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Positive Selection of Anti–Thy-1 Autoreactive B-1 Cells and Natural Serum Autoantibody Production Independent from Bone Marrow B Cell Development

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Abstract

A natural serum autoantibody specific for the Thy-1 glycoprotein (anti–Thy-1 autoantibody [ATA]) is produced by B-1 cells that are positively selected by self-antigen. Here, using ATAμκ transgenic mice we show that cells with this B cell receptor are negatively selected during bone marrow (BM) development. In a Thy-1 null environment, BM ATA B cells progress to a normal follicular stage in spleen. However, in a self-antigen–positive environment, development is arrested at an immature stage in the spleen, concomitant with induction of CD5. Such cells are tolerant and short-lived, different from B-1. Nonetheless, ATA-positive selection was evident by self-antigen–dependent high serum ATA production, comprising ~90% of serum immunoglobulin M in ATAμκ mice. Splenectomy did not eliminate ATA production and transfer of tolerant splenic B cells did not induce it. These findings demonstrate that B-1 positive selection, resulting in the production of natural serum ATA, arises independently from the major pathway of BM B cell development and selection.

Key words: B-1 • ATA • CD5 • IgM • Thy-1

Introduction

Developing B cells pass through several key checkpoints that they must traverse to survive and mature. Premature B cell receptor (BCR)* signaling due to autoreactivity leads to negative consequences, termed "negative selection" or "tolerance" (1–3). Thus, B cells with potentially hazardous autoreactive specificities are prevented from maturing, resulting in a lack of pathogenic serum autoantibody (4–6). However, a type of autoreactive B cell present in normal healthy individuals has been long-recognized as the source of natural autoreactive antibody in serum (7–10). A significant proportion of this autoantibody appears to derive from a specific B cell compartment, B-1. These cells reside preferentially in the peritoneal cavity (PerC) and intestinal lamina propria, in contrast to the majority of B cells that populate the conventional lymphoid organs. B-1 B cells often express CD5 and are enriched for autoreactive specificities, particularly to carbohydrate epitopes on self-glycolipids or glycoproteins (11–14).

Anti–Thy-1 autoantibody (ATA) is one of the classic natural serum antithymocyte autoantibodies (8, 15). The ATA encoded by germline V_H3609-$D_{Q52/FL8.2}$-J_H2/$V_κ21C$-$J_κ2$ Ig heavy and light chain genes derives from the SM6C10 hybridoma made from $CD5^+$ B-1 B cells (16, 17). This ATA recognizes a glycosyl determinant restricted to mouse Thy-1 (CD90) expressed on the majority of thymocytes and a fraction of peripheral T cells, as a species-, cell type–, and development-specific self-antigen (18). The exact glycosyl antigen form is unclear at present, however, thymocytes/T cells are considered as the major source of physiologic self–Thy-1 antigen. Importantly, we previously showed that in $V_H3609μ$ transgenic (Tg) mice, the normal accumulation of $CD5^+$ ATA B cells in the PerC and the production of serum ATA did not occur in the absence of Thy-1, providing a model for B-1 B cell positive selection in association with the production of natural serum autoantibody (16, 17).

The precise details of the development of these ATA B-1 cells remains to be established. In particular, it was unclear whether they arose during the course of normal bone marrow (BM) B cell development. The generation of B-1 cells initially occurs in the fetal liver (12), diminishing as

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**Abbreviations used in this paper:* ATA, anti–Thy-1 autoantibody; BCR, B cell receptor; BrdU, bromodeoxyuridine; FL, fluorescein; PerC, peritoneal cavity; splx, splenectomy; Tg, transgenic; Thy^M, thymocyte plasma membrane.

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primary development shifts to the BM after birth. B-1 cell generation gradually declines during the first several weeks of postnatal life, whereas there is increasing production of follicular B cells, the major population of B cells found in typical lymphoid sites (19, 20). The reason for the decline in B-1 cell generation is a long-standing puzzle. One mechanism is a defect in an early pre–B cell checkpoint, specifically critical in BM B cell development, as seen with V_H11 rearranged heavy chain due to failure of μ heavy chain to assemble with surrogate light chain (21). However, $V_H3609\mu$ heavy chain is capable of assembling with surrogate light chain, generating a pre-BCR (unpublished data), so $V_H3609\mu$ rearranged B cells should complete normal early BM B cell development, with the $V_H3609\mu$ heavy chain becoming associated with diverse Ig light chains. Thus, BM would be predicted to continually give rise to the ATA specificity as a component of the normal repertoire of newly generated B cells available for subsequent selection checkpoints. This study assessed the fate of such newly generated ATA B cells from the BM to spleen, the site of final peripheral maturation, to determine whether positive selection into the B-1 compartment occurs, contributing to natural serum autoantibody production.

Materials and Methods

Mice. C.B17, BALB/c, C.B17 SCID, Thy-$1^{-/-}$.CB17, and $J_H^{-/-}$.CB17 were bred and maintained in our laboratory animal facility. The ATAμ Tg mouse line (ATA1) expressing a V_H3609-μ^a Tg on a C.B17 background was previously described (17). To establish ATAμκ ($V_H3609\mu$/Vκ21C) Tg mouse lines, a Vκ21C rearranged kappa light chain gene was cloned from the SM6C10 hybridoma (16). DNA extracted from the SM6C10 hybridoma was cut with MunI. The rearranged Vκ21C was identified on a Southern blot by hybridizing with a pECκ probe and also with a Vκ21C fragment made by PCR amplification, and then DNA was size-fractionated and a lambda phage library was made with EcoRI precut Zap Express (Stratagene). The phage colonies were screened with the pECκ probe, rescreened with Vκ21C, and then the phage containing the rearranged kappa gene was converted into plasmid (pBK-CMV vector). The Vκ21C gene insert was freed of plasmid DNA using NotI and SalI and then coinjected with purified V_H3609-μ insert into (C3H × C57BL/6)F_1 eggs. Among the first offspring, PCR tail DNA showed 7/49 $3609\mu^+$/Vκ21C^+, all cointegrated. Six Tgμκ lines were then bred to C.B17 mice, selecting H-$2^{d/d}$ at the second generation with continued crossing to C.B17 for several (>4) generations. One founder line, 3369, was used in this study as a representative non-BCR editing ATAμκ Tg line. ATA Tg^+ mice with Thy-$1^{+/-}$ and Thy-$1^{-/-}$ backgrounds were generated by backcrossing with Thy-$1^{-/-}$.CB17 as previously described. Both females and males were used at 6–12 wk for most studies. All mice were maintained under specific pathogen-free conditions. Studies were performed according to institutional guidelines for animal use and care.

Anti-ATA Idiotype Antibody. SM6C10 IgM antibody was purified from ascites followed by absorption with protein A–Sepharose 4B beads (Sigma-Aldrich) to eliminate copurified IgG2b. Sprague-Dawley rats purchased from Taconic were hyperimmunized subcutaneously weekly four times, initially with 200 μg SM6C10 in CFA, next with 200 μg in IFA once, and then with 100 μg in IFA twice. 1 mo after the last immunization, a rat was boosted with 50 μg SM6C10 (soluble, no adjuvant) and spleen cells were fused with SP2/0 3 d later. Antiidiotype specificity was screened by comparing reactivity to SM6C10 and control non-ATA IgM (SM3G11; reference 16) by ELISA assay, by SM6C10 thymocyte staining inhibition activity using FACS®, and by comparing staining of ATAμκ Tg, ATAμ Tg, and non-Tg mouse B cells. Fine specificity was then examined by ELISA using various hybridoma IgMs. The selected hybridoma, P9-19A4, is a rat IgG2a.

Antibody Reagents, Flow Cytometry Analysis, and Cell Sorting. The following hybridoma antibodies were used in this study: IgM (331.12), IgM^a (RS-3.1), IgM^b (AF6-78), IgD^a (AMS-15), CD45R/B220 (RA3-6B2), CD5 (53-7.3), CD21 (7G6), CD23 (B3B4), CD24/HSA (30F1), CD62L/L-selectin (MEL-14), AA4.1 (22), and ATA idiotype (P9-19A4). Most monoclonal antibody reagents used for four to six color flow cytometry analysis were prepared in our laboratory by purifying antibodies and coupling to either fluorescein (FL), PE, allophycocyanin, or biotin (revealed by Texas Red–conjugated avidin). Cy7-PE–coupled antibody was made by first precoupling Cy7 dye (Amersham Biosciences) to R-PE (QuantaPhy). Cascade blue labeling reagent was purchased from Molecular Probes. FL–anti-CD21 was purchased from BD Biosciences. A modified FACStarPlus™ dye laser dual laser flow cytometer (BD Biosciences) was used in most four color analyses and cell sorting. A dye laser/Argon ion laser FACSVantage™ SE (BD Biosciences) with an additional Krypton laser (INNOVA 302C; Coherent) was used for six color analysis and the calcium mobilization experiment.

Thymocyte Plasma Membrane (Thy^M) Isolation. A plasma membrane fraction was prepared using a standard procedure. In brief, thymocytes from 10 mice were suspended in homogenizing buffer (25 mM Tris, 1 mM $MgCl_2$, 30 mM NaCl) at 5×10^8/ml and homogenized. After removing debris, the supernatant was applied to 41% sucrose in homogenizing buffer and centrifuged at 92,000 *g* for 1 h at 4°C. The interphase was harvested and washed twice at 64,000 *g* for 30 min, and then resuspended in 1 ml sterile PBS and stored at −70°C. The yield was 2–3 mg protein (OD_{280}) from 2×10^9 thymocytes.

Cell Culture. Various cell sorter purified spleen B cell ($B220^+$) fractions were cultured in U-bottomed 96-well culture plates (3799; Costar) at 5×10^4 cells in 150 μl/well of culture medium (RPMI 1641, 10% FCS, 50μM 2-ME) with or without stimulation. 2–3 d after culture, cells were harvested, propidium iodide was added, and cells were analyzed by FACS®, determining total cell number, dead cell number, and change in cell size.

Bromodeoxyuridine (BrdU) Labeling and Analysis. The BrdU labeling and analysis together with simultaneous cell surface staining were performed as described elsewhere (23, 24) with a minor modification to facilitate the cell staining procedure in a 96-well flexible plate. Mice were injected with BrdU (Sigma-Aldrich) 0.6 mg /0.2 ml PBS intraperitoneally twice a day for 3 d, and killed 12 h after the last injection for the initial BrdU-labeled cell analysis. Cell surface staining (using PE, allophycocyanin, and biotin/Texas Red–coupled reagents) was performed on ice in a plate (10^6 cells/well for each analysis) and placed at room temperature. After washing twice with PBS, cells were fixed and permeabilized by using commercial solutions at room temperature (30 μl/well for 15 min for each step, Fix and Perm; Caltag Laboratories), DNase I treated (5 mg/ml in DNase solution, pH 5.0, 100 μl/well, for 30 min; Sigma-Aldrich), and then incubated with FL–anti-BrdU (BD Biosciences) in 3% FCS containing staining medium (25 μl/well for 30 min). Between each treatment step,

cells were washed twice with PBS (100–150 μl/well) by multipipette resuspension followed by centrifugation. Anti-BrdU–stained cells were washed with staining medium and analyzed on a FACSVantage™ flow cytometer. Similarly stained/treated cells without the last anti-BrdU staining step and the cells from BrdU-uninjected mice were used as controls to determine the FL background of respective B cell populations.

Calcium Mobilization Assay. The protocol used was described elsewhere (25) with some modifications. Preparation of spleen cells, erythrocyte lysis, and surface staining were all performed at room temperature with deficient RPMI 1640 medium (Irvine Scientific) containing 10 mM Hepes plus 3% FCS ("Indo loading buffer"). Surface stained cells were washed once and resuspended at 5×10^6/ml and loaded with 8 μM Indo-1 a.m. (Sigma-Aldrich) together with Pluronic F-127 (0.01% w/v final; Molecular Probes) for 45 min at 37°C. After washing twice, each 2×10^6 cell sample was resuspended in 0.5 ml per tube and prewarmed to 37°C for 10 min just before analysis. Data were collected for 30 s to establish the baseline violet/blue (405:485 nm) ratio, and then stimulated by the addition of 30 μg/ml Thy^M. Data were collected for a total of 5 min. Rat anti–IgM (B7-6) and A23187 ionophore (Sigma-Aldrich) were used to reveal peak BCR signaling and maximal calcium mobilization. Data were analyzed using FlowJo® software (Tree Star Software).

Serum ATA Titer and ELISA Assay. ATA activity in the serum was tested by thymocyte staining analysis using 1:10 diluted serum in combination with FL–anti-IgM as a second step antibody as previously described (17). To quantitate IgM/κ antibody for total IgM, Id^+ IgM, IgM^a, and IgM^b in an ELISA assay, anti-IgM (331.12)/biotin-anti–mouse κ (187.1), 19A4/biotin-anti–mouse κ (or anti-IgM), anti-IgM/biotin–anti-IgM^a, and anti-IgM/biotin–anti-IgM^b were used as plate coating and second step reagent combinations, respectively, followed by incubation with alkaline phosphatase–conjugated avidin as the third step reagent. TEPC183 (IgM^aκ, ATA id^-), SM6C10 (IgM^bκ, ATA id^+), and H30-2C3 (IgM^aκ, ATA id^+; reference 17) antibodies were used as standards.

Adoptive Cell Transfer. $1-2 \times 10^6$ cell sorter purified B cell ($B220^+$) fractions were injected into the tail vein of recipient mice that had been lightly irradiated (300 rad) 1 d before use. Serum was harvested by orbital bleeding at designated times after injection.

Splenectomy (splx). Adult splx was performed by a standard protocol. In brief, 2-mo-old mice were anesthetized and a small incision was made to expose the spleen. The spleen was removed using a portable cautery unit to burn and seal connecting blood vessels. The body wall was closed by vicryl suture and the skin was closed with wound clips. Manipulated mice were kept in a laminar flow–equipped cage rack but without antibiotics. All mice maintained a healthy appearance.

Results

Self-antigen–dependent CD5 Induction at the Immature $AA4.1^+$ Stage. As previously described (17), the positive selection of ATA B cells is evident in the analysis of PerC cells of $V_H3609\mu$ Tg mice (ATAμ Tg; Fig. 1). ATA B cells, expressing the $V_H3609\mu$ transgene (Tgμ) in combination with endogenous $V_\kappa21C$-$J_\kappa2$, comprised 30–80% of the $Tg\mu^+$ endogenous μ $(Endo\ \mu)^-$ B cells in the PerC of $Thy\text{-}1^+$ ATAμ Tg mice, as detected by the anti-ATA idiotype antibody, 19A4, but such cells were infrequent or absent in $Thy\text{-}1^-$ mice (Fig. 1 A, PerC). This self-antigen–dependent increase in ATA B cells was accompanied by increased natural serum ATA (17). In the same ATAμ Tg mouse, a few ATA B cells were detectable as a cluster of "on-diagonal" cells in the BM and spleen by flow cytometry analysis, exhibiting the highest binding of 19A4 antiidiotype antibody, relative to IgM (Tgμ) level as indicated (Fig. 1 A). >80% of the $19A4^{hi}$-stained cells in this diagonal region had rearrangement of $V_\kappa21C$-$J_\kappa2$, as determined by single cell cDNA kappa chain sequencing. The cells that exhibited a lower 19A4-Id/Tgμ ratio lacked this rearrangement but were enriched for usage of other $V_\kappa21$ gene members or showed $V_\kappa21C$ rearranged to different J_κ segments (not depicted).

ATA B cells comprised ~0.4% of newly formed $Tg\mu^+$ B cells in the BM, regardless of $Thy\text{-}1^+$ or $Thy\text{-}1^-$ background, confirming that ATA B cells are generated in the BM with the expected frequency in ATAμ Tg mice. The ATA B cell frequency was only marginally higher in the spleen of $Thy\text{-}1^+$ mice compared with $Thy\text{-}1^-$ (6 vs. 2% of total $Tg\mu^+$ B cells) in contrast to the PerC. However, although the newly formed ATA B cells in the BM did not express CD5, half of the ATA B cells in the spleen showed self-antigen–dependent CD5 expression at a level comparable to PerC ATA B cells (Fig. 1 B). No other $Tg\mu^+$ $(Endo\mu^-)$ cells, including low 19A4-Id–stained cells in the spleen, showed CD5 induction. Thus, Thy-1 antigen exposure and CD5 induction occur during ATA B cell development from BM. Distinctively, however, the majority of these $CD5^+$ ATA B cells in the spleen were $CD24/HSA^{hi}$ $AA4.1^+$ $CD21^-$ and showed lower expression of lymph node homing receptor CD62L, a phenotype associated with immature B cells (24, 26, 27). In contrast, the $CD5^+$ ATA B cells in the PerC were $CD24/HSA^{med}$ $AA4.1^-$ $CD21^{lo}$ $CD62L^+$, a phenotype typical of long-lived B-1 cells in normal mice (Fig. 1 C). The predominance of immature $CD5^+$ ATA B cells in the spleen continued even in aged mice, suggesting that negative selection might be occurring in the spleen.

Maturational Arrest of ATA B Cells. To explore the possibility of negative selection, $V_H3609\mu/V_\kappa21C\kappa$ Tg mice (ATAμκ Tg) were made, in which all newly formed BM B cells expressed the ATA specificity. All six Tg founder lines showed exclusive expression of the ATA transgene by newly generated B cells in BM. In contrast, B cell profiles in the spleen showed some differences, falling into two patterns depending on founder line (Fig. 2 A): either exclusive maintenance of the ATA BCR but with decreased circulating B cells (represented by 3369 line, as will be detailed in this study), or else a predominance of "off-diagonal" B cells with a lower 19A4-Id/Tgμ ratio, due to a high incidence of BCR editing by expression of endogenous light chains (3345 line). Regardless, common to all lines, ATA B cells in the spleen retained a predominantly $CD24/HSA^{hi}$ and $AA4.1^+$ immature phenotype, a sign of arrest of maturation in the normal $Thy\text{-}1^+$ environment (Fig. 2 A). There was no apparent abnormality in the thymus or peripheral T cell compartment in any of the lines.

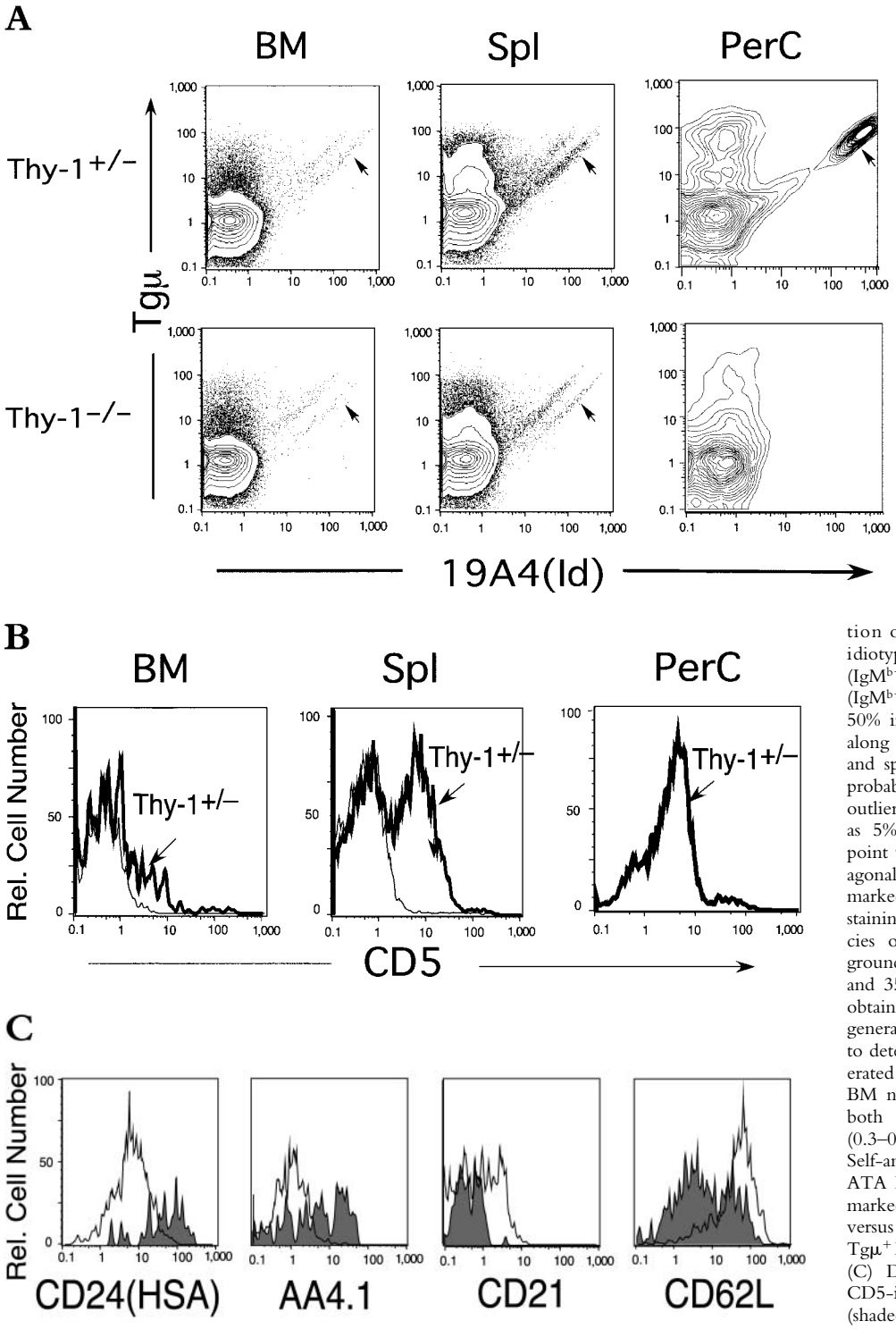

Figure 1. Distinction between spleen and PerC CD5$^+$ ATA B cells. Cell surface staining analysis of ATAμ Tg Thy-1$^{+/-}$ versus Thy-1$^{-/-}$ littermates. (A) Detection of Tgμ$^+$ Id$^+$ ATA B cells by anti-idiotype 19A4 antibody. Endogenous μ$^+$ (IgM^{b+}) cells were gated out in the figure (IgM^{b+} cells; <1% in BM, 2% in spleen, and 50% in PerC) by staining with anti-IgM^b along with the other staining reagents. BM and spleen cell data are presented as 10% probability contour plots with final 10% outliers shown as dots. PerC data are shown as 5% probability contour plots. Arrows point to the 19A4^{hi}-stained ATA B cell diagonal cluster. The Tgμ-positive region is marked by a thin line (>8 units of IgM^a staining). The ATA$^+$/Tgμ$^+$ B cell frequencies of Thy-1$^{+/-}$ versus Thy-1$^{-/-}$ background mice were 6 and 2% in the spleen and 35 and 1% in PerC, respectively. To obtain frequency data specific for newly generated B cells, the BM cells were stained to detect AA4.1$^+$ CD23$^-$ Tgμ$^+$ newly generated B cells together with 19A4. 0.4% of BM newly generated B cells were Id$^+$ on both Thy-1$^+$ and Thy-1$^-$ backgrounds (0.3–0.6% in four independent analyses). (B) Self-antigen–dependent CD5 induction on ATA B cells. CD5 levels of ATA B cells, marked in A, in Thy-1$^{+/-}$ mice (thick line) versus Thy-1$^{-/-}$ mice (thin line). No other Tgμ$^+$ Endo μ$^-$ cells showed CD5 expression. (C) Different surface phenotype by the CD5-induced ATA B cells in spleen (shaded) versus PerC (open).

These AA4.1$^+$ ATA B cells in ATAμκ Tg mice could mature if self-antigen was absent. Fig. 2 B shows analysis of Thy-1–positive and –negative littermates of the non-BCR–editing ATAμκ Tg line 3369. Newly generated B220$^+$ B cells in BM (CD24/HSA^{hi} CD21$^-$ CD23$^-$ AA4.1^{hi}; reference 27) were CD5$^-$ and present at similar frequencies, regardless of Thy-1 exposure (Fig. 2 B, BM). In mice with a Thy-1$^+$ background, the majority of ATA B cells became CD5$^+$ in the spleen and most remained at the CD24/HSA^{hi} CD21$^-$ AA4.1$^+$ immature stage with variable expression of CD23. In contrast, in Thy-1$^{-/-}$ mice, ATA B cells remained CD5$^-$ and the majority progressed to a mature follicular B cell phenotype (CD24/HSA^{low} CD21$^+$ AA4.1$^-$ CD23$^+$; Fig. 2 B, Spleen). Al-

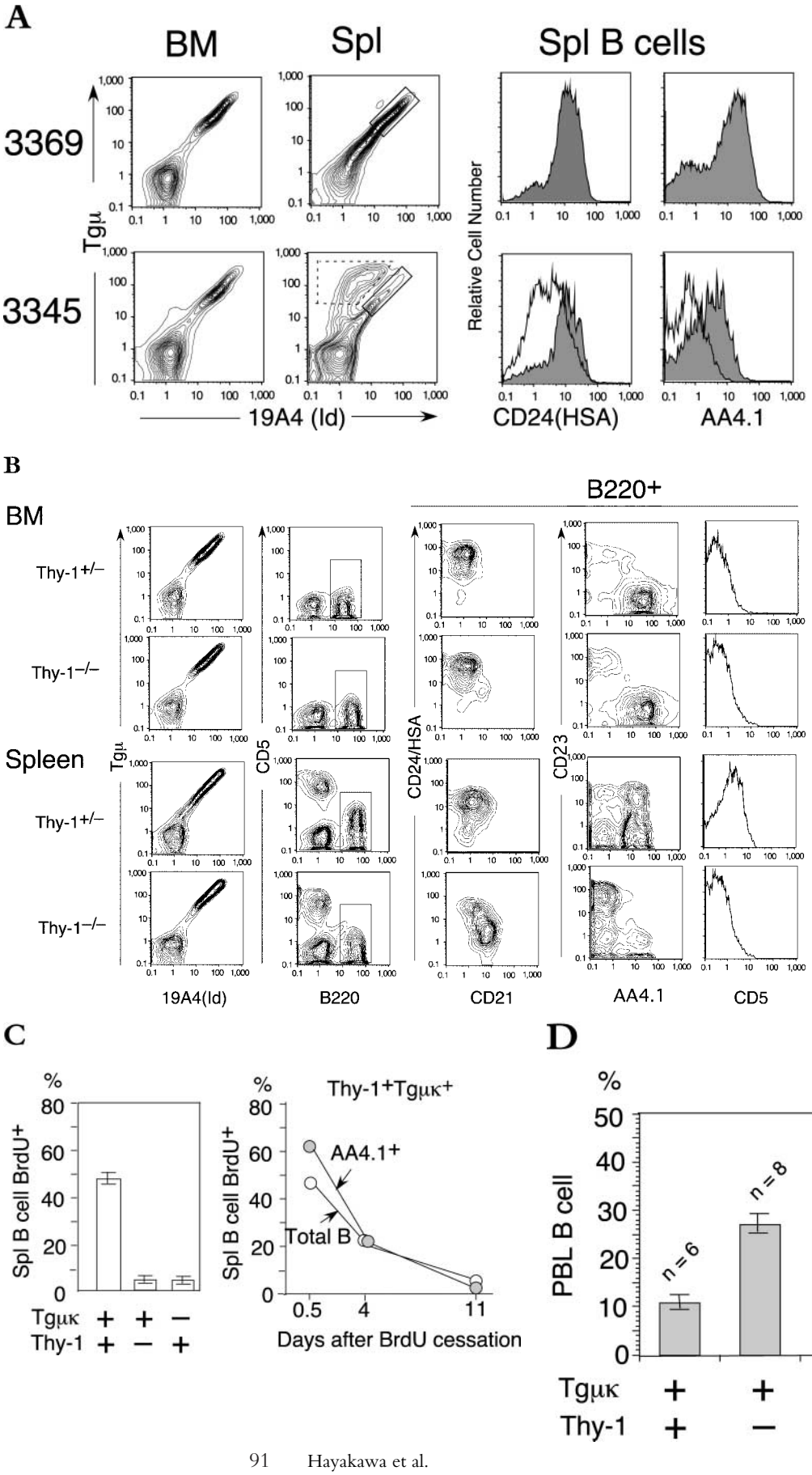

Figure 2. Self-antigen–mediated developmental arrest and rapid turnover of CD5-induced ATA B cells in spleen. (A) Comparison between ATAμκ Tg mice founder lines. Shaded histograms show the immature surface phenotype of spleen ATA B cells (diagonal region) in comparison to the more mature phenotype (solid line histograms) by the BCR-edited cells in the 3345 line (dotted box). (B) Developmental arrest at the AA4.1$^+$ cell stage. Cell surface staining analysis of 3369 ATAμκ Tg Thy-1$^{+/-}$ versus Thy-1$^{-/-}$ littermates. The region of B220$^+$ B cells used for CD24/CD21, CD23/AA4.1, and CD5 analysis is shown in the box. B220$^+$ cells in spleen of Thy-1$^+$ versus Thy-1$^-$ background were 35 and 40%, respectively. (C) Rapid turnover by self-antigen–exposed AA4.1$^+$ ATA B cells. On the left, BrdU$^+$ frequency in spleen B cells (B220$^+$) after 3 d of BrdU injections (0.5 d after the last BrdU injection) is shown. Data from three mice per group is shown. BM B220$^+$ cell BrdU labeling was >95% in all mice. On the right, disappearance of BrdU$^+$ label from AA4.1$^+$ cells after cessation of BrdU injection is shown. Data from total B220$^+$ cells or AA4.1$^+$-gated B cell fraction from Thy-1$^+$ 3369 ATAμκ Tg mouse spleen is shown. The BM B220$^+$ cell BrdU$^+$ cell frequency decreased to 5% 4 d after cessation. Representative data are shown. (D) Lower B cell frequency in PBL resulting from self-antigen exposure. Analysis of PBL of 3369 ATAμκ Tg × C.B17.Thy-1$^{-/-}$ backcrossed littermates. Data from three litters at 5–6 wk of age.

though spleen B cell numbers were similar in Thy-1$^+$ and Thy-1$^-$ mice, ATA B cells in Thy-1$^+$ mice showed a rapid turnover rate in contrast to those in Thy-1$^-$ or non-Tg mice (Fig. 2 C). More than half of AA4.1$^+$ B cells in Thy-1$^+$ ATAμκ Tg mice in the spleen were rapidly labeled by BrdU in 3 d and such labeled cells disappeared quickly, indicating cell death and constant cell turnover. This resulted in fewer circulating B cells in the peripheral blood in Thy-1$^+$ ATAμκ Tg mice in contrast to Thy-1$^-$ background mice (Fig. 2 D).

To test whether these self-antigen–exposed ATA B cells could respond to antigen ex vivo, spleen cells and ATA B cells were purified from mice and stimulated with a soluble Thy-1–containing thymocyte plasma membrane preparation (Thy^M) in vitro (Fig. 3). Although ATA B cells from Thy-1$^-$ mice could be activated and proliferated in response to Thy^M, spleen cells from Thy-1$^+$ ATAμκ Tg mice did not show any sign of activation (Fig. 3 A). This Thy^M–mediated B cell activation of Thy-1$^-$ mouse spleen was BCR specific and could occur independent of T cells, as shown by dose dependent, Thy-1–specific activation of purified ATA B cells as revealed by increased cell size (Fig. 3 B). AA4.1$^-$ CD23$^+$ ATA B cells, the predominant B cell fraction in Thy-1$^-$ mouse spleen, exhibited functional maturity as shown by the longest survival in culture and highest Thy^M-specific response. In contrast, the AA4.1$^+$ B cell fraction, predominant in Thy-1$^+$ mice, showed the poorest survival when Thy-1^M was added to the culture (Fig. 3 C). The effect of antigen preexposure was evident by a high intracellular Ca^{2+} level, already approaching that induced by antigen stimulation of naive B cells as previously reported for tolerant B cells (28). The addition of Thy-1^M only marginally mobilized Ca^{2+} (Fig. 3 D), resulting in eventual cell death upon further culture (Fig. 3 C). These data confirm that AA4.1$^+$ CD5-induced ATA B cells in the spleen were self-antigen exposed and had undergone maturational arrest and tolerance with reduced lifespan as a form of negative selection.

Lastly, we tested the possibility that a more normal diverse B cell repertoire during maturation might spare ATA B cells from negative selection in contrast with the extremely skewed B cell repertoire in μκ Ig Tg mice. However, the generation of 2% ATAμκ Tg BM chimeric mice (cotransferring ATAμκ Tg BM together with an excess of non-Tg BM cells) yielded a similar predominance of AA4.1$^+$ CD5$^+$ ATA B cells in spleen (unpublished data). Taken together, results from studies of ATAμ Tg, ATAμκ Tg, and these BM chimeric mice all indicate that ATA B

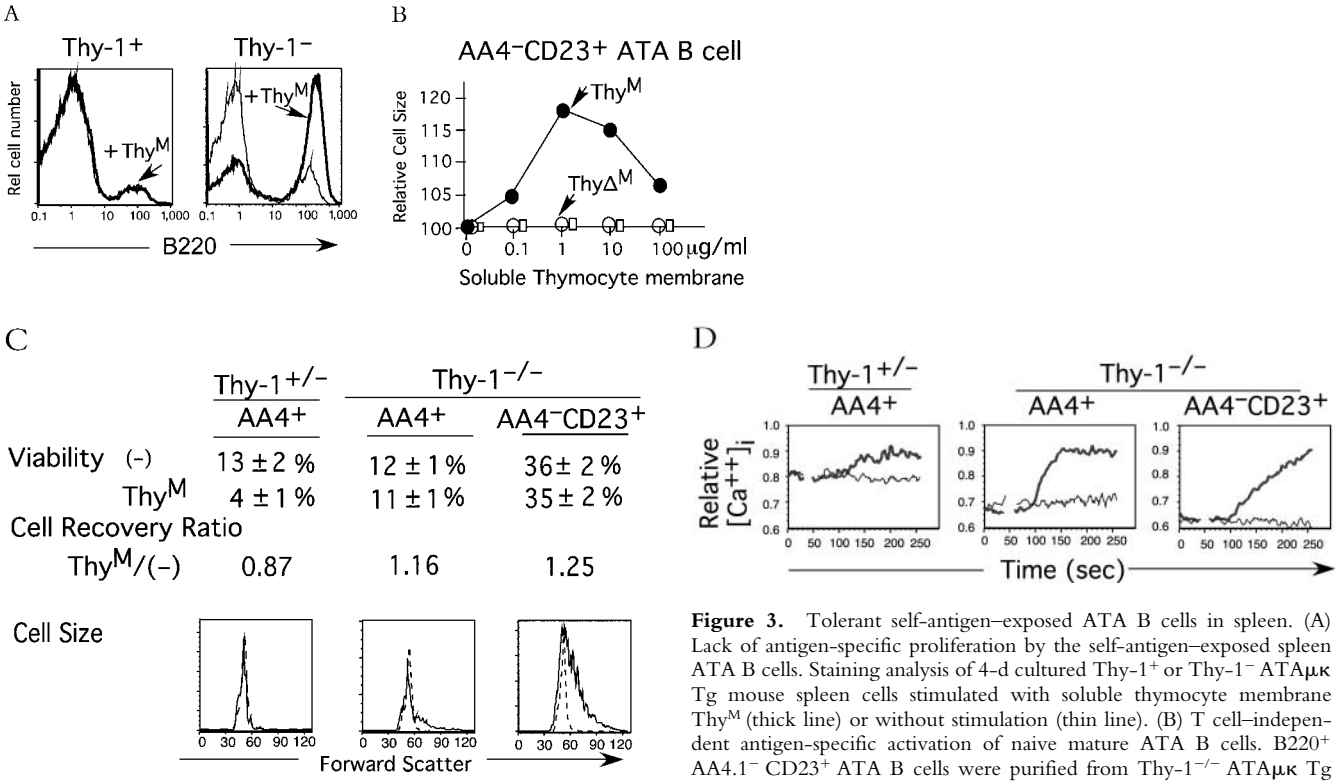

Figure 3. Tolerant self-antigen–exposed ATA B cells in spleen. (A) Lack of antigen-specific proliferation by the self-antigen–exposed spleen ATA B cells. Staining analysis of 4-d cultured Thy-1$^+$ or Thy-1$^-$ ATAμκ Tg mouse spleen cells stimulated with soluble thymocyte membrane Thy^M (thick line) or without stimulation (thin line). (B) T cell–independent antigen-specific activation of naive mature ATA B cells. B220$^+$ AA4.1$^-$ CD23$^+$ ATA B cells were purified from Thy-1$^{-/-}$ ATAμκ Tg mouse spleen and cultured for 2 d. Activation was measured by the increased cell size in mean forward scatter relative to nonstimulated cultures (taken to be 100) by FACS®. Stimulation was with Thy^M (●) or Thy-1$^-$ thymocyte membrane ThyΔ^M (○). Thy^M did not stimulate B cells from non-Tg mice (△). (C) Promotion of cell death of the self-antigen–exposed AA4.1$^+$ B cells by Thy^M. Spleen cells of 3369 Thy-1$^{+/-}$ or Thy-1$^{-/-}$ mice were stained with antibodies to B220, AA4.1 (AA4), and CD23, and respective B220$^+$ cell fractions were purified and then cultured with or without stimulation (Thy^M at 1 μg/ml) for 2 d. Data from triplicate cultures is shown. The cell recovery ratio is the ratio of live cell numbers with/without Thy^M stimulation. Cell size was gated on live cells after culture with (solid line) or without Thy^M (dotted line). (D) Calcium mobilization analysis. Spleen cells were prepared and stained similar to C. Data gated for the designated B cell fractions are shown. Stimulation with Thy^M (thick line) or ThyΔ^M (thin line). Indo-1 violet/blue ratio is shown as relative $[Ca^{2+}]_i$.

cells are negatively selected in the major B cell development pathway from BM.

AA4.1$^+$ Maturational Arrest Comparable to the IgM^{hi} IgD^{lo} Stage. One perplexing aspect of our analysis was the observation that a fraction of tolerant AA4.1$^+$ ATA B cells in the Thy-1$^+$ ATAμκ Tg mice showed the induction of CD23, a phenotype commonly considered as a mature B cell marker (29). Predominance of CD23$^+$ B cells, with coexpression of AA4.1 and CD5, is commonly observed in analyses of self-antigen–exposed ATA B cells in the spleens of mice older than 2 mo. However, CD23 induction can occur as early as the CD24/HSA^{hi} immature IgM^{hi} IgD^{lo} stage, and continues through transitional AA4.1$^+$ stages in normal mice (24, 27). To identify the IgM/IgD phenotypic stage in normal mice that corresponds to this ATA Tg B cell–arrested stage, we performed six color flow cytometry analysis. In normal mice, B cell maturation in the spleen is characterized by quantitative and qualitative changes in the BCR IgM/IgD ratio (30), where newly exported B cells have not yet fully up-regulated expression of IgD. As shown in Fig. 4, the ATA B cell phenotype associated with maturation arrest, i.e., CD24/HSA^{hi} CD21^{lo} and AA4.1$^+$ (with or without CD23 induction), corresponds to the IgM^{hi} IgD^{low} stage (a), but not to other stages. The surface phenotype of the majority of ATA B cells in Thy-1$^-$ mice is comparable to the IgM^{low} IgD^{hi} (Fig. 4 c) mature follicular stage. Thus, negative selection of ATA B cells occurs at the stage corresponding to IgM^{hi} IgD^{low} immature cells in normal mice before additional up-regulation of IgD and follicular B cell generation.

Natural Serum ATA Production Independent of Tolerant B Cell Generation. The analysis of B cell development in the spleen presented above is entirely consistent with negative selection predominating. In accord with this spleen cell tolerance, there were fewer PerC B cells in the ATAμκ lines. Total B cell numbers in PerC were approximately one third of non-Tg mice, suggesting an effect of predominant BM B cell negative selection in the PerC, as with the decrease of B cells in the blood circulation. Most of these PerC ATA B cells in ATAμκ Tg mice were CD11b/Mac-1$^+$, but showed low to undetectable surface CD5 expression compared with spleen CD5$^+$ ATA B cells (unpublished data). This CD5$^{lo/-}$ PerC B cell feature is reminiscent of PerC B cells in another autoreactive B cell model rescued from negative selection due to a decreased autoantigen environment (31).

Analysis of serum ATA, however, revealed a contrasting profile from that expected for negative selection. In conventional negative selection, tolerance induction of autoreactive B cells by exposure to self-antigen is accompanied by reduction of "spontaneous" autoantibody in the serum as a result of premature cell death or induction of anergy (4–6). In contrast, for ATA as a B-1 cell–associated natural autoantibody, the presence of self-antigen positively influenced serum ATA levels, consistently in both ATAμ and ATAμκ Tg mice. All six ATAμκ founder lines showed higher serum ATA titers than the ATAμ Tg lines on a self–Thy-1 antigen–positive, physiologic background (Fig. 5 A). In these ATAμκ Tg mice, ATA constituted ~90% of total serum IgM, whereas in the absence of self-antigen this decreased to 10% in the nonediting line 3369 (Fig. 5 B). Thus, serum ATA production was largely self-antigen dependent, the reverse of what is normally observed in conventional B cell tolerance models of negative selection.

PerC B-1 cells have been known to migrate and differentiate into plasma cells in the intestinal lamina propria (13, 32–34). Histological analysis of the intestinal lamina propria of ATAμκ Tg mice showed plasma cell infiltration at levels significantly higher than transgene negative littermates, whereas there was no significant increase in plasma cells in the spleen, lymph nodes, liver, or BM (unpublished data). This suggested accelerated plasma cell differentiation in the intestinal mucosa resulting from expression of the ATA specificity. Although self-antigen–exposed spleen AA4.1$^+$ ATA B cells did not spontaneously secrete antibody in vitro, they were readily induced to secrete antibody in response to LPS stimulation (unpublished data). B-1 cells in

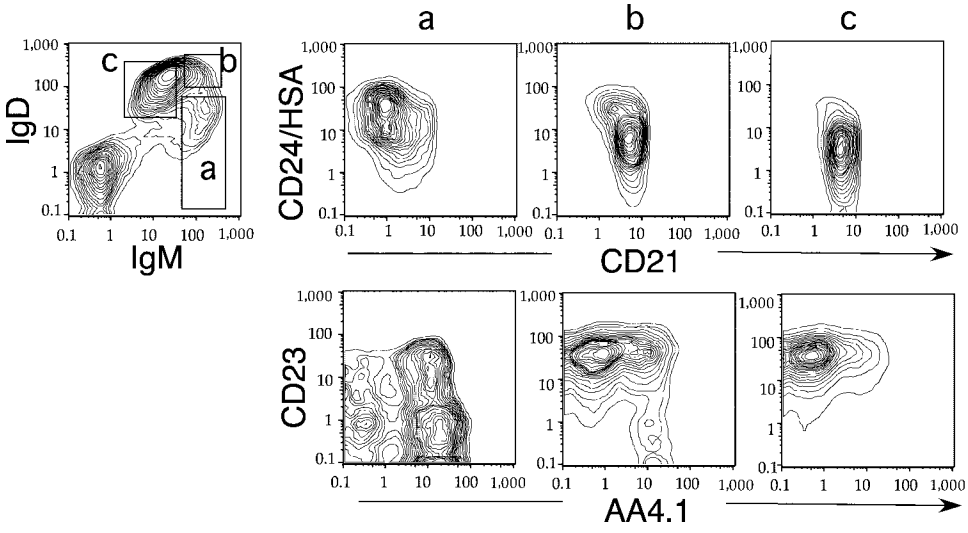

Figure 4. Arrested ATA B cell surface phenotype, comparable to the IgM^{hi} IgD^{lo} cell stage. BALB/c spleen cell analysis by simultaneous six color staining (IgM, IgD, CD24/HSA, CD21, CD23, and AA4.1). Non-naive CD21^{hi} HSA^{med}-stained cells (9% of total IgM$^+$ cells, the majority are IgM^{hi} IgD^{lo} AA4.1$^-$ CD23$^{lo/-}$ marginal zone B cells) were electronically excluded in this figure presentation. The IgM/IgD based regions gated as a, b, and c are shown on the left.

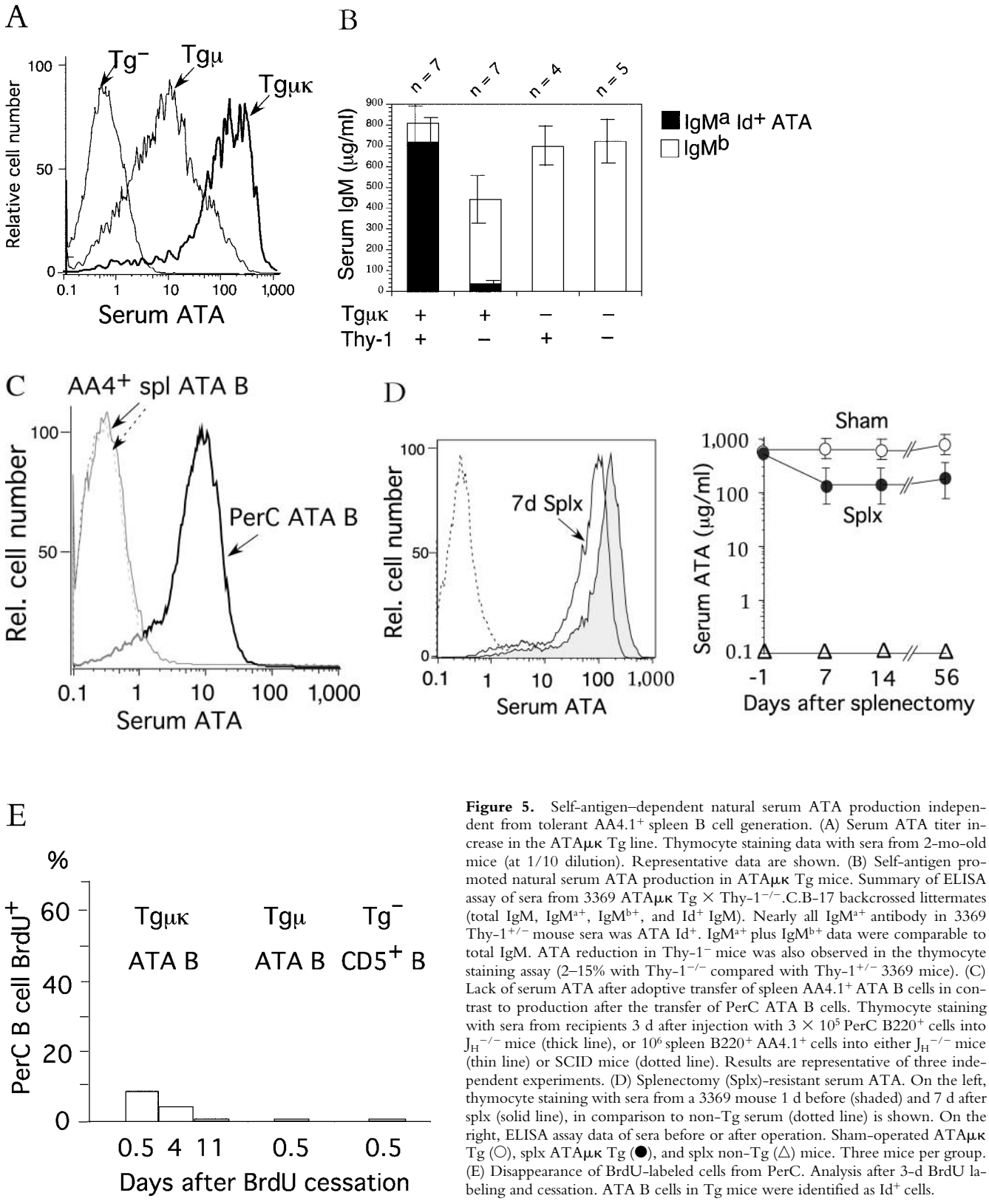

Figure 5. Self-antigen–dependent natural serum ATA production independent from tolerant AA4.1$^+$ spleen B cell generation. (A) Serum ATA titer increase in the ATAμκ Tg line. Thymocyte staining data with sera from 2-mo-old mice (at 1/10 dilution). Representative data are shown. (B) Self-antigen promoted natural serum ATA production in ATAμκ Tg mice. Summary of ELISA assay of sera from 3369 ATAμκ Tg × Thy-1$^{-/-}$.C.B-17 backcrossed littermates (total IgM, IgM^{a+}, IgM^{b+}, and Id$^+$ IgM). Nearly all IgM^{a+} antibody in 3369 Thy-1$^{+/-}$ mouse sera was ATA Id$^+$. IgM^{a+} plus IgM^{b+} data were comparable to total IgM. ATA reduction in Thy-1$^-$ mice was also observed in the thymocyte staining assay (2–15% with Thy-1$^{-/-}$ compared with Thy-1$^{+/-}$ 3369 mice). (C) Lack of serum ATA after adoptive transfer of spleen AA4.1$^+$ ATA B cells in contrast to production after the transfer of PerC ATA B cells. Thymocyte staining with sera from recipients 3 d after injection with 3 × 10^5 PerC B220$^+$ cells into $J_H^{-/-}$ mice (thick line), or 10^6 spleen B220$^+$ AA4.1$^+$ cells into either $J_H^{-/-}$ mice (thin line) or SCID mice (dotted line). Results are representative of three independent experiments. (D) Splenectomy (Splx)-resistant serum ATA. On the left, thymocyte staining with sera from a 3369 mouse 1 d before (shaded) and 7 d after splx (solid line), in comparison to non-Tg serum (dotted line) is shown. On the right, ELISA assay data of sera before or after operation. Sham-operated ATAμκ Tg (○), splx ATAμκ Tg (●), and splx non-Tg (△) mice. Three mice per group. (E) Disappearance of BrdU-labeled cells from PerC. Analysis after 3-d BrdU labeling and cessation. ATA B cells in Tg mice were identified as Id$^+$ cells.

normal and ATAμ Tg mice are also sensitive to LPS (17, 35). Considering that intestinal microfloral bacteria could serve as a potential LPS source in the region, the combination of self-antigen (Thy-1) exposure and subsequent LPS stimulation after migration into the intestinal site was a potential scenario for the induction of Ig secretion.

To distinguish whether natural serum ATA production was a consequence of the vast increase of arrested ATA B

cells in ATAμκ Tg mice or instead derived from B-1 cells generated as a minor population, a cell transfer experiment was performed. AA4.1$^+$ ATA B cells were purified from Thy-1$^+$ ATAμκ Tg mouse spleen and transferred into B cell–deficient $J_H^{-/-}$ mice, providing both normal Thy-1 self-antigen and an intestinal microfloral environment. For comparison, total PerC B cells were harvested and also transferred at the same time because 5–10% of PerC ATA B cells were CD5$^+$, potentially identifying the B-1 cells. As shown in Fig. 5 C, although injection of PerC B cells resulted in serum ATA induction shortly after transfer into $J_H^{-/-}$ mice (within 3 d) that increased additionally by day 7, mice receiving AA4.1$^+$ ATA B cells, even with threefold more cells, did not show significant serum ATA (Fig. 5 C) over a 14 d survey period. Injection of AA4.1$^+$ B cells into SCID mice lacking both B and T cells, in an attempt to increase the chance for escape from Thy-1 antigen–mediated tolerance, also failed to reveal the induction of serum ATA (Fig. 5 C, dotted line). These cell transfer results suggest that although PerC B cells contain the cells responsible for serum ATA production, maturation-arrested B cells in spleen only rarely contribute to serum ATA.

However, there was the possibility that our cell purification and transfer procedures might have promoted the death of short-lived AA4.1$^+$ ATA B cells. Thus, as an alternative approach, we performed splx to directly estimate the extent of spleen B cell contribution to natural serum antibody, taking advantage of the fact that AA4.1$^+$ cells were the major B cell component in spleen of ATAμκ Tg mice. Although splx resulted in some reduction of serum ATA in ATAμκ Tg mice shortly after the operation, the presence of splx-resistant ATA was nonetheless clear and persisted (Fig. 5 D). Splx ATAμκ Tg mice showed levels higher than the average serum ATA of ATAμ Tg mice (Fig. 5, D and A, respectively). Considering the known short half-life of IgM (36), these findings suggest continuing serum ATA antibody production throughout the course of the experiment, without requirement of constant resupply from the spleen, consistent with the cell transfer experiment. The immediate drop in ATA titer was likely due to the loss of preexisting AA4.1$^-$ CD23$^-$ B-1 cells as a minor compartment in the spleen, which may have been generated by development distinct from the primary BM pathway.

In ATAμκ Tg mice, AA4.1$^+$ B cells in the spleen showed 60% BrdU labeling after a short-term pulse (Fig. 2 C). 10% of PerC ATA B cells were BrdU labeled, likely representing cells migrated from the spleen or directly from BM (Fig. 5 E, left). These rapidly labeled cells did not persist in PerC, but rather showed a similar turnover rate as the spleen BrdU$^+$ B cells (Figs. 5 E and 2 B). In contrast to such results with ATAμκ Tg mice, analysis of PerC B cells in ATAμ Tg mice revealed a self-antigen–dependent increase in CD5$^+$ ATA B cells (Fig. 1) and such cells did not incorporate BrdU (Fig. 5 E, middle), consistent with the idea of positive selection and similar to the CD5$^+$ B cells in normal mice (Fig. 5 E, right), known to be long-lived and predominantly noncycling (35, 37). These findings suggest a shift in the balance between positive and negative selection in the PerC B cell population, where negative selection predominates in ATAμκ Tg mice, resulting in a lack of significant ATA B cell accumulation. Serum antibody production observed in our PerC B cell transfer from ATAμκ Tg mice was likely contributed by the B-1 B cells present as a minor population.

Natural Serum Autoantibody in Association with Peritoneal B-1. Dissociation of serum autoantibody production from conventional BM B cell development was additionally substantiated by analysis of Thy-1$^{-/-}$ ATAμκ Tg mice. As shown in Fig. 5 B, in Thy-1$^{-/-}$ ATAμκ Tg mice, the lack of ATA in serum was compensated by non-ATA IgM produced by B cells that rearranged endogenous Ig loci (IgM^b), reaching 70% of the normal IgM level. This serum profile was in clear contrast with the complete absence of B cells bearing endogenous BCRs in most lymphoid organs populated by BM-derived B cells, including the spleen, lymph nodes, mesenteric lymph nodes, and the lymphoid follicles in the gut known as Peyer's patches, but with the notable exception of PerC (Fig. 6 A). The IgM^{a+} B cells in Thy-1$^{-/-}$ mice were all ATA Id$^+$ B cells and consisted predominantly of AA4.1$^-$ CD23$^+$ mature naive follicular B cells, with some naive ATA B cells also detected in the PerC (Fig. 6 A). However, a large proportion of the PerC B cells expressed endogenous IgM^b together with CD5 expression (Fig. 6 B), a phenotype typical of (self)-antigen–exposed B-1 B cells in PerC (Fig. 6 B). In contrast, naive ATA B cells in PerC were CD5$^-$ due to the absence of Thy-1. Thus, there was no B-1 positive selection and a lack of serum antibody contribution. These findings emphasize the significance of a self-antigen–dependent mechanism for the production of natural serum antibody in association with B-1 B cell generation.

Discussion

We have demonstrated here that V_H3609μ/Vκ21C-encoded ATA B cells are negatively selected in the major pathway of BM B cell development. The majority of ATA B cells in the spleen showed maturational arrest. They are short-lived, replaced rapidly from the BM, and are unlikely to be the major source of natural serum ATA. Instead, cells already established outside of conventional lymphoid organs, together with preexisting B-1 cells in the spleen (12, 20), appear to play a significant role in the self-antigen–dependent production of serum ATA. These positively selected B-1 cells could be revealed more readily in PerC of ATAμ-only Tg mice, due to a lower proportion of negatively selected cells, such that CD5$^+$ B cells constitute a significant fraction of the PerC B cells, more similar to normal mice. In contrast, in ATAμκ Tg mice, forced expression of the ATA specificity by all BM immature B cells resulted in predominant negative selection, even in the PerC population, whereas the serum ATA level was intact, independent from negative selection and likely contributed by the presence of infrequent B-1 B cells. Recent reports describing normal or even elevated serum immunoglobulin levels in

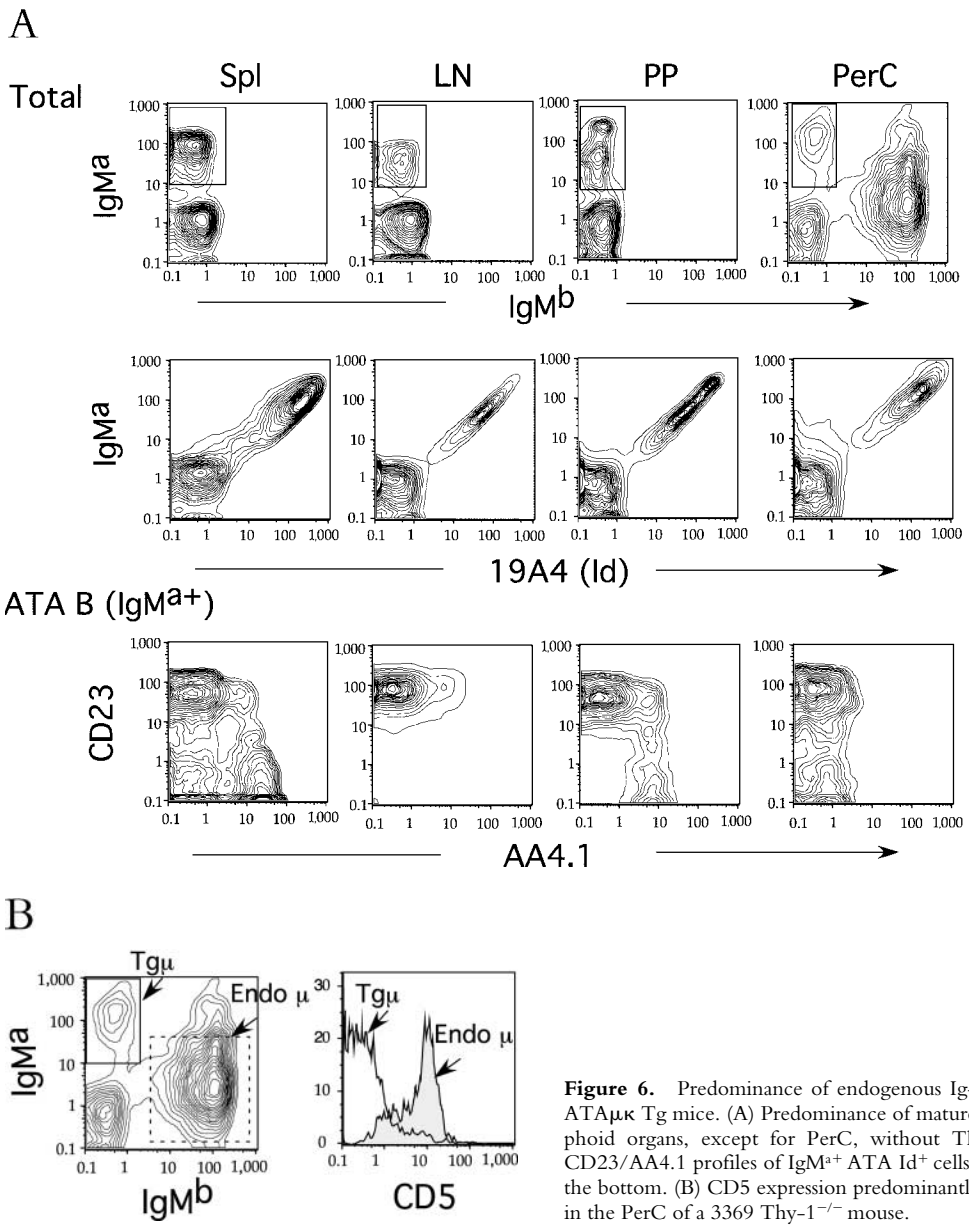

Figure 6. Predominance of endogenous Ig-expressing B-1 cells in PerC of Thy-1$^-$ ATAμκ Tg mice. (A) Predominance of mature naive ATA B cells in conventional lymphoid organs, except for PerC, without Thy-1. Analysis of 3369 Thy-1$^{-/-}$ mice. CD23/AA4.1 profiles of IgM^{a+} ATA Id$^+$ cells (boxed regions at the top) are shown on the bottom. (B) CD5 expression predominantly on the endogenous μ$^+$ (Endo μ) B cells in the PerC of a 3369 Thy-1$^{-/-}$ mouse.

mice lacking most peripheral B cells due to blocked BM development resulting from absence of IL-7 (38) or conditional deletion of Rag-2 (37) are consistent with this notion. Taken together, BM-derived B cell development and selection in conventional lymphoid organs seems unlikely to produce the B-1 cells that participate in the daily production and maintenance of natural serum autoantibody in normal healthy individuals.

CD5 induction, commonly thought to mark B-1 cells, nonetheless occurred for BM-derived ATA immature B cells during negative selection as a result of self-antigen exposure. Thus, CD5 induction can occur during B cell development, but before cell fate is determined, either negatively as shown here, or positively as found with positive selection in T cell development (39). It should be emphasized, however, that these CD5-induced B cells from BM are not equivalent to long-lived B-1 cells generated after positive selection as our serum ATA data indicated, and that the induction of CD5 is not a general consequence for all self-antigen–exposed tolerant B cells. Despite the expectation that negative selection will be frequent during BM B cell development (3), CD5 induction is normally rare (19, 40) and typically absent from, or present at very low levels on, tolerant B cells (28). Only B cells expressing the prototypic ATA κ chain became CD5$^+$ in ATAμ Tg mice. Thus, CD5 induction from immature B cells appears to require specific types of antigen or a specific threshold of BCR signaling. Previously, it was considered that the paucity of CD5 induction during BM B cell development might be due to a relative decrease of B-1–associated specificities in the newly generated B cell repertoire by either increasing diversity in μ-heavy chain rearrangement (41,

42) or altered pre-BCR selection (21, 43). This study suggests that even if generated, B cells with such specificities will likely make little or no contribution to any mature B cell pool in normal mice.

The finding of a positive impact of self-antigen on serum autoantibody levels, first reported for the heavy-only 6C10-μ Tg line and shown here with ATA-μκ Tg mice, stands in sharp contrast to most other models of B cell autoreactivity where the presence of autoantigen has negative effects (4–6, 31, 44, 45). One other previously reported exception is an RF Tg model where the presence of antigen has little effect, either negative or positive, leading to the description of this Tg BCR as "clonally indifferent" in normal mouse background (46, 47). This has been attributed to the relatively low affinity of this BCR for antigen, as a higher affinity RF model shows strong antigen-mediated central tolerance in the presence of antigen (47, 48). The ATA BCR does not appear to exhibit a particularly low affinity for antigen, as the presence of high levels of Tg-encoded serum autoantibody is accompanied by arrested B cell development and/or elimination of the specificity in the spleen by receptor editing. This suggests that the ATA specificity, representative of a B-1–associated natural autoantibody, is regulated in a manner very distinct from previous models of autoreactivity associated with pathogenesis.

We have previously characterized B cell development in another Tg model expressing a prototypic $CD5^+$ B-1–associated specificity to a phosphatidylcholine-associated cryptic determinant on erythrocytes revealed by treatment with the proteolytic enzyme bromelain (49, 50). Unlike the ATA specificity, however, B cells in this BR1-$V_H11/V_κ9$ model did not edit nor show obvious signs of anergy in the spleen, but instead adopted a typical B-1 phenotype and showed evidence of extended survival in culture (50). Although a definitive explanation for this difference remains to be determined, recent analysis of a V_H11 knock-in mouse (unpublished data) indicates that physiological expression of this heavy chain results in accumulation of a fraction of $V_H11V_κ9$ B cells in spleen showing the "arrested development" phenotype, as described here with ATA BCR animals. Quantitative differences in the level of BCR between these lines might be responsible for such a different outcome. In addition, differences in ATA versus phosphatidylcholine B cell development could arise from variations in the expression level of the antigens. These are issues that we are currently investigating.

There has been emerging interest on the origin of natural serum autoantibody and its relevance to antibodies to virus or commensal bacteria. The constitutive presence of neutralizing antibody in serum is essential, protecting against viral or bacterial replication to prevent potentially lethal disease progression, playing a role as a part of innate immunity (51–54). Natural autoantibodies are often reactive to carbohydrate on self-antigens (16, 55), which might be shared with determinants on bacterial, viral, or tumor antigens. These observations have led to the hypothesis that positive selection of the autoreactive B cell repertoire might be an active process to provide protective immunity for immunological surveillance. Previously, it was speculated that these self-antigens might be unique, being inefficient in inducing tolerance (26, 56). However, our study shows that these natural autoantigens can function as tolerogens, similar to classical autoantigens. The transfer of fetal B cell progenitors, but not adult BM B cell progenitors, into the adult mouse environment resulted in B-1 cell positive selection, i.e., accumulation of $CD5^+$ B cells (20). This raises the additional possibility that differences of immature B cell responsiveness that specifically allow B-1 cell positive selection may also account for autoreactive B cell production from such fetal precursors.

Serum ATA is already detectable in the fetus and newborns of ATA Tg mice (17, 57) before BM B cell development is established, supporting the idea that B-1 cell positive selection occurs before birth. Some B-1 cells are maintained throughout life by self-renewal (12) as a persisting fetal/neonatal B-1 cell population, although it is likely that some B-1 cells might be generated continuously from precursors in the BM as a minor divergent pathway. The intestinal lamina propria appears to serve as a major site for B-1 cell differentiation into antibody-secreting cells and class switching to IgA (13, 33) in normal mice. Interestingly, there is a significant level of endogenous (non-ATA) IgA secretion in ATAμκ Tg mice as found with other Ig Tg B cell mouse models (33, 58), suggesting a strong ongoing serum antibody production separate from the majority of BM B cell development. Unique IgA production in the gut lamina propria in mice deficient for the majority of B and T cells has been reported (59, 60). These findings suggest that there are B cell system(s) that actively provide protective immunity independent from the majority of B cell development in the adult. B-1 cell development may represent such a system, uniquely allowing positive selection to self- and/or commensal antigens. This is an important issue to address in future studies.

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