Attenuation of Apoptosis Underlies B Lymphocyte Stimulator Enhancement of Humoral Immune Response

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Abstract

B lymphocyte stimulator (BLyS) is a newly identified monocyte-specific TNF family cytokine. It has been implicated in the development of autoimmunity, and functions as a potent costimulator with antiimmunoglobulin M in B cell proliferation in vitro. Here we demonstrate that BLyS prominently enhances the humoral responses to both T cell–independent and T cell–dependent antigens, primarily by attenuation of apoptosis as evidenced by the prolonged survival of antigen-activated B cells in vivo and in vitro. BLyS acts on primary splenic B cells autonomously, and directly cooperates with CD40 ligand (CD40L) in B cell activation in vitro by protecting replicating B cells from apoptosis. Moreover, although BLyS alone cannot activate the cell cycle, it is sufficient to prolong the survival of naive resting B cells in vitro. Attenuation of apoptosis by BLyS correlates with changes in the ratios between Bcl-2 family proteins in favor of cell survival, predominantly by reducing the proapoptotic Bak and increasing its prosurvival partners, Bcl-2 and Bcl-xL. In either resting or CD40L-activated B cells, the NF-κB transcription factors RelB and p50 are specifically activated, suggesting that they may mediate BLyS signals for B cell survival. Together, these results provide direct evidence for BLyS enhancement of both T cell–independent and T cell–dependent humoral immune responses, and imply a role for BLyS in the conservation of the B cell repertoire. The ability of BLyS to increase B cell survival indiscriminately, at either a resting or activated state, and to cooperate with CD40L, further suggests that attenuation of apoptosis underlies BLyS enhancement of polyclonal autoimmunity as well as the physiologic humoral immune response.

Key words: primary B cell • CD40 • nuclear factor κB • Bcl-2 • autoimmunity

Introduction

B lymphocyte stimulator (BLyS)[1] (1), also called B cell activating factor belonging to the TNF family (BAFF [2]), TNF homologue that activates apoptosis, nuclear factor [NF]-κB, and c-Jun NH₂-terminal kinase (THANK [3]), TNF and apoptosis ligand–related leukocyte-expressed ligand 1 (TALL-1 [4]), and zTNF4 (5) is a recent addition to the TNF family. The expression of BLyS appears to be regulated by IFN-γ and restricted to cells of myeloid origin (1). BLyS has also been shown to bind only to mature B cells (1, 2). This unique combination of cell specificity in expression and targeting distinguishes BLyS from other TNF family cytokines, and suggests a novel role for BLyS in B cell immunity.

Consistent with this possibility, recombinant human BLyS, in either the membrane-bound form or the soluble form containing the extracellular 152 amino acids, functions as a potent costimulator with anti-IgM for proliferation of tonsillar and peripheral blood B lymphocytes in vitro (1, 2). Moreover, intraperitoneal injection of soluble

human BLyS (1) or ectopic expression of BLyS in mice (5–7) led to increases in B cells and plasma cells and elevation of serum Ig levels in the absence of immunization. BLyS transgenic mice further developed severe autoimmunity with characteristics of systemic lupus erythematosus (5–7). Together, these results suggest that BLyS is a key mediator of monocyte signals for the development of polyclonal autoimmunity, and may contribute to the antigen-specific antibody response. The humoral immune response, in most cases, requires T cell help mediated by the interaction between CD40, a TNF family receptor expressed on B cells, and CD40L, its ligand expressed on activated T cells (8). Whether BLyS directly cooperates with CD40L in the humoral immune response has not been elucidated.

The mechanism of BLyS signaling is not well understood. BLyS is a 285–amino acid type II transmembrane protein, with significant homology to a proliferation-inducing ligand (APRIL; >25%), and to a lesser extent, TNF-α, lymphotoxin α, FasL, and TNF-related apoptosis-inducing ligand (TRAIL), in its extracellular domain (1). Two proteins that bind BLyS, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA), have now been identified (5, 9–11). TACI was initially cloned as a TNF family protein expressed on the surface of B cells and ionomycin and phorbol ester–activated T cells (12). BCMA was previously identified as an orphan TNF family protein (13), and was only recently found to bind BLyS (5, 9). Whereas nothing is known about BCMA signaling, TACI appears to signal as a classical TNF family receptor. The extracellular domain of TACI shares homology with death receptor 3 and TNFR1, and its intracellular domain has been shown to associate with the calcium modulator and cyclophilin ligand (CAML [12]), and to bind TNF receptor–associated factor (TRAF)2, TRAF5, and TRAF6 in yeast two–hybrid screens (10). Overexpression of TACI led to the activation of NF-κB in 293 cells (11), and AP-1 and nuclear factor of activated T cells in addition to NF-κB in transformed Jurkat T cells as determined by reporter assays (12). Likewise, BLyS has been shown to activate NF-κB and c-Jun NH$_2$-terminal kinase in A20 lymphoma cells (10). Although these findings in cell lines implicate a role for NF-κB in BLyS signaling, whether NF-κB is activated by BLyS in primary B cells, the physiologic targets of BLyS action, is not known.

To address these questions, we have investigated the role of BLyS in T cell–independent and T cell–dependent antibody responses in vivo, and the cooperation between BLyS and CD40L in the activation of primary mouse B cells in vitro. Evidence is presented to suggest that attenuation of apoptosis is the primary consequence of BLyS signaling in B cells, which underlies the enhancement of humoral immune response and is likely to be mediated by specific NF-κB and Bcl-2 family proteins. These findings have significant implications for BLyS in the conservation of naive B cells, in polyclonal autoimmunity, and in the physiologic humoral immune response.

Materials and Methods

Immunization. BALB/cAnNCR female mice, 6–8 wk of age, were immunized with the indicated amounts of the Pneumovax 23 vaccine (Merck) subcutaneously on days 0 and 7. Purified soluble, recombinant human BLyS synthesized in baculovirus (20 μg [1]) or PBS was administered subcutaneously daily through day 9. The hapten carrier conjugate 2,4-dinitrophenol (DNP)-BSA (100 μg) was administered intraperitoneally on days 0 and 7. BLyS (20 μg) or PBS was administered intraperitoneally daily through day 14. 4-hydroxy-3-nitrophenyl–chicken γ globulin (NP-CGG) was prepared by conjugating (4-hydroxy-3-nitrophenyl) acetyl succinimide ester (Biosearch Technologies, Inc.) to chicken γ globulin (Sigma-Aldrich) at an 18:1 ratio, as described (14). C57BL/6 male mice, 10 wk of age, were immunized intraperitoneally with 75 μg NP-CGG precipitated in a 100 mg/250 μl solution of aluminum hydroxide adjuvant. BLyS (10 μg) or PBS was administered intraperitoneally daily through day 8. For each time point, three to five mice were used for each experimental condition.

ELISA. The relative titers of Pneumovax-specific polyclonal IgM, IgA, and IgG were determined in sera collected on days 0 and 7 by ELISA on 96-well ELISA plates (Nunc) coated overnight at 4°C with 50 μl of Pneumovax (0.1 μg/ml) and blocked with 100 μl of milk for 2 h at 4°C. Serum samples were serially diluted (threefold) beginning with a 1:1,000 dilution across eight wells of the 96-well plate. The blocking agent was removed and the assay plates were washed twice with PBS. Diluted serum samples (100 μl) were added to individual wells and incubated at room temperature for 1 h, after which plates were washed three times with PBS and a biotinylated goat anti–mouse isotype-specific reagent added for 1 h at room temperature. Plates were washed three times and developed using the ABC horseradish peroxidase (HRP) kit (Vector Laboratories) and the appropriate colorimetric substrate (3,3′,5,5′-tetramethylbenzidine). HRP reactions were stopped by the addition of dilute sulfuric acid. OD readings at 414 nm were performed using a UV-MAX ELISA reader (Molecular Devices). Data were analyzed using software provided by Molecular Devices (SoftMax). Values are expressed as relative titers corresponding to the highest reciprocal dilution that yields a detectable signal in the assay. DNP-specific IgM and IgG titers were assayed on sera collected on days 0, 7, and 14. ELISA was performed similarly using plates previously coated with 50 μl of DNP-KLH (100 μg/ml).

NP-specific IgM and IgG titers were assayed on sera collected on days 0 and 8. ELISA plates (Corning) were coated overnight at 4°C with 100 μl/well of a 100 μg/ml solution of 4-hydroxy-3-iodo-5-nitrophenylacetyl succinimide ester conjugated to BSA (NIP-BSA) in Tris-buffered saline (TBS: 20 mM Tris, pH 7.5, 150 mM NaCl). Serial dilutions of the serum were incubated in triplicate on the NIP-BSA–coated ELISA plates at 4°C overnight. Bound antibody was detected by sequential incubation with biotinylated affinity-purified goat anti–mouse IgG or IgM (1:30,000 dilution in 0.5% BSA/TBS; Southern Biotechnology Associates, Inc.) for 2 h at 37°C, followed by a 30-min incubation with peroxidase-conjugated streptavidin at room temperature (1:2,000 dilution in 0.5% BSA/0.05% Tween/TBS; Vector Laboratories). The ELISAs were subsequently developed with an HRP substrate kit (Bio-Rad Laboratories), detected at OD 414 nm on a Titertek Multiskan II, and values were reported as described above.

Enzyme-linked Immunospot. Enzyme-linked immunospot (ELISPOT) was used to quantify the number of plasma cells that were secreting NP-specific IgM or IgG at the single cell level.

Multiscreen-HA membrane plates (Millipore) were coated with 50 μg/ml NIP-BSA overnight. Threefold serial dilutions beginning with a 100-μl suspension of 10^7 B cells per ml were then cultured on precoated HA-multiscreen membrane plates for 3.5 h in complete RPMI at 37°C in a 5% CO_2 chamber. Bound IgG or IgM was detected by sequential incubation with biotinylated affinity-purified goat anti–mouse IgG or IgM (1:20,000 dilution in PBS, 0.1% Tween, 5% FCS) and peroxidase-conjugated streptavidin (1:1,000 dilution in PBS, 0.1% Tween, 5% FCS). Secreting cells were detected using 3-amino-9-ethyl-carbazole tablets (AEC; Sigma-Aldrich) and counted in duplicate.

In Vitro Primary B Cell Differentiation Assay. Primary B cells were isolated from spleens of 8–12-week-old C57BL/6 mice (Taconic Farms). Splenocytes were depleted of red blood cells with ACK lysis buffer (150 mM ammonium chloride, 6 mM calcium bicarbonate, and 10 μM disodium ethylene diamine tetraacetic acid; all from BioWhittaker) and then incubated with rabbit complement (Cedarlane) together with anti-Thy1.2 (hybridoma HO-13-4) and anti-CD4 (hybridoma GK1.5) at 37°C for 20 min to deplete macrophages and T lymphocytes. Discontinuous Percoll (1.129 g/ml; Amersham Pharmacia Biotech) density centrifugation was performed to recover resting B cells at the 60–70% interface (high density) and activated B cells and plasma cells at the 50–60% interface (low density). The resting B cells (1.5×10^5/ml) were cultured with mitomycin C (100 μg/ml)–arrested CD40L-expressing L cells (CD40L cells) at a 10:1 ratio unless otherwise specified, in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), 2 mM nonessential amino acids, and 50 μM β-mercaptoethanol. The feeder layer in cocultures was renewed every 3 d. The stock of CD40L cells was cultured in the same media without β-mercaptoethanol and supplemented with HAT (0.1 mM hypoxanthine, 4×10^{-4} mM aminopterin, and 1.8×10^{-2} mM thymidine; Sigma-Aldrich). Purified recombinant human soluble BLyS was expressed in baculovirus and purified from Sf9 cell supernatant (1). The number of live cells was determined by trypan blue staining and cell counting in triplicate.

Detection of Surface CD40 and Fas and Intracellular IgM. The surface expression of CD40 and Fas was detected with a PE-conjugated rat monoclonal antibody to CD40 and PE-conjugated hamster monoclonal antibody to Fas (BD PharMingen) by flow cytometry using a Becton Dickinson FACSCalibur™. A PE-conjugated rat anti–mouse CD4 (BD PharMingen) was used as an isotype control. Detection of intracellular IgM by immunofluorescence staining was performed essentially as described (15), with a Texas red–conjugated goat anti–mouse IgM polyclonal antibody (Southern Biotechnology Associates, Inc.). The cells were counterstained for 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to localize the nuclei before microscopic analysis.

Annexin V Binding Assay. The binding of annexin V to cell surface phosphatidylserine was assayed on live cells enriched by Ficoll (Amersham Pharmacia Biotech) centrifugation, using an annexin V–FITC apoptosis detection kit (Oncogene Research Products). Samples were analyzed with a FACSCalibur™ according to the manufacturer's specifications.

Cell Cycle Analysis. Cell cycle analysis was performed on $1–3 \times 10^6$ live cells enriched by Ficoll centrifugation. The cells were fixed in 70% ethanol at 4°C overnight, and incubated in 1 ml of PBS containing 50 μg/ml propidium iodide (Sigma-Aldrich) and 100 U/ml ribonuclease A (Sigma-Aldrich) for 30 min at room temperature in the dark. The total DNA content of each cell was analyzed with a FACSCalibur™ using a Cell-Fit program with

gating set to exclude debris and cells containing less than diploid DNA. Each determination was based on the analysis of 10,000 cells. To detect replicating DNA, cells were cultured in the presence of 5 μM of 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich) for 2 h before fixation with 70% ethanol as described above. The fixed cells were resuspended in a solution containing 2 N HCl and 0.5% Triton X-100 for 30 min at room temperature, and neutralized in 0.1 M $Na_2B_4O_7$, pH 8.5. Cells were then washed with PBS containing 5% fetal bovine serum and 0.5% Tween 20, and stained with a mouse FITC-conjugated anti-BrdU monoclonal antibody (Boehringer) at a dilution of 1:50 for 30 min. The stained cells were resuspended in 1 ml of PBS containing 5 μg/ml propidium iodide for 2 h and analyzed by FACSCalibur™ as described above.

Electrophoretic Mobility Shift Assays. To prepare whole cell extracts, viable cells were purified by Ficoll gradient, washed, and resuspended in buffer A (20 mM Hepes, pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1 mM dithiothreitol, 0.4 mM Pefabloc, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin). After incubation on ice for 10 min and clearing by centrifugation at 14,000 rpm for 10 min at 4°C, the supernatant was collected and protein concentration was determined by the Bradford assay (Bio-Rad Laboratories). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (16), using 2.6 μg of protein in each reaction. The NF-κB subunits in the DNA–protein complexes were identified by preincubating extracts with 0.8 μl of polyclonal antisera against NF-κB p50, p52, p65, crel, or RelB (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C before the addition of 20,000 cpm of ^{32}P-labeled H2K probe (5′-GATC-CAGGGCTGGGGATTCCCCATCTCCACAGG-3′). The samples were resolved by electrophoresis on a native 5% polyacrylamide gel in 0.5× TBE (25 mM Tris-Cl, pH 8.3, 25 mM boric acid, and 0.5 mM EDTA) at 200 V, and the gel was dried and autoradiographed.

Immunoblotting. Proteins (15–40 μg, in equal amounts) in whole cell extracts were resolved by electrophoresis on a 12% polyacrylamide-SDS gel, transferred to a polyvinylidine difluoride membrane (PVDF; Millipore), and probed with the following antisera: hamster anti–mouse Bcl-2, rabbit anti–human Bcl-xL, and rabbit anti–human Bak (BD PharMingen). The blots were subsequently incubated with specific biotinylated secondary antibodies, followed by streptavidin-conjugated HRP (Jackson ImmunoResearch Laboratories). The blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Results

BLyS Enhances the T Cell–independent Humoral Immune Response. BLyS increases the serum Ig levels in the absence of immunization and functions as a potent costimulator with anti-IgM for B cell proliferation in vitro, suggesting that it may modulate the humoral immune response independent of T cells. To address this possibility, whether BLyS modulates the antigen-specific antibody response was investigated in mice challenged with the Pneumovax 23 vaccine, a polyvalent vaccine consisting of highly purified capsular polysaccharides from the 23 most prevalent or invasive pneumococcal types of *Streptococcus pneumoniae*.

The serum titers of Pneumovax-specific IgM increased ~10-fold within 7 d of antigen injection (10, 1, or 0.1 mg/

kg), consistent with an IgM-dominant response to a carbohydrate antigen (Fig. 1 A). Coadministration of recombinant soluble human BLyS (20 μg [1]) increased the mean IgM titers 3–10-fold (Student's one-tailed t test: $P < 0.05$; Fig. 1 A), which were maintained for at least 14 d despite discontinuation of BLyS on day 10, and were not significantly increased by a secondary challenge on day 8 (data not shown). As predicted from a T cell–independent antigen, Pneumovax did not elicit a strong IgG response, and the small increases observed were insensitive to simultaneous BLyS exposure (Fig. 1 B). By contrast, BLyS profoundly increased IgA titers, 1,000–10,000-fold at day 7 over that of buffer and Pneumovax-only control groups ($P < 0.05$). The frequency of antigen response also increased, from 2 out of 15 for the Pneumovax-only group to 11 out of 15 for mice injected with BLyS (Fig. 1 C). BLyS therefore enhances the humoral immune response of the IgM and IgA isotypes to the T cell–independent polysaccharide antigens contained in a Pneumovax vaccine.
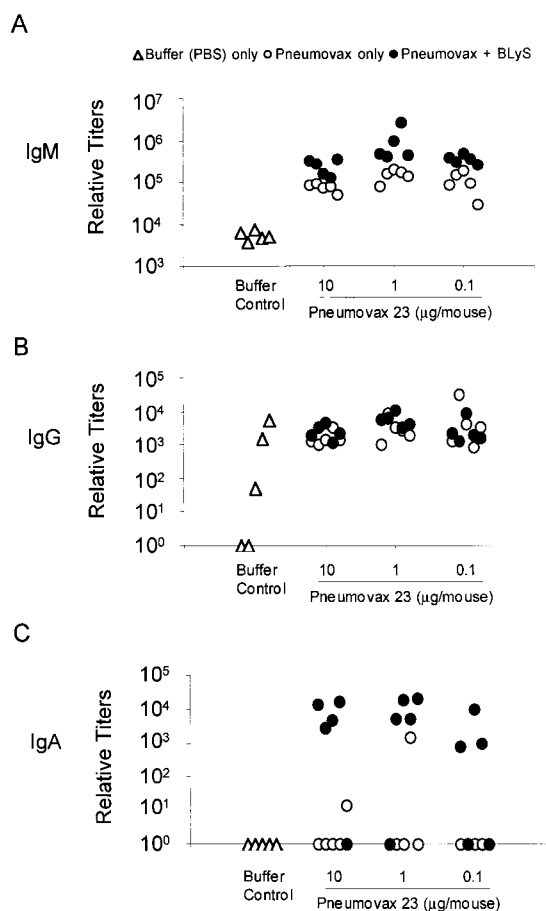

Figure 1. BLyS enhances antibody response to Pneumovax. Primary Pneumovax 23–specific IgM (A), IgG (B), or IgA (C) responses in mice after BLyS administration. Pneumovax-specific Ig titers were determined by ELISA on sera collected from buffer control mice on day 0 (△), from mice immunized with Pneumovax (○), or from mice immunized with Pneumovax and injected subcutaneously daily with BLyS (20 μg/mouse) on day 7 (●).

BLyS Enhances the T Cell–dependent Humoral Immune Response by Increasing Antigen-specific Plasma Cells. To investigate whether BLyS also enhances the T cell–dependent humoral immune response, mice were challenged with the hapten carrier DNP-BSA. Little if any DNP-specific IgM was detected in mice immunized with the antigen only, even on day 14 (Fig. 2 A), consistent with previously observed DNP responsiveness among normal BALB/c mice (data not shown). BLyS treatment alone led to a similarly modest IgM response to DNP, most likely due to increases in nonspecific antibody production (1). However, BLyS (20 μg/mouse) markedly increased the relative titers of serum DNP-specific IgM (5–10-fold) in immunized mice ($P < 0.05$; Fig. 2 A). The relative titers of DNP-specific IgG were increased by either immunization (30-fold) or BLyS treatment (3-fold) by day 14, but not substantially by coadministration with BLyS and DNP-BSA (2-fold; Fig. 2 A). Thus, BLyS markedly enhances the IgM response, and modestly increases the already robust IgG response, to DNP–BSA.

To corroborate these findings, whether BLyS modulates the antibody response to another well-characterized T cell–dependent antigen, NP-CGG (14), was investigated. Compared with PBS treatment, BLyS (10 μg/mouse) increased the relative titers of NP-specific serum IgM twofold ($P < 0.05$) and IgG fourfold ($P < 0.05$) by day 8 (Fig. 2 B). The BLyS enhancement of antibody response to NP-CGG differed significantly from that to DNP-BSA, presumably due to the nature of antigen, the difference in mouse strain, the concentration of BLyS, and the time of serum collection in this study. Despite these differences, BLyS enhances the humoral immune response to two T cell–dependent antigens.

To determine whether BLyS enhances the antibody response by increasing Ig-secreting cells, the number of splenic B cells secreting NP-specific IgM or IgG was quantified by ELISPOT, which assays Ig secretion at the single cell level (Fig. 2 C). BLyS reproducibly increased the frequency of NP-specific IgM and IgG plasma cells (twofold) by day 8 (Fig. 2 C, left). Taking into account the expansion of splenic B cells (see Fig. 3 B), this corresponded to a sixfold increase in the total number of plasma cells secreting NP-specific IgM and IgG ($P < 0.05$; Fig. 2 C, right). The enhancement of NP-specific antibody response by BLyS therefore has its basis in increases of antigen-specific, Ig-secreting plasma cells.

BLyS Attenuates B Cell Apoptosis in a Primary T Cell–dependent Response. Having shown that BLyS increased the frequency and the total number of NP-specific plasma cells, we next asked whether this was due to prolonged cell survival in vivo and in vitro. Immunization with NP-CGG alone resulted in a small increase in the spleen weight (data not shown), which was augmented by the administration of BLyS ($P < 0.05$; Fig. 3 A). This corresponded to a 2.7-fold increase ($P < 0.05$) by day 8 of total B cells isolated by Percoll gradient centrifugation (Fig. 3 B).

The possibility that BLyS enhances B cell accumulation by protection from apoptosis was then considered. Chromatin fragmentation, a signature of apoptosis in transformed

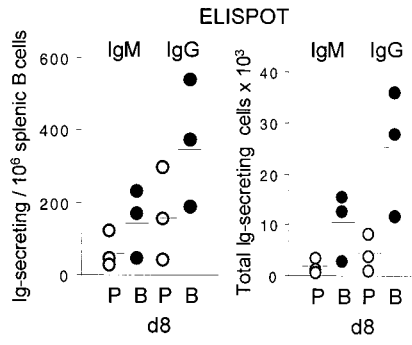## A DNP-BSA
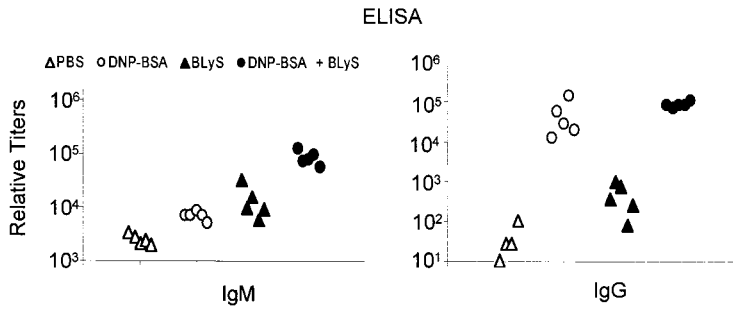
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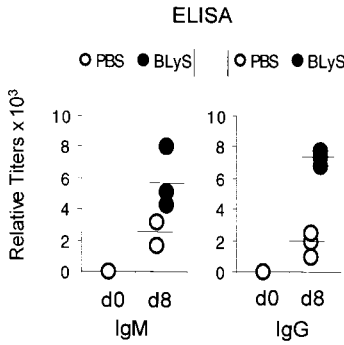
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Figure 2. BLyS enhances antibody response to DNP-BSA and NP-CGG. (A) Primary DNP-BSA–specific IgM and IgG responses in mice after BLyS administration. Serum DNP–specific Ig titers were determined on day 14 by ELISA from PBS control mice (△), DNP-BSA–immunized (○), BLyS alone (▲) or DNP-BSA–immunized and injected intraperitoneally daily with 20 μg/mouse BLyS (●). (B) Primary NP-CGG IgM and IgG responses in mice after BLyS administration. NP-specific Ig titers were determined by ELISA from sera collected from uninjected mice on day 0 (○), or from NP-CGG–immunized mice on day 8, which were coinjected with PBS (○) or 10 μg/mouse BLyS (●). (C) Primary NP-CGG–specific IgM- and IgG-secreting plasma cells after PBS (P) or BLyS (B) administration were determined by ELISPOT assay on day 8. Frequency of plasma cells (left) and total splenic plasma cells (right). These experiments have been performed three times.

cells and Fas–mediated apoptosis of B cells, is not often associated with apoptosis of plasma cells or primary mouse B cells (reference 17 and Chen-Kiang, S., manuscript in preparation). We therefore chose to directly assay Annexin V binding activity, which detects the translocation of phosphatidylserine from the inner to outer leaflet of the plasma membrane characteristic of early apoptotic cells (17, 18). Exposure to BLyS in vivo reduced apoptosis of both high (60–70% interface) and low density (50–60% interface) splenic B cells isolated from NP-CGG–immunized mice, in particular the low density B cells (Fig. 3 C). Because these cells exhibit a lower nuclear to cytoplasmic ratio character-
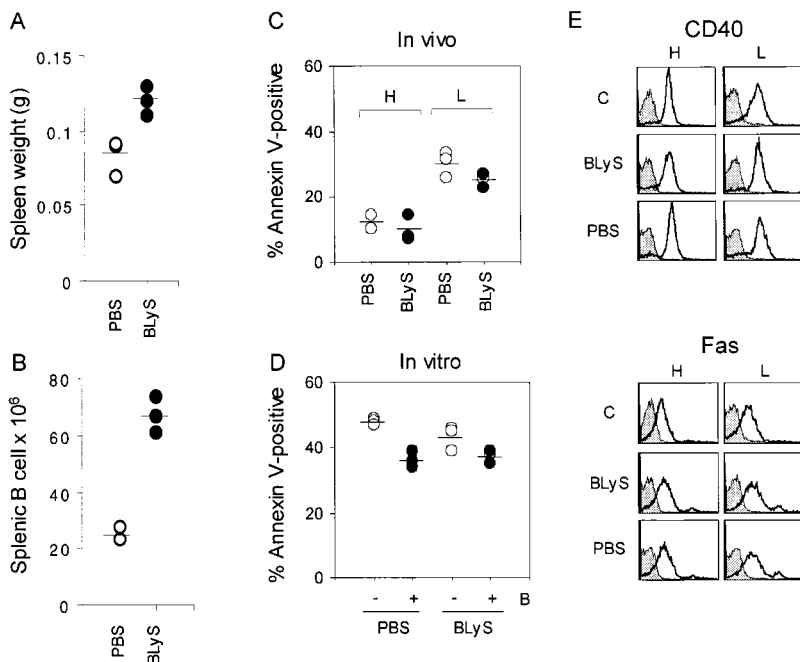

Figure 3. BLyS enhances activated B cell expansion and reduces apoptosis in vivo and in vitro. (A) Spleens were weighed (weights shown in grams) on day 8 of NP-CGG immunization, with coinjection of PBS (○) or 10 μg/mouse BLyS (●). (B) Viable splenic B cells in PBS and BLyS–coinjected mice were determined on day 8 of NP-CGG immunization ($n = 3$). (C) Annexin V binding of high density (H) and low density (L) splenic B cells isolated on day 11 after immunization with NP-CGG and coinjected with PBS (○) or 10 μg/mouse BLyS (●) ($n = 3$). (D) Annexin V binding of low density splenic B cells were isolated on day 8 of NP-CGG–immunized mice, coinjected with PBS or BLyS as indicated, and cultured in vitro for 24 h in media alone (○) or with 100 ng/ml BLyS, $n = 3$ (●). (E) Expression of CD40 and Fas on high (H) and low density (L) B cells without immunization (C), or on day 8 after immunization with NP-CGG coinjected with PBS or BLyS. These experiments have been performed three times.

istic of activated B cells, these results suggest that BLyS protects activated B cells more effectively, presumably due to their higher propensity to undergo apoptosis. To confirm this finding, low density B cells were isolated from immunized mice with BLyS or PBS treatment in vivo and incubated in the presence or absence of BLyS for an additional 24 h in vitro. BLyS (100 μg/ml) significantly reduced apoptosis (25%) in B cells without prior exposure to BLyS ($P <$ 0.05; Fig. 3 D). Although B cells isolated from BLyS-treated mice were less apoptotic, as expected, they were further protected from apoptosis by the addition of BLyS in vitro ($P <$ 0.05; Fig. 3 D). BLyS therefore attenuates apoptosis of antigen–activated B cells in a primary T cell–dependent immune response in vivo and in vitro.

Survival of activated B cells can be enhanced by CD40, which signals cell survival and cell cycle activation, and reduced by Fas, which initiates apoptosis. This raises the possibility that reduction of apoptosis and enhancement of B cell expansion might be secondary to regulation of CD40 or Fas expression by BLyS. However, this is not the case. The expression of CD40 on B cells remained unchanged after immunization and BLyS treatment. Whereas a slight increase in Fas expression on low density B cells was seen after immunization, Fas expression remained constant after BLyS treatment (Fig. 3 E). These results rule out the possibility that BLyS attenuates B cell apoptosis through increasing CD40 or decreasing Fas expression.

BLyS Enhances B Cell Activation and Expansion in Response to CD40L In Vitro.   The marked increases in NP–specific serum IgM and IgG titers and the corresponding plasma cells secreting NP–specific Igs (Fig. 2) suggest that BLyS enhances the T cell–dependent humoral immune response by cooperating with CD40L early in B cell activation. This possibility was investigated in vitro in primary resting mouse splenic B cells cocultured with mitotically-arrested CD40L cells at a 10:1 ratio. Under these conditions, >80% of the resting B cells enter the cell cycle and expand within 36 h. The addition of BLyS (50 ng/ml) profoundly enhanced the expansion of live cells, fivefold by day 6 compared with activation by CD40L alone (Fig. 4 A). This enhancement was BLyS concentration dependent, being optimal between 1 and 10 ng/ml by day 3 (Fig. 4 B). Although the basis for the decline in B cell expansion at higher BLyS concentrations remains to be determined, BLyS efficiently cooperates with CD40L in B cell activation in vitro.

Flow cytometric analysis of the cell cycle (17) showed that BLyS increased the number of cells (24–33%) in the S and G2/M phases of the cell cycle, which represent cycling cells (19) (Fig. 4 C). To corroborate this result, the uptake of BrdU into replicating DNA was determined by pulse-labeling cells for 2 h at 12-h intervals (Fig. 4 D). The presence of BLyS prominently increased the number of replicating cells (BrdU-positive), from twofold (1.5–3%) at the onset of cell cycle entry at 36 h to fivefold (4.6–24%) at 60 h during active replication. BLyS therefore enhances the activation and expansion of B cells in response to CD40L by profound increases in replicating cells.
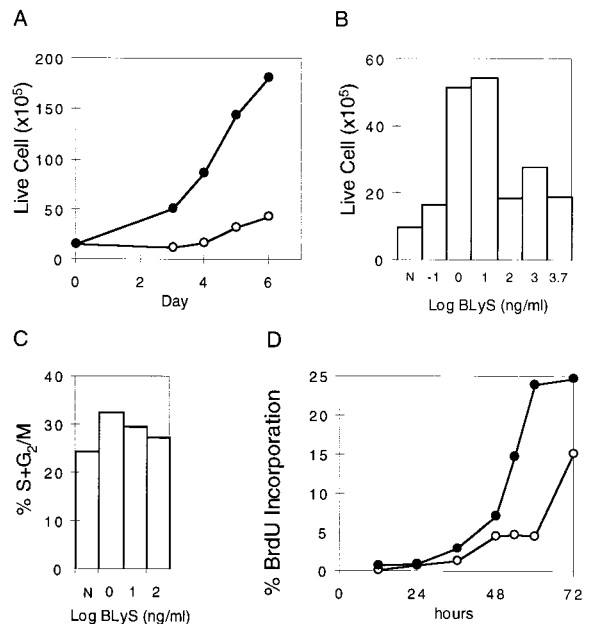

Figure 4.   BLyS enhances the activation of B cells by CD40L in vitro. (A) Resting mouse splenic B cells were cocultured with CD40L cells, in the presence (●) or absence (○) of BLyS (50 ng/ml). The accumulation of live cells was determined on the days indicated, by trypan blue staining and counting in triplicate. This experiment has been performed three times. (B) The accumulation of viable cell number on day 3 of coculture with CD40L cells in the presence of indicated concentrations of BLyS or without BLyS (N). (C) FACS® analysis of DNA content on day 3 of coculture with CD40L cells in the presence of indicated concentrations of BLyS or without BLyS (N), and expressed as the percentage of cells in the S and G2/M phases of the cell cycle. (D) Analysis of BrdU incorporation in a 2-h period, at 12-h intervals of coculturing with CD40L cells in the presence (●) or absence (○) of BLyS (5 ng/ml).

Attenuation of Apoptosis Underlies Cooperation of BLyS with CD40L in B Cell Activation.   The increase in replicating cells was not accompanied by either accelerated cell cycle entry, which occurs 24 h after CD40L activation (Fig. 4 D), or shortening of the cell cycle time as determined by BrdU pulse–chase analysis (data not shown). This, together with the marked reduction of apoptosis in activated B cells in response to BLyS in vivo and in vitro (Fig. 3, C and D), strongly suggests that the enhancement of B cell expansion is secondary to protection of replicating B cells from apoptosis. This hypothesis further predicts that attenuation of B cell apoptosis by BLyS and by CD40L may be functionally redundant.

To address this possibility, resting B cells were activated with increasing concentrations of CD40L by varying the ratio of CD40L cells to B cells (Fig. 5 A). The expansion of live cells, although evident only at the highest CD40L to B cell ratio (1:1) on day 3, was prominently increased by costimulation with BLyS (5 ng/ml; Fig. 5 A, left). Conversely, although protection of B cells from apoptosis by CD40L was concentration dependent, the presence of BLyS effectively reduced cell death even at the lowest CD40L concentration, as determined by trypan blue staining (Fig. 5 A, right). Together with marked increases in BrdU uptake (Fig. 4 D), these results suggest that BLyS co-
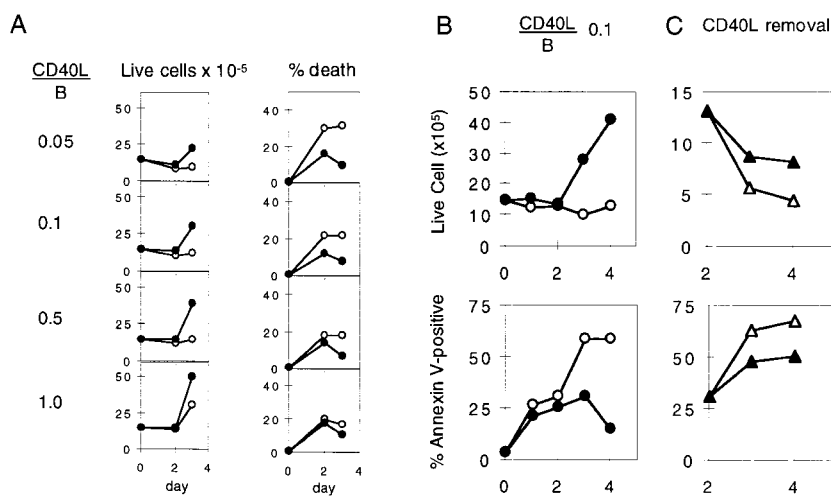
Figure 5. BLyS enhances the expansion of CD40L-activated B cells through the attenuation of apoptosis. (A) The accumulation of live cells and the percentage of dead B cells were determined by trypan blue staining on days of culturing with CD40L cells at the indicated ratios in the presence (●) or absence (○) of BLyS (5 ng/ml). (B) The accumulation of live cells and the percentage of cells expressing annexin V binding activity were determined on B cells continuously cocultured with CD40L cells at a ratio of 10:1, or (C) cultured in media alone after 2 d of coculture with CD40L cells, in the presence (●) or absence (○) of BLyS (5 ng/ml). These experiments were repeated three times.

operates with CD40L in B cells primarily by attenuation of apoptosis, which is amplified by cell replication, leading to augmented B cell expansion.

Analysis of annexin V binding activity confirmed that increased expansion of live cells corresponded to reduced apoptosis by BLyS (Fig. 5 B). To further verify that attenuation of apoptosis is the primary function of BLyS, the BLyS and CD40L signals were uncoupled by removing CD40L cells after 2 d of coculture. It was evident that BLyS significantly protected CD40L-activated B cells from apoptosis (Fig. 5 C). The lack of expansion of activated B cells after CD40L removal further indicated that BLyS alone was insufficient to sustain cell cycle progression. Consistent with its inability to regulate CD40 or Fas expression in vivo (Fig. 3 E), BLyS did not alter the expression of CD40 or Fas on CD40L-activated B cells in vitro (Fig. 6). These results confirm that attenuation of apoptosis underlies cooperation of BLyS with CD40L in B cell activation.

Figure 6. BLyS does not regulate CD40 or Fas expression in vitro. FACS® analysis of CD40 and Fas expression on the indicated days of in vitro culturing in the presence of CD40L, alone or together with BLyS (5 ng/ml). These experiments were repeated three times.

BLyS Significantly Prolongs the Survival of Naive Resting B Cells. The reproducible, albeit modest, reduction in apoptosis of high density B cells in response to BLyS in vivo (Fig. 3 C) suggests that attenuation of apoptosis by BLyS is not restricted to B cells activated by antigen or CD40L. Indeed, BLyS alone (5 ng/ml) significantly protected naive resting splenic B cells from apoptosis, as evidenced by the maintenance of live cells (from 20 to 75%) for 4 d in vitro in the presence of BLyS and the corresponding decrease in annexin V binding activity (Fig. 7 A). Attenuation of apoptosis by BLyS was not accompanied by cell cycle activation (Fig. 7 B), consistent with failure of BLyS to sustain cell cycle progression after CD40L activation (Fig. 5 C). Moreover, BLyS did not enhance IgM synthesis or induce appreciable changes in cell morphology in either resting or CD40L-activated B cells (Fig. 7 C). Thus, BLyS functions to prolong the survival of naive resting B cells in addition to CD40L-activated B cells in vitro.
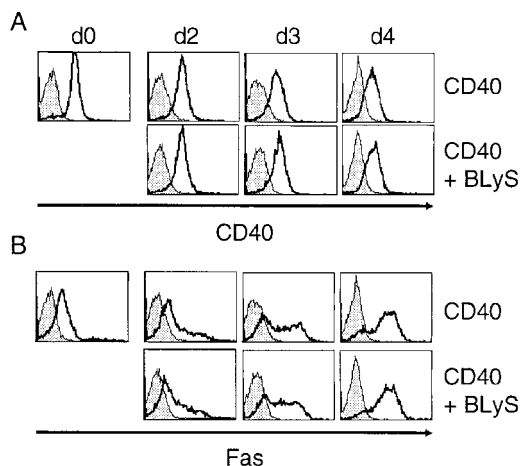
BLyS Regulates Bcl-2 Family Proteins. The Bcl-2 family proteins are differentially regulated in response to various stimuli in B lymphocytes and are crucial for their survival (20, 21). Given that BLyS effectively attenuates B cell apoptosis in either resting or activated states in vivo and in vitro, it was of interest to determine whether BLyS regulates Bcl-2 family proteins. We addressed this question first in B cells after NP-CGG immunization. Of the Bcl-2 family proteins examined, BLyS reduced the expression of Bak modestly in both high and low density B cells without changing the expression of its antiapoptotic partners, Bcl-xL or Bcl-2 (Fig. 8 A and data not shown).

Since BLyS attenuates B cell apoptosis independent of antigen stimulation (Figs. 5 and 7), we then characterized the regulation of Bcl-2 family proteins by BLyS and CD40L in vitro. The expression of Bak was profoundly reduced by CD40L activation, and further diminished to undetectable levels by costimulation with BLyS (Fig. 8 B). Although Bcl-xL was increased and Bcl-2 was reduced by CD40L activation, as expected from their regulation (during B cell activation) in germinal centers (22, 23), they were differentially regulated by BLyS; Bcl-xL expression
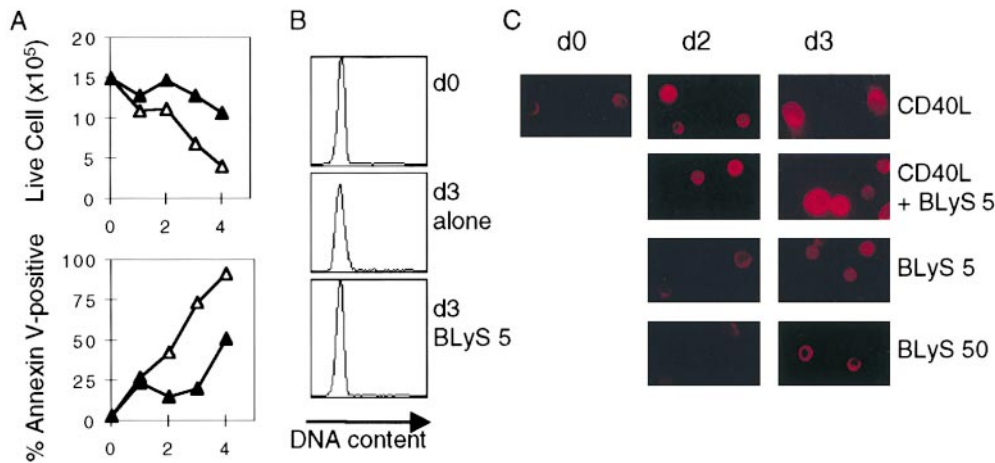
Figure 7. BLyS prolongs the survival of resting B cells in vitro. (A) The number of live resting B cells and the percentage of cells expressing Annexin V binding activity were determined on the days indicated during culture in media in the presence (▲) or absence (△) of BLyS (5 ng/ml). (B) FACS® analysis of DNA content of day 0 and 3 B cells. (C) Immunostaining of intracellular IgM in B cells cocultured with CD40L cells or BLyS (5 or 50 ng/ml), or both, on the days indicated. These experiments have been performed three times.

was not modified, although Bcl-2 expression was partially restored by BLyS costimulation (Fig. 8 B, compare lanes 1, 4, and 5). These results were similar but not identical to those obtained from immunized B cells in vivo, possibly due to the synchronized nature of the in vitro experiments and the absence of antigen activation. Protection of CD40L-activated B cells from apoptosis by BLyS therefore correlates with inverse regulation of the pro- and antiapoptotic proteins of the Bcl-2 family.

BLyS also protects naive resting B cells from apoptosis (Fig. 8 B, lanes 1–3). However, the regulation of Bcl-2 family proteins in this population was different from that of CD40L-activated B cells: BLyS appreciably elevated the expression of Bcl-2 and Bcl-xL without changing the expression of Bak. Taken together, these results suggest that BLyS regulates the ratio of Bcl-2 family proteins to favor cell survival in both resting and CD40L-activated B cells by distinct mechanisms.
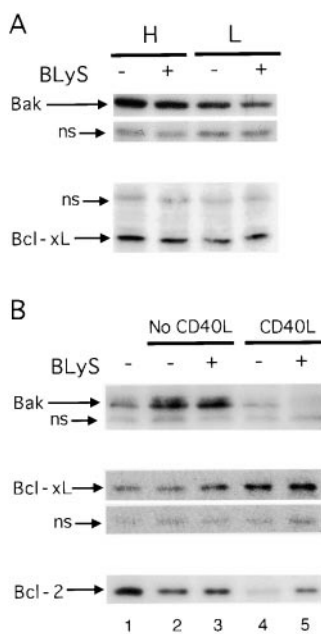

Figure 8. Regulation of Bcl-2 family proteins by BLyS. (A) Expression of Bak and Bcl-xL in high density (H) and low density (L) splenic B cells isolated from mice administered with BLyS or PBS on day 11 of immunization with NP-CGG. (B) Expression of Bak, Bcl-xL, and Bcl-2 on high density splenic B cells, either freshly isolated (lane 1), after 24 h of in vitro culture with media alone (lanes 2 and 3), or after 48 h of coculture with CD40L cells (lanes 4 and 5), in the presence or absence of BLyS. ns, nonspecific bands used as controls for sample loading.

BLyS Preferentially Activates NF-κB RelB and p50 in Resting and Activated B Cells. TNF family proteins frequently activate NF-κB and its downstream antiapoptotic genes (24), including Bcl-xL (25–28). NF-κB has been shown to be activated by BLyS or by overexpression of TACI in cell lines (10, 11), and we have evidence that attenuation of apoptosis by BLyS correlated with regulation of Bcl-2 family proteins (Fig. 8). Whether NF-κB was activated by BLyS in primary B cells was then determined (Fig. 9).

In freshly isolated, resting naive splenic B cells, active NF-κB binding complexes consisting of p50/crel, p50/p65, p50/RelB, and p50/p50 dimers were already present, as indicated by antibody interference analysis in EMSAs (Fig. 9, A and B). Within 1 d of in vitro culturing, these complexes were drastically reduced, correlating with apoptosis presumably due to loss of in vivo stimuli (Fig. 9 A, lane 2). The NF-κB binding activity was sustained throughout the 3 d of coculture with CD40L cells (Fig. 9, A, lanes 3–8, and B, lanes 1–6). Costimulation with BLyS (5 ng/ml) significantly increased the activation of all NF-κB complexes, in particular the p50 homodimer, to threefold by day 3 compared with stimulation with CD40L alone (Fig. 9, A, lanes 3–8, and B, lanes 7–12). BLyS therefore enhances NF-κB activation in primary B cells in cooperation with CD40L.

Because BLyS also prolongs the survival of resting cells (Fig. 7), whether it could activate NF-κB in resting B cells was examined. The NF-κB DNA binding activity present in resting B cells was maintained for 1 h in vitro, but declined by 2 h, and reached an extremely low level by 24 h (Fig. 9 C, lanes 2, 4, and 6). BLyS not only prevented this decline, but with time also enhanced the binding of p50/RelB and p50/p50 dimers (Fig. 9 C, lanes 5–9). A notable exception was p50/p65, whose binding was greatly reduced by 24 h and could not be restored by BLyS. Attenuation of apoptosis in naive resting B cells by BLyS in vitro therefore strikingly correlated with preservation of the in vivo (0 h) NF-κB activity and the enhancement of p50/RelB and p50/p50 activation. Together, these results provide direct evidence for activation of specific NF-κB subunits by BLyS in primary B cells independent of their acti-
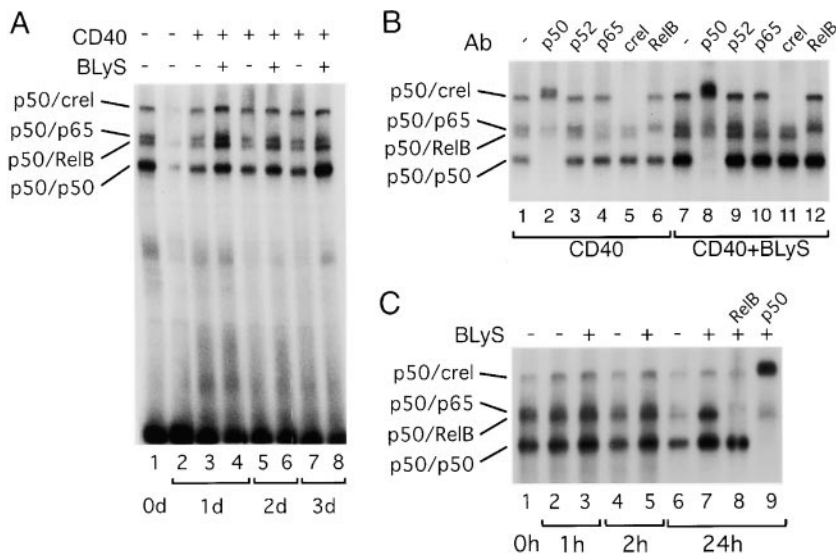
Figure 9. BLyS activates NF-κB in resting and CD40L-activated B cells. (A) EMSA of the NF-κB DNA binding activity present in whole cell extracts prepared from freshly isolated resting splenic mouse B cells (0 d), cells cultured in media alone for 1 d (lane 2), or on days of coculture with CD40L cells in the presence or absence of 5 ng/ml BLyS. The migration of specific NF-κB complexes is marked. (B) EMSA of extracts prepared from B cells cocultured with CD40L cells in the presence or absence of BLyS (5 ng/ml) for 3 d, with or without preincubation with indicated antibodies. (C) EMSA of NF-κB DNA binding activity present in whole cell extracts prepared from freshly isolated resting splenic B cells (lane 1), or cultured for hours in media in the presence or absence of 100 ng/ml of BLyS (lanes 2–9). RelB and p50 lanes represent preincubation of extracts with the antibodies.

vation state, and suggest a role of NF-κB in the attenuation of apoptosis signaled by BLyS.

Discussion

In summary, our results have demonstrated that BLyS enhances the physiologic humoral immune response to both T cell–independent and T cell–dependent antigens, and strongly suggest that attenuation of B cell apoptosis is the underlying mechanism.

Modulation of Physiologic Humoral Immune Response by BLyS

The ability of BLyS to augment antibody response to the polyvalent carbohydrate vaccine Pneumovax demonstrates that it can modulate the physiologic humoral immune response independent of T cells (Fig. 1). Moreover, we show that BLyS enhances the humoral immune response to two T cell–dependent antigens, DNP-BSA and NP-CGG (Figs. 2 and 3). While this manuscript was in review, it was reported that the addition of a chimeric TACI–Fc blocked the increase of serum anti-Pneumovax IgM (10), disrupted germinal center formation, and inhibited antibody response to NP-CGG (11) and to KLH (10). Although these reports show that BLyS is a participant in the humoral immune response, our results indicate that additional BLyS can enhance the magnitude of the response. Thus, these studies complement each other and establish that BLyS modulates both T cell–dependent and –independent humoral immune responses.

We further showed that costimulation with BLyS in vivo led to increased serum titers of antigen-specific Igs of the IgM and IgA isotypes in response to Pneumovax, of predominantly the IgM isotype against DNP-BSA, and of both IgM and IgG against NP-CGG. The common denominator of all three of these responses was an increase in the IgM response, suggesting that BLyS acts early in the humoral immune response. The characterization of the NP-CGG response further demonstrates that the elevation of antibody levels has its basis in the increase in antigen-specific plasma cells, which in turn results from enhanced expansion of activated splenic B cells in response to BLyS (Fig. 3). Our findings provide a cellular basis for further investigation of BLyS response in humoral immunity. They also leave open the temporal and spatial specificity of BLyS action during B cell terminal differentiation. Is the increased accumulation of NP-specific IgG plasma cells solely a consequence of the prior expansion of NP-specific IgM mediated by BLyS? Are B cells subject to BLyS regulation after isotype switch or terminal differentiation to plasma cells? Does BLyS increase the expansion of long-lived plasma cells in the bone marrow? Elucidating the temporal and spatial specificity of the BLyS response as well as the regulation of the BLyS receptors during B cell terminal differentiation would help to elucidate the modulation of the humoral immune response by BLyS.

Cooperation between BLyS and CD40L

Reconstitution of BLyS action on primary B cells in vitro demonstrates that BLyS acts on B cells autonomously, and that it cooperates synergistically with CD40L in B cell activation and expansion (Figs. 4–7). The profound enhancement of CD40L activation by BLyS further suggests a tripartite interaction between BLyS expressed on monocytes, CD40L expressed on T cells, and CD40 and BLyS receptors expressed on B cells. Although BLyS does not regulate either CD40 or Fas expression on B cells (Fig. 6), it is possible that interactions between BLyS and other TNF family proteins expressed on T and B cells occur and can further contribute to the modulation of B cell immune response.

Most importantly, our data strongly suggest that the primary functional consequence of cooperation between BLyS and CD40L is enhanced expansion due to attenuation of apoptosis, as determined by the relative contributions of BLyS to cell cycle progression and cell survival (Figs. 4 and 5). On this basis, we suggest that BLyS signaling lowers the B cell survival threshold, thereby leading to

augmented expansion of B cells after activation by CD40L (see below). This understanding has profound implications for the mechanism of BLyS action in physiologic T cell–dependent antibody response.

Mechanism of BLyS Action in Primary B Cells

BLyS Attenuates B Cell Apoptosis. BLyS has been suggested to enhance B cell proliferation based on enhanced accumulation of activated B cells in germinal centers and plasma cells in BLyS transgenic mice (6), and expansion of anti-IgM stimulated B cells in response to BLyS in vitro (1). Here we suggest that BLyS primarily enhances B cell survival, which secondarily leads to greater accumulation of replicating B cells. The evidence supporting this conclusion is as follows. First, exposure to BLyS reduces apoptosis of NP-CGG–immunized B cells in vivo and in vitro. Second, BLyS prolongs the survival of B cells activated by CD40L in vitro, even after removal of CD40L stimulation. Third, BLyS prolongs the survival of naive resting B cells. Finally, BLyS alone was incapable of activating the cell cycle. Consistent with our conclusions, BLyS was reported to enhance the survival of the heterogeneous splenocyte population in vitro (9). Thus, BLyS amplifies the CD40L activation of B cells by rescuing actively replicating cells which would otherwise have been eliminated by apoptosis.

BLyS reduces apoptosis of naive resting B cells as well as CD40L-activated B cells in the absence of exogenous death signals, and does not enhance survival through activation of CD40 or reduction of Fas expression in vivo and in vitro. On this basis, BLyS is likely to antagonize an as yet unidentified Fas–independent apoptotic pathway(s) operating in primary resting and activated B cells.

Regulation of Bcl-2 Family Proteins by BLyS. In our first attempt to elucidate the BLyS signaling pathway in primary B cells, we have found that BLyS attenuation of apoptosis correlates with changes in the ratio of Bcl-2 family proteins to favor cell survival. In NP-CGG–challenged mice, BLyS reduced the proapoptotic Bak protein without accompanying changes in Bcl-2 or Bcl-xL in B cells. After CD40L activation in vitro, BLyS prominently reduced Bak and partially restored Bcl-2 expression. By contrast, Bcl-xL was modestly, if at all, upregulated by BLyS alone in vitro (Fig. 8). The Bcl-2 family proteins are therefore differentially regulated in primary B cells in the context of immunization and activation state. Bcl-2 is required for the survival of mature naive lymphocytes (29), and Bcl-xL is crucial for the survival of immature lymphocytes (30) and germinal center B cells (23). The impairment in affinity maturation of germinal center B cells in Bcl-xL transgenic mice (31), and in negative selection of autoreactive B cells in Bcl-2 transgenic mice (32), further highlights the critical roles of Bcl-2 family proteins in the humoral immune response. The levels of Bcl-2 protein have been shown to be higher in peripheral B cells isolated from BLyS transgenic mice (6). Our results provide the first experimental evidence correlating regulation of Bcl-2 family proteins in primary B cells by BLyS with cell survival in vivo and in vitro. Although preliminary, they are consistent with a role for Bcl-2 family proteins in mediating the BLyS survival signals in the immune response (6).

Activation of NF-κB by BLyS. The sustained activation of p50 and RelB by BLyS in naive resting B cells and CD40L–activated B cells have provided the first evidence for BLyS regulation of NF-κB in primary B cells (Fig. 9). NF-κB subunits are thought to be differentially used in cell cycle progression and apoptosis in resting and activated B cells (33). Our finding of differential activation of NF-κB subunits by BLyS is consistent with this notion. Although p50 has not been shown to be essential for B cell survival, it is required for basal and specific antibody response as suggested by studies of p50-deficient mice (34). RelB is required for the development of specific dendritic cells (35) and for macrophage-mediated immunity to various pathogens (36). The striking correlation between differential activation of p50 and RelB by BLyS and attenuation of apoptosis implies that these NF-κB subunits specifically mediate BLyS signals for B cell survival. In this regard, it is surprising that the p65/RelA dimer, which is associated with the antiapoptotic effects of NF-κB in various systems (37), is not activated by BLyS. Instead, RelB, which has not yet been shown to have antiapoptotic properties in B cells, is regulated by BLyS. Whether p50 or RelB is required to mediate the BLyS signals for B cell survival in response to BLyS is currently under investigation.

BLyS Attenuation of B Cell Apoptosis and Autoimmunity

Attenuation of apoptosis by BLyS is not restricted to B cells after activation by antigen or CD40L, as BLyS also prolongs the survival of high density B cells after antigen challenge in vivo (data not shown) and naive resting B cells in vitro (Fig. 7). Activation of the cell cycle is therefore not required to prime the primary B cells for BLyS response. This finding implies that BLyS may contribute to both the conservation of the B cell repertoire and the development of polyclonal autoimmune diseases. Consistent with this notion, the addition of soluble BCMA–Ig reduced the total number of B cells in the blood and the peripheral lymphoid organs (9). The deletion or anergy of autoreactive B cells prevents autoimmunity (38), and apoptosis of antigen-specific B cells after activation is crucial for the negative control of the humoral immune response (39). The development of autoimmune disease in BLyS transgenic mice (5–7) could be explained by our findings that BLyS enhances B cell survival indiscriminately. This hypothesis is supported by the finding that TACI–Fc can delay the onset of autoimmunity in at least one mouse model of systemic lupus erythematosus (5). A better understanding of the mechanism of BLyS signaling should facilitate the development of BLyS as a specific target for control of the humoral immune response and for therapeutic interventions of autoimmune diseases.

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