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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 postulating 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.

B lymphocyte tolerance is achieved by the purging of autoreactive B cells at key developmental checkpoints. Central tolerance checkpoints act upon immature B cells to prevent their exit from the bone marrow (BM). The ligation of the BCR by autoantigen at this stage leads to deletion, receptor editing, or functional silencing (1–4). Some self-reactive B lymphocytes escape central tolerance in the BM and gain access to the periphery. Autoreactive cells in the periphery are often rendered less fit to survive (5, 6). Although central B cell tolerance mechanisms are generally well understood, peripheral tolerance mechanisms are less so.

Because determinants of survival in the peripheral B cell pool differ from those in the BM, mechanisms of peripheral tolerance are likely distinct from those of central tolerance. This difference is largely due to the advent of two developmental changes. First, while BCR signaling can still mediate elimination, it also becomes essential for survival (7). Furthermore, when coupled with appropriate costimulatory signals, BCR signaling leads to activation. Second, B cells at late transitional stages begin to acquire receptors for the prosurvival factor B lymphocyte stimulator (BLyS, also known as BAFF) (8–10). Survival signals through the main BLyS receptor (BR3 or BAFFR) are crucial for maintenance of mature marginal zone (MZ) and follicular (FO) naive peripheral B cell subsets (11, 12). This is evidenced by profound mature B cell lymphopenia upon BLyS depletion or in mice with deleted or signaling-defective BR3 (13–17). BLyS is therefore considered a “limiting trophic resource” for the peripheral B cell pool.

The stringent regulation of the size of the peripheral B cell pool as well as levels of available BLyS are suggestive of a “competitive survival” process in the periphery. Indeed, numerous studies have demonstrated that interclonal competition determines relative survival among peripheral B cells (18–20). As such, autoreactive B cells have been suggested to be at a competitive disadvantage within the normal B cell pool (5). This, along with the observation that BLyS overexpressing mice develop autoimmune- nity (21, 22), indicates a role for BLyS in the regulation of peripheral B cell self-tolerance. However, it has yet to be determined whether BLyS-mediated autoimmunity results from a global expansion of the peripheral B cell pool and consequent enhanced survival of autoreactive clones. Alternatively, elevated BLyS levels may selectively promote the survival of autoreactive B cells.

Evidence for the latter possibility first came from studies conducted using a transgenic model of autoreactivity in which transgenic BCRs recognize the artificial “neo–self-antigen” hen egg lysozyme (HEL). By reducing the availability of BLyS in vivo, Lesley et al. (23) demonstrated that B cells recognizing soluble HEL self Ag were “more dependent” on BLyS for their survival than were wild-type B cells. Another study indicated that HEL self Ag-recognizing B cells normally deleted in the presence of competing non–self-reactive B cells were rescued from deletion by excess BLyS. Furthermore, excess BLyS allowed HEL binding B cells access to splenic locales from which they were excluded previously (24).

Whether BLyS selectively promotes the survival of self-reactive B cells that are naturally occurring and autoimmune disease-associated was later examined in another B cell transgenic model of autoreactivity. Anti-chromatin B cells from VH3H9 BCR...
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-azophenylarsenate (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 Vκ1 knock-in mice contain a targeted Vκ1 transgene composed of the combination of gene segments VΗHDCR, DFL16.1, and JΗ2 originally isolated from the dominant Ars responding clone in A/J mice. HKIR Vκ1 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 Vκ1 gene. These mice have been backcrossed to the C57BL/6 (B6) background and have been previously described (28, 29). The double transgenic HKI65/Vκ10 and HKIR/Vκ10 lines were produced by breeding HKI65 and HKIR mice to a conventional L chain transgenic mouse line. The L chain transgenic mouse express the Vκ10A-Jc1-L chain gene of the dominant anti-Ars clone (30). B6 (CD45.2+) and B6.SJL (CD45.1+) mice were purchased from The Jackson Laboratory and were bred in-house. Mice were housed in pathogen-free conditions in the Thomas Jefferson University animal facility and received autoclaved water and food. All mice except BM chimeric mice were 8–12 wk of age when used in experiments.

Adoptive transfers

Mature, resting splenic B cells were isolated from age-matched HKIR/Vκ10 or HKI65/Vκ10 donors via negative selection using anti-CD43 MACS-conjugated magnetic beads (Miltenyi Biotec). B cells from each group were mixed with equal numbers of B cells similarly isolated from B6 spleens. These B cell mixtures (3 × 10^6 total cells) were injected into the retro-orbital sinuses of congenic B6.SJL (CD45.1+) recipients.

In vivo BLyS supplementation

Mice were injected s.c. with 10 μg recombinant human (rh)BLyS (Human Genome Sciences) (31) daily. After 9, 10, or 17 d rhBLyS treatment, mice were sacrificed and splenocytes were analyzed.

CFSE labeling and adoptive transfers

Mature, resting splenic B cells were isolated from rhBLyS-treated B6 or PBS treated B6.SJL donors via negative selection using anti-CD43 MACS-conjugated magnetic beads (Miltenyi Biotec). B cells were adjusted to 10 × 10^6 cells/ml in PBS before addition of an equal volume of 5 μM CFSE in warm PBS. The reaction was quenched 90 s later by addition of 1 vol FCS. Cells were then washed and resuspended in PBS, mixed at a 1:2:1 ratio (B6/B6SJL), and injected into the retro-orbital sinuses of B6 recipients.

Statistical analysis

Statistical significance of data was determined by using a two-tailed, unpaired Student t test in the Microsoft Excel program.

Spleen cell cultures and survival assay

Splenocytes were isolated from B6 mice and naive B cells were enriched by negative selection using MACS and anti–CD43-conjugated magnetic beads (Miltenyi Biotec). B cell purity was always >90% as assayed 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 × 10^6 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 × 10^6) were incubated in 96-well plates with purified anti-CD16/32 (eBioscience; clone 93) to block nonspecific Ab binding. Cells were then stained with combinations of the following Abs and reagents: FITC-PE, -EA647-εFluor 450-, or biotin-conjugated anti-B220 (eBioScience; clone RA3-6B2); FITC- or PE-conjugated anti-IgM (Jackson ImmunoResearch Laboratories); PE-, biotin-, or PerCP-Cy5.5-conjugated anti-CD45.2 (eBioscience; clone 104); allophycocyanin-Cy7–conjugated anti-CD45.1 (eBioscience; clone A20); AF647-conjugated anti-BR3 (eBiocience; clone 7H22-E16); FITC- or allophycocyanin-conjugated anti-CD21/35 (BD Pharmingen; clone 7G6); biotin-, PE-Cy7−, or PE-conjugated anti-CD23 (eBioscience; clone B3B4); PE- or allophycocyanin-conjugated anti-CD93 (eBioscience; clone AA4.1); FITC-conjugated anti-CD43 (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 BAFFR-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% 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 mice. BM cells were freshly isolated from donor mice and were depleted of both mature B and T cells by negative selection using MACS and anti–CD3– and anti–CD19–conjugated magnetic beads (Miltenyi Biotec). Mice received antibiotic treatment starting the night before irradiation and continuing for 2 wk. Food was removed from cages the night before and reintroduced after irradiation.
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 V510A-Jk11 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 V4μ 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/Vk10 and HKI65/Vk10 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 lines. 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/Vk10 and HKI65/Vk10 (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/Vk10 and HKI65/Vk10 mice. This reduction was more pronounced on the weakly autoreactive HKI65/Vk10 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 composition of CD45.2+ donor B cells 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. Peripherally 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 cells were effectively depleted whereas FO B cells were still present although declining in 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

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**Figure 2.** In vitro and in vivo BLyS supplementation. A. Splenic B cells were isolated from B6 mice and cultured with or without 10 ng/ml rhBLyS. The percentages of 7-aminocoumarin D-negative (live) cells were determined at various time points by flow cytometry. The symbols represent means derived from triplicate wells, with error bars (sometimes too small to be seen) indicating SD. Data are representative of three independent experiments. B–E. Mice of each indicated genotype were treated with either daily s.c. injections of rhBLyS or PBS for 9 d. On day 9, spleen cells from mice were isolated and stained with anti-B220, anti-CD21, anti-CD23, and E4 and analyzed by flow cytometry. B and D, Gates were set to show the percentages of MZ (B220+CD21intCD23high) and FO (B220+CD21intCD23low) B cells. C, Gates were set to show the percentages of canonical (B220+E4+) and noncanonical (B220+E4−) B cells. E, Bars represent the mean of percentages of E4+ cells in MZ, FO, and B220+ gates. MZ and FO gates were drawn as depicted in B. The means are derived from three mice per group, with error bars indicating SD. The plots shown are representative results from three independent experiments with at least three mice per group. SSC, side scatter.

**Figure 3.** Survival of adoptively transferred wild-type and HKI canonical B cells. A. Diagram depicting the experimental approach. B, Maturity, resting B6 splenic B cells (1.5×106) were mixed with either 1.5×106 HKIR/Vsx10 splenic B cells (top) or 1.5×106 HKI65/Vsx10 splenic B cells (bottom) and adoptively transferred to syngeneic B6.SJL (CD45.1+). 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.
BLyS and decreased BR3 levels on B cells, particularly in HKI/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.

B cells from rhBLyS-treated B6 mice and PBS-treated B6.SJL mice into B6.SJL recipients at a 1:2 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 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 mean of percentages depicted by E4 + gates on representative plots in A (right plots). The means are derived from three mice per group, with error bars indicating SD. Data are representative of 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/Vk10 mice are unlikely to account for the decreased relative survival of HKI/Vk10 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 (CD45.1) and HKI (CD45.2) allotype system to distinguish wild-type (CD45.1) of developing wild-type B cells. We generated BM chimeric mice described in 42 COMPETITIVE REGULATION OF AUTOREACTIVE B CELLS

HKI canonical B cells are outcompeted in mixed BM chimeric mice 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 in the peripheral blood of chimeric hosts reconstituted solely with either HKIR/Vk10 or HKI65/Vk10 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 HKIR/Vk10 and HKI65/Vk10 mature B cells also decreased in peripheral blood over time. Furthermore, both HKIR/Vk10 and HKI65/Vk10 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 phenotypes. HKIR/Vk10 and HKI65/Vk10 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. 8A and data not shown). Surface IgM downregulation was much more pronounced on HKIR/Vk10-derived B cells than those derived from HKI65/Vk10 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...
analyzed the proportion of HKIR/Vx10- and HKI65/Vx10-derived B cells in the spleen.

After 17 d of 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/Vx10- and HKI65/Vx10-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, B cells began to be outnumbered by wild-type B cells in mixed BM chimeras. In the spleen, the immature compartment was somewhat reduced (Fig. 10C). In contrast, the CD45.2+/CD45.1+ ratio was represented in immature BM subsets as well as late transitional stages in the spleen. The ratios of B220+/CD93+CD45.2+ cells to B220+/CD93+CD45.2− cells were evaluated over time. The means are derived from three mice per group, with error bars indicating SD.

**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 autoreactivity.

Similar to mice expressing anti-HEL H and L chain transgenes along with soluble HEL, HKIR/Vx10 and HKI65/Vx10 transgenic lines exhibit elevated levels of serum BLyS and lower surface BR3 levels on B cells. As HKI/Vx10 mice have lower numbers of
B cells in the periphery than do wild-type mice (N. Nikbakht and T. Manser, unpublished observations), more available BlyS per B cell may cause downregulation of surface BR3. It has been demonstrated previously that B cells downregulate BR3 when exposed to high BlyS levels (23), a finding we reproduced in this study.

However, unlike the case in anti-HEL Ig/HEL double transgenic lines (23), higher serum BlyS levels and lower surface BR3 expression do not correlate with higher BlyS dependency for HKI B cell survival. As such, these differences in BlyS and BR3 levels in HKI double transgenic mice are unlikely to explain the reduced relative survival of HKI/Vk10 B cells in adoptive hosts containing diverse B cell repertoires. Indeed, we found that BlyS supplementation of B6 donor mice enhanced the survival of transfected B cells in BlyS-depleted chimeric mice relative to donor B cells from B6.SJL mice treated with PBS alone. The mechanistic basis of this interesting phenomenon remains to be addressed but would appear to have little or no relevance to the overall conclusions of this study.

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 autoresponsive HKI canonical clones are equally outcompeted by wild-type clones in settings of mature 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 autoantigens 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 B cell populations. 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 outcompeting 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 Scot Fenn for technical assistance and all members of the Manser Laboratory for indirect contributions to this work. We also thank Matthew Farbaugh and the Kimmel Cancer Center Flow Cytometry and Laboratory Animal Facilities.

Disclosures

The authors have no financial conflicts of interest.

References


