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A B-cell subset uniquely responsive to innate stimuli accumulates in aged mice

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We have discovered a distinct mature B-cell subset that accumulates with age, which we have termed age-associated B cells. These cells comprise up to 30% of mature B cells by 22 months. Despite sharing some features with other mature B-cell subsets, they are refractory to BCR and CD40 stimulation. Instead, they respond to TLR9 or TLR7 stimulation and divide maximally on combined BCR and TLR ligation, leading to Ig production and preferential secretion of IL-10 and IL-4. Although similar to follicular B cells in both B-lymphocyte stimulator (BLyS) receptor expression and BLyS binding capacity, these cells do not rely on BLyS for survival. They are neither cycling nor the result of intrinsically altered B lymphopoiesis in aged BM, but instead appear to be generated from mature B cells that exhaustively expand during the individual's lifetime. Finally, they present Ag effectively and favor polarization to a TH17 profile. Together, these findings reveal that while the magnitude of the mature primary B-cell niche is maintained with age, it is increasingly occupied by cells refractory to BCR-driven activation yet responsive to innate receptor stimulation. (*Blood.* 2011;118(5):1294-1304)

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Introduction

Advancing age is accompanied by compromised immune responses and an increased propensity for autoimmunity. While the mechanisms underlying these broad functional changes remain unclear, they likely include age-associated alterations in developing and mature T- and B-cell compartments (reviewed in Maue et al[1] and Cancro et al[2]). Indeed, while hematopoietic stem cell numbers are maintained or enlarged with age, the numbers of cells entering lymphoid lineages are consistently reduced, yielding substantially lower T- and B-cell production rates.[3-7] This is evidenced by reductions in BM progenitor pools, as well as corresponding decreases in differentiation intermediates in the thymus and periphery. Despite this, mature peripheral T- and B-cell numbers are relatively stable,[8,9] but display changes in clonal composition and diversity, as well as differentiative potential.[2,8,10-13] While causal relationships have proven difficult to establish, these shifts in lymphocyte production, composition, and turnover are accompanied by alterations in immune responsiveness. For example, although aged animals develop germinal centers (GCs), both primary and memory humoral responses are blunted, possibly reflecting reduced capacity for class-switch recombination and generation of long-lived plasma cells and memory B cells.[2,14,15] In toto, these changes correlate with increased morbidity and mortality[16] as well as heightened incidence of some humoral autoimmune diseases (reviewed in Hasler and Zouali[17]).

These observations may in part be explained by age-associated changes in the representation of different functional lymphocyte subsets. Indeed, shifts in the proportions of T-cell subsets are a consistent feature of aging, including increased proportions of regulatory T cells,[18] reduced generation of functional memory $CD4^+$ T cells[3,19] and a selective reduction in the turnover of memory-like ($CD44^{hi}$) $CD8^+$ T cells.[5] However, whether functionally distinct B-cell subsets emerge with age is less clear. While the numbers of immature peripheral B cells fall with decreased BM production, robust shifts in mature peripheral B-cell subsets have not been revealed by prior phenotyping strategies.

Here we describe a novel mature B-cell subset that accumulates with age. Although positive for the B-cell coreceptor CD19, these cells express neither CD21/35, CD23, CD43, nor AA4.1. These cells respond poorly to BCR and CD40 ligation, despite having surface IgM levels comparable with follicular (FO) B cells. Instead, they respond to TLR9 and, to a lesser degree, TLR7 ligation, and proliferate most vigorously to combined BCR and TLR stimuli. Unlike FO and marginal zone (MZ) B cells, they do not rely on B-lymphocyte stimulator (BLyS) for survival, but nonetheless express BLyS receptors and sequester BLyS. Furthermore, they are not the product of B lymphopoiesis in the aged microenvironment, nor do they appear to be self-renewing. Instead, cells with these phenotypic characteristics can be derived from FO B cells after exhaustive expansion in vitro and in vivo. Functionally, B cells in this novel subset secrete Ig on TLR, but not BCR, stimulation. Moreover, they preferentially secrete IL-4 and IL-10 and, when acting as APCs, enhance IL-17 production by activated T cells. Together, these findings reveal a shift in functional primary B-cell subsets that likely contributes to the overall features of humoral immunosenescence.

Methods

Mice

C57BL/6, BALB/cBy, (Balb/c × C57BL/6)F_1, and DBA/2 mice ranging in age from 3 to 22 months were obtained from the National Institute on Aging. Congenic female C57BL/6 ($CD45.1^+$) mice were obtained from the National Cancer Institute. OTII transgenic mice (C57BL/6-Tg(TcrαTcrβ)425Cbn/J) were obtained from The Jackson Laboratory. All

Submitted January 13, 2011; accepted April 23, 2011. Prepublished online as *Blood* First Edition paper, May 11, 2011; DOI 10.1182/blood-2011-01-330530.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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animal procedures were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee.

Flow cytometric analyses of cell-surface Ags

mAbs used for staining included: anti-B220, anti-CD19, anti-CD43, anti-CD5, anti-CD11b (Mac-1), anti-CD86, and anti-CXCR5 (all from BD Pharmingen), and anti-CD93 (AA4.1), anti-CD21/35, anti-CD23, anti-IgD, anti-IgM, anti–L-selectin, anti-BR3, anti-MHCII and anti-CXCR4 (all from eBioscience). Secondary stains were PE-Texas Red– (BD Pharmingen) or QDot705-coupled streptavidin (Invitrogen). BLyS binding capacity was measured as described[20] using biotinylated BLyS (Human Genome Sciences Inc). Dead cells were excluded by the Live/Dead Fixable Aqua dead cell stain kit (Invitrogen). Data were collected on a BD LSRII flow cytometer with 4-log scale axes (shown in figures) and analyzed by FlowJo software (TreeStar).

Cell sorting

For magnetic sorting, $CD23^+$ splenocytes were isolated using a MiniMACS column and streptavidin microbeads (Miltenyi Biotec) with biotinylated anti-CD23 (BD Pharmingen). For flow cytometric sorting, splenocytes cleared of RBCs were stained with anti-CD19 (BD Biosciences), anti-CD23 (BD Biosciences), anti-CD43 (BD Biosciences), anti-CD93 (AA4.1, eBioscience), and anti-CD21/35 (eBioscience) for B cells; with anti-CD3 (BD Biosciences), anti-CD4 (BD Biosciences), anti-CD25 (BD Biosciences), anti-CD44 (BD Biosciences), and anti–L-selectin (eBioscience) for T cells, and with anti-CD11c (BD Biosciences) for dendritic cells (DCs). 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes) was used to exclude dead cells. Live, singlet lymphocytes were sorted on a BD Biosciences FACSDiva. Enrichment of $CD23^+$ splenocytes by MACS was routinely $\geq 95\%$. Flow cytometric sorting yielded enrichments between 96% and 100% for each subset (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Cell culture

Sorted B cells were cultured in RPMI medium with 10% FCS as described.[21] For assessment of proliferation, cells were labeled with CFSE (Invitrogen) as described[21] before culture with various reagents: 60 ng/mL rhBLyS (Human Genome Sciences), 10 μg/mL $F(ab')_2$ anti–mouse IgM, μ chain specific (anti-μ; Jackson ImmunoResearch Laboratories), 10 μg/mL anti-CD40 (BD Pharmingen), 10 μg/mL LPS (*Escherichia coli* O111:B4; InvivoGen), 1μM CpG (Genetics Core Facility, University of Pennsylvania), and/or 1 μg/mL CLO97 (InvivoGen). Cells were incubated at 37°C and 5.5% CO_2 for 72 hours. TO-PRO-3 (Invitrogen) was used to indicate dead cells. Sorted naive OTII $CD4^+$ T cells were labeled with CFSE as described,[21] then cultured with sorted B cells at a ratio of 1:1 in presence of 10 μg/mL $(OVA)_{323\text{-}339}$ peptide (GenWay) and reagents to polarize toward T-cell subsets as follows: Th1: anti–IL-4 (10 μg/mL; BD Biosciences), IL-12 (10 μg/mL; PeproTech). Th17: anti–IFN-γ (10 μg/mL; BD Biosciences), anti–IL-4 (10 μg/mL), TGF-β (2.5 ng/mL; R&D Systems), IL-6 (100 ng/mL; R&D Systems). Treg: anti–IFN-γ (10 μg/mL), anti–IL-4 (10 μg/mL), TGF-β (5 ng/mL), IL-2 (100 U/mL; Proleukin). Cells were incubated at 37°C and 5.5% CO_2 for 5 days.

In vivo neutralization of BLyS was performed as described.[22] Anti-BLyS is a monoclonal IgG1 hamster anti–mouse BLyS (Human Genome Sciences Inc).

Quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) from sorted B cells, reverse transcribed (SuperScript system; Invitrogen) and analyzed by qPCR. The following custom primers were made using the Primer-BLAST Primer designing tool from NCBI: TLR7-foward: 5′-AGG CTC TGC GAG TCT CGG TT-3′, TLR7-reverse: 5′-TGC AGT CCA CGA TCA CAT GGG C-3′; TLR9-forward: 5′-ACT TCG TCC ACC TGT CCA AC-3′ and TLR9-reverse: 5′-TCA TGT GGC AAG AGA AGT GC-3′. TaqMan primers (Applied Biosystems) were used to detect cytokine expression. Samples were normalized to GAPDH and represented as fold change over young FO B cells or a mouse universal reference total RNA (Zyagen) using the ΔΔCT method.

Adoptive B-cell transfer

Eight million MACS-enriched $CD23^+$ splenocytes from C57BL/6 ($CD45.2^+$) donors were CFSE-labeled, then injected IV into congenic C57BL/6 ($CD45.1^+$) hosts. Anti-CD45.1 and anti-CD45.2 (eBioscience) permitted discrimination between donor and host cells.

Cell-cycle analysis

Cells were surface stained, then fixed and permeabilized (Invitrogen Fix & Perm Kit) and incubated with 10 μg/mL DAPI at 4°C overnight. Cells were analyzed on an LSRII and doublets excluded.

Ig ELISA

Total Ig in cell-culture supernatants was measured using unconjugated anti–mouse Ig(H+L; Southern Biotechnology Associates) as capture Ab, and HRP-conjugated goat anti–mouse Ig(H+L; Southern Biotechnology Associates) as detection Ab followed by a TMB substrate kit (BD Biosciences).

Intracellular cytokine staining

Phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Fisher BioReagents), ionomycin (500 ng/mL; Cell Signaling Technology), and Golgi stop (BD Biosciences) were added for 5 hours at the end of T-cell culture. After surface staining, cells were fixed and permeabilized by Foxp3 Fix-Perm Kit (eBioscience), then stained with PE-conjugated anti–IFN-γ (BD Biosciences), eF450-conjugated anti-Foxp3 (eBioscience) and PECy7-conjugated anti-IL17A (BioLegend). Data were collected on a BD LSRII flow cytometer with 4-log scale axes (shown in figures) and analyzed by FlowJo software (TreeStar).

Results

A novel B-cell subset accumulates with age

Extensive analyses of surface phenotypes in the mature splenic B2-cell pool ($CD19^+CD43^-AA4.1^-$) of 3-month-old, 12-month-old, and 22-month-old female C57BL/6 mice revealed a subset lacking both CD21/35 and CD23. Both the frequency and number of these cells grew continuously with age (Figure 1A-B), increasing as much as 5-fold between 3 and 22 months of age. By 22 months, this population is evident in all females, ranging from 10%-30% of all mature splenic B cells. Limited analyses suggest that this subset appears with delayed kinetics in aged males (supplemental Figure 2). This B-cell subset was also found in aged female Balb/c, (Balb/c × C57BL/6) F_1 and DBA/2 mice (supplemental Figure 2), indicating that it is neither a strain-specific trait nor the result of extensive homozygosity.

This subset was found in the BM and variably in the blood, but was infrequent in the peritoneal cavity (PEC) or lymph nodes (LNs; supplemental Figure 3). We extensively analyzed the surface phenotype of these cells. They lack CD43, CD5, and Mac-1 expression (Figure 1C), so they are neither B1 B cells nor Bw cells recently described in wild mice ($CD5^-Mac\text{-}1^+B220^{hi}IgM^{hi}IgD^{hi}CD43^-CD9^-$).[23] Furthermore, they express IgM levels similar to FO B cells but lower levels of IgD. They are not activated, because MHC-II and CD86 levels are comparable with FO B cells (Figure 1C). We considered the possibility that these cells represent an "exhausted" cell population. Exhausted T cells, which are immunologically unresponsive and metabolically "depressed," have been identified in chronic infections in both mice

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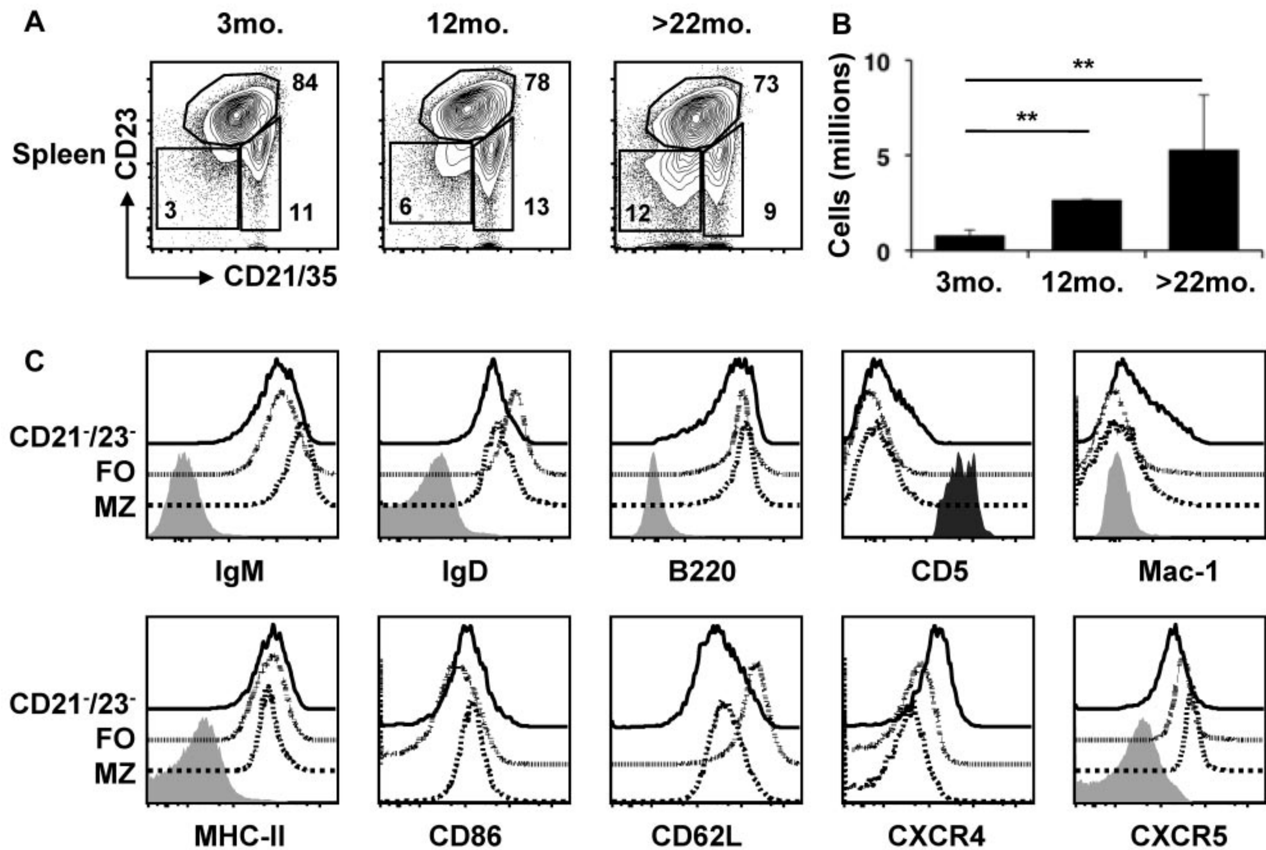

Figure 1. CD21/35⁻CD23⁻ B cells accumulate in aged mice. (A) Cells from spleen were first gated on $CD19^+$ $AA4.1^-$ $CD43^-$ live single lymphocytes. Percentage of FO ($CD21/35^{lo}CD23^+$), MZ ($CD21/35^{hi}CD23^-$), and novel B-cell ($CD21/35^-CD23^-$, ABC) subsets are shown in female C57BL/6 mice at age 3, 12, and > 22 months. (B) Absolute numbers of ABCs in spleen from mice at age 3, 12, and > 22 months; n = 3-5 mice and similar results were observed in 5 independent experiments. The Student *t* test was used for statistical analysis; $^{**}P < .01$. (C) Expression of 10 surface markers on ABCs (dark bold) compared with FO (dotted) and MZ (dashed) B cells from 22-month-old mice. Light gray peaks represent negative controls and the black peak represents the positive control for CD5 on B1 B cells.

and humans.[24-26] Moreover, an IgD^-CD27^- population, possibly corresponding to exhausted memory B cells accumulates in elderly humans.[27,28] Similarly, exhausted B cells ($CD20^{hi}/CD27^{lo}/CD21^{lo}$) were recently defined in the blood of HIV-viremic individuals, characterized by expression of the inhibitory receptor FcR-like-4 (FCRL4) and a profile of trafficking receptors.[29] We therefore assessed the $CD21/35^-CD23^-$ B cells for L-selectin and CXCR5 expression, and found that both are lower compared with FO B cells; however, the level of CXCR4 is higher (Figure 1C). FCRL4 has not been identified in mice and therefore could not be assessed.[30,31] Moreover, Pax5 expression is similar in $CD21/35^-CD23^-$ and FO B cells; and Blimp-1 expression is slightly increased (data not shown)—as reported for both mouse exhausted T cells and human exhausted B cells.[29,32-34]

Together, these findings indicate that $CD21/35^-CD23^-$ B cells do not correspond to any previously defined B-cell subset, but share some characteristics with exhausted B cells. Because they emerge and may eventually predominate with age, we have termed them age-associated B cells (ABCs).

ABCs respond to innate but not adaptive stimuli

We interrogated the activation requisites of ABCs by assessing proliferation and survival after adaptive or innate receptor ligation. Sorted B-cell subsets (supplemental Figure 1) were incubated with varied stimuli for 72 hours in vitro. ABCs showed lower survival and negligible proliferation in response to anti-μ and/or anti-CD40, compared with FO B cells from either aged or young mice (Figure 2). Thus, despite having surface IgM and CD40 levels similar to those of FO B cells, ABCs have impaired responses to stimuli associated with adaptive immune and costimulatory receptors.

In contrast, ABCs proliferated vigorously in response to the TLR9 ligand CpG, and to a lesser but noticeable degree to the TLR7 ligand CLO97 (Figure 3A-B). In accord with this, qPCR analysis showed that ABC express TLR7 and are enriched for TLR9 expression (Figure 3C). Surprisingly, ABCs showed little or no response to the TLR4 ligand LPS, further suggesting that they differ from previously described mature B-cell subsets, particularly MZ B cells, which expand vigorously to LPS challenge.[21] Interestingly, simultaneous TLR and BCR ligation yielded the most vigorous response, resulting in higher proportions of responding cells and more extensive division (Figure 3).

To ask whether ABC exert *trans* regulatory effects that thwart BCR stimulation, we performed in vitro mixing experiments. The results indicate that cocultured ABC do not affect division of young FO B cells to either BCR, TLR9 or combined stimuli (supplemental Figure 4), indicating that ABC do not exert direct *trans* regulatory effects on other B cells.

ABC bind BLyS but do not require BLyS for survival

Homeostasis in the primary B-cell niche is governed by BLyS, whereby FO and MZ B cells compete for available BLyS to survive (reviewed in Treml et al[35]). To determine whether ABC share this

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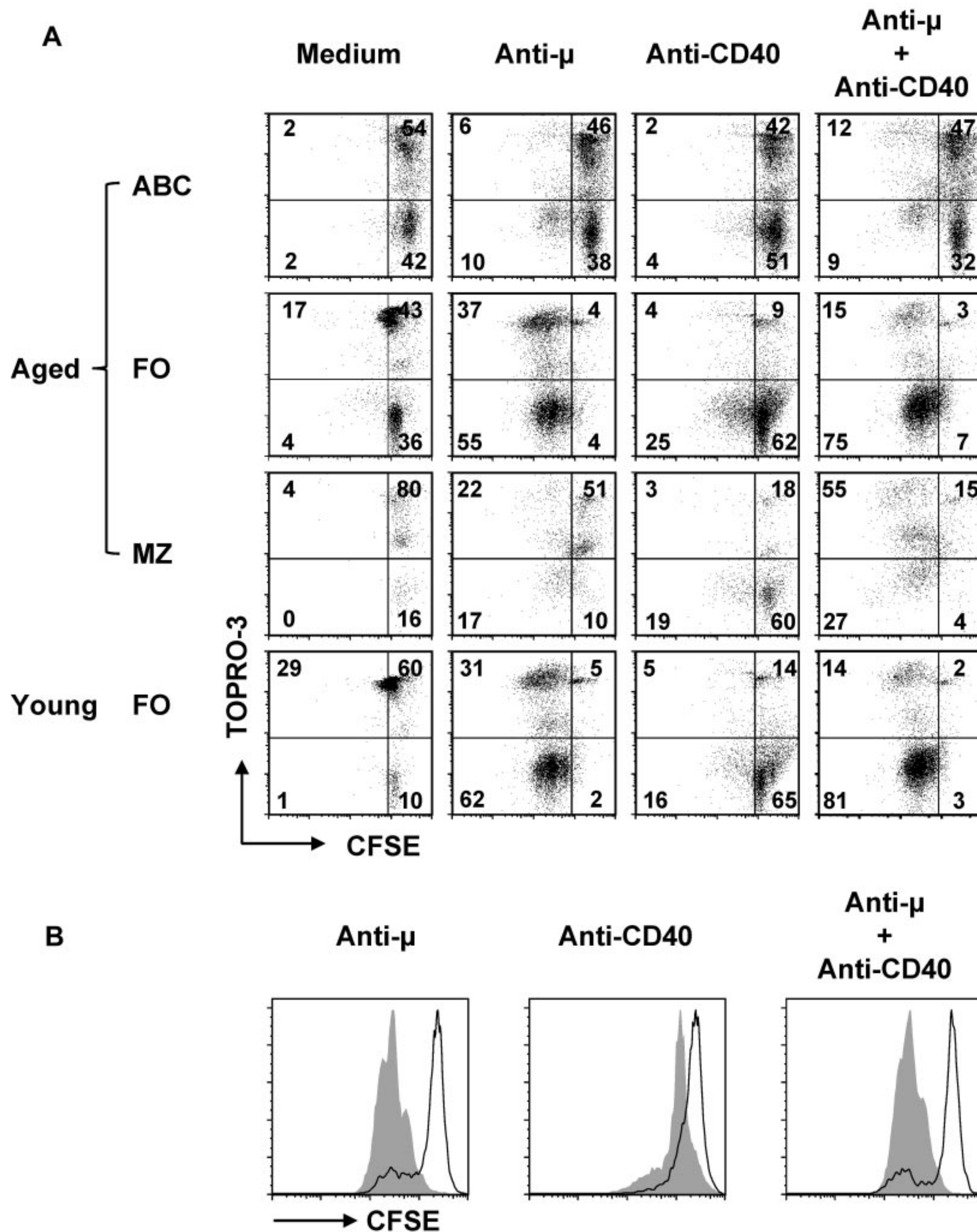

Figure 2. Impaired function of ABCs in vitro. (A) Flow cytometrically sorted splenic CD21/35$^-$CD23$^-$ (ABC) and FO B cells from aged or young mice were labeled with CFSE and then cultured with stimuli shown for 72 hours. Dead cells were stained by TO-PRO-3; CFSE dilution indicates proliferation. (B) Representative CFSE histograms for the same cultures shown in panel A show proliferation of ABCs (bold line) and FO (filled gray) B cells from aged mice. Similar results were found in 5 independent experiments.

niche, we examined their BLyS receptor expression, BLyS binding capacity, and BLyS-dependent survival. Similar to FO and MZ B cells, ABCs express 2 of the 3 BLyS receptors, BR3 and TACI. The level of surface BR3 on ABC is comparable with FO B cells, whereas slightly higher levels of surface TACI were generally observed. None of these subsets express the third BLyS receptor, BCMA (data not shown). In accord with this receptor expression profile, the BLyS binding capacity of ABCs, as assessed by addition of exogenous BLyS ex vivo, mirrors that of FO and MZ cells (Figure 4A).

Because ABCs express BLyS receptors and bind BLyS, we wished to determine whether their persistence, as with FO and MZ B cells, relies on BLyS. Accordingly, we compared BLyS mediated survival of ABCs and FO B cells in vitro and in vivo. Sorted FO and ABC were incubated with or without BLyS for 72 hours (Figure 4B; sorting gates are shown in supplemental Figure 1). In the absence of BLyS, ~ 15% of FO B cells from young mice survived, while ~ 40% of aged FO or ABCs survived. Thus, ABC and FO B cells from aged individuals have greater intrinsic survival capacity. Consistent with prior findings, BLyS significantly enhanced the survival of both young and aged FO B cells. In contrast, survival of ABC was largely unaffected; suggesting that their survival relies minimally, if at all, on BLyS. To interrogate whether ABC are indeed BLyS-independent in vivo, we treated mice with a BLyS neutralizing Ab, then enumerated splenic B-cell subsets 21 days later (Figure 4C). As expected,[22] FO and MZ

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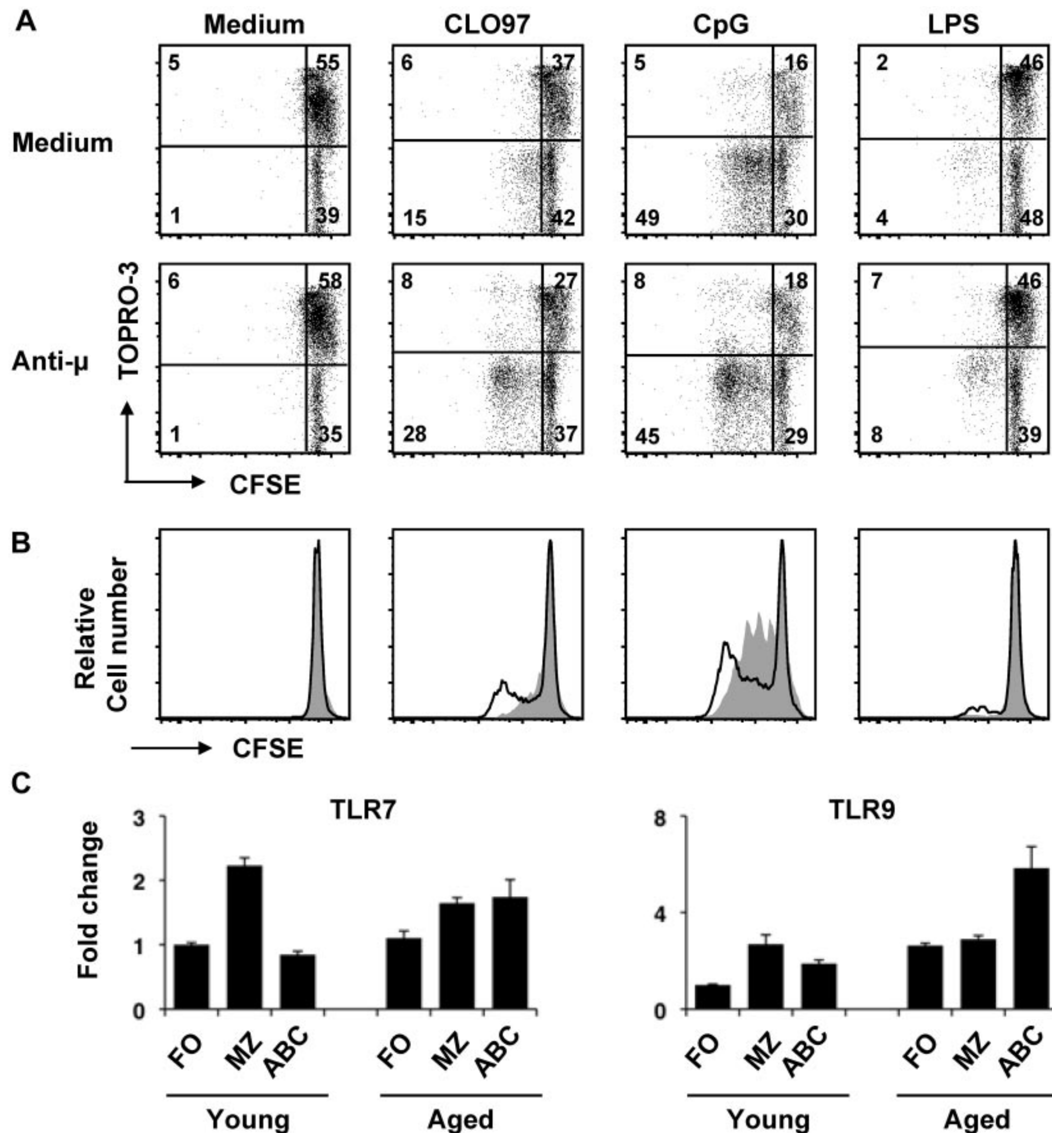

Figure 3. ABCs respond to TLR ligands in vitro. (A) Flow cytometrically sorted splenic ABCs from aged mice were labeled with CFSE then cultured with stimuli shown for 72 hours. Dead cells were stained by TO-PRO-3; CFSE dilution indicates proliferation. Similar results were found in 2 independent experiments. (B) CFSE histograms for the same cultures shown in panel A show proliferation of cells with (bold lines) or without (gray filled histograms) anti-μ. (C) TLR7 and TLR9 expression by qPCR in FO, MZ, and ABCs from young or aged mice. Fold change is relative to the level of each receptor observed in young FO (1.0 = the average level in FO B cells from young mice).

pools in both young and aged mice were depleted after anti-BLyS treatment, although aged FO cells were somewhat less sensitive to BLyS neutralization. In contrast, the number of ABCs was unchanged.

Taken together, these results suggest that ABC may be exceptional competitors for homeostatic space; because they occupy the preimmune B-cell niche by consuming (binding) BLyS, yet do not rely on BLyS for survival. Thus, while overall numbers in the primary B-cell niche are maintained with age, it is increasingly populated by ABC, probably at the expense of the FO subset. Moreover, given the functional attributes of ABC, this ongoing homeostatic displacement might engender increasingly diminished capacity for adaptive immune responses.

ABC are not the product of aged B lymphopoiesis

B-cell genesis slows with age, reflecting both lineage intrinsic and microenvironmental changes.[6-8,36] Thus, ABCs may emerge as the product of altered B lymphopoiesis in the aged. Alternatively, they might reflect the lifelong accumulation of cells generated at low rates from existing mature compartments. To distinguish these possibilities, we assessed peripheral B-cell subsets in aged mice after autoreconstitution after sublethal irradiation (Figure 5A). We reasoned that if ABCs are the product of B-cell genesis in the aged BM, then they should be immediately replenished; whereas if they reflect accumulation, then a peripheral compartment with few ABCs, comparable with that of young adults, should result. After full peripheral lymphoid autoreconstitution (> 12 weeks post irradiation), the numbers and proportions of mature splenic B-cell subsets closely resembled those of young adults, with both the frequency (Figure 5A) and number (Table 1) of ABCs lower than those in untreated aged mice. Thus, we conclude that the aged B-cell progenitor compartment is neither intrinsically nor microenvironmentally skewed toward ABC generation. It remained possible that ABCs represent a small pool of expanding, self-renewing cells. However, cell-cycle analysis revealed that ABCs are quiescent (Figure 5B), making it unlikely that

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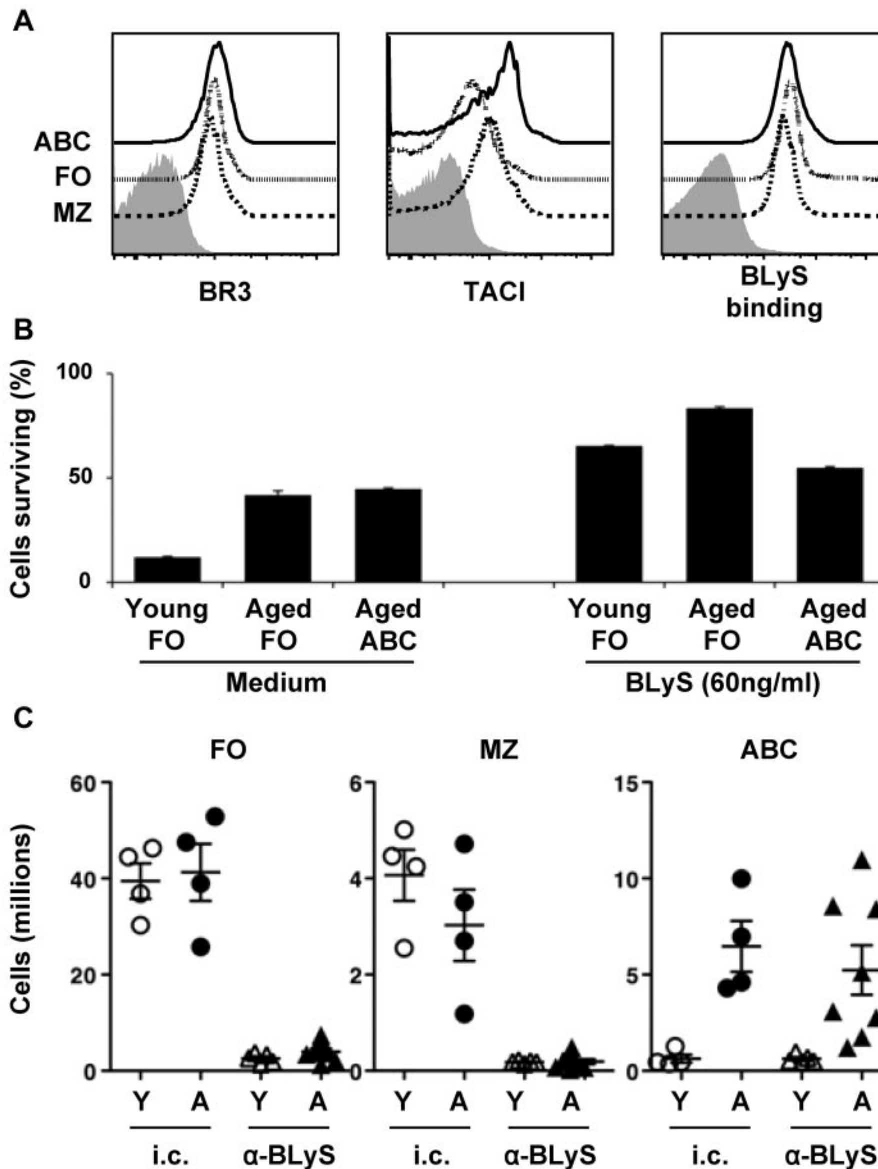

Figure 4. BLyS binding and BLyS requirement of ABCs. (A) Surface expression of BLyS receptors and BLyS binding capacity of ABCs (dark bold) compared with FO (dotted) and MZ (dashed) B cells from aged mice. (B) Flow cytometrically sorted splenic ABC and FO B cells from young and aged mice were cultured separately in the absence (medium only) or presence of BLyS. Living cells were TO-PRO-3 negative. (C) Effect of BLyS neutralization in vivo. Mice were treated with 100 μg of anti-BLyS or isotype control IgG1 IP on days 0 and 5. Numbers of FO, MZ and ABCs are shown at 21 days after the first injection. Mean with SEM are shown; each symbol represents one mouse. Similar results were observed from 3 independent experiments.

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they self-renew at an appreciable rate and confirming the notion that they are not activated.

ABCs can be generated from FO B cells

Our finding that ABCs arise from neither altered B lymphopoiesis nor rapid self-renewal raised the question of whether cells within conventional mature B-cell subsets can yield ABCs. We addressed this question with both in vitro and in vivo approaches. We initially assessed the ability of sorted FO B cells to yield progeny of the ABC phenotype following various combinations of in vitro stimuli (Figure 5C). FO B cells from aged mice divided in response to all stimuli. Whereas the progeny of cells experiencing BCR and CD40 ligation retained CD23 and CD21/35 expression, those resulting from TLR7 or TLR9 stimulation down-regulated CD23 and CD21/35 with each successive division. Moreover, combined TLR and BCR ligation resulted in levels of these surface markers comparable with ABCs. Thus, FO B cells acquire the ABC phenotype after extensive proliferation driven by TLR ligands alone or in combination with BCR ligation, but not after stimulation via BCR or CD40. Interestingly, FO B cells from young mice showed similar phenotypic changes under each stimulation condition (not shown), indicating that cells with the potential to yield ABCs exist in the FO pool regardless of age.

To establish whether $CD23^{+}$ B cells can similarly give rise to ABCs in vivo, we followed the persistence, proliferation, and phenotype of aged and young CD23+ B cells after adoptive transfer. Eight million $CD23^{+}$ B cells from aged or young C57BL/6 donors were loaded with CFSE and transferred separately into otherwise untreated young congenic recipients. One week after transfer, donor cells had retained their FO

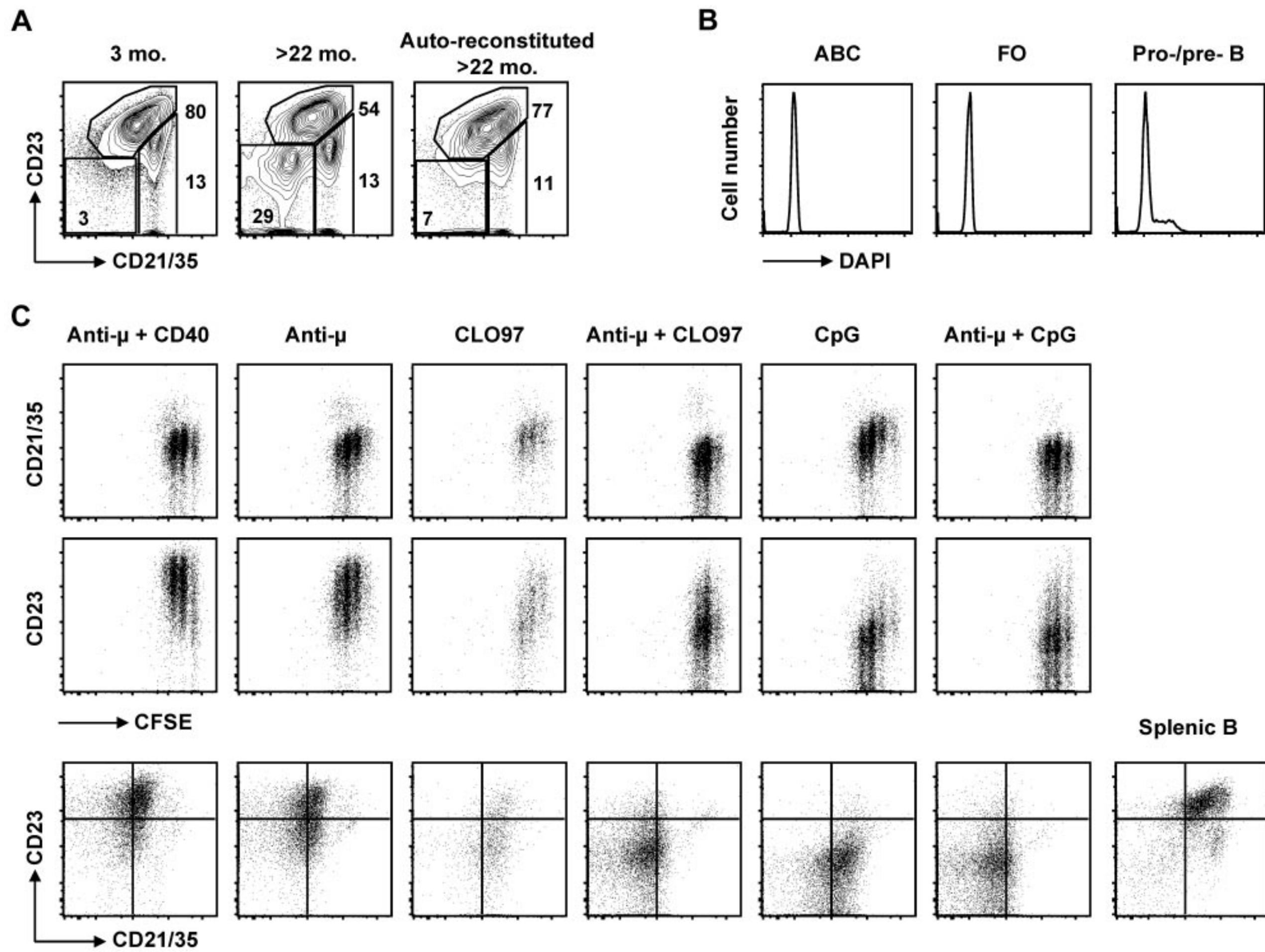

Figure 5. ABCs accumulate with age and can be generated from FO B cells on TLR stimulation in vitro. (A) Percentages of B-cell subsets in untreated young, aged, and autoreconstituted aged mice. The latter group of mice was sublethally irradiated (500R) on day 0 and allowed to reconstitute for 12 weeks. Gating strategy is the same as that described for Figure 1A. Similar results were observed in 3 independent experiments. (B) Cell-cycle analysis by DAPI for splenic ABC, FO, and BM pro-/pre- B cells from aged mice. (C) FO B cells from aged mice were sorted and CFSE-labeled, then cultured with stimuli shown for 3 days. Dead cells were excluded by DAPI staining. CD21/35 and CD23 expression on live cells is shown; CFSE dilution indicates cell division. $CD19^+$ splenic B cells were analyzed on the same day as a reference for CD21/35 and CD23 expression (right-most plot).

phenotype, and had not undergone proliferative expansion (not shown). One month after transfer, the proportions and numbers of B cells recovered were similar regardless of B-cell donor age (Figure 6A). However, the recovered cells had an altered phenotypic distribution (Figure 6B), with some acquiring ABC characteristics. CFSE dilution patterns revealed that some cells had divided (Figure 6C), and that only those that had divided extensively acquired the ABC phenotype (Figure 6D).

Based on these findings, we favor the idea that a small proportion of the FO B-cell pool undergoes exhaustive expansion to yield ABCs. It remains possible that additional B2 lineage compartments, including late transitional or MZ precursor subsets, can also expand to produce ABCs, but these would represent a small proportion of overall ABC formation given their relative representation in peripheral B-cell pools. In any case, months to years would be required for ABCs to achieve significant numbers, as is indeed observed over the lifespan of a mouse.

Table 1. Numbers of mature B-cell subsets after autoreconstitution in aged mice

	ABC	FO	MZ
Autoreconstituted aged mice	1.6 ± 0.9	24 ± 10	3.3 ± 1.2
Nontreated aged mice	9.5 ± 5.9*	17 ± 11	3.9 ± 1.3
Nontreated young mice	0.9 ± 0.3	21 ± 6	4.4 ± 0.7

Splenic B-cell subsets were gated as described in Figure 1. Cell numbers (in millions) are presented as mean values of 4 to 5 mice per group ± SD. Cell numbers in each subset were compared with those of nontreated young mice. The Student *t* test was used for analysis.

ABC indicates age-associated B cell; FO, follicular; and MZ, marginal zone.

*$P < .01$.

ABCs secrete Ab and characteristic cytokines in response to innate stimuli

To explore the functional characteristics of ABCs, we assessed their ability to elaborate Ab after stimulation through innate and adaptive receptors. Sorted ABC, FO, and MZ cells were cultured for 3 days with anti-µ, anti-CD40, TLR ligands, or combinations thereof (Table 2). ABCs generated significantly less Ab after BCR and/or CD40 ligation compared with young FO B cells, consistent with their lack of activation and expansion in response to adaptive stimuli (Figure 2). In contrast, TLR9 stimulation alone or in combination with anti-µ yielded substantial Ab production, significantly more than aged FO cells under the same conditions. Interestingly, concomitant BCR stimulation profoundly dampened

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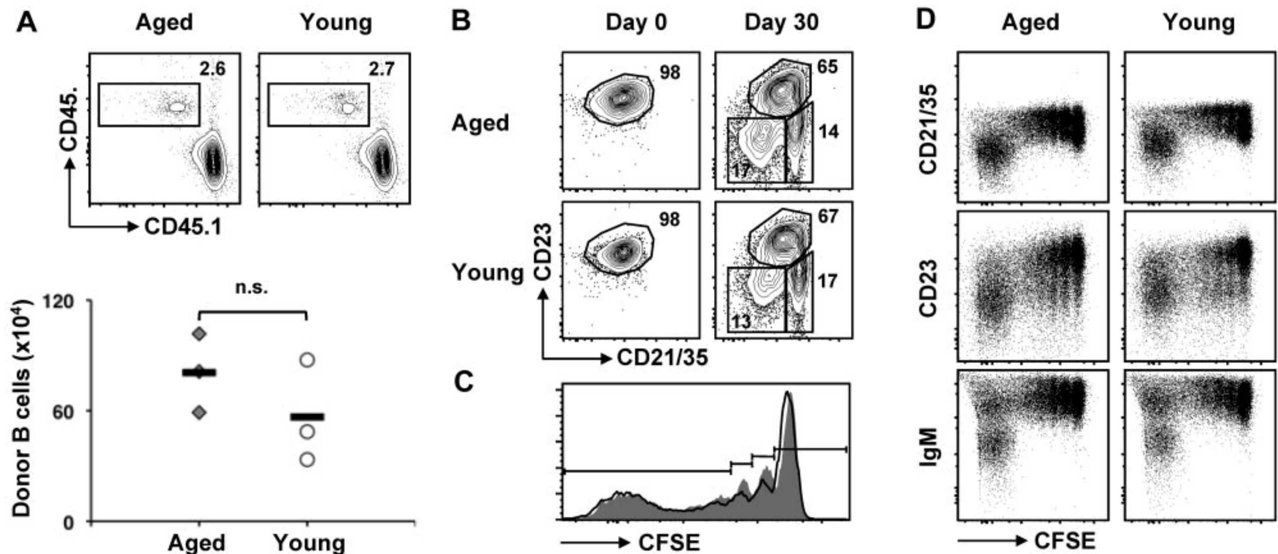

Figure 6. FO B cells from both aged and young mice can give rise to ABCs after transfer into replete hosts. MACS-sorted CD23$^+$ B cells (8×10^6) from aged or young mice were labeled with CFSE, then injected separately into young congenic C57BL/6 hosts. Recipient mice were analyzed 1 month after transfer. (A) Percentages and numbers of donor cells 1 month after transfer. Top plots show live lymphocytes, CD19$^+$B220$^+$ gated, of donor (CD45.2$^+$) and host (CD45.1$^+$) phenotype. Absolute numbers of donor cells (bottom graph) were not significantly different (n.s.). Similar results were found in 5 experiments. (B) Surface-staining phenotype of CD45.2$^+$ B cells before (left) and 1 month after transfer (right) shows ABCs (CD21/35$^-$CD23$^-$) and MZ (CD21/35^{hi}CD23$^-$) subsets of donor origin after 1 month. (C) CFSE dilution histograms of B cells from aged (filled gray) and young (bold line) donors recovered 1 month after transfer. (D) The dilution of CFSE in B cells from aged and young donors accompanied by surface expression of CD21/35, CD23, and IgM.

TLR-driven Ab production by young and aged FO and aged MZ B cells, whereas ABCs resisted this inhibition.

Cells with reduced CD21 and CD23 surface expression, as well as robust proliferation to combined TLR and BCR stimulation, are attributes that have been associated with autoantibody production in mouse models.[37,38] Given the similar phenotype and activation characteristics of ABCs, as well as the increased propensity for autoantibody formation with age, we asked whether ABCs are enriched for autoreactive specificities by analyzing the same supernates for dsDNA-binding Ab. Neither ABCs nor FO B cells generated dsDNA binding activity under any of the stimulation conditions, whereas MZ B cells produced some dsDNA binding Ab after TLR activation (not shown). Thus, ABCs, at least according to the phenotypic criteria used here, are not enriched for anti-dsDNA specificities.

B-cell cytokine production has recently been recognized as an important aspect of effector function, particularly in terms of guiding T-cell fate after Ag presentation (reviewed in Lund and Randall[39]). Accordingly, we assessed cytokine production by activated ABCs, as well as the impact of ABC presentation on T-cell polarization. Sorted subsets from aged animals were cultured with various stimuli and the expression of IFN-γ, IL-4, IL-6, and IL-10 assessed by qPCR. Compared with FO or MZ B cells, ABCs preferentially elaborate IL-4 and IL-10 after either TLR7 or TLR9 stimulation (Figure 7A).

Table 2. Ig secretion by B-cell subsets upon stimulation in vitro

	Young FO	Aged FO	Aged MZ	ABC
Medium	2.3 ± 0.5	4.8 ± 0.4	1.6 ± 0.4	4.4 ± 1.8
Anti-μ	40 ± 5	21 ± 3*	12 ± 2*	5.5 ± 2.4*
Anti-CD40	42 ± 2	24 ± 3*	61 ± 10	17 ± 5*
Anti-CD40 + anti-μ	50 ± 6	25 ± 4*	45 ± 8	20 ± 4*
CLO97	154 ± 27	68 ± 18*	1710 ± 249*	77 ± 34†
CLO97 + anti-μ	50 ± 11	29 ± 2†	154 ± 41†	63 ± 14
CpG	230 ± 34	253 ± 25	1271 ± 159*	323 ± 89
CpG + anti-μ	66 ± 20	31 ± 7†	185 ± 5*	236 ± 70†

Cells were sorted (supplemental Figure 1) and cultured for 3 days with stimuli shown. Total Ig concentration (nanograms per milliliter) in supernatants was measured by ELISA. Data are presented as mean values of triplicate cultures per each condition ± SD. Ig secretion by the different B-cell subsets was compared with young FO B cells in the same stimulation conditions. The Student *t* test was used for statistical analysis.

ABC indicates age-associated B cell; FO, follicular; and MZ, marginal zone.

*$P < .01$.

†$.01 < P < .05$.

ABCs can present Ag and potentiate Th17 polarization

Because ABCs express MHC class II at levels similar to FO B cells (Figure 1), yet exhibit distinct activation and cytokine production profiles, we interrogated the fate of T cells after Ag presentation mediated by ABCs. In these studies, CFSE-loaded OTII T cells were cultured under different polarizing conditions in the presence of either aged FO or ABCs pulsed with cognate OVA peptide. ABCs presented Ag to T cells under all polarizing conditions, enabling equivalent T-cell expansion. However, ABCs were more potent inducers of the Th17 profile, as indicated by significantly higher proportions of IL-17A–producing cells in these cultures (Figure 7B).

Discussion

These studies reveal a unique B-cell subset, termed ABCs, that accumulates with age, comprising up to 30% of mature peripheral B cells in 22-month-old mice. ABCs are phenotypically distinct from previously described B-cell subsets and, although they express receptors for the homeostatic cytokine BLyS, their survival is BLyS independent. Functionally, they are refractory to activation via adaptive immune receptors, yet responsive to innate receptor stimulation. ABCs preferentially produce IL-4 and IL-10 following these stimuli, and when functioning as APCs enhance polarization toward the Th17 phenotype. Together, these findings suggest that

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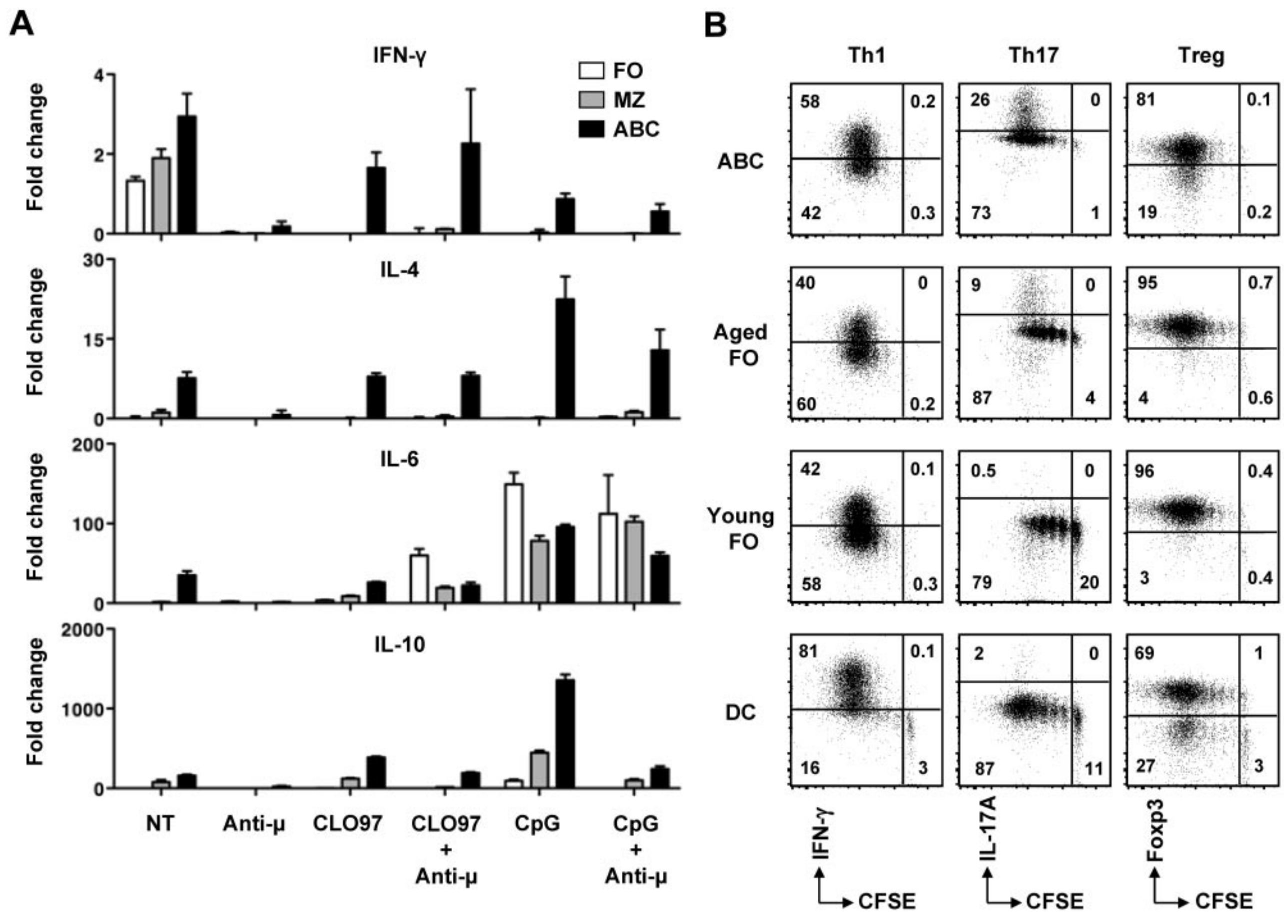

Figure 7. Cytokine expression and T-cell polarization on Ag presentation by ABCs. (A) Sorted ABCs and aged or young FO B cells were cultured with stimuli shown for 3 days. RNA was extracted and analyzed for expression of cytokines by qPCR. Fold change is relative to the expression level of each gene in standard total mouse RNA. (B) CFSE-labeled naive OTII CD4$^+$ T cells (L-selectin^{hi}CD44^{lo}) were cultured with polarizing cytokines and Ab for 5 days, in the presence of young or aged FO or DC (CD11c^{hi}) that had been pulsed with ovalbumin$_{323\text{-}339}$ peptide. Intracellular expression of cytokines and CFSE dilution were analyzed on gated CD4$^+$ OTII T cells.

the mature B-cell compartment is increasingly occupied by an exceptionally robust B-cell subset that may, in part, contribute to poor adaptive humoral responses and increased propensity for inflammation and autoimmunity associated with aging.

The numbers and proportions of ABCs increase progressively with age in inbred mice and F_1 hybrids, suggesting their emergence is a general age-associated process. We favor the idea that ABCs are the progeny of mature B cells that have undergone extensive expansion. Supporting this possibility, cells with an ABC phenotype can be generated by the extensive division of mature B cells after innate stimulation in vitro, as well as adoptive transfer into replete hosts. Alternatively, ABCs might result from B-cell genesis in the context of an aged microenvironment. Indeed, unique developmental lineages are revealed after the predominant B2 generative pathway wanes with age, and homeostatic pressures can alter BM output and selection.[40,41] However, because ABCs fail to reappear in irradiated 22-month-old mice after autoreconstitution, we consider this possibility unlikely. Furthermore, because ABCs are quiescent, the bulk of their accumulation probably reflects slow but continuous generation, rather than autonomous proliferative expansion.

If extensive proliferation of a few mature B cells is in fact responsible for the gradual generation of ABCs throughout life, the question arises as to what factors stimulate or predispose this small group of cells to undergo such expansion. Persistent, Ag-driven expansion might be responsible, yet ABCs are unlike currently defined mouse memory B-cell populations, particularly in terms of IgD expression. Alternatively, ABCs may be derived from intrinsically robust primary B cells engaged in protracted homeostatic expansion. In this regard, it is noteworthy that ABCs express BLyS receptors at levels similar to FO and MZ B cells, yet do not rely on BLyS to survive. Indeed, successful competition for BLyS signaling underlies homeostasis in the primary B-cell pools, whereby the overall size of the peripheral pools is circumscribed by the amount of available BLyS (reviewed in Miller et al[42]). Because mature B-cell numbers remain relatively constant with age, it is tempting to speculate that ABCs may impact the selection or survival of cells in the FO or MZ subsets by sequestering BLyS, thereby displacing less fit clonotypes. Moreover, BLyS independence would explain the persistence of a peripheral B cell that is unable to respond to BCR cross-linking, as these cells would be free of the need for signaling cross-talk between BCR and BLyS receptors to sustain downstream survival pathways.[35]

The basis for profoundly blunted responses to BCR and CD40 ligation among ABCs is not yet clear. It reflects neither a lack of appropriate receptors nor a global proliferative block, because ABCs express IgM, IgD, and CD40 at levels similar to FO or MZ B cells, and divide robustly after TLR stimulation. Moreover, this does not reflect the production of *trans* acting factors that negate BCR-driven activation, because their presence did not affect proliferation among cocultured FO B cells. Thus, these characteristics may instead reflect intrinsic changes in BCR signaling. For

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example, reduced CD21/35 expression on ABCs might be partially responsible, because CD21 is required for normal thymus-dependent (TD) humoral responses (reviewed in Fearon and Carroll[43]) and can amplify BCR signaling events.[44] Consistent with this possibility, CD21 is down-regulated on anergic B cells,[44] and a dysfunctional B-cell subset with low CD21 surface expression has been reported in HIV-viremic individuals[29] as well as in common variable immunodeficiency patients.[45] Despite being refractory to BCR cross-linking alone, some aspects of BCR signaling are either intact or rescued by downstream cross-talk, because simultaneous BCR ligation augments the extent of proliferation seen with TLR stimulation. This could reflect an additive increase in signal strength, because simultaneous BCR and TLR stimulation increases NF-κB activation and downstream kinase phosphorylation events.[46] Alternatively it may be the product of more complex cross-talk or reprogramming in these downstream pathways.[47]

Regardless of the exact basis for these unusual activation and signaling attributes, ABCs are refractory to stimulation through receptors generally associated with adaptive, T-dependent (TD) responses. Accordingly, it is tempting to speculate that ABCs play a role in the damped efficacy of adaptive humoral responses observed with age. Whereas reduced numbers of Ag-responsive B cells per se are unlikely to impact total Ab production in a primary adaptive response, this refractory ABC population will nonetheless compete for Ag and engage in cognate interactions with activated T cells, yielding changes in the quality of ensuing responses. For example, the propensity of ABCs to engender Th17 differentiation may be at the expense of T follicular helper cell generation,[48] thus thwarting interactions necessary for affinity maturation and memory cell formation. Moreover, because Th17 cells favor CD8 T-cell activation in some systems,[49] redirected polarization because of ABC presentation might indirectly contribute to the CD8 T-cell clonal expansions observed with age.[1,4] Similarly, while not enriched for autoreactive Abs themselves, such *trans* activities may enable ABCs to contribute to age-associated tendencies for autoimmunity. Indeed, augmented Th17 activity is implicated in autoimmune and inflammatory diseases, including age-associated colitis in both mice and humans.[50]

While understanding the full role of ABCs in immunosenescence awaits further investigation, our findings identify them as a phenotypically and functionally unique B-cell subset that occupies an increasing proportion of the primary B-cell niche with age.

Acknowledgments

The authors thank Philippa Marrack and Anatoly Rubtsov for insightful discussion, and the Penn Flow Cytometry & Cell Sorting Facility for expert technical assistance.

This work was supported by US Public Health Service grants AG030227 and AI073939 (M.P.C.).

Authorship

Contribution: Y.H. performed the experiments, with assistance from P.O. for cell isolation, cell culture, and qPCR, and from M.S.N. for real-time PCR; Y.H. and M.P.C. designed the research; and Y.H., J.L.S. and M.P.C. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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