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Withdrawal of Sex Steroids Reverses Age- and Chemotherapy-Related Defects in Bone Marrow Lymphopoiesis

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A significant decline in immune function is characteristic of aging. Along with the involution of the thymus and associated impaired architecture, which contributes to profound loss of naïve T cell production, there are also significant declines in B cell development and the progenitors that support lymphopoiesis. These collectively lead to a reduced peripheral immune repertoire, increase in opportunistic infections, and limited recovery following cytoablation through chemo- or radiotherapy. We have previously shown that sex steroid ablation (SSA) causes a major reversal of age-related thymic atrophy and improves recovery from hematopoietic stem cell transplant. This study focused on the impact of SSA on the B cell compartment and their progenitors in middle-aged and cyclophosphamide-treated mice. In both models, SSA enhanced the number of lymphoid progenitors and developing B cells in the bone marrow (BM) as well as reversing age-related defects in the cycling kinetics of these cells. Enhanced BM lymphopoiesis was reflected in the periphery by an increase in recent BM emigrants as well as immature and mature plasma cells, leading to an enhanced humoral response to challenge by hepatitis B vaccine. In conclusion, SSA improves lymphoid progenitor and B cell recovery from age- and chemotherapy-induced immunodepletion, complimenting the effects on T cells. Since SSA has been achieved clinically for over 25 years, this provides a novel, rational basis for approaching the need for immune recovery in many clinical conditions. The Journal of Immunology, 2009, 182: 6247–6260.

It has been well documented that there is a general deterioration in the adaptive immune system with age (1, 2). This deterioration is most evident as thymic atrophy, which is characterized by greatly disrupted thymic stromal architecture leading to a profound decline in thymopoiesis (3, 4). Age-related immune system decline is not, however, restricted to the thymus as there are also significant declines in B lymphopoiesis and humoral immunity (5–7). Furthermore, significant declines have also been observed in early lymphoid progenitors with age. There is also growing evidence that with age there is an intrinsic defect in hematopoietic stem cells and their ability to commit to the lymphoid lineage (8, 9).

Although all blood cells ultimately derive from bone marrow (BM) resident hematopoietic stem cells (10), there is still considerable debate about the identity of the earliest committed lymphoid progenitor (11–13). There have been at least three early hematopoietic precursors identified that contain both B and T cell potential, all of which fall within the Lin"IL-7Rα" fraction of BM. The common lymphoid progenitor (CLP) 1, which exhibited both T and B cell potential in an in vivo competitive setting, was the first of these to be described (14). More recently, two B220⁺ populations have been discovered which have potent B cell and limited T cell potential: the pre-TCR⁺c-kit⁻ CLP-2 (15) and the CD19⁺c-kit⁺ early progenitor with lymphoid and myeloid potential (EPLM) (16). Both of these progenitors were shown to exhibit bi-potent lymphoid ability in vivo and in vitro systems. The transition from lymphoid progenitor to early B cell has not yet been fully elucidated, but it seems apparent that there is a requirement for IL-7 (17–20) and it is associated with a down-regulation of c-kit and an associated up-regulation of B220 (21), implicating the involvement of CLP-2 and EPLM, both of which express B220.

BM development of B cells is a well-understood process that can be defined by three distinct stages based on expression of CD43 and surface IgM (22), in order of progression; pro-B cell (CD43⁺IgM⁻), pre-B cell (CD43⁺IgM⁺), and imm-B cell (CD43⁻IgM⁺). Based on expression of CD24 and B220, B cells that have been recently exported from the BM can be tracked in the periphery as being B220lowCD24high (23). Furthermore, activated B cells and plasma cells can be characterized as expressing B220 and CD138/syndecan-1 with or without surface IgG (24, 25).

Although not affected by a morphological defect as obvious as that with the atrophic thymus, there are still significant declines in BM lymphopoiesis with age. Within the lymphoid progenitor populations, there is a large reduction in the number and frequency of CLP-1 cells, a decline in their responsiveness to IL-7 (26), as well as a decrease in their proliferative potential (27). The number of B-lineage cells in the BM also decreases during aging at a similar rate to thymic involution (28). This can be predominantly attributed to the pre-B cell subset, the most numerous of the developing B cells, which decreases in both number and frequency with age.
Possible mechanisms include an increase in apoptosis (32) as well as a decreased proliferation in response to IL-7, compared with young mice (26, 33). There is impaired V to DJ recombination (34) and a decrease in the total expression of RAG-1 and RAG-2 in the BM (7, 28) and in purified pro-B cells (35). This decline in RAG expression may lead to a failure in the transition to the pre-B cell stage, thereby leading to a reduction in numbers (36).

Linked to these defects are age-related alterations in the BM microenvironment that supports B lymphopoiesis, such as decreased IL-7 production (37, 38). Interestingly, despite TGF-β acting as a negative regulator of IL-7 activity (39), in senescence-accelerated mice, there was actually a slight decrease in the amount of TGF-β protein in the stroma (40).

Despite defects in the generation and export of new B cells with age (30, 41), there is little change in the total number of B cells in the periphery due, in part, to a greater life span and homeostatic self-renewal of existing peripheral B cells (30, 37), analogous to the expansion of peripheral T cells (42). However, a decline in exported B cells from the BM does lead to a severely limited B cell Ab repertoire with age (6, 43, 44) and may underlie the poor humoral response to vaccines in aged patients (45). Furthermore, the recovery of the BCR repertoire from cyclophosphamide (Cy)-induced immunodepletion is severely compromised in aged mice (37).

These declines in BM lymphopoiesis, along with thymic atrophy, become more evident from the onset of puberty, predominantly due to elevated levels of sex steroids (46–48). Treatment with estrogen decreases B lymphopoiesis and causes a rapid decline in IL-7 responsiveness (49), as well as selectively depleting CLP-1 cells and a population of lymphoid-committed precursors within the LSK fraction of BM (50). Similar effects can be observed with androgen treatment (51). Although both androgen and estrogen receptors are found on developing B cells as well as the supporting stroma (48, 52), androgenic suppression of B lymphopoiesis is mediated indirectly through the BM stroma and its down-regulation of IL-7 and up-regulation of TGF-β (51). It has also been noted that removal of sex steroids, through either ovariectomy (53) or through transgenic mice such as the testicular feminized (tmf) mouse, enhances B lymphopoiesis (54). This study examines in more detail the efficacy of sex steroid ablation (SSA) to regenerate B-lymphopoiesis in the middle-aged and following Cy treatment.

Materials and Methods

Animals

Inbred C57BL/6, BALB/c, or RAG-1−/− mice were obtained from the Animal Resources Centre, Monash Animal Services, or the Baker Institute Precinct Animal Centre. Young mice were between 6 and 8 wk old and middle-aged mice were 9 mo old in this study, which, while still considered middle-aged, already possess a degenerated immune system. Mice were maintained at the Precinct Animal Centre and Mouseworks. Mice were allowed to acclimatize for 7 days before experimentation, which was performed according to animal experiment ethics committee guidelines and approval.

Surgical SSA

Mice were anesthetized and a small incision was made in the scrotum. The testes were exposed, ligated with dissolvable sutures, and removed. The wound was closed with dissolvable sutures. For surgical stress control, sham-SSA was performed as above, but without removal of the testes. In Cy experiments, mice were SSA on the day of the second injection.

Cy treatment

Mice were injected i.p. with endoxin (Baxter Pharmaceuticals) at a dose of 100 mg/kg body weight per day for 2 days (total dose of 200 mg/kg).

Flow cytometric analysis

Single-cell suspensions of freshly dissected BM were obtained by flushing tibiae and femurs with cold PBS supplemented with 0.2% BSA with a 26-gauge needle. The resulting marrow was then suspended by gently pipetting through a 22-gauge needle. Single-cell suspensions of spleen were obtained by mechanical digestion using frosted glass slides. Released cells were washed with cold PBS supplemented with 0.2% BSA. Cells were recovered by centrifugation at 470 χ gmax for 5 min at 4°C. Cell counts were determined by gating viable cells based on cell size using a Z2 Coulter Counter (Beckman Coulter).

The following fluorochrome-labeled Abs against murine Ags (as well as appropriate isotype controls) were used: FITC or allophycocyanin-conjugated anti-IgM (II/41), PE-conjugated anti-CD43 (S7, FITC, PE, or PerCP-conjugated anti-CD45R (RA3-6B2), PE-conjugated anti-CD127 (A7R34), allophycocyanin-conjugated anti-CD117 (2B8), biotin-conjugated anti-CD24 (M1/69), FITC-conjugated polyclonal anti-IgG, and PE-conjugated anti-CD138 (28-1-2). Secondary reagents used were PerCP or allophycocyanin conjugated to streptavidin.

Early lymphoid progenitor lineage mixture consisted of the following Abs conjugated to FITC or PE: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD11b (M1/70), anti-CD11c (HL3), and anti-Gr-1 (RB6-8C5).

To detect proliferating B cells and lymphoid progenitors, cells were surface labeled as above and then fixed and permeabilized using a commercial Fix and Perm Kit as per the manufacturer’s instructions (BD Biosciences). Cells were then labeled with anti-Ki-67 FITC. To detect apoptosis, cells were washed in annexin V binding buffer and stained with FITC-conjugated annexin V. Apoptotic cells were gated on live cells expressing subset specific markers and positive for annexin V staining.

All conjugates were purchased from BD Biosciences except anti-CD127 PE, which was purchased from Chemicon International. All cells were analyzed on a multiparameter FACSCalibur (BD Biosciences) using CellQuest Pro software (BD Biosciences).

Hepatitis B vaccination schedule

At 2 or 6 wk after surgery, mice were vaccinated with s.c. injections of 500 μl of H-B-VaxR II (CSL) at a concentration of 5 μg/ml in the presence of aluminum hydroxide. This was repeated three times 2 wk apart to give a total of three vaccinations per mouse. Fourteen days following the final vaccination, mice were sacrificed and serum was collected for analysis of Ab titer.

Serum collection

At 2 and 6 wk after surgery, 500 μl of blood was collected by cardiac puncture. Blood was allowed to clot for 4 h at room temperature. Samples were then centrifuged at 855 χ gmax for 3 min. Serum was collected and frozen at −20°C until ELISA was performed.

ELISA

Abs against hepatitis B vaccine peptides were detected using ELISA. Maxisorb Immun plates (Nunc) were coated with 100 μl of hepatitis B small surface Ag (provided courtesy of H. Netter, Monash University, Clayton, Australia) at a concentration of 500 ng/ml overnight at 4°C. Before ELISA was performed, the plate was blocked with 10% skim milk powder in PBS for 2 h to block any nonspecific binding of Ag. Plates were incubated with 100 μl of 2-fold serum dilutions for 1 h at 37°C. Specific Abs were detected by reaction with HRP-labeled goat anti-mouse IgG (Sigma-Aldrich) followed by enzyme substrate (H2O2/β-phenylelenediamine) and visualized by measuring the absorbance at 492 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer.

Statistical analysis

Statistical analysis was performed with the nonparametric, unpaired Mann-Whitney U test using SPSS software. A p ≤ 0.05 was considered to be statistically significant.

Results

Age-related declines in BM lymphopoiesis are rapidly reversed following SSA

To examine the effect of SSA on lymphoid progenitors and developing B cells, middle-aged (9 mo) mice were surgically SSA, which reduces serum testosterone levels to ~1% of normal levels within 6 h (55): B lymphopoiesis was determined by flow cytometric analysis (Fig. 1B) for 6 wk following surgery. Confirming
FIGURE 1. SSA rapidly restores lymphopoiesis in middle-aged BM to levels observed in the young. A, Total cellularity of BM from 2-mo, 9-mo ( ), and sham-SSA ( ) or SSA ( ) 9-mo mice 4, 7, 10, 14, 28, and 42 days after surgery. B, Representative flow cytometry profiles for CLP-1, CLP-2, EPLM, and developing B cell subsets of an untreated 2-mo mouse. C, Cellularity of lymphoid progenitor cells (CLP-1, CLP-2, EPLM) with age and following SSA. D, Absolute cell number of B cell subsets. Results are expressed as mean ± SEM of 6–10 mice for each group at each time point. Data are representative of three or more independent experiments. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 compared with sham-SSA 9-mo mice. ∧, p ≤ 0.05; ∧∧, p ≤ 0.01; and ∧∧∧, p ≤ 0.001 compared with 2-mo mice.
previous reports, we found that total BM cellularity of two femurs and two tibiae combined per mouse were increased with age (29), albeit marginally (Fig. 1A). Following SSA, there was no change in total BM cellularity at early time points; however, from 14 through to 28 days following surgery, there was a significant increase compared with sham-SSA controls (Fig. 1A).

Within the CLP-1 population (identified as Lin<sup>-</sup>IL-7Rα<sup>-</sup>c-kit<sup>-</sup>), we confirmed previous reports that with age there is a significant decrease in both the number (Fig. 1C) and frequency (data not shown) (26, 27). The cellularity of CLP-2 (Lin<sup>-</sup>IL-7Rα<sup>-</sup>B220<sup>-</sup>c-kit<sup>-</sup>) (15) and EPLM (Lin<sup>-</sup>IL-7Rα<sup>-</sup>B220<sup>+</sup>c-kit<sup>-</sup>) (16) cells reflected the age-related decrease observed within the CLP-1 subset (Fig. 1C). Following SSA, a steady increase in the number and frequency (data not shown) of all of these progenitor subsets was evident from day 7 and, within the CLP-2 and EPLM subsets specifically, restoration to normal young numbers occurred by days 7 and 10, respectively. Furthermore, this increase in the number of CLP-2 and EPLM cells was maintained for at least 6 wk following surgery and briefly reached hypertrophic levels compared with untreated young mice at day 14.

By 9 mo of age, we found that the total number and frequency of B cells (as measured by expression of B220) had decreased significantly compared with that at 2 mo of age (Fig. 1D). Following SSA, total B cells in the BM were shown to be increased as early as 4 days after SSA and this increase, in both frequency (data not shown) and number, was maintained over all time points examined, reaching an equivalent number to young mice by 10 days after SSA (Fig. 1D). Individual developing B cell subsets were subsequently examined using expression of CD43 and surface IgM as markers of B cell development. The most immature B cell subset, the pro-B cell (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>), was found to significantly decrease in number and frequency with age, confirming

![FIGURE 2](http://www.jimmunol.org/) Enhanced lymphopoiesis in lymphoid progenitors is a result of altered cycling kinetics. A, Proportion of Ki-67<sup>+</sup> CLP-1 and CLP-2 cells in BM of 2-mo, 9-mo ( ), and sham-SSA ( ), or SSA ( ) 9-mo mice 4, 7, 10, 14, and 28 days after surgery. B, Proportion of apoptotic CLP-1 and CLP-2 cells as measured by staining with annexin V with age and following SSA. C, Ratio of number of proliferating to apoptotic CLP-1 and CLP-2 cells that is analogous to efficiency of lymphopoiesis. Results are expressed as mean ± SEM of 6–10 mice for each group at each time point. Data are representative of three or more independent experiments. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 compared with sham-SSA 9-mo mice. ∧, p ≤ 0.05; ∧∧, p ≤ 0.01; ∧∧∧, p ≤ 0.001 compared with 2-mo mice.
FIGURE 3. Increased number of developing B cells can be attributed to altered cycling kinetics. A, Proportion of Ki-67$^+$ total B, pro-B, pre-B, and imm-B cell subsets in BM of 2-mo, 9-mo ( ), and sham-SSA ( ) or SSA ( ) 9-mo mice 4, 7, 10, 14, and 28 days after surgery. B, Proportion of developing B cells stained with annexin V indicating apoptosis. C, Ratio of the number of proliferating to apoptotic developing B cells, a qualitative measurement that is analogous to the efficiency of B lymphopoiesis. Results are expressed as mean ± SEM of 6–10 mice for each group at each time point. Data are representative of three or more independent experiments. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001 compared with sham-SSA 9-mo mice. ∧, p ≤ 0.05; ∧∧, p ≤ 0.01; ∧∧∧, p ≤ 0.001 compared with 2-mo mice.
previous reports (26, 32). However, although there was no change at the earliest time point examined following SSA, there was a sustained increase in the number (Fig. 1D) and proportion (data not shown) of pro-B cells from 7 days following SSA. These cells did not, however, reach young levels. Among the most numerous population of developing B cells, the pre-B cell (B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>+</sup>) was a profound decrease in their number and frequency with age, confirming previous studies (26, 32). Furthermore, after SSA this subset follows and contributes to the trend of total B cells, showing a sustained increase in both number and frequency by 4 days, reaching levels which even exceeded the young by 10 days (Fig. 1D). The imm-B cells (B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>+</sup>) show a similar, but delayed trend, with a significant increase by 10 days after SSA.

**SSA induces proliferation of lymphoid progenitors and developing B cells in the BM of middle-aged mice**

To determine possible mechanisms behind enhanced B cell numbers following SSA, we investigated whether there was increased proliferation (Ki-67 expression) or decreased apoptosis (annexin V). Within the precursor populations, we found a decrease with age in the number (data not shown) and percentage of Ki-67<sup>+</sup> CLP-1 and CLP-2 cells, indicating a reduced rate of proliferation (Fig. 2A). SSA restored the proliferative state of CLP-1 almost to levels observed in young mice proportionally by day 4 and numerically by day 7 (data not shown). CLP-2 cells were similar, with the proportion expressing Ki-67 being restored to young levels by day 10 and in number by day 14 (data not shown). There was also an increase in the proportion of apoptosing (annexin V<sup>+</sup>) CLP-1 and CLP-2 cells with age and subsequent decline by day 7 after SSA (Fig. 2B). The efficiency of lymphopoiesis, as reflected by the ratio of proliferating to apoptotic cells, showed a decline with age in CLP-2 and CLP-2 populations and a reversal following SSA by days 7 and 14, respectively (Fig. 2C).

Developing B cell subsets mirrored the kinetics observed within the more primitive lymphoid progenitors, with age-related decreases in both the proportion (Fig. 3A) and number (data not shown) of proliferating total B cells; these were reversed following SSA, being comparable to young levels by day 7 following SSA (Fig. 3A). All developing B cell subsets examined reflected this trend, particularly the pre-B and imm-B cell subsets. We found a significant increase in the rate of apoptosis among developing B cells with age that was similarly reversed as a result of SSA (Fig. 3B). Again, the ratio of Ki-67<sup>+</sup>:annexin V<sup>+</sup>, reflecting B cell survival efficiency, was significantly decreased with age and restored following SSA to untreated levels as early as 4 days following surgery (Fig. 3C).

**RAG-1 is required for SSA-mediated increases in lymphoid progenitors but not B lymphopoiesis**

We next examined the impact of SSA in RAG-1-deficient (RAG-1<sup>−/−</sup>) mice which have a developmental defect that allows early B lymphopoiesis to develop normally but there is a block between the pro-B to pre-B cell transition, with only few pre-B cells emerging and a total lack of imm-B cells (56).

Among the less mature CLP-1, CLP-2, and EPLM populations (Fig. 4A), we found significantly fewer cells in the BM of these RAG-1<sup>−/−</sup> mice. Neither of these were enhanced by SSA, demonstrating that the effects of SSA on CLP-1 and CLP-2 cells are RAG dependent despite the fact that it is generally considered more important later in the pro-B to pre-B cell transition (56). Within the pro-B cell subset, which was unaffected by the loss of RAG expression, SSA induced a significant increase in the number and frequency of cells compared with sham-SSA controls (Fig. 4B). Further progression to the pre-B cell compartment, however, was blocked, consistent with the literature.

**SSA-mediated regeneration of BM lymphopoiesis leads to enhanced peripheral B cells**

Consistent with previous reports (30, 37), we found little change in the number of B cells in the spleen with age. This is no doubt due to the peripheral clonal expansion of existing B cells as a result of the decline in export of naive B cells from the BM, ultimately resulting in a compromised repertoire. Following SSA there was no immediate effect on peripheral B cells, indicating that SSA does not directly impact on circulating B cells. By day 14, however, there was a significant increase in the number of B cells that was maintained over 6 wk (Fig. 5A). The dependence on BM export for SSA-mediated increases in peripheral B cells was confirmed by examining recent BM emigrants (RBEs), as measured by CD24 (human serum albumin) expression. These RBEs increased from day 14 through to day 42 (Fig. 5B) in SSA mice compared with both sham-SSA and untreated 2-mo controls. To further dissect the peripheral B cell compartment, CD138<sup>+</sup>IgG<sup>+</sup> and CD138<sup>−</sup>IgG<sup>+</sup> plasma cells were examined (Fig. 5C). Significant increases in both of these subsets were observed by day 14 with CD138<sup>−</sup>IgG<sup>+</sup> cells surpassing untreated young levels by day 14 (Fig. 5C).
Taken together, these results indicate that although increases in peripheral B cells are dependent on BM export, an increase in the mature CD138\(^{+}\)/IgG\(^{+}\)/H11001 plasma cells also at day 14 may indicate there is also a minor effect on existing plasma cells or their immediate activated precursors, that have already encountered Ag (Fig. 5C).

To examine the functional capacity of the Ab response following SSA, middle-aged mice were challenged with the hepatitis B virus (HBV) vaccine and humoral function was measured by ELISA. Despite an increase in the absolute number of CD138\(^{+}\)/IgG\(^{+}\)/H11001 plasma cells, there was little difference in the humoral response 2 wk following SSA. By 6 wk, however, the Ab titer was significantly enhanced compared with sham-SSA controls, approximating the level of normal young mice (Fig. 5D). To determine the number of responders to the HBV vaccine, baseline absorbance for unvaccinated control mice were subtracted from the absorbance from test mice. At all titers and, within all groups, the test mice showed a higher absorbance, indicating that all animals responded to the HBV vaccine. This was observed at both time points and in all groups. The impact of SSA, however, did increase the Ab titer when adjusted this way such that at 6 wk the average titer for sham-SSA mice was 180 (range between 80 and 320) vs 1208 (range between 80 and 2056; data not shown) for SSA mice.

Recovery in BM lymphopoiesis from Cy treatment is dramatically enhanced as a result of SSA in young mice

Administration of the clinically common anti-neoplastic chemotherapeutic agent Cy caused a major loss in total BM cellularity by day 2 after treatment which gradually recovered by day 5 to normal levels followed by a hypertrophic effect that peaked at day 7 (Fig. 6A). Following SSA, BM cellularity was restored at similar
levels to sham-SSA controls, but there was a larger hypertrophic peak at day 28 with total cellularity significantly higher at days 14, 28, and 42 (Fig. 6A). In terms of the recovery of the earliest lymphoid progenitors from chemotherapy, we found a significant decrease in the frequency (data not shown), and number (Fig. 6A) of CLP-1, CLP-2, and EPLM subsets with the nadir at day 2. This was followed by a rapid restoration of cellularity by day 4 (CLP-1) and day 7 (CLP-2 and EPLM). Following SSA, there was no change in the number or frequency (data not shown) of CLP-1 cells at the earliest time points examined; however, over days 4–5 these cells significantly increased. A second wave of enhanced CLP-1 numbers occurred at days 28 and 42, indicating that
following chemotherapy treatment, SSA causes several waves of CLP-1 progenitors to differentiate. The CLP-2 fraction recovered at day 7, 3 days later than CLP-1 cells, suggesting a possible precursor-progeny relationship. Removal of sex steroids dramatically increased CLP-2 numbers by day 4 and this was followed by a second increase from day 14 and maintained to day 42.

A similar trend was observed in total B cell number and frequency with the exception that the lowest point in the B cell number was delayed by 2 days in pro- and pre-B cells and up to 5 days in imm-B cells (Fig. 6B). This was followed by a gradual increase in the number and frequency so that by day 14 normal levels are restored. This was significantly enhanced following SSA with hypertrophic numbers of B cells reached by day 14, simultaneous with CLP-2 but delayed behind CLP-1 cells, and maintained through to day 28 among pro-B cells and day 42 in pre-B and imm-B (Fig. 6B).

**Cell cycle kinetics of lymphopoiesis following SSA in Cy-treated mice**

Examining the rate of proliferation and apoptosis within the lymphoid progenitor and developing B cell subsets in the BM indicated one potential mechanism for SSA-mediated recovery following Cy treatment. Within the lymphoid progenitors, there was a significant increase in proliferation and decrease in apoptosis immediately following Cy administration (Fig. 7, A and B). However, although proliferation of CLP-1 and CLP-2 cells was further enhanced following SSA, there was little difference in apoptosis between SSA and sham-SSA controls until day 42 (Fig. 7B). Nevertheless, when efficiency of B cell maintenance was
FIGURE 8. Efficiency of lymphopoiesis in developing B cells is improved as a result of SSA in a chemically induced model of immune depletion. A. Proportion of proliferating total B, pro-B, pre-B, and imm-B cells in BM from untreated 2-mo (□) and Cy-treated sham-SSA (■) or SSA (■) 2-mo mice 3, 7, 14, 28, and 42 days after surgery. B. Proportion of apoptotic developing B cells at days 7, 14, and 42 after Cy administration. C. Ratio of proliferating to apoptotic cells at days 7, 14, and 42 after Cy treatment. Results are expressed as mean ± SEM of six mice for each group at each time point. Data are representative of three or more independent experiments. *, p ≤ 0.05; **, p ≤ 0.01 SSA compared with sham-SSA mice. ∧, p ≤ 0.05; ∧∧, p ≤ 0.01 SSA compared with untreated 2-mo mice. #, p ≤ 0.05; ##, p ≤ 0.01 sham-SSA compared with untreated 2-mo mice.
examined, there was still a significantly enhanced ratio of proliferating to apoptotic CLP-1 and CLP-2 cells from day 14 after treatment (Fig. 7C).

In contrast to the progenitors, we found a decrease in the percentage of all B cell subsets expressing the proliferation marker Ki-67 (Fig. 8A) immediately following Cy treatment and an increase in the level of apoptosis among developing B cells (Fig. 8B). Following SSA, the rate of proliferation was significantly enhanced above sham-SSA levels in a stepwise fashion, starting with the pro-B cells from day 7 to 28, pre-B cells from days 14–28, and imm-B cells from day 14–42 (Fig. 8A). Conversely, apoptosis declined significantly from day 7 in pro-B cells and day 14 in pre-B and imm-B cell subsets (Fig. 8B). These changes in the cycling kinetics and apoptosis of developing B cells are reflected as a sharp decline in the ratio of proliferating to apoptotic cells following Cy administration. Following SSA, this ratio is significantly improved compared with sham-SSA controls and untreated animals by day 7 in pro-B cells and day 14 for pre-B and imm-B cells (Fig. 8C).

SSA-mediated recovery of B lymphopoiesis in the BM aids peripheral emigration 14 days after treatment

To examine whether these changes in the BM are transferred into the periphery, we looked at the peripheral B cell number and function was examined. Following SSA, there was no immediate change in the number of B cells in the spleen of young Cy-treated mice (Fig. 9A). This indicates that there was no direct effect of SSA on the number of peripheral B cells. However, 14 days following Cy treatment, there was a significantly increased number of B cells in the spleen of SSA mice in comparison to sham-SSA controls that was maintained over all time points examined (Fig. 9A). These cells are likely to have recently emigrated from the BM.

**FIGURE 9.** Changes in BM lymphopoiesis as a result of SSA are converted into the periphery in a chemically induced model of immune depletion. A, Total splenic and B cell number from untreated 2-mo (●) and Cy-treated sham-SSA (○) or SSA (◂) 2-mo mice 7, 14, 28, and 42 days after surgery. B, Number of B220<sup>lo</sup>CD24<sup>hi</sup> RBEs at days 14 and 42 after Cy treatment. C, Number of immature IgG<sup>−</sup> and mature IgG<sup>+</sup> plasma cells 14 and 42 days after Cy treatment. D, Specific Ab titer against HBV vaccine peptide at 14 and 42 days after SSA from pooled untreated age-matched controls (▲, broken black line) and Cy-treated 2-mo sham-SSA (●, broken gray line) or SSA (◂, solid line) mice. Results are expressed as mean ± SEM of 6–10 mice for each group. Data are representative of two or more independent experiments. *, p ≤ 0.05; **, p ≤ 0.01; ***p ≤ 0.001 compared with sham-SSA 9-mo mice. ∧, p ≤ 0.05; ∧∧, p ≤ 0.01; ∧∧∧, p ≤ 0.001 compared with untreated age-matched mice.
by the increase in B220\textsuperscript{low}CD24\textsuperscript{high} expressing cells; however, by 42 days after SSA, there was no difference in the putative RBEs between SSA mice and sham-SSA controls (Fig. 9B). There was also a significant increase in CD138\textsuperscript{+} IgG\textsuperscript{+} plasma cells, a population that is only minimally depleted as a result of Cy due in part to their relatively quiescent nature at both 14 and 42 days following SSA. This indicates there may be a direct effect on those B cells that have already encountered Ag and have entered a quiescent memory stage. There was a significant increase in the number of CD138\textsuperscript{+} IgG\textsuperscript{+} plasma cells 14 but not 42 days after SSA. Interestingly, even the sham-SSA mice exhibited a hypertrophic level of these cells at 14 days, indicating that Cy, or its damage, can promote their temporary expansion. These results were functionally corroborated by the humoral response to HBV vaccine, which showed a severe defect with Cy treatment to the vaccine. Although there was no effect of SSA at day 14 in the humoral response, by day 42, the SSA response had been restored to untreated levels while the sham-SSA control response was still limited (Fig. 9C).

Discussion

There is considerable evidence that age-related declines in lymphopoiesis, both in the BM and thymus, are mediated primarily by the effects of sex steroids (46–48, 51, 52, 57–59). We and others have previously shown that SSA, through either surgical or chemical means, regenerates the middle-aged or chemically involuted thymus (60–66) as well as enhances peripheral T cell function (66) and recovery following both autologous (67) and allogeneic (68) BM transplant. The effect of age on the immune system is not, however, restricted to the thymus, with substantial evidence of a profound decrease in BM lymphopoiesis (26, 27, 29, 33, 35, 40, 58, 69, 70). Similar to the thymus, while androgen receptors are expressed on both developing B cells as well as on the supporting BM microenvironment (52), the effect of sex steroids are predominantly mediated through the stromal component (51), although lymphoid progenitors not only express sex steroid receptors (59) but their function is potently regulated by sex steroids (50, 58).

The data in the present study corroborate previous research indicating a profound age-related decline in B cell development in the BM. We confirm that there is a significant decrease in pre-B cells with age and also a substantial decrease in the earlier pro-B and downstream imm-B cells (26). We further show that decreases in the pre-B cell subset are likely attributed to an increase in the rate of apoptosis with age (32) and a decrease in the proliferative potential of upstream CLP and pro-B cells (26, 27, 33). This decline in BM lymphopoiesis explains the decrease in B cell production and export in middle-aged mice and may also contribute to the decline in intrathymic progenitors and thymopoiesis (71).

Our previous studies on the effects of sex steroids on lymphopoiesis and the impact of their removal on thymic function and BM transplantation formed the basis for the present studies on BM lymphoid progenitors and B lymphopoiesis. The data demonstrate that following removal of sex steroids (in this case surgically, a process analogous to chemical SSA in the clinic), there is a rapid and sustained increase in developing B cells and their upstream lymphoid progenitors. However, although SSA significantly increased all lymphoid progenitor and developing B cell subsets compared with age-matched sham-SSA controls, the observation that CLP-1 and pro-B cells fail to reach normal young levels suggests that sex steroids are not solely responsible for the age-related degeneration in these cells. Despite this, CLP-2/EPLM and pre-B cells were all increased to hypertrophic levels compared with untreated young mice following SSA, the kinetics of which are consistent with these cells being the progeny of CLP-1 and pro-B cell, respectively (15, 16). As early as 4 days after surgery, we found a significant increase in pre-B cells, earlier than the precursor pro-B cell population, indicating a direct effect of SSA on this subset. This also suggests that the pro-B cell population is more sensitive to the effects of sex steroids than other developing B cell subsets. Pro-B and imm-B cells showed no significant change until days 7 and 10, respectively, which suggests that changes in these subsets are dependent on an increased pool of their respective upstream progenitors, lymphoid-primed multipotent progenitors in the case of pro-B cells and CLPs or pre-B cell precursors in the case of imm-B cells. The progression from pre-B to imm-B cells also appears to be enhanced as a result of SSA with significant increases in the number and frequency of both pre-B and imm-B cells until at least 42 days after SSA.

Withdrawal of endogenous sex steroids reversed age-altered cycling kinetics by returning the rate of proliferation and apoptosis among all lymphoid precursors and developing B cell subsets to levels seen in normal young animals. This result was reflected in the ratio of proliferating to apoptotic cells as a measure of the efficiency of B lymphopoiesis, which was significantly reduced with age and reversed by SSA. Although SSA was able to return this ratio to levels observed in young animals, the mechanisms behind this alteration are not yet known. From previous reports, however, it is likely that at least some of these effects are indirectly mediated by BM stromal microenvironment and the cytokines produced therein (72).

The enhanced survival and proliferation potential among developing B cells support the notion that SSA acts at the level of increasing the supply of B cell precursors to undergo selection events. Previous studies suggest that, despite the decline in B cell export from the BM (30, 41), there is no age-related change in the number of B cells in the periphery due to peripheral clonal expansion (30), an event which leads to a subsequent decline in the repertoire (44). We observed a significant increase in peripheral B cells following SSA, predominantly a result of B220\textsuperscript{low}CD24\textsuperscript{high} RBEs, suggesting that the peripheral repertoire may have been restored. However, although increases in CD138\textsuperscript{+} IgG\textsuperscript{+} plasma cells can be attributed to a progression from RBEs, increases in the number of mature CD138\textsuperscript{+} IgG\textsuperscript{+} plasma cells indicate that there may also be direct effects of SSA on peripheral B cells. Functionally challenging peripheral B cells with HBV vaccine, however, indicated that despite an increase in the number of mature plasma cells at 2 wk, there was no functional change in the humoral response until 6 wk after SSA, by which time there is likely to have been de novo activation of newly formed naive B cells.

Expression of the gene RAG-1 is essential for B and T cell development with RAG-1-deficient mice exhibiting a block in the progression from pro-B to pre-B, the time of Ig rearrangements, but little effect on lymphoid progenitors (73, 74). We observed a significant impairment in pre-B cells but not pro-B cells; however, although there was a significant loss of lymphoid precursors, it was not complete. This indicates that although RAG-1 is required for mature B cell development, it is less important in the maintenance of the lymphoid progenitor compartment. Fourteen days following the removal of sex steroids, when the most profound impacts of SSA are observed, there were significantly more EPLM and pro-B cells in the SSA mice, yet no change in the CLP-1 or CLP-2 populations. RAG-1 expression is thus required for SSA-mediated increases within CLP subsets but not in the pro-B cell subset.

In the aging model, SSA-mediated expansion of pre-B cells preceded that of lymphoid progenitors by several days. By analogy with the thymus where developing thymocytes condition their microenvironmental stromal niches (called “cross-talk”; reviewed in Refs. 75 and 76), these more “mature” RAG-dependent developing
B cells may, at least in part, condition the BM stromal niches to facilitate production of these precursors. As such, it will be important to resolve any aberrations in the BM niche of RAG-1/−/− mice which may allow EPLM and pro-B cells, but not CLPs, to develop and expand following SSA.

One very practical application of this study was the SSA-induced recovery of B lymphopoiesis following treatment with the anti-neoplastic chemotherapeutic agent Cy (77). We have previously shown a significant enhancement of T cells along with the supporting thymic stroma following SSA in this model of immunodepletion (Ref. 60 and G. L. Goldberg, J. A. Dudakov, N. Seach, J. J. Reisger, T. Ueno, K. Vlahos, M. V. Hamnett, L. F. Young, T. S. P. Heng, R. L. Boyd, and A. P. Chidgley, manuscript in preparation). In the BM after Cy, there were similar decreases in the developing B cell compartment as within the thymus. This was particularly evident within the pre-B cells, the most numerous developing B cell, making it analogous to the intrathymic double-positive (CD4−CD8+) population, which is the most significantly affected developing T cell population (Ref. 60 and manuscript in preparation). Cy also effected all developing B cells to some degree, with the most profound effects observed within the pre-B and imm-B cell subsets, both of which are virtually ablated between days 3 and 5 following treatment. Following SSA, the first populations to recover were the lymphoid progenitors and early pro-B and pre-B cell precursors at day 4. This was carried through the developmental process with increases in the number and frequency of all subsets by day 14 and maintained through to day 42. Although recovery of B lymphopoiesis is relatively fast in sham-SSA controls, the rate within the SSA group is significantly enhanced and even reaches hypertrophic levels compared with untreated controls, an observation comparable to that seen in the thymus (60).

In fact, in the Cy model of immunodepletion, regeneration of the B cell compartment occurs in a wave. This may be due to the fact that in this model, as distinct from the aging model where there are more subtle changes at the B cell subpopulation levels, there is severe depletion of virtually all cells. Hence, in the Cy model, there is a wave of rejuvenation occurring from the more resistant cells, whereas in the aging model there is a more synchronous repopulation occurring simultaneously from all of the preexisting cells. The latter is not possible in the Cy model because many of the precursor cells have been depleted as a result of the therapy, the regeneration of the earliest B cell precursors being critical for regeneration of the later, more mature, developing B cells.

Interestingly, changes in the cycling kinetics of developing B cells followed a maturing lineage progression, with the earliest increases observed in the pro-B cell subset at day 7, followed by the pre-B and imm-B cells by day 14 no doubt reflecting the precursor-prenymph transition. Furthermore, the imm-B subset continued to remain increased by SSA compared with sham-SSA mice at day 42. Similarly, the earliest cell subset to show a decline in apoptosis following SSA was the pro-B cells at day 7, followed by the pre- and imm-B cell subsets by day 14. Within the chemical immunodepletion regime, the recovery of the progenitors (EPLM, CLP-1, CLP-2) occurred 2–3 days earlier than the reversal of age-induced BM defects. The most likely explanation for this is that Cy-treated mice were young and hence had a more efficient stromal niche than the middle-aged mice, which would require time to regenerate.

Taken together with other work from our laboratory and the well-documented regeneration of thymopoiesis following SSA, these observations contribute to establishing a clinically appropriate conditioning regimen for regeneration of lymphopoiesis in middle-aged and/or chemically immunodepleted patients.

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Disclosures

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