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Cutting Edge: BLyS Enables Survival of Transitional and Mature B Cells Through Distinct Mediators

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These studies characterize BLyS responsiveness and receptor expression among transitional and mature peripheral B cells. The results show a maturation-associated increase in BLyS binding capacity that reflects differential expression patterns of the three BLyS receptors. Accordingly, BLyS administration enforces only late transitional and mature peripheral B (MB) cell compartments. Furthermore, bromodeoxyuridine labeling and cell cycle analyses show these effects are mediated through enhanced proportional survival of cells traversing the T2, T3, and MB cell stages, rather than by causing proliferation or slowing transit within these subsets. Despite similar effects on survival, BLyS up-regulates the antiapoptotic genes A1 and bel-x<sub>L</sub> in MB cells but not immature B cells. Together, these findings show that, while BLyS influences B cell survival in several peripheral differentiation subsets, the downstream mediators differ, thus providing the first direct evidence for an established B lineage survival system whose intermediates change as B cells mature. The Journal of Immunology, 2002, 168: 5993–5996.

B lymphocytes transit several differentiation stages as they leave the bone marrow to mature in the periphery, and only a fraction of these marrow émigrés ultimately survive (1, 2). Deletion of self-reactive cells contributes to attrition (3, 4) and immature B cells are differentially susceptible to induced cell death in vitro (5, 6), but shifts in B lineage survival pathways during normal B cell maturation have not yet been described. In contrast to negative selection events, some cell losses reflect failure to meet minimal BcR signaling requisites, suggesting that specificity-dependent positive selection also plays a key role (7–9). Once mature, B cells have an average life span of 80–120 days, but clonotypic longevity varies, subject to relative fitness in competition for viability-promoting cues (10, 11).

BLyS lymphocyte stimulator protein (BLyS; trademark, Human Genome Sciences) (4, 5) profoundly influences peripheral B cell homeostasis and selection (12–18), but the relative roles of expansion, survival, and differentiation rates in these activities, as well as whether BLyS acts similarly on newly formed and mature peripheral B cells, remain unknown. Therefore, we have examined BLyS binding, receptor expression, and activity in each peripheral maturation subset.

Materials and Methods

Mice

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures were conducted in accord with the Animal Welfare Act.

Abs and flow cytometry

Cytokinefluorometric analyses were conducted as described (1, 2). The allophycocyanin-conjugated anti-AA4.1 was provided by Dr. D. Allman (University of Pennsylvania, Philadelphia, PA).

Kinetic analysis

Mice were treated with 10 µg rBLyS s.c. daily. After 4 days of BLyS treatment, mice also received i.p. injections of 0.5 mg bromodeoxyuridine (BrDU<sup>3</sup>, Sigma-Aldrich, St. Louis, MO) twice daily, and splenocytes were analyzed at successive intervals thereafter as described (1).

Cell cycle analysis

Mice received 10 µg rBLyS i.p. daily for 8 days. Splenic B cell subsets were sorted directly into cold 95% ethanol and kept at −20°C for >24 h. Flow cytometric analysis for DNA content was performed following a 30-min incubation in PI buffer (0.1% glucose in PBS, 100 U RNase, and 1 µg/ml propidium iodide). Doublets were excluded based on size.

B cell subset isolation and culture

Immature B cells were prepared from irradiated autoreconstituting mice as described (1). RBC-depleted splenocytes were treated with 100 µg/ml M 2-ME, and 100 U/ml penicillin/streptomycin. Mature B cells were prepared from normal mice by magnetic selection for CD23<sup>+</sup> B cells. T1, T2, T3, and mature spleen B cell subsets were isolated by FACs from untreated mice. Cells were cultured in RPMI 1640 medium with 10% FBS (HyClone Laboratories, Logan, UT), 2 mM glutamine, 15 mg/ml 1% oxaloacetic acid, 5 mg/ml sodium pyruvate, 20 U/ml insulin, 1% nonessential amino acids, 50 µM 2-ME, and 100 U/ml penicillin/streptomycin. Immature B or mature B (MB) cells were cultured at 4 × 10<sup>9</sup> cells/ml in 24-well plates with or without 100 ng/ml rBLyS.

Abbreviations used in this paper: BrDU, bromodeoxyuridine; MB, mature B; BCMA, B cell maturation Ag; TACI, transmembrane activator and cAML interactor.
Semiquantitative RT-PCR gene expression analysis

RNA was isolated using TRIzol reagent (Life Technologies, Rockville, MD). RNA (1 μg) was pretreated with RNase-free DNase I, then reverse transcribed using random hexamers (250 ng) and Superscript II reverse transcriptase (Life Technologies). Each RT-PCR sample consisted of 1/20 of template reverse transcriptase reaction mixture in a 50-μl PCR with Taq polymerase (1.5 U; Roche, Basel, Switzerland) and 0.4 μM gene-specific primers. As an endogenous reference standard for comparing starting template CDNA, 18S ribosomal RNA was coamplified with transmembrane activator and c-AML interactor (TACI), A1, or bax using a QuantumRNA 18S kit (Ambion, Austin, TX). Aliquots (6 μl) were collected at successive cycles, analyzed by agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Eugene, OR), densitometrically imaged, and analyzed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Semi-quantitative RT-PCR was graphed as cycle number vs log (density), and the linear portions of the curves were compared and normalized to an 18S ribosomal RNA internal standard. Densitometric values of other gene-specific RT-PCR were multiplied by correction factors derived from the 18S rRNA RT-PCR results, and in turn plotted as cycle number vs log (adjusted density) for comparison.

PCR primers had the following sequences: murine B cell maturation Ag (BCMA)-1 and BCMA-3 primers were as reported by Madry et al. (19); murine TACI sense 5'-gccaacgtgtagcact-3', TACI antisense 5'-gctcaactggaggatcgct-3'; murine BR3 sense 5'-gccaagcaacagctctgctc-3', BR3 antisense 5'-gccgcatggagcttttcgttg-3'; murine BR3 sense 5'-ggaggatgcaggtgag-3', antisense 5'-ggaggatgcaggtgag-3'; bax primers and "classic" 18S primers were purchased as a relative RT-PCR kit (Ambion).

Results and Discussion

BLyS binding capacity and receptor expression shift with maturation

Marrow B lineage subsets were resolved according to Hardy et al. (21) and analyzed for BLyS binding. No appreciable binding was observed in fractions A through D, but fraction E (IgM<sup>+</sup> AA4.1<sup>+</sup> B220<sup>+</sup>subclass) displayed clear BLyS binding (Fig. 1A). Within fraction E, a small population of CD23<sup>+</sup> cells bound BLyS with greater average intensity than CD23<sup>-</sup> fraction E cells. The basis for this is presently unclear but might suggest alternative maturation pathways that diverge within this fraction. Mature recirculating B lymphocytes (fraction F) displayed bright BLyS binding comparable to that seen in mature splenic B cells (see below, Fig. 1B).

Splenic maturation stages were divided according to Allman et al. (2), yielding three transitional subsets: T1 (CD23<sup>+</sup> IgM<sup>high</sup> AA4.1<sup>+</sup>), T2 (CD23<sup>+</sup> IgM<sup>high</sup> AA4.1<sup>+</sup>), and T3 (CD23<sup>+</sup> IgM<sup>low</sup> AA4.1<sup>+</sup>). BLyS binding was demonstrable in all transitional subsets. Although the average intensity was somewhat greater in the T2 and T3 subsets, all distributions were dispersed, suggesting considerable heterogeneity in BLyS binding characteristics within these pools. BLyS binding intensity was highest and tightly distributed among MB cells (Fig. 1B).

Together, these data indicate that BLyS binding activity ensues concomitant with surface IgM expression in the bone marrow and increases with maturation. These results could indicate generally increasing levels of all three BLyS receptors with maturation, or might instead reflect the composite of dissipate, individually regulated receptor expression patterns. Therefore, we determined the expression patterns of BCMA, TACI, and Bcmd/BR3 in sorted B cell subsets using semiquantitative RT-PCR (Fig. 2). After normalization, BCMA transcripts were most prominent in the T1 subset, with lowest expression in the MB cell subset. In contrast, TACI displayed a reciprocal expression pattern, whereby MB cells had nearly 10-fold as much TACI as the T1 subset. While BR3/ Bcmd transcripts were detectable in all subsets, the T1 subset exhibited significantly lower levels than all later differentiation stages.

These maturation-associated variations in BLyS binding and receptor expression suggested BLyS might influence immature B and MB cells differently. For example, Bcmd/BR3 may be the principal receptor required for recruitment and maintenance of the

![FIGURE 1.](http://www.jimmunol.org/)

**FIGURE 1.** BLyS binding in bone marrow and splenic B cell differentiation subsets. Bone marrow (A) or splenocytes (B) were harvested and stained as previously described (17). Subsets were resolved as shown in the *A* left plot of each panel, and the surface binding of biotinylated BLyS was assessed in each subset (center histograms of each panel). Immature marrow B cells (fraction E) were further resolved into CD23<sup>+</sup> and CD23<sup>-</sup> groups, and their corresponding BLyS binding is shown in the *B* right histograms. CD23<sup>+</sup> splenic transitional B cells were further resolved as T2 and T3 subsets by IgM<sup>high</sup> vs IgM<sup>low</sup> criteria, and their BLyS binding is depicted in the *right histograms.* Data are representative of five experiments. Negative controls shown are identically stained B220<sup>+</sup> splenocytes. In addition, fluorochrome-coupled streptavidin without biotinylated BLyS yielded similar negative control histograms, and preincubation with excess unlabeled BLyS competitively inhibited biotinylated BLyS staining (data not shown).

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** Patterns of BLyS receptor expression within immature and mature peripheral B cell subsets. RNA from FACS-sorted subsets was subjected to RT-PCR for the BCMA, TACI, and Bcmd/BR3 transcripts. Three replicates of each subset yielded similar results. Gel images (A) were subjected to densitometric analysis and adjusted for amount of 18S RNA amplified relative to other subset samples, and the log<sub>10</sub> of adjusted density was plotted (B).
follicular B cell pool, because mice lacking this receptor have a severe follicular B cell deficiency (18, 22–24); whereas signals via TACI and BCMA may play dominant roles in earlier or alternative maturation subsets. Furthermore, BLyS-induced effects on these subsets might proceed through different downstream mediators. To assess these possibilities, we determined the magnitude, production rate, turnover rate, and mitotic activity of each peripheral differentiation subset during exogenous BLyS treatment.

**BLyS enhances survival among late immature and mature peripheral B cells**

Despite their BLyS binding capacity, neither immature bone marrow B cells (data not shown) nor the peripheral T1 subset (Fig. 3A) changed appreciably during exogenous BLyS administration. Marked increases were observed in both the T3 and MB cell subsets (p < 0.01), and a milder but reproducible effect (p < 0.05) was seen in the T2 subset (Fig. 3A).

The basis for these increases was established by in vivo BrdU labeling. These analyses revealed significant increases in the number of labeled cells per day entering the T2 (p < 0.05), T3 (p < 0.01), and mature (p < 0.01) peripheral populations during BLyS administration (Fig. 3B). In contrast to these increased absolute labeling (production) rates, no significant differences in the proportional labeling (renewal) rates were observed (Fig. 3B), indicating that BLyS does not extend residency time in any of the transitional subsets. While we have also included the short-term proportional labeling plot of mature peripheral B cells to strengthen our argument against increased mitotic activity (below), this is too short a time frame to assess MB cell turnover, because the average life span of MB cells is >80 days. In fact, we previously showed that BLyS receptor mutations increase MB cell turnover (22), indicating that MB cell life span is indeed influenced by BLyS.

Because transitional B and MB peripheral subsets are quiescent (2), enhanced transit from each subset’s predecessor pool was likely responsible for enhanced production rates. Nonetheless, because BLyS has been reported to facilitate B cell proliferation in vitro, it remained possible that these increases reflected proliferation. We directly addressed this possibility by examining the effect of BLyS on the proliferative activity of splenic B cell subsets. Following 8 days of continuous BLyS treatment, transitional and mature splenic B cells were isolated by cell sorting and stained for DNA content (Fig. 3D). Negligible proportions (<0.5%) of cells were observed in the G2 + M gate among all transitional subsets of untreated control mice, in accord with Allman et al. (2). The proportion of cells in cycle was not significantly altered by exogenous BLyS administration (Fig. 3D). Moreover, enhanced division within these populations should have yielded increased short-term proportional BrdU labeling, which was not observed (Fig. 3C).

Together, these findings suggest that enhanced survival is a primary activity of BLyS in vivo. Accordingly, we favor the notion that BLyS regulates peripheral B cell numbers in two ways: by varying the proportion of cells lost to death during late transitional B cell development, as shown here, and by serving as the primary determinant of mature follicular B cell survival, as evidenced by our studies in the B cell-deficient A/WySnJ mouse (18, 20).

**Only MB cells up-regulate Bcl-xL and A1 in response to BLyS**

Members of the Bcl-2 family influence lymphocyte survival (25), and a relationship between BLyS-mediated survival and bcl-2 family member expression has been suggested (26, 27). Therefore, we investigated how BLyS affects A1, bcl-2, bcl-xL, and bax expression in immature and mature peripheral B cells in vitro.

Among MB cells, the expression of A1 and bcl-xL increased 2-to 7-fold in the presence of BLyS, whereas bcl-2 and bax transcript
levels did not change. In contrast, none of the bcl-2 family members examined were up-regulated when total transitional B cells (T1–T3) were cultured with BLyS (Fig. 4). We have further determined that the T2/T3 fraction up-regulates A1 and bcl-xL <3-fold in the presence of BLyS (data not shown).

These results are consistent with our previous studies that showed A1 is up-regulated as developing B cells enter the mature peripheral pool, and are in general accord with reports that BLyS can activate Bcl-2 family members. Moreover, these findings provide the first demonstration of a B lineage-specific survival system whose receptors and downstream mediators correlate with maturation subset. Because transitional B cells are targets of specificity-based selection and are differentially sensitive to death via BcR ligation, it is tempting to speculate that BLyS-mediated survival mechanisms are integral to these processes. For example, alternative differentiation and survival pathways for emerging B cells might be determined by independently controlling BCMA, TACI, and Bcmd/BR3 expression through adaptive vs innate immune receptors. Determining the nature and extent of these relationships will likely prove key to understanding the survival, selection, and sorting processes active during peripheral B cell maturation.

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References