B cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging

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Abstract

Aging is associated with a decline in B-lymphopoiesis in the bone marrow and accumulation of long-lived B-cells in the periphery. These changes decrease the body’s ability to mount protective antibody responses. We show here that age-related changes in the B lineage are mediated by the accumulating long-lived B cells. Thus, depletion of B-cells in old mice was followed by expansion of multipotent primitive progenitors (MPPs) and common lymphoid progenitors (CLPs), a revival of B-lymphopoiesis in the bone marrow, and generation of a rejuvenated peripheral compartment that enhanced the animal’s immune responsiveness to antigenic stimulation. Collectively, our results suggest that immunosenescence in the B-lineage is not irreversible, and that depletion of the long-lived B cells in old mice rejuvenates the B-lineage and enhances immune competence.
Introduction

The aging of the immune system involves many physiological changes that are collectively referred to as “immune senescence”. These changes affect both the innate and the adaptive immune system, often resulting in an immunodeficient state that is currently incompletely understood. The most important immunological manifestations in aging include poor responsiveness to new or evolving pathogens, and reduced efficacy of vaccination \(^1,2\). Primary causes of this immune incompetence are the decline in production of naïve lymphocytes in the bone marrow (BM) and thymus, and the expansion and increased survival of antigen-experienced memory lymphocytes. The outcome of these changes is a marked reduction in the diversity of the peripheral B cell repertoire and in the capacity of the body to mount protective antibody responses (reviewed in \(^3,7\)). Most attempts to enhance vaccination efficacy in aging are based on improving the delivery of antigen, but have yielded limited success \(^8\). Thus, while generation of a memory compartment constitutes an essential process for the function of the immune system throughout life, the expansion of this compartment in aging limits the ability of the immune system to respond to new antigenic challenges.

The B lineage undergoes dramatic age-related alterations in its cellular composition. Old mice show a marked decrease in the frequency of precursor B cells and in B-cell production in the BM, whereas in the periphery there is a significant reduction in naïve follicular B cells, reduction in B cell diversity, and accumulation of long-lived antigen-experienced B cells \(^7,9,10\). In addition, intrinsic defects have been shown in the response of B cells from aged mice or humans, including: decreased class switch recombination (CSR) and expression of activation induced cytidine deaminase (AID) and E47 \(^11\), reduced activation and expression of phosphotyrosine
kinases and protein kinase C, decreased expansion of B cells in response to antigen, and reduced number and volume of germinal centers (GCs). These findings may explain why in aging the antibody response to new antigens is poor in quality and in quantity.

The mechanisms underlying the cellular alterations in aging are unclear. Most existing data support the idea that hematopoietic stem cells (HSCs) acquire intrinsic defects with aging that suppress B lymphopoiesis. Accordingly, the peripheral B-cell compartment adapts to these changes by accumulating long-lived cells, thus maintaining the peripheral B-cell numbers unchanged. Another hypothesis is that age-related alterations in the B lineage are mediated by the accumulation of long-lived peripheral B cells with age, which suppresses B lymphopoiesis in the BM. This hypothesis stems from earlier studies showing that B lymphopoiesis in the BM of young/adult mice is enhanced after ablating the peripheral B cell compartment by sublethal irradiation. A major implication of this hypothesis is that the potential to reactivate B lymphopoiesis exists in old mice, and the recent identification of a subpopulation of HSCs in the BM of old mice that retains an equivalent potential to differentiate into lymphoid and myeloid lineages supports this. Hence, the present study attempts to test the second hypothesis and to determine whether aging in the B lineage is mediated by the accumulating long-lived B cells.
Methods

Ethics Statement - All mouse studies described in the present study were approved by the committee for the supervision of animal experiments at the Technion.

Mice – Mice used were C57Bl6 mice that are normal, or carrying a targeted conditional Baff-r locus (BAFF-R\textsuperscript{FL})\textsuperscript{25}, or transgenic for Mx-cre\textsuperscript{26}, or R26R-EYFP transgene for the EYFP-Cre reporter system\textsuperscript{27}, or Balb/c transgenic for human CD20 (hCD20 Tg)\textsuperscript{28}. The mice were housed and bred at the animal facility of the Faculty of Medicine, Technion. For the experiments we considered old mice to be ≥20 months old and young mice to be 3-4 months old. In addition, old mice were pre-screened by staining of blood samples to ensure they have an "old-like" B cell phenotype before treatment. An "old-like" B cell phenotype in C57Bl6 mice and the hCD20 Tg mice was determined when frequency of newly-generated AA4.1-expressing B cells in peripheral blood was <5% of the B220+ cells (see supplementary figure S4). In some experiments, 3-83Tg mice\textsuperscript{29} were used and "old-like" B cell phenotype in these mice was determined by staining of peripheral blood for the loss of transgenic receptor expression in >50% of the B cells using anti-3-83 idiotype antibody, as described in\textsuperscript{9}.

B cell Depletion – In order to deplete B cells in vivo, mice were injected ip with the following mixture of monoclonal antibodies at 150 µg/mouse each: rat anti-mouse CD19 (clone 1D3), rat anti-mouse B220 (clone RA36B2), and mouse anti-mouse CD22 (clone CY34). After 48 hours, the mice were injected with a secondary antibody mouse anti-rat kappa (clone TIB216) at 150 µg/mouse. To deplete B cells in the hCD20 Tg model, mice were injected ip with 1mg/mouse of mouse anti-human CD20 monoclonal antibodies (clone 2H7) as described\textsuperscript{28}. Injected mice were bled.
from the tail vain at different time intervals to determine depletion efficiency and the kinetics of B cell return by flow cytometry (see supplementary figure S5). In some experiments, mice were sacrificed to determine depletion efficiency by flow cytometry and by absolute numbers in different lymphoid organs.

**Flow cytometry** – single-cell suspensions of from BM, spleen, lymph node, peritoneal cavity and peripheral blood were stained for surface marker expression using FITC-, PE-, APC, Pacific blue, Cy5 and biotin-conjugated antibodies, followed with streptavidin PerCP. Data were collected on a FACSCalibur™ (BD Biosciences, Immunocytometry Systems, Mountain View, CA, USA) or on FACSLSRII (BD Biosciences) and analyzed using the CELLQuest™ or FlowJo software (Tree Star, Ashland, OR, USA). Analysis of B cell populations and hematopoietic progenitors were conducted as described 16,30.

**Immunization and poly(I)poly(C) administration** – To activate the Mx-cre in vivo for ablation of \(\text{loxP}\)-flanked loci, mice received i.p. injections of three doses of poly(I)-poly(C) (400 μg/mouse each time) (InvivoGene, San Diego, CA) on days 0, 3, and 6. Deletion efficiency was determined by flow cytometry using the EYFP-Cre reporter system as described 27. In some experiments, mice were injected ip with NP-CGG (50μg/mouse) in alum adjuvant. Mice were bled from the tail vain and anti NP IgG1 antibodies in serum were quantified by standard ELISA using NP-BSA-coated plates.
**Statistical analysis** - The statistical significance of differences between experimental groups was determined using unpaired Student's t test, with differences considered significant at $p<0.05$. 
Results

**Chronic B-cell deficiency prevents the age-related decline of B lineage precursors in the BM**

To study whether long-lived B cells mediate age-related decline in B lymphopoiesis, we first used a mouse model enabling us to ablate B cells and establish a chronic deficiency of peripheral B cells. For these experiments we generated mice that are homozygous for a targeted loxP-flanked Baff-r allele (BAFF-R^{FL/FL}) and transgenic for cre-recombinase driven by the type-I interferon responsive promoter (Mx-cre). BAFF-BAFF-R signaling is essential for the survival of mature B cells, but dispensable for B lymphopoiesis in the BM. In these mice, the administration of the interferon inducer poly(I)-poly(C) results in the ablation of BAFF-R (deletion efficiency in the spleen 80-90% determined by EYFP-Cre-reporter system, supplementary figure S1C), the depletion of about 50% of the B cells as measured in the peripheral blood and spleen, and the appearance of many newly generated B220+/AA4.1+ B cells in peripheral blood and spleen (supplementary figure S1A and S1B).

In old BAFF-R^{FL/FL}/Mx-cre mice, the peripheral B-cell deficiency that was induced upon the ablation of BAFF-R stimulated significant B lymphopoiesis in the BM, that was similar to the B lymphopoiesis of young mice (figure 1A). We found a high frequency of proB (B220+/CD43+/IgM-), preB (B220+/CD43-/IgM-), and immature B (B220^{lo}/IgM+) cells in the BM of old BAFF-R^{FL/FL}/Mx-cre mice, but not of old control BAFF-R^{FL/+}/Mx-cre mice, following treatment with poly(I)-poly(C). Further quantification analysis of the B lineage subsets confirmed these finding. Thus, old BAFF-R^{FL/FL}/Mx-cre mice treated with poly(I)-poly(C) for B cell depletion had similar numbers of B220+ cells in the BM as those found in the BM of young mice.
(figure 1B), with about 75% of them being newly generated and residing in the BM (~15% proB, ~38% preB, ~20% immature) and only 25% recirculating (B220(high)/IgM+/AA4.1-) (Fig. 1C, right 2 pie charts). In contrast, about 10 fold less B cells were found in the BM of the old, poly(I)-poly(C)-treated, control BAFF-RFL/+/Mx-cre mice (figure 1B), and 88% of them were recirculating (figure 1C), which is in agreement with previous studies that showed an age-related decline in the number of precursor B cells in the BM. A similar low number of B cells (3x10^6±2x10^6) was detected in the BM of old, untreated BAFF-RFL/FL Mx-cre mice (figure 1B), indicating the development of age-related changes in B lymphopoiesis in these mice. The poly(I)-poly(C) treatment had no effect on B lymphopoiesis in the young BAFF-RFL/FL/Mx-cre mice (not shown).

These results indicate that when long-lived B cells do not accumulate, frequencies of precursor B cells in the BM do not significantly decline with age, thereby ensuring a continuous flow of B cells to the periphery. This may imply the existence of a feedback cross-talk mechanism between the B cells in the periphery and progenitors in the BM, which regulates B lymphopoiesis in aging.

**Active B cell depletion rejuvenates the B lineage in old wild-type mice**

Because our results implicated the age-related decline in B lymphopoiesis with the accumulating population of long-lived B cells, we next tested whether active depletion of these cells could revive B lymphopoiesis in aged wild-type mice. In these experiments, we used a mixture of antibodies to deplete the B cells in vivo and followed the reconstitution of the peripheral B cell compartment by analyzing peripheral blood samples over time. With this treatment we demonstrate a 80-90% depletion of the peripheral B cells, as analyzed in peripheral blood, spleen, peritoneal
cavity and lymph nodes (supplementary figure S2B and S2C). A significant advantage of this depletion approach is the fact that most of the primary antibodies in this mixture (specific for CD19 and B220) are derived from rat and are rapidly cleared when injected into mice (within 5 days, supplementary figure S2A). Moreover, this antibody depletion mixture does not substantially modify total numbers of developing B cells (proB, preB, immature) and their relative frequency in the BM (supplementary figure S3A and S3B, respectively). Hence, the rapid clearance of the antibody mixture and its effectiveness in selective depletion of peripheral B cells, but not progenitor B cells in the BM, indicate that the process of B lymphopoiesis in the injected mice is not repressed. This conclusion is also supported by the fact that young mice rapidly reconstitute the B cells in peripheral blood and spleen following depletion (supplementary figure S2D and S2E).

The results in figure 2B show that reconstitution of B cells in the peripheral blood of old mice after B-cell depletion took >50 days. This slow reconstitution time reflected the poor B lymphopoiesis in the aged BM, as was also reported following lymphoablation by irradiation or treatment with cyclophosphamide ⁹,¹⁹, or in BM chimeras ¹⁵. In order to find whether the chronic B cell deficiency had reactivated B lymphopoiesis, these old mice were subjected to second and third rounds of B cell depletion (each round of depletion was introduced only after >80% reconstitution of the B cells in peripheral blood). The results in figure 2B reveal a profound decrease in the reconstitution time with each additional depletion circle. Thus, the reconstitution time of the B cells after the second round of depletion was decreased by >70%, and full reconstitution was established within 18-30 days. After the third round of depletion, the reconstitution time was decreased by 85%, achieving complete B-cell
reconstitution within only 8 days (figure 2B), which is similar to the time of B-cell return in young wt mice (supplementary figure S2D).

The profound reduction in the time of B-cell return after multiple depletions suggested that the depletion of peripheral B cells and the consequential chronic B cell deficiency had stimulated enhanced B lymphopoiesis in the BM of the old mice. Indeed, analysis of the BM of the treated mice revealed that B lymphopoiesis had been reactivated as revealed by the increase in absolute number of B220+ cells, relative to the number of B220+ cells in the BM of the old untreated mice (figure 2D). Further analysis revealed a revival of B lymphopoiesis in the BM of old mice that had been subjected to multiple rounds of B-cell depletion, with profound increase in the frequencies and absolute numbers of pro/preB and immature B cells (figure 2C and 2E). The increase in precursor frequencies was further confirmed by AA4.1 staining (figure 2C, lower panel). These frequencies and absolute number of precursor B cells were similar to those in the BM of young mice (figure 2C and 2E).

In the spleen, we found that the treated old mice completely restored the size of the B cell population as revealed by the absolute B cell numbers (figure 2D). Moreover, Cambier and colleagues have shown an accumulation of antigen-experienced long-lived B cells bearing the phenotype PanCD45+/B220lo in the spleen of old wt mice 9 (figure 2F middle panel, arrows indicating the antigen-experienced PanCD45+/B220lo population relative to the mature PanCD45+/B220+ population). Figure 2F shows that the PanCD45+/B220lo cells were eliminated in the old mice treated for B cell depletion and replaced with a population of mature B cells that are PanCD45+/B220+. These observations indicate that the potential to produce a high output of B lymphocytes is retained in the aged BM and that the rate of B lymphopoiesis is dictated by long-lived peripheral B cells.
B cell depletion stimulates expansion of early hematopoietic progenitor cell populations to reactivate B lymphopoiesis in the BM.

To further understand at which stage of BM development aging was reversed by B cell depletion, we analyzed different populations of hematopoietic progenitor cells in young, old and old B-cell depleted mice. To do so, we used transgenic mice expressing human CD20 specifically in the B lineage (hCD20 Tg mice), starting from the late preB stage. A significant advantage of using these mice is that effective long-term chronic depletion of peripheral B cells is obtained by a single injection of mouse anti human monoclonal antibodies specific to human CD20, without causing any depressing effect on B lymphopoiesis in the BM or resulting in major alterations in T cell subsets in the periphery, as had been described. The use of these mice is also important to validate our results obtained in the BAFF-R^{FL/FL}/Mx-cre and in wild-type mice treated with B cell depleting antibody mixture, in a third experimental setting.

After B cell depletion, complete B cell reconstitution in hCD20 Tg mice lasted 60-70 days, as described. Figure 3 indicates that, as observed in the previous experimental settings shown in figures 1 and 2, the long-term peripheral B-cell deficiency that was induced in old hCD20 Tg mice stimulated significant B lymphopoiesis in the BM. Thus, while most of the B220+ cells in the BM (lymphocyte gate) of old hCD20 Tg mice are circulated (B220^{hi}/IgM+/AA4.1-), in old hCD20 Tg mice treated for B cell depletion there is a profound increase of up to 10 folds in frequencies of precursor B cells (pro/preB B220+/IgM-/AA4.1+ and immature B220+/IgM+/AA4.1+).
The frequencies of common lymphoid progenitors (CLPs, Lin-/c-Kit+/IL-7R+) and multipotent primitive progenitors (MPPs, Lin-/c-Kit+/CD34+/Flt3+) in the BM of these mice were also studied by flow cytometry. In agreement with earlier studies \(^{10,16,33}\), we found that both of these populations are decreased in BM of old mice. In contrast, we found a significant increase in the frequencies of CLPs (figure 4 A and B) and MPPs (figure 4 B and C) in BM of the B-cell depleted old mice, to levels that are similar to those found in young mice. Thus, we conclude that peripheral B cell depletion and the consequential induction of chronic B cell deficiency stimulate expansion of early BM progenitor cell populations to reactivate B lymphopoiesis in aged mice.

**B cell depletion rejuvenates the peripheral B cell repertoire in aged mice**

We next tested whether B-cell depletion rejuvenates the peripheral repertoire. Cambier and colleagues used an immunoglobulin transgenic (Ig-Tg) mouse model (3-83Tg mice \(^{29}\)) to measure age-associated changes in the B-cell repertoire \(^{9}\). In young 3-83Tg mice, about 90% of the splenic B cells express the transgenic receptor \(^{29}\). This peripheral repertoire changes with aging due to selection of long-lived cells bearing non-transgenic receptors, and in old 3-83Tg mice, it is dominated by B cells that express endogenous Ig genes (\(^{9}\) and figure 5A). Similar to the old wt mice, we found that B-cell depletion revived B lymphopoiesis in the old 3-83Tg mice, as revealed by the significant increase in absolute number of B cells in the BM (figure 5B) and by the >15-fold increase in the frequency of newly generated B220+/AA4.1+ cell that express the transgenic receptor (stained with specific anti-idiotype antibody, ID+) in the BM (figure 5C). In the spleen of the treated mice we found that most of the reconstituting B cells (>90%) express the transgenic receptor and that the long-lived
PanCD45+/B220lo compartment had been replaced with a population of mature B cells that are PanCD45+/B220+ (figure 5D). We conclude that elimination of the long-lived antigen-experienced B cells in old 3-83Tg mouse re-activates B lymphopoiesis in the BM to re-construct a new Ig-Tg peripheral repertoire that is not different than that of a young 3-83Tg mouse 29.

**Rejuvenated B lineage in old mice confers an enhanced immune responsiveness**

One of the most important changes in aging is the failure to mount protective antibody responses to vaccination and to infectious agents, due to a reduction in the diversity of the peripheral repertoire 3,5,7. Because the peripheral compartment of the aged mice was replaced and rejuvenated after B-cell depletion, we next tested whether this new compartment conferred an increased competence to mount an antibody response against a new antigenic challenge. As shown in figure 6, old wt mice produced a very poor anti-NP IgG1 response compared with young mice (66.5±19.8 relative to 347.3±47.7, P<0.01). In contrast, a significant 3-4-fold increase in anti-NP IgG1 antibody titers was found in old wt mice that were subjected to B-cell depletion (161.3±44.8 in old depleted relative to 66.5±19.8 in the old, p=0.03). These levels, however, were still lower than those of the young mice (p=0.01). Hence, the reconstituted B-cell compartment in old mice restored in part the capacity to mount an antibody response to a new antigenic challenge.
Discussion

The present study shows that B lymphopoiesis can be reactivated in aging, thus indicating that aging in the B lineage is not an irreversible process. Our data support the idea that in aged mice there is a feedback mechanism between the peripheral B cell compartment and the progenitor compartment in the BM in controlling B cell output and its persistence. Earlier studies to demonstrate such a feedback mechanism in the B lineage used young (2-3 month old) mice and came to controversial conclusions. Thus, Park and Osmond 21,23 and Cancro and Allman 22 demonstrated homeostatic regulation, whereas Freitas and colleagues 34 suggested that B cell production in the BM is autonomously regulated. The results and conclusions of the present study do not support or contradict any of these previous works since our study was conducted in old mice, where dramatic alterations in the peripheral B cell compartment develop physiologically, including accumulation of long-lived memory cells and a significant reduction in naïve follicular B cells 7,9,10. Hence, this feedback mechanism may develop with age and may not be relevant to young mice used in the earlier studies 21-23,34.

We show here that when long-lived B cells do not accumulate, B lymphopoiesis does not decrease significantly with age. Moreover, upon elimination of peripheral B cells in aged mice, B lymphopoiesis in the BM is reactivated, indicating that the long-lived peripheral B cells mediate the age-related alterations in B lymphopoiesis. This conclusion is validated here in 3 different experimental settings: BAFF-R FL/FL/Mx-cre, wild-type mice treated with B cell depleting antibody mixture, and hCD20 Tg mice. The results we show here very well fit with the concept of hematopoiesis in which early cell lineages adapt their output to demand 35,36. There are several examples of homeostatic regulations in the hematopoietic system which do
not substantially change with aging, including the effect of G-CSF on proliferation and mobilization of stem cells \(^{37}\), red blood cell production during physiological and pathological changes \(^{36}\), and the ability of BM precursors to generate well-functioning CD4 T cells in mice \(^{38}\). Thus, in the three experimental settings used here, the chronic demand or the acutely-induced demand for B cells in the periphery prevented or reversed age-related alterations in the B lineage and imposed continuous B lymphopoiesis in the BM of old mice.

We show here that chronic B cell deficiency, imposed by depletion of the peripheral B cells, reverses age-related alterations in hematopoietic progenitor content in aged BM. Earlier studies have shown that frequencies and differentiation of MPPs and CLPs are decreased in old mice \(^{10,16,33,39}\), providing an explanation to the reduced production of B and T lymphocytes in aging. Upon B cell depletion, however, we found expansion of these progenitor cell populations and reactivation of B lymphopoiesis. Hence our results indicate plasticity in the size of CLP and MPP populations, which is determined by the peripheral demand for B cells in aging. Other studies, however, have shown that age-related alterations in the hematopoietic progenitor content in the BM and in the B lineage reflect acquisition of intrinsic defects \(^{15,16,18}\) and DNA damage in HSCs \(^{17}\), which is a progressive and irreversible process \(^{15,19}\). We propose that acquisition of these defects in HSCs may be regulated by the peripheral demands for B-cell. For example, the decreased demand for B cell production in aged mice due the accumulation of long-lived cells may lead to selection of mutated slow cycling HSCs, whereas chronic demand for B cells enforces continuous B cell production and this may select for rapid cycling, unmutated HSCs.

However, the finding that early progenitor populations (MPPs and CLPs) can re-expand and that B lymphopoiesis can be reactivated in aging indicates that even if
HSCs do accumulate defects, these defects do not abolish the potential to produce B lineage cells in the old BM. This may suggest that low frequency of unmutated HSCs exist in the old BM and are selected to expand and differentiate into B lineage cells upon B cell depletion. Though, the restoration of B lymphopoiesis in the aged mice may not occur rapidly, and a long-term deficiency may be necessary to measurably accomplish it. This possibility is supported by recent studies proposing that age-related alterations in the B lineage are associated with changes in the clonal composition of the HSCs, rather than with changes in individual stem cells\(^{40}\), or dictated by clonal expansion of functionally distinct HSC population\(^{24}\). In agreement with this, we suggest that long-term deficiency may be necessary for expansion of unmutated, lymphoid-biased HSCs in order to measurably detect expansion of MPP and CLP populations and reactivation of B lymphopoiesis in old mice in vivo, as we show here.

The results described here are in apparent contradiction with earlier studies showing that BM or purified HSCs from old mice failed to reconstitute the peripheral B cell compartment upon short-term of B cell ablation by cyclophosphamide or in BM chimeras\(^{9,15,19}\). This failure, however, can be explained by the low frequency of lymphoid-biased HSCs in the old BM used in these experiments, as opposed to that found in the BM of young mice\(^{24,40}\). In fact, our finding that peripheral B cell reconstitution in old mice after one round of depletion lasted >50 days is in agreement with these studies. However, we propose here that the chronic B cell deficiency, obtained following one round of B cell depletion (this study) or after BM or HSCs transfer into irradiated mice or in cyclophosphamide-treated mice\(^{9,15,19}\), reactivates B lymphopoiesis in the BM, but long-term is necessary to allow progenitor (MPP and CLP) and precursor expansion to restore it into full capacity and to measurably detect...
it. In agreement with this, we show here that the rate of B cell reconstitution in old mice increases after the second and the third rounds of depletion, reflecting the increase in frequencies of lymphoid-biased HSCs and progenitors upon the peripheral demand. This observation may not have been detected in the previous studies since peripheral B cell reconstitution was followed right after induction of the peripheral demand but not after repeated or long-term deficiency as we did here.

An alternative possibility for reactivation of B lymphopoiesis is that HSCs are capable to clear the accumulated defects to reverse cellular senescence. There are studies proposing that environmental factors and epigenetic modifications affect the entrance into cellular senescence. Since changes introduced by environment or by epigenetic mechanisms are reversible, it has been thought that cellular senescence can be reversed. Indeed, this was demonstrated in a study in which young and old mice shared their circulatory system by establishing parabiotic pairings. This study demonstrated that exposing aged skeletal stem cells to factors present in young serum, restored their proliferation and regenerative capacity.

Enhancing the immune competence to mount antibody responses to new antigens is of major importance to the elderly population. We show here that mice treated for B cell depletion developed significantly increased antibody responses to NP-CGG challenge. The interpretation of this finding is that the peripheral compartment that is reconstructed in old mice after B cell depletion is more competent in recognition and responsiveness to new antigenic challenge. Yet, the antibody responses mounted by the rejuvenated repertoire were still lower compared to the responses developed in young mice. This may reflect age-related alterations in T cells and in innate immune cells, which have not been replaced in these mice, perhaps contributing to the incomplete restoration of immune responsiveness.
Similarly, rheumatoid arthritis patients treated with B-cell depletion therapy generate a diverse repertoire of B cells that is mainly derived from newly generated B cells in the BM\textsuperscript{44,45}. In old mice, the rejuvenated repertoire produced a significant increase in antibodies to a new antigenic challenge. It will be interesting to find whether old patients treated for B cell depletion can also mount an increased antibody response to new antigens.

Finally, an important question that arises here is how chronic B cell deficiency is transcribed into a sensing mechanism of feedback regulation allowing a cross-talk between the peripheral and the progenitor B cell compartments. It is tempting to speculate that a soluble factor, which is produced in the periphery (perhaps by long-lived B cells), has an inhibition/activation capacity on B lymphopoiesis in the BM (such as erythropoietin in the homeostasis of red blood cells\textsuperscript{36}). The observation that MPP and CLP populations are also expanding upon B cell depletion suggests that the sensing mechanism operates at a high hierarchy of the hematopoietic system to increase proliferation and/or commitment to the B lineage. The existence of such regulatory factor is supported by a recent study proposing that age-related alterations in HSCs result from defects in niche cells that are systemically regulated by insulin-like growth factor-1\textsuperscript{46}. B cell depletion is also associated with reduction of serum immunoglobulins\textsuperscript{28}, but this factor appears not to affect B cell generation in the BM\textsuperscript{47-49}. It is also possible that competition on niche space in the BM between developing, mature and plasma cells plays a role in regulation of B cell development\textsuperscript{50}. Studies to understand how this cross-talk is mediated are now conducted.
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Authorship and Conflict of Interest Statements

Z.K., S.Na. and S.Nu. designed and performed the research, analyzed the data and contributed to the writing the manuscript. K.G., T.I. and T.L. preformed experiments and analyzed data. Y.S. and M.S.S. contributed new analytic tool. D.M. is PI in this study and contributed in designing the research data analysis and writing the manuscript. All authors declare no competing financial interests.
References

Figures legends

Figure 1. Ablation of BAFF-R induces chronic B cell deficiency and prevents aging of B lymphopoiesis in the BM of old mice. Young (4 months) and old (20 months) C57Bl6 BAFF-R FL/FL/Mx-cre and young and old (age-matched) control C57Bl6 BAFF-R FL/+Mx-cre mice received i.p. injections of three doses of poly(I)-poly(C) (400 μg/mouse each time) to ablate the loxP-flanked BAFF-R allele. Prior to treatment old mice were pre-screened to have <5% AA4.1+ B cells in peripheral blood as an indication for an "old-like" B cell phenotype. After 90 days, BM was analyzed for B lymphopoiesis. (A) BM cells were stained and analyzed by FACS with a lymphocyte gate as defined by light scatter. The IgM vs. AA4.1 plots were gated for CD19+/B220+ cells, and the B220 vs. CD43 plots were gated on IgMneg cells. (B) Absolute numbers of CD19+/B220+ cells in the BM of the indicated mice. The B cell numbers were calculated based on the total number of nucleated cells purified from two femurs and two tibias (n=3 mice in each group). (C) Quantification of B-cell subsets in the BM. The relative proportion of each B-cell subset was determined in the B220-gated population. Shown are representative results of young (n=3) and old (n=3) control BAFF-R FL/+Mx-cre mice and two different Old BAFF-R FL/FL/Mx-cre mice (from n=3).

Figure 2. B-cell depletion revives the B lineage in aged mice. Old B10D2 (20 months) wt mice (n=5) were subjected to three rounds of B-cell depletion. In each round, mice were first injected ip (day 0) with a mixture of monoclonal antibodies specific to B220 (150μg/mouse), CD19 (150μg/mouse) and to CD22 (150μg/mouse), followed by a second ip injection of rat anti-mouse kappa monoclonal antibodies (150μg/mouse) 48 hours later (more details on the effectiveness and the specificity of
the depletion is detailed shown in supplementary figure S2). Blood samples were
collected at various times after depletion and analyzed for B-cell frequency
(%B220+/CD19+ cells). The second and the third rounds were administered to each
mouse only after >80% of the initial B-cell numbers had been restored. (A) Peripheral
blood staining of representative young and old mice. Prior to treatment old mice were
pre-screened to have <5% AA4.1+ B cells in peripheral blood as an indication for an
"old-like" B cell phenotype (see also supplementary figure S4). (B) Kinetics of B-cell
reconstitution after depletion. The time-course of B-cell return after each depletion
cycle is shown for three (out of 5) individual mice. The histogram in each plot
corresponds to the B-cell frequency in peripheral blood before the first depletion. (C)
FACS analysis of the B lineage cells in the BM of mice subjected to three rounds of
depletion. Mice were analyzed 10-14 days after restoring 100% of the B cell counts in
peripheral blood (100-120 days after first depletion was administered). BM cells were
stained and analyzed by FACS with a lymphocyte gate as defined by light scatter. The
IgM vs. AA4.1 plot (bottom) was gated for B220+ cells. Representative plots of five
mice are shown. (D, E) - Absolute numbers of B220+ cells in BM and spleen (D), and
of specific B cell populations in the BM (values represent mean ± SD, x10⁶) (E). The
B cell numbers were calculated based on the total number of nucleated cells purified
from two femurs and two tibias (n=5 mice in each group). An asterisk (*) indicates a
significant difference p<0.05. (F) - FACS analysis of the B lineage cells in the spleen
of mice subjected to three rounds of depletion. Spleen cell analysis for B220 and
PanCD45 was conducted on gated IgM+ cells. Gates pointed by arrows are for the
PanCD45+/B220+ and the PanCD45+/B220lo populations. Representative plots of
five mice are shown.
Figure 3. B cell depletion in old hCD20 Tg mice re-activates B lymphopoiesis in the BM. Old (20 months) hCD20 Tg Balb/c mice (pre-screened to have <5% AA4.1+ B cells in peripheral blood as an indication for an "old-like" B cell phenotype) were injected ip with 1mg/mouse of mouse anti-human CD20 monoclonal antibodies (clone 2H7). B cell depletion was confirmed by staining of peripheral blood 5 days later. Mice were followed for 70 days until >90% reconstitution of peripheral blood B cells was achieved. Mice were then analyzed by flow cytometry for the B lineage in the BM and compared with old (age-matched) and young (4 months old) hCD20 Balb/c controls). The BM cells were analyzed with a lymphocyte gate as defined by light scatter. Plots shown are representative of 4 mice in each group.

Figure 4. B cell depletion expands populations of BM hematopoietic progenitor cells in old mice. Old (20 months) hCD20 Tg Balb/c mice (pre-screened to have <5% AA4.1+ B cells in peripheral blood as an indication for an "old-like" B cell phenotype) were treated for B cell depletion by ip injection of mouse anti-human CD20 monoclonal antibodies (clone 2H7). BM cells were analyzed by flow cytometry 70 days later for the frequencies of Lin-/c-Kit+/IL7R+ cells (CLPs) and Lin-/c-Kit+/CD34+/Flt3+ cells (MPPs), and compared with old (age-matched) and young (4 months old) hCD20 Balb/c controls. (A) –Representative FACS profiles of the indicated mice showing CLPs. (B) - BM frequencies of CLPs from the young, old, and old-depleted mice (n=4 in each group). (C) –Representative FACS profiles of the indicated mice showing MPPs. (D) - BM frequencies of MPPs from the young, old, and old-depleted mice (n=4 in each group). Statistically different differences are specified.
Figure 5. B-cell depletion in old 3-83Tg mice revives B lymphopoiesis in the BM and rejuvenates the peripheral repertoire. Old B10D2 3-83Tg mice (20 months) (confirmed to have an old-like B-cell phenotype by blood-sample staining) were treated for B-cell depletion. This was achieved by an initial ip injection with a mixture of monoclonal antibodies specific to B220 (150μg/mouse), CD19 (150μg/mouse) and to CD22 (150μg/mouse), followed by a second ip injection of rat anti-mouse kappa monoclonal antibodies (150μg/mouse) 48 hours later. Mice were bleed 3 days after last injection to ensure >90% B cell depletion in peripheral blood. After 65 days, the BM and spleen were analyzed for the B lineage by flow cytometry. (A) Peripheral blood staining of representative young and old 3-83Tg mice. Old mice were pre-screened to have <50% B cells in peripheral blood expressing the transgenic receptor. (B) Absolute numbers of B220+ cells were calculated based on the total number of nucleated cells purified from the BM (two femurs and two tibias) or spleen (n=4 mice in each group). An asterisk (*) indicates significant difference p<0.05. (C) BM cells were analyzed by flow cytometry with a lymphocyte gate as defined by light scatter. Expression of the transgenic receptor was detected using an anti-3-83 clonotype specific monoclonal antibody 54.1. (D) Spleen cells from the indicated mice were analyzed by flow cytometry. Analysis for B220 and PanCD45 was conducted on gated CD19+ cells. The plots shown are representative of 4 mice in each group.

Figure 6. Old mice with a rejuvenated peripheral repertoire mount an increased anti-NP IgG1 response. Old C57Bl6 wt mice (22 months) (pre-screened to have <5% AA4.1+ B cells in peripheral blood as an indication for an "old-like" B cell phenotype) were subjected to one round of B-cell depletion and were immunized i.p. with NP-CGG 70 days later. Prior to immunization, these mice were bled to confirm
reconstitution of $>80\%$ of B220$^+$ in peripheral blood. Old, age-matched, and young C57Bl6 mice (4 months) that were untreated for B cell depletion were used as controls and immunized with NP-CGG at the same time. The amount of anti-NP-specific IgG1 antibodies in the serum was determined by ELISA 7 days later, using an IgG1 standard curve for reference. The plot shows titers of anti-NP IgG1 antibodies of individual mice (old depleted n=9, old untreated n=11, young untreated n=7 and naïve n=4) collected from 4 different experiments and run simultaneously, and the mean and standard errors of each group. Student’s t-test was conducted to examine statistical significance between antibody titers of different groups.
Figure 1

A. BM cells, lymphocyte gate

B. BM, gated CD19+/B220+ cells

C. BM, gated IgM+ cells

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Figure 2

A. Peripheral blood B220+ cells

B. % B cells in blood

C. young old old depleted

BM cells, lymphocyte gate

BM cells, gated B220+ cells

D. E.

F. young old old depleted

Spleen cells, gated IgM+ cells

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Figure 3

**hCD20 Tg mice**
(BM cells, lymphocyte gate)

<table>
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<th>young</th>
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<td>2 28</td>
<td>21 11</td>
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<tr>
<td>AA4.1</td>
<td>78 10</td>
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</tr>
<tr>
<td>B220</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Figure 4

A. young  old  old depleted
   c-Kit
   IL-7R
   Lin- gated

B. % BM CLP cells
   young  old  old depleted
   p<0.05

C. Lin-/c-Kit+ gated
   PI3
   CD34

D. % BM MPP cells
   young  young depleted  old  old depleted
   p<0.05

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Figure 5

A. 3-83 Tg mice
(peripheral blood, lymphocyte gate)

B. total B220+ (x10⁶)

C. 3-83 Tg mice
(BM cells, lymphocyte gate)

D. 3-83 Tg mice
(spleen cells, lymphocyte gate)
Figure 6

![Graph showing serum anti-NP IgG1 (µg/ml) levels for naive, young, old, and old depletion groups with NP-CGG.

The graph displays the following:
- y-axis: Serum anti-NP IgG1 (µg/ml)
- x-axis: NP-CGG
- Data points for each group (naive, young, old, old depletion)
- Median values
- p-values: p<0.01, p=0.03, p=0.01
B cell depletion reactivates B lymphopoiesis in the BM and rejuvenates the B lineage in aging

Zohar Keren, Shulamit Naor, Shahar Nussbaum, Karin Golan, Tomer Itkin, Yoshiteru Sasaki, Marc Schmidt-Supprian, Tsvee Lapidot and Doron Melamed