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*J Immunol* 2011; 187:37-46; Prepublished online 1 June 2011;
doi: 10.4049/jimmunol.1003924

http://www.jimmunol.org/content/187/1/37

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/06/01/jimmunol.1003924.DC1

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Cellular Competition Independent of BAFF/B Lymphocyte Stimulator Results in Low Frequency of an Autoreactive Clonotype in Mature Polyclonal B Cell Compartments

Neda Nikbakht,* Thi-Sau Migone,† Chris P. Ward,† and Tim Manser*

The peripheral B cell prosurvival cytokine BAFF/B lymphocyte stimulator (BLyS) has been proposed to participate in the regulation of immunological tolerance. Selective elimination or reconstitution of B cells expressing transgene-encoded, autoreactive BCRs upon systemic BLyS depletion or supplementation, respectively, was observed in two separate studies. Such findings led to a model positing a higher dependency of autoreactive B cells on BLyS. We tested this model by exploiting two targeted IgH transgenic mice (H chain knock-in [HKI]) that produce large numbers of follicular (FO) B cells that are either weakly or strongly autoreactive with nuclear autoantigens. Even though HKI B cells do not exhibit classical features of anergy, we found that mature, naive, autoreactive HKI B cells are outcompeted for representation in the periphery by a polyclonal B cell population. However, this is not due to a higher dependency of HKI B cells on BLyS for survival. Additionally, excess BLyS does not rescue HKI B cells from selective elimination. These findings suggest that some autoreactive FO B cells can fully develop while in competition with non-autoreactive cells for BLyS, but remain at a competitive disadvantage for other trophic factors that regulate peripheral stability. As such, our data indicate the existence of peripheral tolerance mechanisms that regulate the frequency of autoreactive FO B cells independent of the BLyS pathway. The Journal of Immunology, 2011, 187: 37–46.
transgenic mice are outnumbered by wild-type B cells when they develop as part of a diverse B cell repertoire. Furthermore, VH3H9 B cells exhibit conventional features of anergy, including reduced lifespan, exclusion from follicular locales, and attenuated activation (6, 25). Treatment of mice with BLYS increased the numbers of these B cells in a competitive environment. However, BLYS supplementation did not resolve the anergic state of these B cells, as auto-Ab production and spleen localization of these cells remained unaffected (26). More recent studies by Nemazee and colleagues (27) showed that reduction of BLYS activity in VH3H9 mice via expression of ΔBLYS, a natural inhibitor of BLYS, lowered B cell numbers but did not dramatically alter the BCR repertoire of these mice, suggesting only subtle changes in the frequency of highly autoreactive B cells.

Given these apparently inconsistent results, we sought to conduct additional studies in another BCR transgenic model of B cell autoreactivity to natural autoantigens to examine the role of BLYS dependency in peripheral B cell tolerance. B cells in H chain knock-in (HKI) mice express BCRs reactive with p-azophenylarsanate (Ars) as well as nuclear autoantigens. HKI autoreactive B cells are distinguished from those produced in most other autoreactive BCR systems in that they are potentially pathologic yet they are capable of entering the mature peripheral B cell compartment and reside in lymphoid follicles (28). Furthermore, two different lines of HKI transgenic mice have been generated that contain B cells with ~10-fold different avidities for nuclear autoantigens. We used this system to determine whether HKI B cells exhibited increased dependency on BLYS and whether the extent of this dependence correlated with the level of autoreactivity.

Materials and Methods

Mice

HKI65 Vικ knock-in mice contain a targeted Vικ transgene composed of the combination of gene segments VHIdCR, DFL16.1, and JH2 originally isolated from the dominant Ars responding clone in A/J mice. HKIR Vιλ knock-in mice are identical to HKI65 mice except for the presence of an arginine mutation in position 55 of the CDR2 region of the transgenic Vιλ gene. These mice have been backcrossed to the C57BL/6 (B6) background and have been previously described (28, 29). The double transgenic HKI65/Vιλ knock-in mice express the VH3H9 chain transgenic mice are outnumbered by wild-type B cells by negative selection using MACS and anti–CD43-conjugated magnetic beads (Miltenyi Biotec). B cell purity was always >90% as assessed by flow cytometry. Cells were cultured in RPMI 1640, 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 5 mM 2-ME in 24-well plates (2 x 10^5 cells/well) with or without 100 ng/ml rhBLYS. Survival was assayed using an annexin V apoptosis detection kit (BD Biosciences). Live cells were identified as being 7-aminoactinomycin D-negative.

In vivo BLYS neutralization

One or two 100-μg doses of the hamster anti-mouse BLYS mAb 10F4 (Human Genome Sciences) were injected i.p. Control mice were injected i.p. with 100 μg ChromPure hamster IgG (Jackson ImmunoResearch Laboratories).

Flow cytometry and mAbs

Single-cell suspensions of splenocytes or total BM cells were prepared. Cells (1–2 x 10^6) were incubated in 96-well plates with purified anti-CD16/32 (eBioscience; clone 93) to block nonspecific Ab binding. Cells then were stained with combinations of the following Abs and reagents: FITC-, PE-, or biotin-conjugated anti-B220 (eBioscience; clone RA3-6B2); FITC- or PE-conjugated anti-CD45RA (Jackson ImmunoResearch Laboratories); PE-, biotin-, or PerCP-Cy5.5–conjugated anti-CD45.2 (eBioscience; clone 104); allophyocytocyanin-Cy7–conjugated anti-CD45.1 (eBioscience; clone A20); AF647-conjugated anti-BR3 (eBioscience; clone 7H22-E16); FITC- or allophyocytocyanin-conjugated anti-CD21/55 (BD Pharmingen; clone 7G6); biotin-, PE-Cy7–, or PE-conjugated anti-CD3 (eBioscience; clone B3B4); PE- or allophyocytocyanin-conjugated anti-CD3 (eBioscience; clone AA4.1); FITC-conjugated anti-CD3 (BD Pharmingen; clone S7); and biotin-conjugated E4 (made in-house). Biotin conjugates were detected using streptavidin-conjugated PerCP-Cy5.5 or PE-Cy7 (eBioscience). Flow cytometry analysis was conducted on FACScalibur or LSR II flow cytometers (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

BLYS and anti-BLYS assays

Serum BLYS was measured by ELISA using murine BAFR-Fc (Alexis) as a capture reagent and an anti-murine BLYS mAb (16D7; Human Genome Sciences) as a detector. Samples were diluted to a final concentration of 10% matrix on the assay plate. The limit of detection was 0.8 ng/ml. Serum anti-BLYS (10F4) levels were measured by ELISA; the limit of quantification was 0.075 μg/ml.

Isolation of PBLS

One hundred microliters of blood was collected into heparin containing tubes, diluted with an equal volume of RPMI 1640, and incubated for 2 min with 9 ml ACK lysis buffer. Cells were then collected after centrifugation and washed twice with PBS before use.

Construction of BM chimeras

B6.SJL mice were exposed to two consecutive doses of 550 cGy gamma radiation, 4 h apart, using a Gammacell 40 laboratory irradiator (Nordion International). Four to 8 h after administration of the second dose, various combinations of BM cells were injected into the retro-orbital sinuses of congenic B6.SJL (CD45.1+) recipients. Cells (1–2 x 10^6) were incubated in 96-well plates with purified anti-CD16/32 (eBioscience; clone 93) to block nonspecific Ab binding. Cells then were stained with combinations of the following Abs and reagents: FITC-, PE-, or biotin-conjugated anti-B220 (eBioscience; clone RA3-6B2); FITC- or PE-conjugated anti-CD45RA (Jackson ImmunoResearch Laboratories); PE-, biotin-, or PerCP-Cy5.5–conjugated anti-CD45.2 (eBioscience; clone 104); allophyocytocyanin-Cy7–conjugated anti-CD45.1 (eBioscience; clone A20); AF647-conjugated anti-BR3 (eBioscience; clone 7H22-E16); FITC- or allophyocytocyanin-conjugated anti-CD21/55 (BD Pharmingen; clone 7G6); biotin-, PE-Cy7–, or PE-conjugated anti-CD3 (eBioscience; clone B3B4); PE- or allophyocytocyanin-conjugated anti-CD3 (eBioscience; clone AA4.1); FITC-conjugated anti-CD3 (BD Pharmingen; clone S7); and biotin-conjugated E4 (made in-house). Biotin conjugates were detected using streptavidin-conjugated PerCP-Cy5.5 or PE-Cy7 (eBioscience). Flow cytometry analysis was conducted on FACScalibur or LSR II flow cytometers (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

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Results

BLyS serum levels in HKI transgenic mice and surface BR3 levels on HKI autoreactive clonotypes

In HKI65 mice, the targeted transgene-encoded H chain is coexpressed with an endogenous V\(\kappa\)10A-Jκ1 L chain gene in ~10–12% of B cells to reproduce the BCR expressed by the major Ars responding clonotype in A/J mice termed “canonical” (28–30). The BCR expressed by this clonotype also has weak reactivity for intracellular autoantigens (32, 33).

In the HKIR line, canonical BCRs also are expressed by 10–12% of total B cells yet they exhibit substantially increased affinity for nuclear autoantigens due to an R mutation in the targeted transgenic V\(\kappa\)1 gene (34). Canonical HKIR Abs stain nuclei in ANA assays and possess 10-fold higher avidity for dsDNA and ssDNA in ELISA assays than do canonical HKI65 Abs (29). In the HKIR/V\(\kappa\)10 and HKI65/V\(\kappa\)10 double-transgenic lines, >95% of B cells express canonical BCRs. Canonical BCRs in all four HKI lines are detectable using the monoclonal anti-clonotypic Ab E4.

Although BLyS levels are tightly regulated in normal mice, previous studies demonstrated the presence of unusually high levels of BLyS in the serum of anti-HEL–Ig transgenic mice expressing soluble HEL (23). Furthermore, B cells in these mice expressed lower levels of BR3. As such, we investigated whether BLyS serum or B cell surface BR3 levels are altered in the HKI transgenic mice. Although serum BLyS levels in single transgenic lines HKIR and HKI65 were not significantly different from those measured in wild-type mice, serum BLyS levels were higher in double transgenic lines HKIR/V\(\kappa\)10 and HKI65/V\(\kappa\)10 (Fig. 1A). Surface BR3 expression was not significantly different on HKIR or HKI65 canonical B cells compared with wild-type controls (Fig. 1B). BR3 expression was reduced on the surface of B cells in HKIR/V\(\kappa\)10 and HKI65/V\(\kappa\)10 mice. This reduction was more pronounced on the weakly autoreactive HKI65/V\(\kappa\)10 B cells (Fig. 1C).

In vivo BLyS supplementation in HKIR and HKI65 mice

Canonical B cells constitute ~10–12% of the B cell pool in the single transgenic lines, whereas they make up >95% of the B cells in the double transgenic lines. Therefore, we hypothesized that higher levels of BLyS are required to maintain canonical B cells and that BLyS may be limiting the size of the canonical B cell population in HKIR and HKI65 mice. To test this hypothesis we supplemented these mice with exogenous BLyS and determined whether the percentages of canonical B cells increased after this treatment. We first verified that rhBLyS enhanced in vitro naive B cell survival. rhBLyS significantly increased the survival of resting mature B cells after 2 and 3 d in culture (Fig. 2A). We then established an in vivo BLyS supplementation regimen enabling us to detect the preferential expansion of the highly BLyS sensitive MZ B cell subpopulation. After 9 d rhBLyS treatment of wild-type mice, significant expansion of the MZ B cell compartment was observed (Fig. 2B).

We then similarly treated HKIR and HKI65 mice with rhBLyS. Surprisingly, after 9 d rhBLyS treatment, neither the canonical HKIR nor the canonical HKI65 B cell pools preferentially expanded (Fig. 2C and data not shown). This was despite the expansion of the MZ B cell compartment in these mice (Fig. 2D). Although the MZ compartment is normally reduced in HKI lines, particularly in HKIR, as compared with B6 mice, the MZ population size increase in HKI mice was similar to the expansion observed in B6 mice. Finally, the proportion of E4+ (canonical) B cells in both the FO and MZ compartments of HKIR and HKI65 mice did not change as a result of BLyS supplementation (Fig. 2E). Therefore, preferential expansion of the canonical B cell compartment does not take place in response to BLyS supplementation.

Naive B cell competition

Although our initial observations suggested that increased BLyS levels did not provide a competitive advantage for HKIR and HKI65 canonical B cells, we were concerned that the enforced expression of the transgenic H chain in HKI mice influenced naive B cell competition. Canonical B cells compete with an oligoclonal repertoire in HKI mice, as all noncanonical HKI B cells express an identical H chain. As such, we created a system in which canonical B cells were in competition with B cells expressing a diverse wild-type BCR repertoire.

We adoptively transferred equal numbers of wild-type and canonical mature B cells into syngeneic hosts and then monitored the relative survival of each transferred population. We used the CD45.1/CD45.2 allotype system to distinguish donor from host cells and E4 staining to distinguish canonical from wild-type donor cells (Fig. 3A). Five days after adoptive transfer, chimeric mice were sacrificed and the relative survival of each transferred population was determined. Although nearly equal numbers of HKI and wild-type B cells were injected, HKIR E4+ cells comprised only ~11% of donor cells 5 d after transfer (Fig. 3B). Similarly, weakly autoreactive HKI65 E4+ cells were equally outcompeted and constituted 10–11% of donor cells on day 5 after transfer (Fig. 3B).

In vivo BLyS depletion

To further evaluate whether changes in BLyS levels preferentially altered peripheral stability of HKI B cells, we opted for a BLyS depletion strategy that produces rapid effects on B cell survival. Administration of two doses of the hamster anti-BLyS mAb 10F4 reduced circulating serum BLyS levels to undetectable levels after 5 d (Fig. 4A). Furthermore, anti-BLyS treatment resulted in depletion of most peripheral B cells in wild-type as well as in HKIR and HKI65 mice. Peripheral B cell numbers began to decline significantly by day 7 after treatment (Fig. 4B). On day 15 after treatment the peripheral B cell pool was reduced to <20% of its original size (Fig. 4C). Serum levels of BLyS continued to remain undetectable at this time (Fig. 4A).
Different degrees of BLyS dependency were observed among B cell subpopulations on day 5 after anti-BLyS treatment. As expected from the BLyS supplementation results, the MZ B cell compartment exhibited high sensitivity to BLyS depletion. Five days after anti-BLyS treatment, MZ B cell numbers (Fig. 4D). We, therefore, chose day 5 after treatment as the optimal analysis time point.

**In vivo BLyS depletion in settings of adoptive transfers**

If autoreactive HKI B cells are highly BLyS-dependent, depleting BLyS should cause a pronounced loss of adaptively transferred canonical B cells relative to wild-type cells. Furthermore, this outcompetition should be more stringent for highly autoreactive HKIR canonical B cells as compared with less autoreactive HKI65 canonical B cells.

As in the experiments described above, we adoptively transferred equal numbers of naive B cells from B6 and either HKIR/Vsx10 or HKI65/Vsx10 donors to B6.SJL (CD45.1\(^+\)) congenic recipients (Fig. 3A). Within each group, half of the resulting chimeric mice received a single dose of anti-BLyS at the time of transfer and half received an isotype control Ab. In groups receiving isotype control, HKIR/Vsx10 and HKI65/Vsx10 E4\(^+\) cells comprised ~11% of total donor cells at the time of analysis, demonstrating as before that canonical B cells from both sources were equally outcompeted by wild-type B cells (Fig. 5A, upper panel, 5B). However, this composition remained unaltered in recipients receiving anti-BLyS treatment (Fig. 5A, lower panel, 5B). Importantly, BLyS neutralization was effective in these recipients, as they exhibited undetectable levels of serum BLyS and ~40% reduction in total B cell numbers. Furthermore, the highly BLyS-sensitive MZ B cell compartment was completely depleted in these recipients (data not shown). Therefore, neither HKIR nor HKI65 canonical B cells exhibited higher sensitivity to BLyS neutralization in a setting of competition with wild-type B cells.

**Evaluating the impact of pre-exposure to BLyS on naive B cell competition**

The above results raised the possibility that decreased survival of donor B cells in adoptive hosts was influenced by higher serum
BLyS and decreased BR3 levels on B cells, particularly in HKIR/Vk10 double transgenic mice. To test whether exposure to higher BLyS levels in donor mice influences survival of donor B cells in BLyS-deficient hosts, we treated a cohort of B6 mice with rhBLyS for 10 d until the MZ B cell pool expanded (Supplemental Fig. 1A). We then CFSE-labeled and adoptively transferred naive B cells from rhBLyS-treated B6 mice and PBS-treated B6.SJL mice into B6.SJL recipients at a 1.2:1 ratio. This ratio was selected to compensate for expansion of the MZ pool in rhBLyS-treated mice, as MZ B cells fail to survive adoptive transfers (Supplemental Fig. 1B). The resulting chimeric mice either received a single dose of anti-BLyS at the time of transfer or received an

**FIGURE 5.** In vivo BLyS depletion in settings of adoptive transfers. A, Mature, resting B cells (1.5 × 10⁶) isolated from B6 splenocytes were mixed with either 1.5 × 10⁶ HKIR/Vx10 splenic B cells (first and third panels from top) or 1.5 × 10⁶ HKI65/Vx10 splenic B cells (second and fourth panels from top) and adoptively transferred into syngeneic B6.SJL (CD45.1+) recipients. Recipients received either a single dose of 100 μg anti-BLyS Ab (bottom two panels) or a dose of isotype control (upper two panels) on the day of transfer. Five days later the chimeric mice were sacrificed and their splenocytes were stained with mAbs specific for the indicated markers and analyzed by flow cytometry. The plots shown are representative results from three independent experiments.

**FIGURE 4.** In vivo BLyS depletion. A, Serum murine BLyS levels measured by ELISA in B6, HKIR, or HKI65 mice that received two doses of 100 μg anti-BLyS Ab 10F4 i.p. on day 0 and day 5 are shown in black bars (each bar represents one mouse). Gray bars indicate serum BLyS levels of mice that received isotype control Ab. B and C, Mice of the indicated genotypes received anti-BLyS treatment as described above and were sacrificed on day 7 (B) or day 15 (C) after treatment. Splenocytes from anti-BLyS-treated mice or those of B6 mice that received the isotype control were stained with anti-B220 and E4 and analyzed by flow cytometry. The bars represent the ratio of the mean for numbers of B220⁺ B cells in each group divided by the mean of B220⁺ cells in the control group. There were at least three mice per group and the error bars represent SD. D, B6 mice receiving a single dose of either 100 μg anti-BLyS Ab or isotype control were sacrificed on day 5 after treatment and their spleen cells were stained with mAbs specific for the indicated markers and analyzed by flow cytometry. The plots shown are representative results from three independent experiments.
isotype control Ab. In mice receiving isotype control, the ratios of transferred B cells remained constant 3 and 5 d after transfer (Fig. 6A, upper panel, 6B, left). In contrast, this ratio changed in favor of rhBLyS-pretreated donor B cells in hosts receiving anti-BLyS treatment (Fig. 6A, lower panel, 6B, right). This experiment indicates that pre-exposure to excess BlyS provides a survival advantage for donor B cells in BlyS-deficient wild-type environments. As such, the increased serum BlyS levels in HKI/Vκ10 mice are unlikely to account for the decreased relative survival of HKI/Vκ10 B cells in adoptive hosts.

**HKI canonical B cell competition during development**

The data presented above demonstrate that both weakly and strongly autoreactive mature HKI clones are equally outcompeted by mature B cells expressing a wild-type BCR repertoire. However, we found no role for BlyS in this competitive process. To investigate the nature of this competition further, we focused on developing HKI B cells. Specifically, we evaluated the frequency of HKI canonical B cells while they matured in the presence of developing wild-type B cells. We generated BM chimeric mice reconstituted with equal numbers of progenitors from wild-type and either HKIR/Vκ10 or HKI65/Vκ10 donors. We used the CD45.1/CD45.2 allotype system to distinguish wild-type (CD45.1) and HKI (CD45.2) donor-derived cells (Fig. 7B).

We analyzed B cells in the peripheral blood of chimeric mice at different time points following reconstitution. All mature B cells in the peripheral blood of chimeric hosts reconstituted solely with either HKIR/Vκ10 or HKI65/Vκ10 progenitors expressed CD45.2 (Fig. 7A). However, when mixtures of equal numbers of wild-type and HKI progenitor cells were used to reconstitute lethally irradiated hosts, most peripheral blood B cells were of wild-type origin (CD45.1) (Fig. 7B). The percentages of both HKI/Vκ10 and HKI65/Vκ10 mature B cells also decreased in peripheral blood over time. Furthermore, both HKI/Vκ10 and HKI65/Vκ10 B cells were outnumbered to the same extent by wild-type B cells (Fig. 7B).

We previously observed that in both single and double transgenic mice, HKI autoreactive B cells appear to escape central tolerance via downregulating their surface BCRs yet otherwise display full phenotypic maturity (28). Therefore, it was possible that HKI canonical B cells were outcompeted in BM chimeric mice due to a failure in BCR downregulation. We tested whether HKI B cells in BM chimeric mice and HKI transgenic mice exhibited similar characteristics. HKI/Vκ10 and HKI65/Vκ10 progenitor-derived B cells (CD45.2) were stained by the E4 Ab, exhibited characteristics of mature FO B cells, and expressed lower levels of surface IgM in spleen and peripheral blood (Fig. 8; data not shown). Surface IgM downregulation was much more pronounced on HKI/Vκ10-derived B cells than those derived from HKI65/Vκ10 progenitors, a phenomenon that also occurs in HKI transgenic mice (28).

**In vivo BlyS supplementation in chimeric mice**

Given the results obtained from BM chimeras, we decided to evaluate whether the outcompetition of developing HKI B cells was BlyS-dependent to ensure that our previous results were not influenced by the environment in which HKI B cells underwent primary development. It was possible that transitional HKI canonical B cells acquired heightened BlyS dependency as they passed through certain immature developmental stages when in competition with wild-type developing B cells. If developing HKI B cells fail to repopulate the periphery as efficiently as do wild-type B cells due to heightened BlyS dependency, supplementing BM chimeric mice with exogenous BlyS should provide a competitive advantage to HKI-derived B cells. To test this idea we treated BM chimeric mice constructed as above with rhBlyS and

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**FIGURE 6.** Impact of BlyS pretreatment on B cell survival in BlyS-deficient hosts. A, Two cohorts of B6 (CD45.2) and B6.SJL (CD45.1) donor mice were treated with daily s.c. injections of either rhBlyS or PBS, respectively, for 10 d. On day 11, mature, resting B cells were isolated from donor spleens and labeled with CFSE. Labeled B cells from rhBlyS- and PBS-treated donors were mixed at a 1:2:1 ratio and adoptively transferred into B6 recipients (4 × 10⁶ cells/recipient). Recipients received either a single dose of 100 μg anti-BlyS Ab (bottom panels) or a dose of isotype control (upper panels) on the day of transfer. Five days later the chimeric mice were sacrificed and their splenocytes were stained with anti-B220, anti-CD45.2, and anti-CD45.1 and analyzed by flow cytometry. The ratios of B220 +CD93 + gates on representative plots in A and B show the percentage of B cells with different isotypes within the B220 +CD93 + gate. The plotted data are representative of at least three mice per group.

**FIGURE 7.** HKI canonical B cells are outcompeted in mixed BM chimeric mice. A and B, Lethally irradiated B6.SJL (CD45.1) hosts received BM from mice of the genotypes indicated in the diagram. PBLs from BM chimeric mice were isolated and stained with anti-B220, anti-CD93, and anti CD45.2 at various times after reconstitution and analyzed by flow cytometry. The ratios of B220 +CD93 +CD45.2 + cells to B220 +CD93 +CD45.2 − cells were evaluated over time. The mean values are derived from three mice per group, with error bars indicating SD (sometimes too small to be seen).
analyzed the proportion of HKIR/V<sub>k</sub>10- and HKI65/V<sub>k</sub>10-derived B cells in the spleen.

After 17 d rhBLyS treatment, elevated levels of rhBLyS were detected in the sera of chimeric mice (Fig. 8B). B cells in rhBLyS-treated mice exhibited downregulation of surface BR3 (Fig. 8C), in agreement with previous reports (23). As expected, the highly BLyS-dependent MZ B cell pool was expanded (Fig. 9A). However, the percentages of HKIR/V<sub>k</sub>10- and HKI65/V<sub>k</sub>10-derived mature B cells in the spleen of chimeric mice on day 17 did not differ from the percentages observed in peripheral blood prior to treatment. Furthermore, these percentages closely resembled those obtained from control chimeric mice treated with PBS (Fig. 9B).

**Immature and mature B cell subsets in BM chimeric mice**

We next investigated at which B cell developmental stages HKI B cells began to be outnumbered by wild-type B cells in mixed BM chimeras. In the BM, we resolved pre-B, pro-B, immature, and mature populations as described (Fig. 10A) and determined the relative contribution of HKIR/V<sub>k</sub>10 progenitors to each compartment (Fig. 10C) (35, 36).

In the spleen, we resolved transitional and mature B cells using the maturity marker CD23 and immature B cell marker CD93 (Fig. 10B). We divided the splenic transitional B cell pool into early (T1) and late (T2/T3) transitional subsets based on CD23 cell surface expression as described (36). The T2 and T3 subsets were evaluated together, as surface IgM downregulation does not allow distinction of the two subsets among HKI canonical B cells. We then determined the relative contribution of HKIR/V<sub>k</sub>10 progenitors to splenic T1, T2/T3, and mature compartments (Fig. 10C).

**Discussion**

Homeostatic regulatory processes mediated by competition for limiting survival factors determine the size of the peripheral B cell pool. Resource competition has also been proposed as an underlying mechanism of peripheral B cell tolerance (19, 20, 37). As such, autoreactive B cells in the periphery are rendered less fit to survive via outcompetition by non–self-reactive B cells. Because BLyS is required for survival of most naive B cells, competition for BLyS has been proposed as a regulator of peripheral tolerance (22, 23, 38). In this study, we tested whether the survival of self-reactive B cells specific for nuclear autoantigens was highly BLyS-dependent. Our analyses revealed that autoreactive HKI B cells are outcompeted by a polyclonal B cell population, but this competition is not BLyS-dependent and is not correlated with level of autoactivity.

Similar to mice expressing anti-HEL H and L chain transgenes along with soluble HEL, HKI/V<sub>k</sub>10 and HKI65/V<sub>k</sub>10 transgenic lines exhibit elevated levels of serum BLyS and lower surface BR3 levels on B cells. As HKI/V<sub>k</sub>10 mice have lower numbers of
We investigated the relative extent of BLyS dependency of canonical HKI compared with noncanonical B cells in three different competitive settings by either depleting or supplementing BLyS levels in vivo. Throughout these experiments, we used changes in MZ B cell numbers as an internal control for indicating heightened BLyS dependency, as BLyS is particularly important for the generation and maintenance of the MZ B cell compartment (38). First, we manipulated BLyS levels in single transgenic HKI mice in which canonical HKI B cells coexist with an oligoclonal B cell population. Canonical and noncanonical B cells in these mice express the transgenic H chain and differ only in regard to L chain expression. Second, we transferred mature, naive HKI canonical and wild-type B cells into wild-type hosts. In this experiment, adoptively transferred canonical and wild-type B cells competed among themselves as well as with the host B cell compartment. In the third setting, we used equal numbers of progenitors from HKI double transgenic mice and wild-type mice to seed the hematopoietic compartment of irradiated hosts. This experiment allowed us to determine whether the primary development of canonical HKI B cells in the context of limited repertoire diversity influenced their competitive “fitness” as mature B cells in a polyclonal environment.

In all three settings, while canonical HKI B cells were outnumbered by other B cells, altering in vivo BLyS levels did not preferentially affect the pool size of canonical HKI B cells. In contrast, depleting or supplementing BLyS in these settings led to selective contraction or expansion of the MZ B cell pool as well as analogous changes in the total peripheral B cell pool. Our results indicate that both weakly and strongly autoreactive HKI canonical clones are equally outcompeted by wild-type clones in settings of mature B cell adoptive transfers and BM chimeras. Importantly, data from BM chimeras demonstrated that HKI canonical clones are outcompeted in the spleen and BM at both mature and early transitional stages whereas they tend to accumulate in late transitional stages. It was previously shown that competition for BLyS is most severe during late transitional stages of B cell development (39, 40). Therefore, it appears that the stage at which canonical HKI clones are outcompeted does not correlate with a stage of generally heightened BLyS dependency.

Clarification of reasons for the disparate findings regarding a potential role for BLyS in peripheral B cell tolerance in the HEL–HEL–Ig system and in the studies reported in this article and in the VH3H9 anti-chromatin BCR system will require more detailed future investigation. One possibility is that in the HEL system in vivo autoantigen concentrations are much higher than the concentration of endogenous ligands for nucleic acid-binding BCRs, resulting in more extensive BCR cross-linking on developing B cells in the former case. Because endogenous HKI BCR ligands are likely nucleic acid-based but are otherwise poorly defined, altering their in vivo concentrations experimentally would be difficult. Nonetheless, despite the fact that canonical HKIR and HKI65 BCRs have ~10-fold different avidities for DNA and chromatin, and downregulation of surface BCR levels is far more pronounced on canonical HKIR as compared with HKI65 B cells, we observed no differences in peripheral survival of the two types of B cells in any of our experiments.

It must also be considered that either the chemical nature or the valence of the autoantigen in these systems differs in ways that impact the mechanism of peripheral tolerance to which B cells are subjected. For example, nucleic acid-binding BCRs can mediate internalization and delivery of nucleic acid-based autoantigens to the endosomal, nucleic acid-specific TLRs, resulting in stimulation of MyD88-dependent as well as other signaling pathways (41, 42). The autoepitope valence on nucleic acid-based autoantigens is...
likely to be much higher than on protein autoantigens such as HEL, producing different levels of BCR cross-linking that, in the former case, would be relatively insensitive to autoantigen concentrations. These issues will only be resolved via additional studies of the regulation of other B cell clonotypes specific for a variety of different types of autoantigens.

If inadequate competition for BLYS does not render HKI canonical B cells less fit to survive, then what does? The resource competition model of peripheral tolerance predicts that HKI autoreactive clones must be poor competitors for shared peripheral resources. Because our data make BLYS an unlikely candidate as the mediator of this competition, it is possible that HKI canonical clones inadequately compete for another major survival resource in the periphery, such as Ag receptor ligands. Continued BCR signaling is required for peripheral B cell survival (7, 20), and interclonal competition for BCR ligands in the periphery has been demonstrated by the work of Freitas and colleagues (18, 19, 43). This group found that monoclonal transgenic B cells failed to populate the periphery as efficiently as do polyclonal wild-type B cells in mixed BM chimeras. However, they were able to change this composition in favor of monoclonal B cells by supplementing BM chimeras with ligands for the transgenic BCRs (19). Perhaps stimulation by various self Ags is more available to diverse polyclonal repertoires compared with monoclonal HKI B cells. The reduced BCR levels characteristic of HKI canonical B cells may exacerbate this situation. However, this scenario is complicated by the fact that BCR levels on HKIR canonical B cells are lower than on HKI65 canonical B cells, yet both clones are equally outcompeted by polyclonal B cell populations. Additionally, this hypothesis begs the question of how certain BCR ligands would promote survival while others would induce tolerance.

Alternatively, it must be considered that the expression of transgene-encoded BCRs per se renders HKI clones less competitive. This is supported by the fact that we did not observe a correlation between the levels of autoreactivity and outcompete in mixed BM chimeras. Developing B cells expressing transgenic BCRs undergo an accelerated path to maturity without transiting through developmental checkpoints operating on wild-type B cells as they rearrange their BCR genes (44, 45). Therefore, BCR transgenic B cells may not achieve a state of maturity fit for survival in a competitive periphery. The survival disadvantage of autoreactive B cells in various BCR transgenic mouse models may be exacerbated by the accelerated, nonphysiological development of BCR transgenic B cells. Such a situation would confound evaluation of the effects of the affinity and specificity of transgene-encoded BCRs on the peripheral lifespan of B cells expressing them.

Acknowledgments
We thank Scott Fenn for technical assistance and all members of the Manser Laboratory for indirect contributions to this work. We also thank Matthew Farabaugh and the Kimmel Cancer Center Flow Cytometry and Laboratory Animal Facilities.

Disclosures
The authors have no financial conflicts of interest.

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