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Cutting Edge: B Cell Receptor Signals Regulate BLyS Receptor Levels in Mature B Cells and Their Immediate Progenitors

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This study examines how B lymphocyte stimulator (BLyS) receptor expression and responsiveness are influenced by B cell receptor (BcR) signaling. Our results show that resting and BcR-stimulated B cells are dependent on BLyS for survival and that B cells remain BLyS responsive during BcR-induced activation. Further, BcR ligation up-regulates expression of the BLyS receptor B cell maturation defect (BLyS-R3) (Bcmd/BR3), but not other known BLyS receptors. Finally, the coupling of BcR signaling with Bcmd/BR3 expression is limited to late transitional and mature B cells. Together, these findings establish the coupling of BcR signaling with Bcmd/BR3 expression as a fundamental aspect of follicular B cell selection, survival, and activation. The Journal of Immunology, 2003, 170: 5820–5823.

B lymphocyte stimulator (BLyS) profoundly influences peripheral B cell homeostasis and selection (1–4). B cell numbers increase with BLyS treatment, and BLyS-transgenic mice display hypergammaglobulinemia and spontaneous autoantibody formation (1, 3–5). Conversely, in vivo inhibition of BLyS with soluble receptors yields rapid diminution of peripheral B cell pools (5), and follicular B cells are severely depleted in BLyS knockouts (6). Among the three BLyS receptors, B cell maturation defect/BLyS-R3 (Bcmd/BR3) plays the dominant role in maintaining peripheral B cells, because Bcmd/BR3 mutants have a 10-fold reduction in peripheral B cells (7–9) whereas mice lacking B cell maturation Ag (BCMA) or transmembrane activator and cAML interactor (TACI) have normal or elevated B cell numbers, respectively (10, 11). We previously showed that mature B cells continuously compete for BLyS to survive (12) and that BLyS signals determine the proportion of cells that successfully traverse the T2 and T3 transitional stages to join the mature B cell pool (13). Thus, BLyS controls peripheral B cell numbers both by varying the proportion of cells that complete transitional B cell development and by serving as the primary determinant of mature B cell longevity.

The BcR also plays a crucial role in B cell differentiation and survival. Immature B cells are targets of BcR-mediated selection, as evidenced by the significant cell losses and repertoire shifts associated with late B cell maturation (14–16). Further, continued BcR expression is requisite for mature B cell survival (17), and both BcR specificity and signaling can dictate relative survival advantage (18, 19). Despite these parallels, the relationship between BcR signaling and BLyS responsiveness remains unexplored. We herein show that BcR ligation selectively up-regulates expression of Bcmd/BR3. Further, the coupling of BcR signaling with Bcmd/BR3 expression is limited to late transitional subsets and mature follicular B cells. We also find that resting and BcR-stimulated B cells are dependent on BLyS for survival and that B cells remain BLyS responsive during BcR-induced proliferative responses.

Materials and Methods

Mice

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

Recombinant BLyS and derivatives

Recombinant human BLyS (rBLyS) and biotin-conjugated BLyS were provided by Human Genome Sciences (Rockville, MD). Biotinylated BLyS was revealed by a second incubation with Streptavidin Red 670 (Invitrogen, Carlsbad, CA).

B cell subset isolation, culture, and stimulation

Mature splenic B cells were prepared by MACS as described (13). Transitional and mature splenic B cell subsets for BLyS receptor expression analyses were isolated by FACS as described (13, 20). Immature or mature B cells were cultured at 1 × 10^6 cells/ml as described previously (13). In some experiments, mature B cells were loaded with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) in PBS. After a 3-min incubation, excess carboxyfluorescein diacetate succinimidyl ester or deacetylated carboxyfluorescein diacetate succinimidyl ester (CFSE) was quenched with an equal volume of FCS, and cells were washed once before culture. Various doses of F(ab)2 goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) were used for stimulation, in the presence or absence of 50 ng/ml soluble rBLyS (provided by Human Genome Sciences). Soluble CD154 (CD40L) was obtained from the supernatants of transfected J558L cells that express a CD154:CD8a fusion protein (21) and were used at an empirically optimized dilution of 1/5. A 1/80 dilution of supernatant containing anti-CD8 Ab (53,6-72) were added to cross-link the fusion protein (21). CFSE-labeled cells were also incubated with...
1 nM TO-PRO-3 (Molecular Probes, Eugene, OR) immediately before acquisition.

Semiquantitative RT-PCR analyses

Semiquantitative RT-PCR for BlysR expression was performed as previously described (13). BlysR expression was normalized to ribosomal 18S levels using a QuantumRNA Classic 18S primer kit (Ambion, Austin, TX). Cycle numbers used for normalization were chosen based on the determined linear range for each experiment.

Results

**BlysR mediates survival of BcR-stimulated B cells**

Early studies indicated that BlysR enhances $[^{3}H]Tdr$ uptake among anti-$\mu$-stimulated B cells (1), and others have proposed that BlysR signaling induces transitional cells to differentiate (22), suggesting that BlysR might deliver proliferative or inductive signals for B cell expansion and differentiation, similar to costimulatory TNF family members such as CD154. However, recent in vivo results showed that BlysR increases peripheral B cell numbers through enhanced survival among transitional and mature B cells, rather than through proliferative expansion (13); and prior in vitro studies had also suggested survival as a primary and perhaps exclusive action of BlysR (23). These seemingly disparate observations could indicate mechanistically different roles for BlysR in BcR-stimulated vs quiescent B cells. Alternatively, BlysR might afford survival in both cases, with the increased number of mitotic events in BcR-stimulated cultures reflecting enhanced proportional survival within successive cohorts of daughter cells before their next division. Therefore, before examining the effects of BcR ligation on BlysR responsiveness and receptor expression, we directly assessed these possibilities by simultaneously tracking the proliferation and survival of anti-$\mu$-stimulated mature B cells in the presence of either BlysR or CD154.

Mature B cells cultured without anti-$\mu$ (Fig. 1A, left) underwent little division, and more than one-half of the input cells were dead after 72 h. In accord with previous reports (24), addition of CD154 alone engendered several divisions and significantly reduced proportional cell death (Fig. 1A, right). In contrast, BlysR alone fostered no proliferation but yielded a 4- to 5-fold increase in viability (Fig. 1A, middle). Experiments with higher doses of BlysR yielded similar results (not shown).

Mature B cells cultured with anti-$\mu$ alone yielded the expected proliferation accompanied by considerable cell death within each daughter cohort (Fig. 1, B and C, left). Supplementation of anti-$\mu$-stimulated cultures with CD154 enhanced the extent of proliferation, yielding larger proportions and numbers of cells in later divisions than anti-$\mu$ alone, as well as fewer cells in the nondividing pool (Fig. 1, B and C, right). This synergistic effect was most pronounced at suboptimal concentrations of anti-$\mu$ (Fig. 1B) but remained perceptible even at high anti-$\mu$ concentrations. In contrast, supplementation with BlysR yielded identical division profiles to those seen with anti-$\mu$ alone, consistent with a lack of intrinsic comitogenic activity. However, the proportion and number of viable cells within each division were substantially enhanced in the presence of BlysR. Analyses of the absolute numbers of cells recovered (not shown) substantiated the proportional analyses, ruling out differential cell losses between the various treatment groups as the basis for these observations.

**BcR stimulation increases BlysR-binding capacity**

To determine whether stimulated cells remain responsive to BlysR after Ig stimulation, we incubated B cells with rBlysR after an initial 24-h pulse of anti-$\mu$. The results indicated that BcR-stimulated cells remain responsive to BlysR, because the proportion of cells viable after 72 h was clearly enhanced, despite the deferred BlysR addition (Fig. 2A). The results show enhanced viability within both the initially cultured cohort and the first daughter generation, but effects on subsequent cohorts cannot be established because the withdrawal of anti-IgM at 24 h afforded only a single round of division.

Continued BlysR responsiveness after BcR ligation prompted us to ask whether the enhanced survival of BcR-stimulated B cells reflected levels of BlysR binding comparable to those of resting B cells or whether BcR ligation per se influences BlysR binding. Mature splenic B cells were stimulated in vitro with either anti-$\mu$ or LPS. Cells were harvested after 18–26 h, and their BlysR-binding capacity was assessed by staining with biotinylated rBlysR protein. BlysR-binding capacity increased substantially after anti-$\mu$ stimulation, yielding a shifted and well-defined peak with a median fluorescence intensity of 62, compared with a median fluorescence intensity of 25 in unstimulated controls (Fig. 2B, middle). Preliminary kinetic analyses indicate that increased BlysR-binding capacity is first apparent between 10 and 15 h poststimulation (not shown). This sharp increase in binding capacity was not a general feature of proliferating B cells, because LPS stimulation failed to

**FIGURE 1.** BlysR enhances survival during anti-$\mu$-induced proliferation of mature B cells. Mature (CD23$^+$) splenic B cells loaded with CFSE were cultured either without anti-$\mu$ stimulation (A) or with various concentrations of anti-$\mu$ (B and C). Some cultures of each type were further supplemented either with 50 ng/ml recombinant BlysR (middle) or with soluble CD154 (right). After 72 h, cells were harvested, stained with TO-PRO-3, and analyzed cytofluorometrically. Results are representative of triplicates from at least two independent experiments for each treatment group.
BLyS protein, followed by Streptavidin Red 670. Dashed lines are unstained controls. Results are representative of three independent experiments.

FIGURE 2. BcR-stimulated B cells remain BLyS responsive, reflecting enhanced BLyS-binding capacity. A, CD23⁻ B cells were cultured in the presence of 10 µg/ml anti-µ for 24 h. Cultures were then washed and maintained for an additional 48 h either without further supplementation (left plot) or with 50 ng/ml rBLyS (right) and then analyzed cytofluorometrically. Results are representative of four independent experiments. B, CD23⁻ B cells were cultured for 24 h without stimulation (left histogram), with 10 µg/ml anti-µ (middle histogram), or with 10 µg/ml LPS (right histogram). Cells were harvested, and BLyS-binding capacity was assessed cytofluorometrically using biotinylated BLyS protein, followed by Streptavidin Red 670. Dashed lines are unstained controls. Results are representative of three independent experiments.

yield a similarly uniform shift in BLyS-binding capacity (Fig. 2B, right).

BcR signaling selectively up-regulates Bcmd/BR3

The BcR-mediated enhancement of BLyS-binding capacity could indicate a general up-regulation of all BLyS receptors or might instead reflect differential receptor regulation. To distinguish these possibilities, we performed semiquantitative RT-PCR for each known BLySR after stimulation of mature B cells. In accord with our previous findings (13), both TACI and Bcmd/BR3 expression were readily detectable in untreated mature B cells, whereas BCMA expression was low to nil (Fig. 3, right). After anti-µ stimulation, the expression of Bcmd/BR3 increased substantially, ranging from 3- to 12-fold. In contrast to this marked effect on Bcmd/BR3 expression, BcR ligation altered neither BCMA nor TACI message levels. Moreover, LPS stimulation had no positive or consistent effect on the expression of any BLySR among mature B cells, although in some experiments LPS appeared to down-regulate Bcmd/BR3 expression (not shown).

BcR-coupled Bcmd expression ensues in transitional subsets

Follicular B cells transit several immature developmental stages after marrow egress, which can be separated into three transitional subsets based on expression of AA4.1, CD23, and surface IgM intensity (20). Because we had previously shown differential expression of BLySRs within the three transitional populations (13), we assessed whether BcR signaling is coupled with Bcmd/BR3 expression at these differentiative stages.

Control transitional populations yielded results consistent with our previous studies (13): B cells within the T1 subset expressed high levels of BCMA but relatively low levels of TACI and Bcmd/BR3, whereas the T2 and T3 subsets showed the opposite pattern of expression (Fig. 3A). Treatment with anti-µ had no effect on the expression of BLySRs among cells in the T1 subset (Fig. 3, left). In contrast, the more mature T2 and T3 subsets displayed marked increases in Bcmd/BR3 expression after BcR ligation (Fig. 3, middle), similar to that observed among mature follicular B cells.

Discussion

The discovery that BcR signaling is selectively coupled to Bcmd/BR3 expression reveals a previously unappreciated mechanistic link between BcR- and BLyS-mediated processes associated with the T2 and T3 transitional subsets, as well as mature follicular B cells. For example, selection based on BcR specificity occurs during late transitional development (14, 15), yet BLyS signaling via Bcmd/BR3 is also critical to determining the proportion of cells that successfully traverse this developmental stage (4, 6, 7, 12, 13, 22). Because the coupling of BcR signaling and Bcmd/BR3 expression distinguishes transitional cells attempting to enter the mature follicular B cell pool, BcR-mediated selection among these newly formed B cells likely reflects the onset of BcR-driven Bcmd/BR3 expression as the primary determinant of survival. This view is consistent with
previous observations that BLyS-binding capacity among transitional cells is lower and more disperse than among mature follicular B cells and that BLyS-induced up-regulation of anti-apoptotic bcl-2 family members is most pronounced among mature B cells (13).

The findings herein also forge a relationship between BcR-mediated effects on B cell life span and the recent finding that mature B cells compete for BLyS to survive. Surface BcR expression is required for mature B cell viability (17). Further, BcR specificity dictates relative competitive advantage, because neither transgenic clonotypes nor BcR-signaling mutants that would otherwise survive can compete effectively with normal B cells in mixed marrow chimeras (19, 25). The coupling of BcR signaling with Bcmd/BR3 expression suggests a mechanism whereby the basal BcR signaling requisite reflects an obligate minimum level of Bcmd/BR3 expression, and a further survival advantage is afforded those clonotypes with BcRs that engender the highest relative levels of Bcmd/BR3. Several previous findings support this suggestion. First, the notion that BLyS signaling via Bcmd/BR3 is a downstream effector of BcR-mediated survival signals predicts that blocked BcR signaling will override the viability promoting effects of slg. This is consistent with previous findings in BLyS knockouts (6), soluble BLySR administration experiments (5), and bcmd mutants (7–9); all of which yield a shortened B cell life span despite normal slg expression. Moreover, we previously showed that the life span of B cells in mice heterozygous for a bcmd loss of function mutation is intermediate to those in normal and homozygous mutant mice (12). This observation supports the notion that 2-fold changes in Bcmd/BR3 activity, analogous to the range of BcR-mediated shifts in BLyS binding shown here, yield significant changes in B cell longevity.

It is tempting to speculate that BcR-mediated selection during transitional development and BcR-mediated homeostatic survival are mechanistically identical processes. This model would suggest a continuous rather than stepwise selective process, such that the marked cell losses seen during transitional differentiation reflects the imposition of requisite BcR-mediated Bcmd/BR3 expression on a previously unselected population, which is followed by more protracted competition among the mature B cells that endured initial selection.

Finally, the increased proportional survival within initial cohorts of activated mature B cells suggests a role for BLyS in determining the effective burst size reached by responding primary B cell clones. This likely influences the magnitude of primary humoral responses, as well as the cells available to nucleate germinal centers as memory progenitors. More detailed kinetic analyses of these processes will be required to assess these possibilities, as well as the potential role of this regulatory circuit in the selection and maintenance of memory B cell subsets.

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