 6C, λ 5 heterozygous bone marrow B cells were enriched for PC reactivity (~3-fold) and λ 5 knockout bone marrow B cells had ~7-fold increased PC reactivity compared to wild-type young adult bone marrow B cells. These results indicated that deficiencies in SLC expression in young adult mice was sufficient to cause, directly or indirectly, 1) significant losses of immature B cells; 2) alterations in the phenotype and composition of immature B cells; and 3) increased PC reactivity among B cells.

In old mice as well as young adults, deficiency in $\lambda 5$ expression coincides with increased T15^{neg} anti-PC B cell reactivity

In BALB/c mice, old age has been shown to be associated with increased incidences of PC-reactive B cells and, in particular, a substantial increase in PC-reactive B cells that fail to express the T15 idiotype^{8–10,32}. This results in the overall anti-PC antibody responses in old BALB/c mice becoming predominantly T15^{neg}^{8–10,32}. We asked if loss of pre-B cells in old BALB/c mice, concomitant with reduced SLC expression, was associated with changes in PC reactivity and loss of T15 idiotype predominance.

In old BALB/c mice, we have seen increased PC reactivity among both immature bone marrow B cells and splenic FO B cells²⁰. Notably, while in young adults PC reactive ASC were highly T15⁺, FO B cells from “severely pre-B depleted” old BALB/c mice generated PC reactive ASC that were predominantly T15^{neg} (Fig. 7A). In those severely pre-B depleted old BALB/c mice where T15 expression was reduced, the decrease seen in T15 idiotype expression among anti-PC ASC reflected an ~3-fold reduction in T15⁺ anti-PC ASC, but was mainly the result of a substantial (~7-fold) increase in T15^{neg} anti-PC ASC generated from old FO B cells (Fig. 7B).

Note, however, that even in severely pre-B cell depleted old BALB/c mice, there was variability in the extent of T15 idiotype loss; indeed, one of five old mice examined retained high T15 idiotype expression comparable to that seen in young BALB/c mice (Fig. 7A). This variability among individual aged mice in T15 idiotype expression has been previously reported³². In contrast to severely pre-B depleted old BALB/c mice, those that had more moderate loss of pre-B cells retained high T15 expression among PC reactive B cells, similar to that seen in young adults. Therefore, extensive pre-B cell loss, coincident with decreases in SLC, was associated with increases in T15^{neg} PC reactive B cells in most, but not all, old mice.

Would reduction in pre-B cells and deficiency of SLC also coincide with diminished expression of the T15 idiotype in young adult mice? As shown in Figure 7C, the expression of the T15 idiotype varied among PC reactive antibodies derived from splenic FO B cells from individual young adult (2–4 month) $\lambda 5$ knockout mice. All wild-type mice had relatively high T15 idiotype expression among PC reactive antibodies (average, ~65%), consistent with previous results^{8–10,32}; in addition, a slight majority (~60%; 5 of 8) of $\lambda 5$ knockout mice generated PC reactive antibodies that were mainly T15⁺. However, unlike wild-type mice, ~40% (3 of 8) $\lambda 5$ knockout mice produced mainly T15^{neg} PC reactive antibodies (Fig. 7C).

These results show that 1) in wild-type young adults, the T15 idiotype is predominant among PC reactive antibodies; 2) the disruption of the preBCR checkpoint due to deficiency of $\lambda 5$ SLC still permits T15 idiotype dominance; however, 3) within the $\lambda 5$ SLC deficient young adult mouse population, a subset of mice now can be identified that show predominance of T15^{neg} PC reactive antibodies derived from splenic FO B cells.

DISCUSSION

In old age, mice of various strains show inhibited development of B cell precursors and new B cells^{1,2,18–22}. The consequences of this for humoral immunity are not well understood. However, in old mice the antibody repertoire appears to change, becoming more autoreactive^{1,2}. Previously, it has been shown in old BALB/c mice that superimposed on this general autoreactivity is an increased B cell response to the apoptotic cell self-antigen/ bacterial antigen phosphorylcholine (PC)^{8–10,20}. As shown herein, loss of B cell precursors in old mice, while affecting several stages of pro-B/pre-B cell development, is particularly extensive at the preBCR checkpoint. This is not surprising as the reduced expression of SLC would be expected to compromise effective formation of the preBCR and limit early pre-B cell clonal expansion. However, early pre-B cells within Hardy Fraction C' (preBCR checkpoint) in old mice continue to exhibit extensive proliferation consistent with preBCR signaling. Immunoglobulin μ heavy chains differ in their variable regions and, consequently, in the capacity to associate with SLC and form the preBCR^{23–28}. These differences in preBCR assembly directly correlate with extent of proliferation²⁸. This suggests that those few pre-B cells which do transit the preBCR checkpoint in old mice undergo clonal expansion. Conceivably, the μ heavy chains utilized by this reduced pool of pre-B cells in old mice represents those μ heavy chains most capable of associating with SLC and do so even when SLC levels are highly reduced. Alternatively, a subset of μ heavy chains appears capable of surface expression and signaling in the absence of the SLC^{42,43}, although the characteristics and number of such μ heavy chains is not known. In old age, selection for those pre-B cells whose μ heavy chains retain capacity to form the preBCR when SLC is low or increased representation of pre-B cells expressing particular μ heavy chains which signal in the absence of SLC may occur. This would likely alter the repertoire of μ heavy chains used in new B cells and affect the specificity of immature B cells.

An example of the altered antibody repertoire seen in old mice among new B cells is the increased reactivity to PC, as shown herein and previously^{9,10}. Increased PC reactivity among B cells in old BALB/c mice was first seen in the earliest immature B cells, e.g., CD23[−] immature B cells, and probably represents altered expression of antibody specificities during new B cell generation from precursor B cells. Increased B cell reactivity to PC could be replicated in young mice that were partially or fully deficient in SLC λ 5 expression. Since both young adult λ 5 deficient mice and old mice have reduction in the SLC λ 5 proteins, it is tempting to suggest that changes in the PC reactive B cell repertoire in these mice result from alterations in the expression of the preBCR.

Immature B cells expressing surface CD23 were particularly biased for recognition of PC in both young and old BALB/c mice. The production of new B cells in the bone marrow is reduced⁴⁴ and transit of newly derived B cells from bone marrow to spleen is compromised in old mice⁴⁵. This is consistent with the very low levels of recently derived T1 transitional B cells in spleens of old mice. However, we noted that newly formed CD23⁺ transitional T2 B cells (identified by BrdU labeling) were disproportionately observed in the old spleen. While the reasons for this are not known, in preliminary experiments we have noted that CD23[−] immature bone marrow or splenic transitional (T1) B cells from young donors home poorly to the spleens of old mice upon adoptive transfer i.v. (~60% reduced homing

compared to that in young mice over 1–2 days), a finding consistent with the results of Wols, et al.⁴⁵. In contrast, CD23⁺ immature bone marrow or transitional (T2) B cells from young donors homed to old spleen nearly as well as to young spleen (~10% average reduction in homing efficiency). Notably, the CD23⁺ immature B cell pool is highly enriched in PC reactivity (Fig 5A).

Continued migration of new CD23⁺ immature B cells from bone marrow to spleen in old mice may contribute to increased PC reactivity within the FO mature B cell pool. This is consistent with mathematical modeling of BrdU incorporation data which suggests that both a bone marrow immature B cell pool (speculatively, the CD23⁺ immature B cells) as well as splenic T1 transitional B cells contribute to the T2 transitional B cell pool in the spleen⁴⁶

Finally, reductions in SLC λ 5, and concomitant reduced generation of new pre-B cells and B cells, was associated with loss of T15 idiotypic dominance, in both a fraction of young adult mice deficient in SLC λ 5 as well as in most old mice where SLC λ 5 was highly reduced. This suggests that reduced preBCR and new pre-B cell generation is associated with diminished T15 dominance among splenic PC reactive B cells in old age.

Other studies, using transgenic models which bypass the preBCR checkpoint, show that limiting B lymphopoiesis, independent of alterations in the preBCR, is sufficient to not only cause reduced new B cell output, but also changes in the peripheral B cell repertoire. The studies of Johnson, et al.⁴⁷, and Guiterrez, et al.⁴⁸, suggest that reduced production of new B cells leads to the predominance of antigen experienced (including autoreactive) B cells within the spleen in old age. Conversely, mutant mice (CD19 deficient, CD79 deficient, or BLyS deficient), with restricted capacities for B cell development, fail to undergo expansion of antigen experienced B cells in old age⁴⁹. In our studies, changes in PC reactivity and idiotype occur among naïve FO splenic B cells in old mice and not necessarily only in antigen experienced B cell populations. Further studies are required to understand the interplay of reduced B lymphopoiesis, changes at the preBCR checkpoint, and alterations in mature B cell selection, expansion, and homeostasis in determining the B cell antibody repertoire in old age.

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Research Highlights

- Fewer pre-B cells pass the pre-B cell receptor (preBCR) checkpoint in old mice.
- Reduced pre-B cells coincide with increased anti-PC B cell reactivity in old mice.
- PC reactive B cells in old mice have decreased T15 idiotype expression.
- Like old mice, B cells from preBCR deficient young mice have higher PC reactivity.
- Like old mice, preBCR deficient young mice have reduced T15 idiotype expression.

Figure 1. Pre-B cells in old mice are decreased coincident with reduced $\lambda 5$ surrogate light chain expression, but early pre-B cells maintain extensive mitotic activity

Panel A: Bone marrow cells from 24–27 mo. old (n=3) and 2–4 mo. old (n=6) BALB/c mice were individually stained for Hardy Fractions as described in Materials and Methods. Bone marrow represents combined femur/tibia pairs. *Panel B:* SLC $\lambda 5$ protein was detected in the cytoplasm of pro-B cells in the bone marrow of individual old (22–31 months) BALB/c mice (dark circles). Results are also shown for young adult BALB/c mice (open circles). The relative levels of SLC $\lambda 5$ were determined by comparing the mean fluorescence intensities (MFI) of cytoplasmic $\lambda 5$ staining in old pro-B cells vs. that seen in young adults where the latter was set as “1”. Late stage pre-B cells in combined femur/tibia pairs of old and young mice were determined as described in Materials and Methods. *Panel C:* Hardy Fractions from old (24–27 months) and young BALB/c (2–4 months) mice (n=3 old; n=6 young) were assessed for % cells in S/G2/M stages of mitosis by Hoescht DNA staining and fluorescence flow cytometry analysis. *, p<0.05.

Figure 2. Heterogeneity in immature B cells in young and old mice: Detection of CD23⁻ and CD23⁺ immature B cell subsets

Panel A: Representative surface staining of bone marrow AA4.1⁺ CD19⁺ immature B lineage cells for IgM and CD23 in both young adult (~2–3 mo.) and aged (~24–26 mo.) BALB/c mice. Proportions shown are within the AA4.1⁺ CD19⁺ populations. In this example, the AA4.1⁺ CD19⁺ cells were 32% of bone marrow cells in young and 9% in old BALB/c mice used. The IgM⁻ CD23⁻ AA4.1⁺ CD19⁺ cells are pro-B/pre-B cells. Therefore, in this example, the old BALB/c mouse shown has 3% pro-B/pre-B cells in bone marrow vs. 16% in the young adult BALB/c mouse. *Panel B:* The numbers of CD23⁻ and CD23⁺ immature (IgM⁺ AA4.1⁺ CD19⁺) B cells in bone marrow (combined femurs/tibias) of individual young adult (n=9) and severely pre-B cell depleted aged (n=10) BALB/c mice. The ratios shown are for CD23⁻ vs. CD23⁺ immature B cells. **, p < 0.01; ****, p < 0.0001.

Figure 3. CD23 and CD43/S7 surface staining identify overlapping immature B cell subsets in young and old BALB/c mice

Panel A: B cells defined as immature (surface IgM⁺ AA4.1⁺ CD19⁺) were also stained for CD23 and for CD43/S7 in both young adult (2–3 mo.) and aged (24–26 mo.) BALB/c mice.

Panel B: The percentages of CD43/S7⁺ cells among CD23⁻ and CD23⁺ immature B cell subsets in young adult (n=3) and severely pre-B cell depleted (see Fig. 1) aged (n=5) BALB/c mice.

Panel C: The numbers of CD43/S7⁻ CD23⁻, CD43/S7⁺ CD23⁻, CD43/S7⁻ CD23⁺, and CD43/S7⁺ CD23⁺ immature (IgM⁺ AA4.1⁺ CD19⁺) B cells in bone marrow (combined femurs/tibias) of individual young adult (n=3) and severely pre-B cell depleted aged (n=5) BALB/c mice. The percentages indicated are the levels of each immature B cell subset in old BALB/c mice compared to young adults (100%). *, p<0.05; **, p<0.01.

Figure 4. BrdU labeling of immature B cell subsets in bone marrow and spleens of old mice
Panel A: Bone marrow and splenic IgM⁺ AA4.1⁺ CD19⁺ immature or transitional B cells (T1/T2) were identified as CD23⁻ and CD23⁺ subsets in young (3–5 mo.) (n=5) and old (22–25 mo.) (n=3) BALB/c mice. In bone marrow, CD23⁻ immature B cells with relatively high IgM surface expression equivalent to that of CD23⁺ immature B cells were gated and analyzed. These old mice were ~60% depleted of pre-B cells on average. Total CD23⁻ and CD23⁺ immature and transitional (T1/T2) B cells in bone marrow (femur/tibia pairs) and spleen are shown. *Panel B:* Young and old BALB/c mice (same as panel A) were fed BrdU in their drinking water over a 3 day period. Bone marrow and splenic IgM⁺ AA4.1⁺ CD19⁺ immature or transitional B cells (T1/T2) were identified as CD23⁻ and CD23⁺ subsets and BrdU incorporation was measured via fluorescence flow cytometry as in Materials and Methods. The number of total BrdU⁺ cells in bone marrow and spleen are shown for each subset. Bone marrow represents individual femur/tibia pairs.

Figure 5. Elevated PC reactivity in immature B cells in old BALB/c mice
Immature CD23⁻ and CD23⁺ (IgM⁺ AA4.1⁺ CD19⁺) B cells were isolated from bone marrow of individual young (2–4 mo.) and old (24–27 mo.) BALB/c mice. Isolated immature B cell subsets were stimulated with LPS and resultant antibody secreting cells (ASC) with reactivity to PC, as well as total IgM ASC, were identified in ELISpot assays. Results shown are for 3–6 separate individuals per group. *, p < 0.05.

Figure 6. Young adult $\lambda 5$ SLC deficient mice show decreased immature B cells, but increased B cell reactivity to PC

Panel A: The representation of CD23⁻ and CD23⁺ immature (IgM⁺ AA4.1⁺ CD19⁺) B cells within the bone marrow (femur/tibia pairs) of 2–6 mo. old wild-type ($\lambda 5^{+/+}$; WT) C57BL/6 mice (n=12); $\lambda 5$ heterozygous deficient ($\lambda 5^{+/-}$) (n=3) mice; and $\lambda 5$ homozygous deficient ($\lambda 5^{-/-}$) (n=13) mice. *Panel B:* Expression of CD43/S7 was determined among CD23⁻ and CD23⁺ immature B cells from wild-type (n=4), $\lambda 5^{+/-}$ (n=4), and $\lambda 5^{-/-}$ (n=5) young adult mice. *Panel C:* The frequency of antibody secreting cells (ASC) detected by ELISpot for reactivity to phosphorylcholine (PC) for bone marrow B cells from $\lambda 5^{+/-}$ (n=3), and $\lambda 5^{-/-}$ (n=10) young adults relative to those of wild-type mice (set to 1). *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 7. Reduction of pre-B cells and the SLC protein $\lambda 5$ correlates with losses in T15 idiotype among PC reactive antibodies in both old and young mice

Panel A: LPS stimulated FO splenic ASC from old (24–27 mo.) and young (2–4 mo.) BALB/c mice producing antibody reactive with PC and/or with anti-T15 idiotype specific mAb AB1-2 were detected by ELISpot assay. The proportions of PC reactive ASC (maximum 100%) that were T15⁺ (AB1-2 reactive) are shown. The old BALB/c mice assayed were designated as “severely pre-B cell depleted” (“sev pre-B”; n=5) or “moderately pre-B cell depleted” (“mod pre-B”; n=6) as per Figure 1. Values for young adult control mice (n=9) are also shown. *Panel B:* For old BALB/c mice, of severe or moderate pre-B cell depletion, relative levels of anti-PC antibodies with either T15⁺ or T15^{neg} idiotypes were calculated by multiplying the %T15 idiotype by the relative anti-PC ASC. Relative anti-PC ASC were determined by setting reactivity in young BALB/c FO B cells equal to 1.0. *Panel C:* Anti-PC antibodies were generated from LPS stimulated FO splenic B cells from individual young adult (4–5 month) wild-type and $\lambda 5^{-/-}$ mice. T15 idiotype expression was determined by reactivity with anti-T15 idiotype antibody AB1-2 in ELISA assays. *, p<0.05; **, p<0.01; ***, p<0.001.