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Aging-Dependent Exclusion of Antigen-Inexperienced Cells from the Peripheral B Cell Repertoire

Sara A. Johnson,* Stephen J. Rozzo,† and John C. Cambier*†

Aging is accompanied by greatly reduced B cell production in the bone marrow, yet peripheral B cell numbers do not decline. We hypothesize that this may reflect filling of the peripheral pool with B cells that are long-lived as a consequence of specificity for, and chronic stimulation by, environmental Ags. To begin to explore this possibility, we analyzed the effects of aging on B cell population dynamics in the anti-H2\textsuperscript{kb}-3-83\textsuperscript{mu}D Ig-transgenic mouse. We predicted that, because they presumably do not bind environmental Ags, B cells bearing the transgenic receptor may be lost in aged animals. As seen in nontransgenic animals, total splenic B cell numbers remained constant with age in the Ig-transgenic animals despite reduced B cell production. Importantly, although the few newly produced B cells in the bone marrow of aged mice are 3-83 positive, the peripheral compartment of these mice is dominated by B cells that express endogenous Ig genes rather than the transgenes. This population includes large numbers of marginal zone-like and CD21\textsuperscript{low}/CD23\textsuperscript{low}/IgM\textsuperscript{low} B cells, as well as elevated numbers of CD5\textsuperscript{+} B cells. Many of these cells express only non-B220 CD45 isoforms, suggesting that they may be memory cells. A significant proportion of aged transgenic animals produce autoantibodies that are reactive with ssDNA, dsDNA, or histones. Results support the hypothesis that, in the face of severely reduced production with age, B cells are selected based on reactivity to environmental Ags, accumulate, and display activated phenotypes. Cells bearing 3-83-transgenic receptors are excluded from this population due to their specificity. Beyond their importance in aging, these findings define a novel form of receptor revision in which B cells are selected rather than deleted based on Ag reactivity.


These changes include a skewing of V-gene use (20–23), increased accumulation of CD5\textsuperscript{+} B cells (24, 25), and decreased inducibility of the costimulatory molecule CD86 (B7.2) (26). The latter may be attributable to age-related defects in signal transduction through the B cell Ag receptor (BCR) (27, 28). Finally, while peripheral B cell numbers are similar in young and aged individuals, bone marrow (BM) production of B cells declines sharply with age (29–32). Furthermore, Kline et al. (33) have demonstrated that peripheral B cells in aged mice have a much longer life span than their counterparts in young animals, explaining how total B cell numbers can be maintained despite decreased generation of new cells. The mechanism(s) underlying this shift in B cell longevity in aged animals is unclear; however, long-lived B cells, often displaying phenotypes characteristic of BCR-induced activation (e.g., marginal zone (MZ), B1, and memory), are also found in young animals. It seems plausible that with advancing age the peripheral pool becomes dominated by B cells that have received Ag receptor signals, resulting in their long-term survival. Thus, in aged animals, the Ab response to any new immunological insult would be derived from this Ag-experienced B cell pool. This may provide an explanation for findings that the Ab response in aged animals is lacking in quality, rather than quantity, with serum Ig levels of low affinity, often appearing oligoclonal and enriched in autoantibodies (34–39).

To test this hypothesis, we examined the effect of aging on specificity-constrained B cells from anti-H2\textsuperscript{kb}-3-83\textsuperscript{mu}D Ig-transgenic (Tg) mice. If maintenance of the B cell compartment in aged individuals is dependent upon BCR stimulation, then aged 3-83\textsuperscript{mu}D mice should have severely reduced numbers of Tg receptor-expressing B cells in the periphery. Results demonstrate that, as in non-Ig-Tg mice, B cell production slows dramatically with aging, yet total splenic B cell numbers (as defined by CD19 expression)

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3 Abbreviations used in this paper: BCR, B cell Ag receptor; BM, bone marrow; MZ, marginal zone; Tg, transgenic; T1, transitional type 1; WT, wild type; FO, follicular.
are equivalent in aged and young 3-83μo mice. However, activated, e.g., MZ-like, cells dominate the peripheral B cell pool in aged 3-83μo mice. Importantly, these cells express receptors encoded by endogenous Ig genes rather than the Tg receptor. In addition, many of these cells express only non-B220 CD45 isoforms, and a subset also expresses CD5. A significant proportion of animals produce autoantibodies that are reactive with ssDNA, dsDNA, or histones. Data presented support the hypothesis that, in the face of severely reduced generation, B cells are selected based on their reactivity to environmental Ags, accumulate, and display activated phenotypes. In the 3-83 model these B cells are only found within the nonallelically excluded population. Immuneosenescence may result, in part, from forced reliance on this abnormal repertoire for generation of protective immune responses.

Materials and Methods

Animals and tissue preparation

3-83μo Ig-Tg mice (40) on a B10.D2 background, as well as B10.D2 mice, were bred and housed for 2–3 mo in the National Jewish Medical and Research Center animal care facility (Denver, CO). Both male and female mice were studied. Single-cell suspensions were prepared from young (2–3 mo of age) and aged (>22 mo) spleen and BM and red cells lysed using ACK (150 mM NH4Cl, 10 mM KHCO3, 100 mM N2, EDTA). PBL were isolated from heparinized peripheral blood by centrifugation through Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Uppsala, Sweden). In all cases, cells remaining after erythrocyte depletion were washed twice in IMDM and viability was assessed by trypan blue exclusion. Total cell recovery was determined using a Coulter Counter (Coulter Electronics, Hialeah, FL).

Irradiation and autoreconstitution

B cell compartments were depleted in both young (2–3 mo of age) and aged (>22 mo) 3-83μo mice by sublethal gamma irradiation (500 rad). B cell repopulation of peripheral lymphoid organs was assessed at 21 and 28 days and numbers of CD19+ cells were determined by flow cytometry.

Cell surface staining

To block nonspecific Ab binding to the endogenous FcR, cells were resuspended at 1 x 107 per milliliter in IMDM containing 5 μg/ml of the anti-FcR mAb, 2.4G2, before cell surface staining. Blocked cells were washed twice in staining buffer (PBS with 2% FCS and 0.2% sodium azide), incubated for 30 min at room temperature with an optimal amount of conjugated (tri-color, allophycocyanin, PE, FITC, or biotin) Ab, and washed again. Cells were stained in a second step with streptavidin-allophycocyanin, PE, or FITC as appropriate. After washing, cells were analyzed by four-color flow cytometry (FACSCalibur; BD Biosciences, La Jolla, CA). Except where noted, all density plots and fluorescence histograms were pretagged to include only live cells expressing the B cell-specific marker CD19.

Abs for cell surface staining

The following Ab and secondary conjugates were used: anti-CD19 (6D5) tri-color conjugate, goat anti-mouse Ig PE conjugate, streptavidin-PE, and streptavidin-FTTC (Caltag Laboratories, Burlingame, CA); anti-B220 (RA3-6B2) allophycocyanin conjugate, anti-CD5-PE (53-7.3), anti-CD23-FTTC (B3B4), anti-IgM-biotin (AF6-78), anti-IgM-FTTC (AF6-78), anti-IgM-biotin (DS-1), anti-CD21/35-FTTC (7G6), anti-CD80-FTTC (16-10A1), anti-CD86-FTTC (GL1), anti-CD69-biotin (H1.2F3), anti-CD45RA-biotin (DNL-1.9), anti-CD24-PE (M1/69), anti-CD43-FTTC (S7), anti-BP1-PE (6C3), and anti-α/β L chains-biotin (R26-46) (BD PharMingen, San Diego, CA); anti-CD32/16 (2.4G2), anti-3-83 idiootype-FTTC (54.1), anti-3-83 idiootype-biotin (54.1), anti-pan CD45-FTTC (I3/2.5), anti-IgM-FTTC (B76), anti-IgD-FTTC (JAI2.5), anti-493-biotin (gift from A. Rolin, Basel Institute of Immunology, Basel, Switzerland), and anti-κ L chain-FTTC (187.1), all prepared from hybridoma supernatants as previously described (41).

Serological assays

Autoantibody levels were determined by ELISA as previously described (42–44). Briefly, Immunol II microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with calf thymus total histones, heat-denatured DNA (Sigma-Aldrich, St. Louis, MO), or chromatin at 2.5 μg/ml in PBS (pH 7.2) and postcoated with 1 mg/ml gelatin. Serum samples were diluted 1/300 in PBS with 0.5% Tween supplemented with 5 mg/ml bovine y globulins (Sigma-Aldrich) and gelatin, and added to Ag-coated wells for 90 min. After washing, wells were incubated with peroxidase-labeled Ab for mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). After 90 min, substrate was added and OD was determined with an automated spectrophotometer (Dynatech Laboratories) at 405 nm. Abs to dsDNA were measured similarly, except plasmid dsDNA (pGEM) was biotinylated and bound to streptavidin-coated microtiter plates; streptavidin-only wells were used as a control. All assays were performed in duplicate and were quantified against a standard curve obtained with mAbs (45).

To measure total IgG and IgM and IgM allotypes, sera were diluted 1/10,000 and incubated on Immulon II plates coated with polyclonal affinity-purified goat anti-mouse IgG (γ-chain specific; Kirkegaard & Perry Laboratories), IgM (μ-chain specific; BioSource International, Camarillo, CA), IgM+ (BD PharMingen), or IgM- (BD PharMingen), as outlined above. Peroxidase-conjugated, affinity-purified goat anti-mouse IgG (γ-chain specific; Kirkegaard & Perry Laboratories) or IgM (μ-chain specific; BioSource International) were used for detection, ODs were determined, and results were compared with the appropriate standard curve generated with purified polyclonal mouse IgG (Cappel, Durham, NC) or IgM (Chemicon International, Temecula, CA).

Statistical analysis

The Student t test, assuming equal variances, was used to determine the statistical significance of differences in mean cell number (see Fig. 1C) or mean percentage (see Figs. 2B, 4B, 7B, and 8B) of BM and splenic subpopulations.

Total Ig and autoantibody levels (see Fig. 9) were compared using the nonparametric Dunn procedure of the Kruskal-Wallis test.

Results

Reduced B cell production in aged Ig-Tg mice

To determine whether Ig-Tg mice undergo the aging-dependent decrease in B cell production reported previously (29–32) for wild-type (WT) animals, we assessed the ability of aged and young 3-83μo Ig-Tg mice to regenerate B cells following sublethal gamma irradiation. As shown in Fig. 1A, autoreconstitution of the CD19γ compartment in both BM (Fig. 1A, left panel) and spleen (Fig. 1A, right panel) was delayed in the aged animals (Fig. 1A, dotted bars). These data are consistent with those reported by Li et al. (32) and strongly suggest that B cell production is reduced in aged 3-83μo mice. To further validate this contention, we examined ex vivo BM B cells from untreated 3-83μo and B10.D2 (WT control) mice. In our system, use of conventional markers of B cell maturation described by Hardy et al. (46) is not effective because premature expression of the Ig transgenes prevents the use of L/H chains as indicators of developmental stage, and CD43 staining is very weak in mice of B10.D2 background. Therefore, we used CD19 as our marker of maturational stage based on the findings of two other groups (Sato et al. (47) and S. Levy, unpublished observations), delineating the CD19hi low subpopulation as pre- and immature B cells, and the CD19hi high subpopulation as pro- and mature (recirculating) B cells.

As shown in Fig. 1B, staining with anti-CD19 Abs revealed a diminution of the CD19hi low subpopulation in both aged Ig-Tg and WT animals. Analysis of a large group of animals confirmed that this diminution in CD19hi low cell numbers was statistically significant: p = 0.008 for 3-83μo and 0.03 for B10.D2 (Fig. 1C). In contrast, total CD19+ and CD19hi high cell numbers were not significantly different between young and aged animals (Fig. 1C). The latter finding differs from previous reports that mature recirculating B cells increase in the BM with age (29–31, 33, 36, 48). However, it is unclear from this literature whether the reported increase in the number of mature B cells in the BM with age is due to a change in the absolute number of cells or rather an apparent increase due to a change in proportionality consequent to the large decrease in

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pre-B cell numbers. The data we have presented in Fig. 1C indicate that changes in the absolute number of mature B cells in the BM are, in fact, small and not statistically significant. However, we would agree that mature B cells make up a greater proportion of the marrow of aged mice (IgM vs IgD, data not shown). Notably, the ~4-fold reduction in total CD19⁺ cell numbers recovered from 3-83μδ, as opposed to WT, animals can be attributed to the more rapid transit of Tg receptor-expressing cells (that have preformed BCR) through B cell maturational stages.

Examination of additional phenotypic markers confirmed that the CD19low subpopulation contained both pre- and immature B cells, expressing high levels of CD24 (Fig. 3A). Pre-B cells were further distinguished by assessing BP-1 expression (data not shown). In contrast, the CD19high subpopulation expressed low levels of CD24 (Fig. 3A), thus confirming the presence of both pro- and mature recirculating B cells. Mature (recirculating) B cells were further distinguished by assessing IgD expression (data not shown). Importantly, as pro-B cells comprise only 3–4% of total BM cells (46) their contribution to the CD19high cell numbers were negligible. With the exception of absolute cell numbers, no differences (e.g., in levels of CD24 expression) were noted between samples from aged vs young animals. Thus aged Ig-Tg mice, like aged WT mice, are subject to mechanisms that impair B cell production.

Cells that express endogenous H chain genes dominate splenic B cell populations in aged 3-83μδ-Tg mice

Analysis of peripheral B cell compartments demonstrated equivalent numbers of B cells, defined as CD19⁺ cells, in the spleens of aged and young 3-83μδ mice (Fig. 2B, upper panel). However, significant numbers of these cells failed to stain with the anti-3-83 idiotypic Ab, 54.1, while staining strongly with Abs specific for IgM (Fig. 2A). Analysis of a large group of 3-83μδ mice revealed that a significantly greater proportion (mean, 64 vs 11%) of peripheral B cells in aged mice are Id⁺ (p = 0.001), and showed that this occurs in the vast majority of aged animals (Fig. 2B, lower panel). Moreover, the majority of Id⁺ cells expressed endogenous IgM⁺ H chains rather than the Tg IgM⁺ (Fig. 2C).

A total of 3–10% of splenic B cells from young 3-83μδ mice were found to express endogenous BCR as a result of incomplete allelic exclusion, and this number increased dramatically as the 3-83μδ mice aged (Fig. 2). This could be explained by selective expansion or survival of the few IgM⁺-expressing B cells in the periphery, or by increased failure of allelic exclusion in the BM with age. To address this issue, idiotype expression was investigated in the BM. Fig. 3B shows that most pre/immature B cells (CD19lowCD24high) in the BM from aged 3-83μδ mice are Id⁺ (Fig. 3B, lower left panel) and that these cells do not simultaneously...
express Tg and endogenous H chains (Fig. 3B, middle left panel, bottom row), demonstrating that allelic exclusion is equivalent in developing B cells from young and aged animals. Furthermore, recirculating (CD19<sup>high</sup>CD24<sup>low</sup>) B cells from the same animal are largely Id<sup>−</sup> (Fig. 3B, middle right panel, bottom row) and, like their pre/immature counterparts, do not simultaneously express both Tg and endogenous H chains (Fig. 3B, right panel, bottom row). Interestingly, a small proportion (in this case ~20%) of IgM<sup>+</sup>Id<sup>−</sup> cells are also IgM<sup>κ</sup>− (Fig. 3B, bottom row). This loss of idioype, while transgene H chain expression (µα) is retained, must reflect alternate L chain expression. Taken together, data presented in Figs. 1–3 demonstrate that, despite continued generation of Id<sup>+</sup> B cells in the BM, Id<sup>−</sup> cells accumulate in the spleen over time, suggesting peripheral selection of cells expressing non-Tg BCR.

IgM<sup>−</sup>Id<sup>−</sup> splenic B cells from aged 3-83µδ mice display an Ag-experienced phenotype

Activated B cells and their clonal descendants have a longer life span than resting B cells (49). Thus, the periphery of aged mice may be filled by B cells that were activated by environmental Ags. It is intuituive that use of endogenous H chains, but not 3-83µδ transgenes, would provide the range of Ag specificity from which cells that recognize such Ags could be selected most readily by stimulation. To explore the possibility that the IgM<sup>−</sup>Id<sup>−</sup> splenic B cells of aged 3-83µδ mice arise as a consequence of B cell activation, we assessed their expression of a variety of cell surface markers known to be modulated upon BCR engagement. Although the IgM<sup>−</sup>Id<sup>−</sup> B cells present in the spleens of aged 3-83µδ mice did not express markers typical of acute B cell activation such as CD69, CD80, and CD86 (data not shown), a significant proportion of these cells expressed CD5 (Fig. 4A, left panels). Analysis of a large group of 3-83µδ mice revealed that a significantly greater proportion (mean, 41 vs 4%) of peripheral B cells in aged mice are CD5<sup>+</sup> (p = 0.01), and showed that this occurs in the vast majority of aged animals (Fig. 4B, left panel).

Surprisingly, many of the IgM<sup>−</sup>Id<sup>−</sup> B cells failed to express, or expressed only low levels of, the B cell-specific CD45 isoform, B220 (Fig. 4A, right panels). Analysis of a large group of 3-83µδ mice revealed that a significantly greater proportion (mean, 41 vs 4%) of peripheral B cells in aged mice are B220<sup>low</sup> (p = 0.0001), and showed that this occurs in the vast majority of aged animals (Fig. 4B, right panel). Changes in CD45 isoform expression have been shown to occur as a function of activation in both B and T cells, with low m.w. isoforms dominant in memory cells (50–54). CD45 is a transmembrane protein tyrosine phosphatase.

FIGURE 2. Cells that express endogenous H chain genes dominate splenic B cell populations in aged 3-83µδ-Tg mice. Spleen cells and PBL from 3-83µδ mice were stained with anti-CD19, idiootype, and IgM, IgM<sup>κ</sup>, or IgM<sup>κ</sup> Abs and analyzed by flow cytometry. All populations shown were pregated to include only live, CD19<sup>high</sup> cells. A, Expression of idioype vs IgM on splenic B cells from young (upper panel) or aged (lower panel) animals. B, upper panel, Quantitation of total CD19<sup>+</sup> cell numbers in spleen of aged (dotted bar; n = 10) or young (open bar, n = 4) 3-83µδ animals. SEM is indicated for each. B, lower panel, Quantitation of the percentage of idioype-negative B cells in PBL or spleen of aged (n = 44) and young (n = 12) animals. Means (horizontal bars) and statistically significant p values are indicated. C, Expression of idioype vs IgM<sup>κ</sup> (left panels) or IgM<sup>κ</sup> (right panels). Cell subpopulations are identified with rectangular regions, and percentages are indicated.
that is required for normal T and B lymphocyte Ag receptor-mediated cell activation (for review see Ref. 55). Notably, eight CD45 isoforms can be generated as a consequence of alternative splicing of exons 4–6 (or A–C, respectively) (56). To determine whether aged 3-83 μδ mice express CD45 isoforms other than B220 (RABC), cells were stained with a pan-specific anti-CD45 Ab or Abs specific for certain exon/glycosylation combinations; e.g., the anti-CD45RA Ab recognizes not only the RA isoform but also RABC, RAB, and RAC isoforms. As shown in Fig. 5, far left, >95% of splenic B cells from aged animals were found to express CD45, but only half of these cells were reactive with the B220 (CD45RABC)-specific mAb RA3-6B2. Furthermore, B cells from aged 3-83 μδ mice, in contrast to those from young 3-83 μδ mice, express a variety of lower-m.w. CD45 isoforms, as evidenced by the double-negative populations present in, e.g., Fig. 5, middle left panel, bottom row, which shows that 25% of cells do not stain with the anti-RA Ab, and thus must be RO, RB, RC, RBC, or a glycosylation variant. Taken together, data presented in Figs. 4 and 5 suggest that a significant proportion of splenic B cells in aged 3-83 μδ mice have undergone BCR-induced activation.

Id− and B220low B cells accumulate with age in 3-83 μδ mice
To assess the time of onset and rate of accumulation of Id− and B220low B cells, we analyzed expression of these markers by peripheral blood B cells from groups of animals (n = 4) ranging in age from 3 to 23 mo (Fig. 6). Significantly numbers of Id− B cells were detected in the peripheral blood of mice as young as 5 mo of age and increased steadily thereafter (Fig. 6, shaded bars). The loss of B220 followed a similar pattern but appeared to be slightly delayed relative to the accumulation of Id− cells (Fig. 6, dotted bars). In view of findings that B220low− cells occur only among Id− cells (Fig. 4A, right panels), this pattern suggests a sequential event, where Id− cells replace B220 with low-m.w. isoforms of CD45 during peripheral selection.

MZ-like cells are increased in aged 3-83 μδ mice
Splenic B cells present in adult mice can be divided into transitional (further divisible into transitional type 1 (T1) and transitional type 2), follicular (FO), and MZ based on surface marker expression (57). In non-Tg mice MZ B cells account for 5–10% of splenic B cells and are characterized as IgMhighIgDlowCD21highCD23low (58). Importantly, experimental evidence suggests that MZ B cells are long lived, possibly as a function of having seen Ag. Therefore, we examined splenic B cells from aged 3-83 μδ mice for the presence of these subsets. As illustrated in Fig. 7A, right panels, staining with anti-CD21/35 in combination with anti-CD23 Abs revealed that approximately one-third of splenic B cells from this aged 3-83 μδ mouse are FO, compared with two-thirds in the young 3-83 μδ mouse. Analysis of a large group of 3-83 μδ mice revealed that a significantly smaller proportion (mean, 32 vs 70%) of splenic B cells in aged mice are FO (p = 0.001), and showed that this occurs in the majority of aged animals (Fig. 7B). Additional staining indicated that the reduced numbers of CD21highCD23low FO B cells in aged animals could be attributed to a reduction of transitional type 2 (IgMhigh) cells (data not shown). In contrast, CD21highCD23low MZ B cells are increased to >30% in some
Aged non-Tg mice display alterations in peripheral B cell population dynamics similar to those seen in aged 3-83μδ mice

To confirm that the changes seen in B cell populations, e.g., loss of B220 and accumulation of non-FO B cells, is not due to the Ig transgene, phenotypic analysis was performed on non-Tg mice of the same strain, B10.D2. Results demonstrated trends similar to those seen in the 3-83μδ mice. Specifically, in aged B10.D2 mice, most splenic B cells failed to express, or expressed only low levels of, the B cell-specific CD45 isofrom, B220 (Fig. 8A, left panels). Analysis of a large group of B10.D2 mice revealed that a significantly greater proportion (mean, 25 vs 3%) of splenic B cells in aged mice are B220low/− (p = 0.01), and showed that this occurs in the majority of aged animals (Fig. 8B, left panel). Furthermore, MZ-like B cell numbers are also increased in aged B10.D2 mice (Fig. 8A, right panels). Analysis of a large group of B10.D2 mice revealed that a significantly smaller proportion (mean, 48 vs 71%) of splenic B cells in aged mice are FO (p = 0.004), and showed that this occurs in the majority of aged animals (Fig. 8B, right panel). In some aged animals non-T1 CD21low/− CD23low/− and/or CD5− B cells are also increased (data not shown).

Autoantibody production is increased in aged 3-83μδ mice

The dominance of peripheral B cell populations in aged mice by long-lived cells (33) with Ag-experienced phenotypes (Figs. 4–8) suggested that these cells might be subject to chronic stimulation by endogenous Ags, including autoantigens. To begin to explore this possibility, we tested sera from both 3-83μδ and B10.D2 mice for the presence of autoantibodies. As shown in Fig. 9, upper left panel, significant quantities of IgG anti-dsDNA Abs were detectable in 3-83μδ mice (Fig. 9, C) at 12 (median, 0.14 U/ml; p = 0.001), 18 (median, 0.42 U/ml; p = 0.0002), and 24 (median, 0.23 U/ml; p = 0.0001) mo of age. IgG anti-dsDNA Abs were also increased in aged B10.D2 mice (Fig. 9, D) at 12 (median, 0.13 U/ml; p = 0.004), 18 (median, 0.04 U/ml; p = 0.01), and 24 (median, 0.16 U/ml; p = 0.0001) mo of age (Fig. 9, lower left panel). This is consistent with the findings of Eaton-Bassiri et al. (60), who investigated aged BALB/c mice. IgG anti-dsDNA Abs are widely accepted as pathogenic in lupus-like disease (42, 44); however, sera from 7-mo-old (New Zealand Black × New Zealand White)F1 mice assayed in parallel had a median value of 6.24 U/ml. Thus, although the level of anti-dsDNA Abs in the sera of aged mice is significant, it is not pathological.

Additionally, significant quantities of IgG anti-ssDNA Abs were detectable in 3-83μδ mice (Fig. 9, C) at 18 (median, 1.95 U/ml; p = 0.0001) and 24 (median, 1.01 U/ml; p = 0.0005) mo of age (Fig. 9, upper right panel). IgG anti-ssDNA Abs were also increased in aged B10.D2 mice (Fig. 9, D) at 12 (median, 1.27 U/ml; p = 0.0001), 18 (median, 0.94 U/ml; p = 0.0001), and 24 (median, 1.04 U/ml; p = 0.008) mo of age (Fig. 9, lower right panel). Similar increases in anti-histone titers were detected with age in both 3-83μδ and B10.D2 mice, but no significant differences were noted for anti-chromatin Abs (data not shown).

Total IgG levels were increased in 18-mo-old (median, 208 ng/ml; p = 0.01) and 24-mo-old (median, 209 ng/ml; p = 0.01) 3-83μδ mice (data not shown). Total IgM was also increased in 18-mo-old (median, 0.18 μg/ml; p = 0.01) and 24-mo-old (median, 0.31 μg/ml; p = 0.0002) 3-83μδ mice (data not shown). The kinetics of appearance of autoantibodies in the serum (Fig. 9) was strongly correlated with the accumulation of Ig− and B220low/− B cells in the periphery (Fig. 6) of aged 3-83μδ mice. Not surprisingly, total serum IgM levels were increased at 18 (median, 0.92 U/ml; p = 0.0007) and 24 (median, 0.90 U/ml; p = 0.03) mo of age (data not shown). Thus, at least a proportion of nonallelically

FIGURE 4. IgMId− B cells from aged 3-83μδ mice display an Ag-experienced phenotype. Spleen cells and PBL from 3-83μδ mice were stained with anti-CD19, idiotype, and CD5 or B220 Abs and analyzed by flow cytometry. All populations shown were pregated to include only live, CD19+ cells. A, Expression of idiotype vs CD5 (left panels) or B220 (right panels) on splenic B cells from young (upper panels) or aged (lower panels) animals. Cell subpopulations are identified with rectangular regions, and percentages are indicated. B, left panel, Quantitation of the percentage of CD5-positive cells among PB or splenic B cells of aged (n = 40) and young (n = 8) animals. B, right panel, Quantitation of the percentage of B220-negative cells among PB or splenic B cells of aged (n = 55) and young (n = 12) animals. Means (horizontal bars) and statistically significant p values are indicated.

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-aged 3-83μδ animals, compared with <5% in young (Fig. 7A, right panels). Notably, the MZ B cells are Id− (Fig. 7A, left panels), indicating that these cells belong to the accumulating, nonallelically excluded B cell population. The CD21low/− CD23low/− subpopulation, which, in some aged 3-83μδ mice, comprised greater than one-third of splenic B cells, was found to be Id− IgMlow/493−, indicating that these cells are not B1 B cells (IgMhigh) or T1 B cells (493+ ) (59) (data not shown). This reduction in transitional B cells is consistent with decreased BM output of B cells shown in Fig. 1. In contrast, the CD21low/− CD23low/− subpopulation comprised <20% of splenic B cells in young 3-83μδ animals and were found to be Id− IgMhigh/493+ T1 B cells (data not shown). The CD21low/− CD23low/− IgMlow/493− B cell subpopulation seen in the aged animal has not been recognized or functionally defined in previous studies. However, because these cells are larger in size (as defined by light scatter; data not shown), Id−, and IgMlow, they may be chronically activated B cells that have down-modulated surface IgM. As noted previously (Fig. 4), CD5− B cells are also frequently found in the spleens of aged 3-83μδ mice. These cells are CD23low/− and CD21int.
excluded B cells selected to populate the periphery of aged 3-83/H9262/H9254 mice are autoreactive.

Discussion

Profound changes in immune responsiveness occur as higher species age, yet the molecular mechanisms underlying these changes remain unclear. Many investigators have described changes in T cell generation and function and have attributed failed Ab responses to lack of T cell help rather than B cell dysfunction. Data reported in this work demonstrate that peripheral B cells from both aged Ig-Tg and WT mice are phenotypically distinct from those found in young counterparts and suggest that these unique B cell populations arise and are maintained as a consequence of chronic Ag stimulation. Specifically, our data document an age-associated decline in B lymphopoiesis correlated with accumulation of peripheral B cells that, in the case of the 3-83/H9262/H9254 mouse, express endogenous H chains rather than transgene-encoded receptors. These cells express several markers indicative of previous Ag stimulation, including CD21, low m.w. isoforms of CD45, and CD5. Autoantibodies are produced in a significant proportion of these mice. Similar modifications were seen in aged non-Tg mice. Taken together, these results suggest that cells reactive to environmental Ags are selected from the repertoire by virtue of stimulation-induced extension of their life span. Humoral immune responses occurring later in life presumably derive from these populations, resulting in Abs of low affinity.

Reports from multiple groups document reduced B cell production in the BM as a function of age, yet the cause of this reduction remains unclear. The proposed “defect” has been attributed to a quantitative decrease in the number of B cell progenitors (31, 61), changes in the BM microenvironment (32, 62), and limited

FIGURE 6.  B220low/− B cells from aged 3-83μ6 mice express alternate CD45 isoforms. Spleen cells from 3-83μ6 mice were stained with anti-CD19, B220, and pan-CD45, CD45RA, CD45RB, or CD45RC Abs, and analyzed by flow cytometry. All populations shown were pregated to include only live, CD19+ cells. Expression of B220 vs pan-CD45 (left panels), vs CD45RA (middle left panels), vs CD45RB (middle right panels), and vs CD45RC (right panels) on splenic B cells from young (upper panels) or aged (lower panels) animals. Cell subpopulations are identified with rectangular regions, and percentages are indicated. Data are representative of analyses of >10 animals.

FIGURE 5.  B220low/− B cells from aged 3-83μ6 mice express alternate CD45 isoforms. Spleen cells from 3-83μ6 mice were stained with anti-CD19, B220, and pan-CD45, CD45RA, CD45RB, or CD45RC Abs, and analyzed by flow cytometry. All populations shown were pregated to include only live, CD19+ cells. Expression of B220 vs pan-CD45 or CD45RA or CD45RB or CD45RC on splenic B cells from young (upper panels) or aged (lower panels) animals. Cell subpopulations are identified with rectangular regions, and percentages are indicated. Data are representative of analyses of >10 animals.

FIGURE 6.  Id− and B220low/− B cells accumulate with age in 3-83μ6 mice. Peripheral blood was collected from 3-83μ6 mice aged 3, 5, 11, 17, and 23 mo (n = 4 for each group), and lymphocytes were stained with anti-CD19, idio, and B220 Abs and analyzed by flow cytometry. Graph depicts the percent of CD19+ cells that were Id− (shaded bars) or B220low/− (dotted bars) for each age group. SEM is indicated for each.

FIGURE 7.  MZ-like cells are increased in aged 3-83μ6 mice. Spleen cells from 3-83μ6 mice were stained with anti-CD19, CD23, idio, or CD21/35 Abs and analyzed by flow cytometry. All populations shown were pregated to include only live, CD19+ cells. A, Expression of CD23 vs idio (left panels) or CD23 vs CD21/35 (right panels) on splenic B cells from young (upper panels) or aged (lower panels) animals. Cell subpopulations are identified with rectangular regions, and percentages are indicated. B, Quantitation of the percentage of FO B cells among splenic cells of aged (n = 10) and young (n = 4) animals. Means (horizontal bars) and statistically significant p values are indicated.
expression of recombination-activating gene 1/2 (63, 64), VpreB (65), and/or A5 (66). In toto, these data point to a problem in the pro- to pre-B cell transition, which clearly reduces pre-B cell numbers and, ultimately, total splenic B cell emigrés. Importantly, our finding that aged 3-83μΔ Tg mice also have a much-reduced ability to generate new B cells indicates minimally that factors in addition to expression of these proteins must limit B cell production in older animals. If recombination-activating gene, VpreB, or A5 were solely limiting, production should have been rescued by the Ig transgenes (67). Experimental evidence from our laboratory (S. A. Johnson and J. C. Cambier, unpublished observation) and others (68, 69) demonstrates that pro/pre-B cells from aged mice (3-83μΔ and WT) proliferate poorly in response to IL-7. Further investigation is needed to determine the molecular mechanism(s) underlying B cell developmental defects in aged mice.

It has been well documented that, despite decreased B cell generation in the BM, total splenic B cell numbers are equivalent in aged and young non-Tg mice. This apparent contradiction can be explained in part by the extended life span of B cells in aged animals (33). However, none of the previous studies have addressed the mechanism(s) by which such long-lived, possibly self-renewing, B cells might arise. This phenomenon could reflect the selective accumulation of B cells that express receptors that are reactive with environmental Ags as described by Grandien et al. (70). A common feature of long-lived B cells (e.g., MZ, B1, and memory) from non-aged animals is that they display characteristics indicative of BCR-mediated activation. Thus, it seems reasonable that similar mechanisms may contribute to the age-associated accumulation of long-lived B cells.

As a result of imperfect allelic exclusion, 3–10% of B cells from young 3-83μΔ mice express the endogenous IgM H chain and fail to stain with anti-idiotype Abs. This number increases dramatically in the periphery as the 3-83μΔ mice age. One could argue that use of endogenous, polyclonal H chains would, by providing B cells with a greater range of Ag specificity, increase the likelihood of BCR recognition and response to environmental Ags in the periphery. Given enough time, even a low-frequency occurrence of this stimulation could result in the selection and accumulation (as opposed to proliferation) of significant numbers of long-lived IgM+Id- B cells, particularly in an aged environment that lacks normal competition for B cell niches. The slow incorporation of BrdU into peripheral B cells from aged mice shown by Kline et al. (33) is consistent with this hypothesis, as is the appearance of autoantibodies in the sera of aged 3-83μΔ mice at a frequency similar to that found in aged non-Ig-Tg mice.

In addition to the loss of cells bearing the transgene-encoded BCR, our data document previously unreported changes in B cell CD45 isoform expression as a function of age. The fact that the loss of the B220 isoform is limited to the IgM+B cell population is consistent with reports that low-m.w. isoforms of CD45 serve as a marker of cellular activation (50–53). Furthermore, several studies have suggested that different CD45 isoforms have distinct functions (71–74), and that in some cases low-m.w. isoforms may attenuate Ag receptor signaling (75). One might expect that non-B220 CD45 isoforms expressed on B cells from aged mice may attenuate BCR signal transduction, and this attenuation might prevent peripheral deletion of B cells with specificity for environmental Ags.

Our findings also show a previously unreported increase in cells of MZ-like phenotype in aged animals. Martin and Kearney (76) have suggested that MZ B cells use a restricted set of BCR genes, are positively selected into the mature B cell pool as a result of

FIGURE 8. Aged non-Tg mice display alterations in peripheral B cell population dynamics similar to those seen in aged 3-83μΔ mice. Splenic cells from B10.D2 mice were stained with anti-CD19, B220, and pan-CD45 or CD23 and CD21/35 Abs and analyzed by flow cytometry. A. Expression of B220 vs pan-CD45 (left panels) or CD23 vs CD21/35 (right panels) on splenic B cells from young (upper panels) or aged (lower panels) animals. Cell subpopulations are identified with rectangular or circular regions, and percentages are indicated. B, left panel. Quantitation of the percentage of B220-negative cells among splenic B cells of aged (n = 13) and young (n = 7) animals. B, right panel. Quantitation of the percentage of FO cells among splenic B cells of aged (n = 11) and young (n = 6) animals. Means (horizontal bars) and statistically significant p values are indicated.

FIGURE 9. Autoantibody production is increased in aged 3-83μΔ mice. Sera from 3-83μΔ (upper panels, ○) and B10.D2 (lower panels, ●) mice aged 3, 12, 18, and 24 mo were analyzed by ELISA for the presence of Abs reactive with dsDNA (left panels) or ssDNA (right panels). Median values (horizontal bars) and statistically significant p values are indicated.
encounter with self or commensal Ags, and function as “natural immune memory” that protects against particulate blood-borne Ags.MZ B cells function in a T-independent manner and are rarely recruited into germinal centers (77). Notably, aged mice, as compared with young, form fewer germinal centers (26). Significantly, enlargement of the MZ B cell compartment has also been reported in A5-deficient mice and IL-7R-deficient mice in which B lympho- poiesis is impaired (76, 78), as in aging.

Consistent with studies of WT mice (20, 24, 25), aged 3-83μ0 mice display increased numbers of splenic B cells expressing CD5. However, these cells are likely not true B1 B cells, in that they express levels of IgM equivalent to FO cells and do not express detectable levels of CD43 (data not shown). This population is reminiscent of splenic B cells observed in the V(D)J_9 mouse, which bear an Ag receptor specific for phosphatidylcholine. Splenic B cells from this mouse are CD5^+ IgM^+ (equivalent to B2) CD43^-, while peritoneal B cells from the same mouse appear as classic B1 (CD5^+ IgM^hiCD43^-) (79). This may be important in the context of the B-0 hypothesis described by Haughton et al. (80), which states that B1 and B2 cells originate from a common precursor, and that commitment to either subset occurs after the expression of surface Ig and is Ag driven. Commitment to the B1 pathway occurs only upon low-affinity BCR cross-linking, likely under specific conditions such as the absence of T cell help, as in aging. This requirement for entry into the B1 pathway selects for cells bearing surface Ig that has low affinity for environmental Ags. In the case of aged 3-83μ0 mice, some cells that express endogenous BCR may receive sufficient signal to become CD5^+ but insufficient signal to convert to the classic peritoneal B1 phenotype.

Finally, our data show reduced numbers of mature FO B cells in aged animals, presumably consequent to the shorter half-life of these cells. This lack of FO B cells could, in part, account for the limited ability of aged animals to respond to T-dependent Ags.

In conclusion, our studies provide a dissection of the B cell subsets present in aged mice and reveal previously unrecognized alterations in population dynamics. Significantly, these alterations in population dynamics seem to be a consequence of reduced B lymphopoiesis and accumulation of B cells with specificity for environmental Ags.

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References
