

B Lymphocyte Stimulator Activates p38 Mitogen-Activated Protein Kinase in Human Ig Class Switch Recombination

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B lymphocyte stimulator (BLyS), a member of the tumor necrosis factor ligand superfamily, has potent costimulatory activity on B cells. To investigate BLyS signaling in Ig class switching, we examined whether BLyS could control stress-activated protein kinases in human B cells as well as whether BLyS could induce human Ig class switch recombination (CSR) and expression of activation-induced cytidine deaminase (AID). BLyS induced the phosphorylation p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) in human B cells. As evidence of Ig class switch, BLyS plus interleukin (IL)-4 induced generation of switch circle transcripts (CTs) to γ 1–2, γ 4, and epsilon, whereas BLyS plus IL-10 induced γ 1–2 CTs only. BLyS strongly induced AID expression in the presence of IL-4. Treatment with SB203580, a specific inhibitor of p38 MAPK signaling, almost completely reversed BLyS-induced CSR and AID expression in human B cells. The switch vector assay also showed that BLyS induced CSR in the presence of IL-4 in Ramos 2G6 human B cells and that SB203580 reversed CSR. These results indicate that BLyS-activated p38 MAPK plays an essential role in BLyS-induced AID-expression and CSR in human B cells.

Keywords: B lymphocytes; BLyS; Ig isotype switching; p38 MAPK

B lymphocyte stimulator (BLyS, also called TALL-1, THANK, or BAFF) is a potent co-activator of B cells *in vitro*, and *in vivo* induces B cell proliferation and Ig secretion (1). It has been suggested that BLyS likely plays an important contributory role in autoimmune disease pathogenesis and propagation by virtue of its ability to promote B cell survival, expansion, and differentiation. Elevations of BLyS have been observed in the patients with systemic lupus erythematosus (SLE) (2). The levels of BLyS in serum from patients with primary Sjögren's syndrome that correlate with the level of autoantibodies were significantly higher than those in healthy control subjects (3).

BLyS binding to B cells results in the activation of nuclear factor (NF)- κ B (4). However, the intracellular signaling of BLyS remains less well defined in human B cells. Recently, Litinskiy and coworkers have reported that BLyS induced CD40-independent Ig class switch recombination (CSR) and plasmacytoid differentiation (5), whereas CSR is generally thought of as highly dependent upon engagement of CD40 on B cells. CD40 signaling can activate multiple kinases and signal pathways, including NF- κ B, p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) (6, 7).

Activation-induced cytidine deaminase (AID), which is an RNA-editing enzyme, is required for CSR and hypermutation (8). Murine AID expression is induced or regulated by several components that drive Ig CSR—e.g., cytokines, CD40 engagement, lipopolysaccharide (LPS), and a combination of these factors (9)—little is known about the regulation and intracellular signaling involved in human AID expression. The potential role of BLyS in inducing AID expression via p38MAPK signaling has previously been unexplored.

Although the ability of BLyS to produce Ig CSR independent of CD40 is of potential importance, as it may provide yet another link between innate and adaptive immune responses, the details of how BLyS signaling is involved in Ig CSR remain poorly understood. In this study, BLyS-induced CSR was confirmed by employing a novel switch vector assay in Ramos 2G6 human B cells and measuring switch circle transcripts (CTs) in human B cells. We examined whether BLyS could induce the phosphorylation of p38 MAPK and JNK during BLyS-induced CSR and AID expression in human B cells. We used specific inhibitors of p38 MAPK signaling, JNK, and extracellular signal-regulated kinase (ERK) signaling to demonstrate the pathways involved in these events. We also were able to quantify the amount of BLyS-induced CSR and signaling by employing the switch vector assay.

MATERIALS AND METHODS

Reagents

Human interleukin (IL)-4 and IL-10 were obtained from R&D Systems (Minneapolis, MN). BLyS was a gift from Human Genome Sciences (Rockville, MD). Anti-CD40 mAb G28.5 were purchased from ATCC (Rockville, MD). Anti-p38 MAPK Ab, anti-phosphorylated p38 Ab, anti-JNK Ab, anti-phosphorylated JNK Ab, anti-ERK Ab, and anti-phosphorylated ERK Ab were purchased from Cell Signaling (Beverly, MA). PD98059, SB203580, and SP600125 were obtained from Calbiochem (San Diego, CA). Restriction endonucleases, ligase, and mung bean nuclease used for construction of switch vector came from Promega (Madison, MI) and New England Biolabs, Inc. (Beverly, MA).

Cells, Cell Lines, and Cell Culture

Human B cells were isolated from healthy volunteers following methods previously described (10). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation on Ficoll-Hypaque. T cells were depleted by resetting with 2-amino-ethylisothiuronium bromide-treated sheep red blood cells. The resultant B cells were separated by discontinuous Percoll gradient centrifugation. After monocytes/macrophages and NK cells were removed, this B cell population was typically 96% CD20-positive as determined by flow cytometry. B cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (Omega, Tarzana, CA).

The human B lymphoma cell line Ramos 2G6 (ATCC, Rockville, MD) was maintained and cultured in complete RPMI 1640. For transfection, 10 μ g of plasmid DNA that had been predigested with Ase I was mixed with one million cells in 0.2 ml of RPMI and then subjected to electroporation (200 V, 0.975 mF). Selection of stable transfected

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cell lines was achieved by Geneticin (GIBCO BRL, Gaithersburg, MD) selection beginning 2 d later with concentration being increased over 4 wk to 1.5 mg/ml. The DNA switch construct XF-5a that can be used to monitor ongoing DNA recombination has been described previously (11).

Gel Electrophoresis and Western Blot Analysis

Samples containing an equal amount of protein were boiled with electrophoresis sample buffer for 3 min and separated using SDS-PAGE. The separated proteins were transferred electrophoretically to membranes (Millipore, Bedford, MA). The membranes were blocked at room temperature for 1 h in pH 7.4 phosphate-buffered saline with 1% bovine serum albumin, incubated with primary Abs for 1 h at room temperature, washed, and followed by incubation with HRP-labeled secondary Abs for 1 h. The blots were developed using enhanced chemiluminescence reagents (Amersham) and exposed to BioMax film from Eastman Kodak Co.

RNA Extraction and RT-PCR

Total mRNA was obtained from stimulated and unstimulated cells using Trizol reagent (GIBCO BRL). RNA suspended in 0.1% diethylpyrocarbonate-treated water was digested with DNase I (Sigma) to remove possible contaminating DNA, and then extracted with phenol/chloroform followed by precipitation in ethanol. Total RNA (1 μ g) was reverse-transcribed to cDNA. All polymerase chain reaction (PCR) assays were done in 50- μ l reaction volumes containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 1 μ M of each primer, and 2.5 U Taq polymerase (Promega). For detection of AID and GAPDH, PCR was conducted with 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The forward primer (5'-TAGACCCTGGCCGCTGCTACC-3') and reverse primer (5'-CAAAAGGATGCGCCGAAGCTGTCTGGAG-3') were used to amplify AID as a 382-bp PCR product (12).

Switch Circle RNA Transcripts

After total mRNA was obtained and reverse-transcribed to cDNA, CTs were amplified with the forward primers I γ 1/2 5'-GGGC TTCCAAGCCAACAGGGCAGGACA-3', I γ 4 5'-TTGTCCAGG CCGGCAGCATCACCAGA-3', and I ϵ 5'-GACGGGCCACACCA TCCACAGGCACCAAATGGACGAC-3' together with the reverse primer C μ 5'-GTTGCCGTTGGGGTGCTGGAC-3'. I γ 1/2-C μ (608 bp), I γ 4-C μ (358), and I ϵ -C μ (408) CTs were amplified for 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min (5). These assays detect both γ 1 and γ 2 CTs as a single product while distinguishing from γ 4 and ϵ .

Switch Vector Assay

We have recently established a switch vector assay in Ramos 2G6 human B cell line to quantify the induced CSR measuring the frequency of green fluorescence protein (GFP)-positive cells (11). When stimulated to undergo DNA excision/repair equivalent to CSR, the excised DNA will form deleted switch circle that express GFP. The number of cells undergoing vector switching can be quantified by measurement of the expression of GFP. Expression of GFP in unstimulated or stimulated cell lines stably transfected with the switch vector was measured by either single- or dual-color flow cytometry (FACS Core laboratory, UCLA) as described (13). FACS data were analyzed with fetal calf serum expression software (Deno Novo software Inc, Thornhill, ON, Canada).

Densitometric Analysis

The images of ethidium-stained gels were recorded using the gel documentation system Speedlight Platinum (Lighttools Research, Encinitas, CA). The intensity of each band was measured with the Bio Image software Basic Quantifier (Genomic Solutions Inc., Ann Arbor, MI). The samples were diluted to 50, 20, 10, and 5% before PCR amplification. The final quantitative estimates for the densitometric analysis were taken from the linear part of the curve.

RESULTS

BLyS Induces Phosphorylation of p38 MAPK and JNK

To determine whether BLyS activates stress-activated kinase, we examined BLyS induced-phosphorylation of p38 MAPK and

JNK (Figure 1). Human B cells were stimulated with BLyS for various times, and the resulting cell lysates were subjected to immunoblotting with anti-phosphorylated JNK Ab and anti-phosphorylated p38 MAPK Ab. As shown in Figure 1A (*top*), following BLyS stimulation of B cells, there was rapid (within 10 min) and sustained cell activation and phosphorylation of p38 MAPK on tyrosine-182. Similarly, BLyS induced JNK activation, as evidenced by phosphorylation on threonine-183 and tyrosine-185 by (Figure 1B, *top*). As a negative control, we examined ERK-phosphorylation on tyrosine-204, and BLyS failed to induce phosphorylation of ERK (data not shown).

BLyS Induces CSR

CSR generated an extrachromosomal reciprocal switch DNA recombination product, a so-called "switch circle" which contained the I_H promoter of the targeted downstream C_H gene, the intervening deleted DNA fragment, and C μ (Figure 2A). The I_H promoter in the switch circle drives transcription of a chimeric I-C μ RNA product, referred as CTs (14). We tested the ability of BLyS alone or, when combined with either IL-4 or IL-10, to induce CSR to γ 1-2, γ 4, and/or epsilon by measuring switch CTs (I γ 1/2-C μ , I γ 4-C μ , I ϵ -C μ) in resting and stimulated human B cells (Figure 2B). In the presence of IL-4, BLyS induced γ 1-2, γ 4, and epsilon (I γ 1/2-C μ , I γ 4-C μ , and I ϵ -C μ) CTs. On the other hand, BLyS plus IL-10 generated γ 1-2 CTs (I γ 1/2-C μ) only. BLyS plus IL-10 did not result in the appearance of γ 4 or epsilon CTs.

The Effect of SB203580 on BLyS-Induced CSR in Human B Cells

Having shown that BLyS can induce CSR and the concomitant phosphorylation of p38 MAPK and JNK, we sought to determine whether MAPK signaling is directly involved in BLyS-induced CSR. To accomplish this aim, we tested the ability of SB203580 (a specific inhibitor of p38 MAPK signaling), SP600125 (JNK inhibitor), and PD98059 (a specific inhibitor of ERK signaling) to interfere with CSR to epsilon as measured by the appearance of the appropriate CTs. Inhibition of p38 MAPK by SB203580 showed a marked and dose-dependent inhibitory effect on BLyS-induced CSR (Figure 3). Although inhibition of ERK did not result in any effect on BLyS induced CSR, densitometric analysis provided that the inhibitory effect of SP600125 on CSR was 35%.

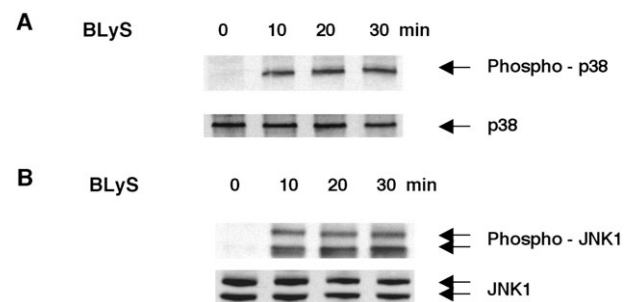


Figure 1. BLyS induces the phosphorylation of p38 MAPK and JNK. Primary human B cells were cultured with BLyS (500 ng/ml) for the times indicated. Equal amounts of cell lysate were subjected to SDS-PAGE followed by blotting with anti-phosphorylated p38 MAPK Ab (A), anti-phosphorylated JNK Ab (B). The same samples were probed with anti-p38 MAPK, and anti-JNK as shown in the lower part of each panel so as to measure the total amount of kinase protein present in the samples.

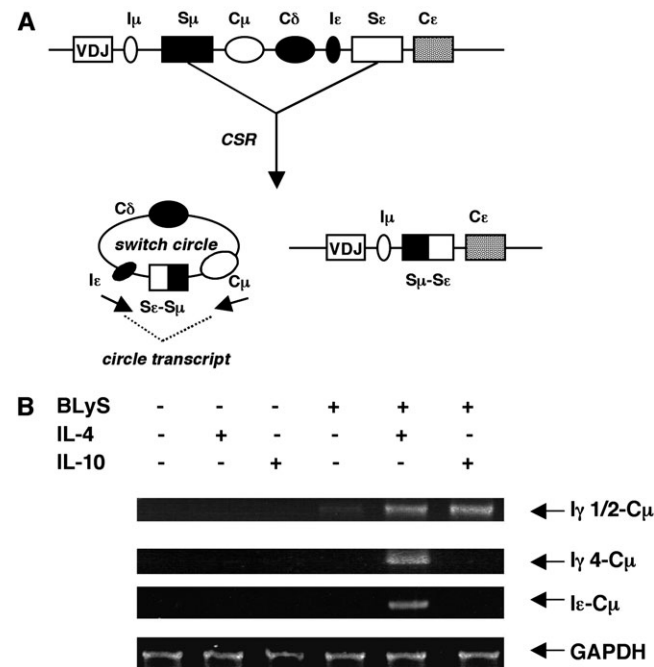


Figure 2. BLyS induces CSR. (A) Diagram of CSR from C μ to C ϵ . Filled and open rectangles are S μ and S ϵ , respectively. The V-shaped dot line indicates the part of the circle transcripts that are amplified using the primers shown by the arrows. (B) The effect of BLyS on the expression of CTs. Primary B cells were cultured for 4 d using the indicated conditions of BLyS (500 ng/ml), IL-4 (5 ng/ml), and/or IL-10 (100 ng/ml). RNA was prepared and CTs (I γ 1/2-C μ , I γ 4-C μ , I ϵ -C μ) were amplified using synthesized cDNAs by pairs of specific primers. GAPDH was used as an internal control for the RT-PCR.

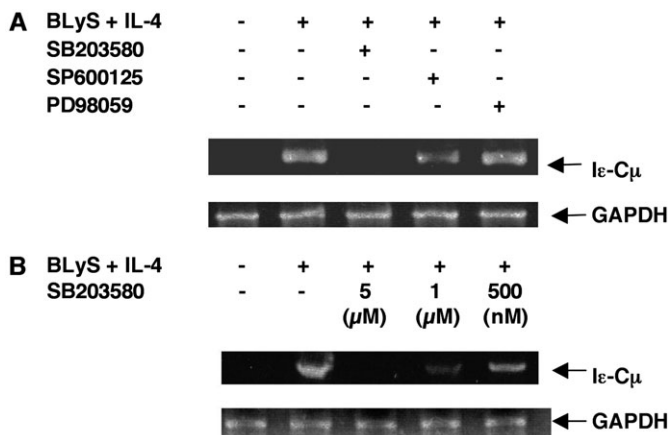


Figure 3. Suppression of BLyS-induced CSR by the p38 MAPK inhibitor SB203580. (A) The effect of SB203580 of BLyS-induced CSR in human B cells. Human B cells were pretreated without or with SB203580 (10 μM), SP600125 (20 μM), or PD98059 (10 μM) for 45 min, followed by culture and stimulation with BLyS (500 ng/ml) and IL-4 (5 ng/ml) for 4 d. RNAs were prepared and CTs (Iε-C μ) were amplified. (B) Dose response of SB203580 inhibition of BLyS-induced CSR. Culture conditions were the same as in A except that the concentration of SB203580 was varied as indicated. One of three separate experiments giving similar results is shown.

SB203580 also inhibited CSR to γ 1–2 whether it was induced by BLyS plus IL-4 or IL-10, respectively (data not shown).

BLyS-Induced Expression of AID Is Blocked by SB203580

Because AID plays a critical role in CSR and V H gene somatic hypermutation (SHM) (8), we examined the role of BLyS in AID mRNA induction in human B cells. BLyS plus IL-4 showed marked induction of AID mRNA expression (Figure 4A), whereas it was difficult to detect any induced AID mRNA expression by BLyS or IL-4 alone. Because we had shown that inhibition of p38 MAPK by SB203580 blocked BLyS-driven CSR, we tested whether SB203580 blocked BLyS-dependent AID mRNA expression in human B cells. Indeed, SB203580 blocked human B cell BLyS-induced AID expression in a dose-dependent manner. AID induction by BLyS was almost completely inhibited by 1 μM SB203580 (Figure 4B).

BLyS Induces Switch Recombination Activity in Switch Constructs in an MAPK-Dependent Fashion

We have previously established a switch recombination assay by using GFP as an indicator for substrate switch recombination (SSR) in human B cell line Ramos 2G6. IL-4 or BLyS alone did not induce SSR, as indicated by a lack of GFP expression from the switch construct (XF-5a) (Figure 5B, top three panels). BLyS, in the presence of IL-4, induced SSR in a dose-dependent manner (Figure 5B, bottom three panels), with the optimal concentration of BLyS being 500 ng/ml, similar to the BLyS concentration for optimal CSR and AID induction in human B cells (data not shown).

We tested the effects of the kinase inhibitors SB203580, SP600125, and PD98059 in this Ramos switch recombination assay, which allows sensitive assessment of the effect on CSR. SB203580 inhibited BLyS-induced SSR in a dose-dependent manner, significantly reducing it at concentrations at or above 1 μM, and completely blocking it at or above 5 μM (Figure 6). PD98059 was without effect while SP600125 showed a maximum of 50% inhibition at 10 μM. Because GFP expression measures

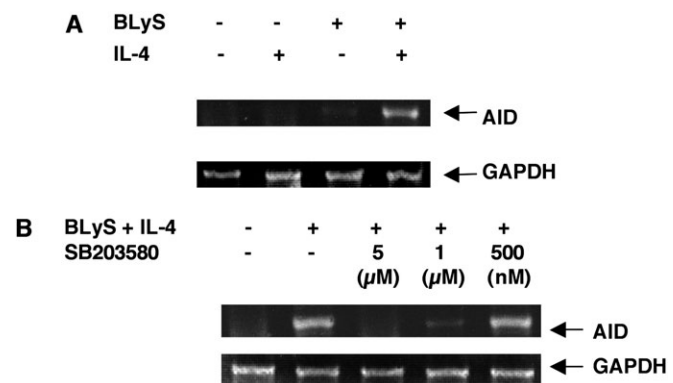


Figure 4. Induction of AID expression by BLyS and IL-4 and its inhibition by SB203580. (A) The effect of BLyS and IL-4 on AID expression in human B cells. Primary B cells were cultured as indicated for 2 d with BLyS (500 ng/ml) and IL-4 (5 ng/ml). RNAs were prepared and cDNAs were synthesized, followed by the amplification by the primer pairs for AID. GAPDH was used as an internal control for RT-PCR. (B) Dose response of SB203580 inhibition of BLyS plus IL-4 induced AID expression. Human B cells were pretreated without or with SB203580 (5 μM, 1 μM, and 500 nM) for 45 min, and then the cells were stimulated with BLyS (500 ng/ml) and IL-4 (5 ng/ml) for 2 d. The RNAs were prepared and cDNAs were synthesized and amplified for AID and GAPDH.

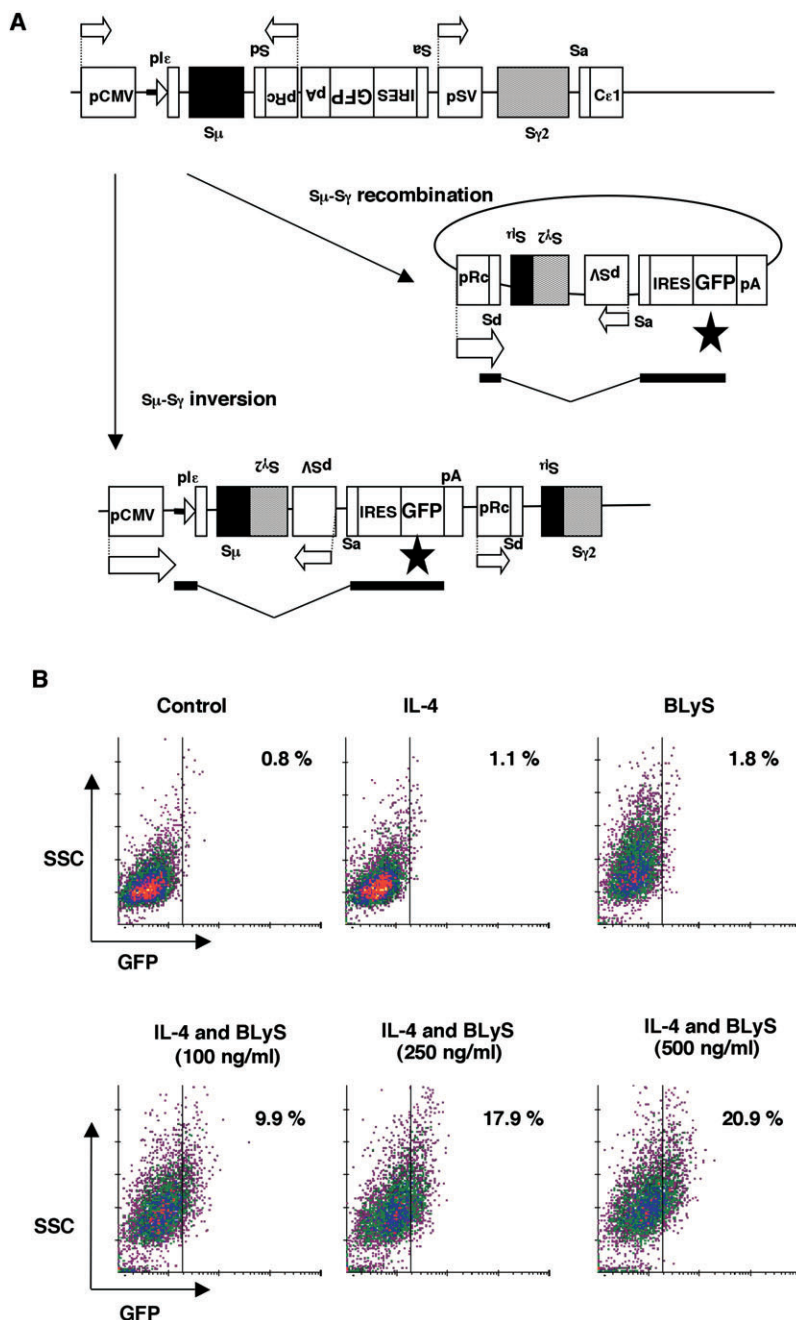


Figure 5. BLyS induces substrate switch recombination (SSR) in Ramos B cells (A) Schematic diagram of the switch construct and its recombination events as deletion or inversion. After switch recombination or inversion between the S_{μ} and $S_{\gamma 2}$ regions, the IRES-GFP expression unit, now comes under the transcriptional control of the pRc/RSV-LTR or pCMV that leads to GFP expression (filled star). Each open arrow indicates the promoter transcriptional site and direction. IRES = Internal Ribosomal Entry Site. pCMV = CMV promoter; pSV = SV40 promoter; pRc = RSV LTR promoter; Sd = splicing donor site; Sa = splicing acceptor site. (B) Dose response of BLyS plus IL-4 induced SSR. Ramos 2G6 cells (1×10^5 cells/ml) permanently transfected with the switch vector shown in A were cultured in the presence of media and IL-4 (5 ng/ml) plus varying doses of BLyS (100 ng/ml, 250 ng/ml, 500 ng/ml) as indicated. After 4 d, SSR was measured based on the expression of GFP by cells containing vectors that had undergone switch recombination. The frequency of GFP-positive cells is shown.

both deletional and inversional recombination events in the constructs (Figure 5A), the effect of SB203580 in the SSR assay reflects the inhibition of recombination activity that includes both deletional and inversional events.

The Effect of BLyS on CD40-Induced SSR

We examined whether BLyS could effect CD40-induced switch recombination, because CD40 has been shown to play a critical role in Ig CSR *in vitro* and *in vivo*. Use of our human vector-based switch recombination assay made it possible to quantify BLyS-induced SSR under a variety of conditions. As shown in Figure 7A, in the absence of α CD40 Ab or at 10 ng/ml of α CD40 Ab, BLyS significantly increased CD40-induced SSR in a dose-dependent manner. In these conditions, the optimal dose of BLyS for SSR was 500 ng/ml. In contrast, at 100 ng/ml of α CD40 Ab, BLyS failed to further enhance CD40-induced SSR. At

100–1,000 ng/ml of α CD40, BLyS did not enhance SSR, and at 1,000 ng/ml, BLyS led to a relative decrease in CD40-induced SSR (data not shown).

We also examined the effect of SB203580 on BLyS- and CD40-induced SSR. Because SB203580 significantly reduced BLyS-induced SSR at concentrations at or above 1 μ M (Figure 6), Ramos 2G6 cells were pretreated with or without 1 μ M SB203580 and the level of SSR was assessed as GFP expression. As shown in Figure 7A, there was significant difference in the GFP expression induced by BLyS between cells treated with SB203580 and those without SB203580. On the other hand, at a concentration of 1 μ M, SB203580 had little effect on CD40-induced SSR, showing that BLyS-induced SSR is more sensitive to SB203580 than CD40-induced SSR. At a concentration of 5 μ M, SB203580 almost completely blocked BLyS-induced SSR

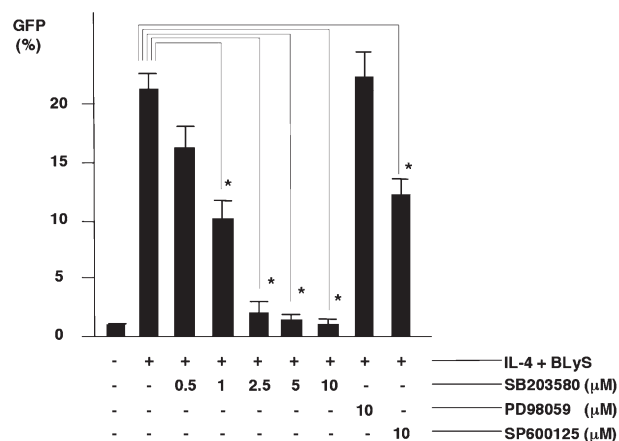


Figure 6. Inhibition of p38 MAPK by SB203580 suppresses BlyS-induced SSR in Ramos B cells. Ramos 2G6 cells containing the switch vector were treated with SB203580 (5 μM, 1 μM, 500 nM), SP600125 (10 μM), or PD98059 (10 μM) for 45 min. Thereafter, the cells were cultured for 4 d with BlyS (500 ng/ml) and IL-4 (5 ng/ml). SSR was measured as the frequency of GFP positive cells as is shown. * $P < 0.05$.

(Figure 6), and also reduced CD40-induced SSR by 50% (data not shown).

DISCUSSION

In the present study, we demonstrated that BlyS activated p38MAPK and JNK in human B cells, and that when combined with IL-4, BlyS induced CSR and AID expression. BlyS plus IL-4 induced the generation of CTs for γ 1 and 2, γ 4, and epsilon; when combined with IL-10, BlyS augmented generation of γ 1 and 2 CTs. Similar to other tumor necrosis factor-like ligands, the BlyS monomer is a β -sandwich that oligomerizes to form a homotrimer (15). BlyS receptor 3 (BR3), TACI, and BCMA have been identified as BlyS receptors.

BlyS induction of the phosphorylation of p38 MAPK and JNK has not been reported previously. Our data strongly indicate that BlyS-activated p38 MAPK plays a pivotal role in BlyS-induced AID expression and CSR in human B cells. Treatment with SB203580, an inhibitor of p38 MAPK signaling, completely blocked BlyS-induced CSR, whereas SP600125, JNK inhibitor, partially blocked CSR.

BlyS strongly induced fresh human B cell AID expression in the presence of IL-4, and this effect was almost completely reversed by 5 μM SB203580. Similarly, in Ramos B cells, BlyS induced SSR of an Ig switch construct in a dose-dependent manner in permanently transfected Ramos B cells in the presence of IL-4. Again, SB203580 was able to almost completely block this BlyS-induced SSR.

Low levels of $I\gamma$ 1/2 -C μ mRNA were detected when human B cells were stimulated by BlyS alone as measured by CTs (Figure 2B). We cannot exclude the possibility that these human B cells isolated from PBMC may be making low levels of cytokines, e.g., IL-10 that promote CSR. On the other hand, our switch construct system revealed that BlyS itself did not induce SSR (Figure 5B). In this latter system, there was definitive direct evidence of the participation of BlyS in inducing CSR. Thus, we were able to show that BlyS strongly induced CSR in the presence of IL-4 in both fresh human B cells and in our switch construct system.

MAPKs couple extracellular signals to transcriptional responses in the pathways linked to membrane Ig (16). p38 MAPK and JNK are activated in a variety of the signaling cascades such as those induced by inflammatory cytokines that are angiogenesis activators and mitogens for Kaposi's sarcoma cells and B cells (17). Similarly, p38 MAPK is required for CD40-induced proliferation and CD40-induced CD54/ICAM-1 expression, although CD40 is able to induce gene expression via both p38 MAPK-dependent and -independent pathways (7). Optimal transcriptional response of IL-4-inducible promoters has been shown to require costimulatory signals through CD40-stimulated intracellular kinases such as p38 MAPK and the dominant-negative p38 MAPK inhibited IL-4-regulated response (18). The effects of BlyS on B cell survival or proliferation have been reported

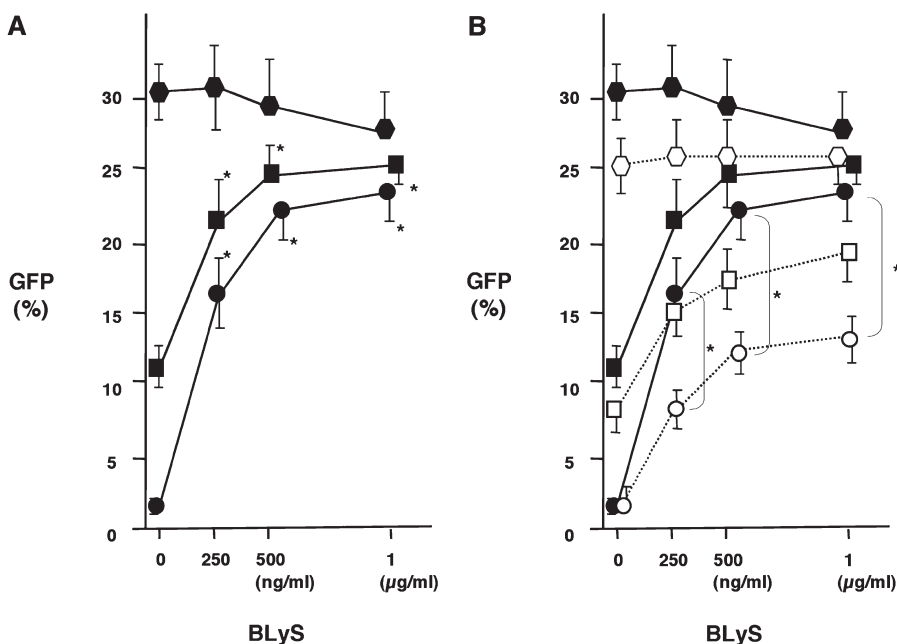


Figure 7. The effect of BlyS on CD40-induced SSR. (A) Dose response of BlyS induction of CCR with or without CD40 engagement. Ramos 2G6 cells containing the switch vector were cultured for 4 d with IL-4 (5 ng/ml) with varying doses of α CD40 (none, closed circle; 10 ng/ml, closed square; or 100 ng/ml, closed hexagon). BlyS at different concentrations was added as noted (250 ng/ml, 500 ng/ml, 1 μg/ml) and the level of SSR in the cultures was assessed as GFP expression on flow cytometry. The significance is shown when compared with GFP expression without BlyS in each condition respectively (* $P < 0.05$). (B) The effect of SB203580 on BlyS- and CD40-induced SSR. Ramos 2G6 cells were pretreated with SB203580 (1 μM) (open circle, square, or hexagon) or without (filled circle, square, or hexagon) for 45 min, and then the cells were stimulated with IL-4 (5 ng/ml) with varying doses of α CD40 (none, circle; 10 ng/ml, square; or 100 ng/ml, hexagon). BlyS at different concentrations was added and the level of SSR was assessed as GFP expression (* $P < 0.05$).

(19–20). However, a direct role of p38 MAPK in CSR has not identified previous to the present study with BLyS.

BLyS rapidly and transiently enhances the p50/p65 DNA binding activity and induces phosphorylation of I κ B α characteristic of the classical NF- κ B pathway. Also, BLyS promotes the processing of p100 to p52 and sustained formation of p52/RelB complexes via the alternative NF- κ B pathway (21). NF- κ B p50 can control the CSR to γ 3 (22) and NF- κ B can specifically control CSR to γ 1, but not to epsilon (23). Since the p38 MAPK pathway is required for NF- κ B p65 transactivation (24) and treatment with SB203580 inhibited BLyS-plus-IL-4-induced CSR to epsilon (Figure 3), p38 MAPK may well participate in CSR through NF- κ B pathway or other pathways, including a putative switch recombination-mediated function or a 3' Ig α -enhancer-mediated function (25–26). Although the full intracellular signaling pathway for BLyS-induced CSR remains to be elucidated, we did find that BLyS induced the phosphorylation of p38 MAPK, which is required for CSR.

On the other hand, BLyS induced the phosphorylation of JNK, and JNK is coupled to CD40-, CpG DNA-, and B cell receptor-mediated signals in B cells (27–28). CD40 plays an important role in B cell proliferation, survival, memory, and Ig CSR. TNF receptor-associated factor2 (TRAF2) is essential for CD40-induced CSR and activation in B cells (29). TNF receptor superfamily signals require TRAF2 for activation of JNK (30). Because the JNK inhibitor SP600125 partially blocked BLyS-induced CSR, it may be that JNK plays a role through TNFR superfamily signals such as CD40 and BLyS in CSR.

AID is essential for human Ig CSR because mutations in the AID gene cause type II hyper-IgM immunodeficiency (31). The AID gene-encoded product appears to be the only lymphoid-specific factor required for CSR and SHM, as CSR and SHM can be reconstituted in fibroblasts that ectopically express AID (32–33). Although AID expression has been shown to be induced by costimulation with TGF- β , IL-4, and CD40 engagement in a murine B cell lymphoma line, the intracellular signaling involved remains unknown. It has been reported that IL-4-induced STAT6 binding to a site in the 5' upstream region of the AID gene and NF- κ B synergize with signals delivered via the IL-4 receptor that activate STAT6 to induce optimal AID gene expression (34). In addition, the p38 MAPK pathway is necessary for NF- κ B signaling (24) and has been found to directly regulate the activity of the transactivation domain of STAT6 (18). Although we did not demonstrate directly that the actions of BLyS on class switching are dependent upon AID, our data showed that BLyS, in the presence with IL-4, induced AID expression in B cells; our data also demonstrated the requirement for p38 MAPK for this effect. The ectopic expression of AID has been shown to induce CSR in an artificial switch construct in fibroblasts at a level comparable to that in stimulated B cells (32).

BLyS acts on mouse primary splenic B cells autonomously, and directly cooperates with CD40 stimulation in B cell activation *in vitro* by protecting replicating B cells from apoptosis (20). It was unknown whether BLyS would affect CD40-induced CSR in human B cells. In our switch recombination assay, in which we can quantify SSR, BLyS increased CD40-induced SSR in a dose-dependent manner only with suboptimal levels of CD40 stimulation of CD40 (10 ng/ml α CD40 Ab), having no effect on the CSR with optimal CD40 engagement (100 ng/ml α CD40 Ab). These data suggest that BLyS and CD40 engagement induce CSR via the same or very similar intracellular signaling pathway, with both of them activating p38 MAPK and NF- κ B (which are essential for CSR), although we have demonstrated that BLyS-induced SSR was more sensitive to SB203580 than CD40-induced SSR.

It is becoming evident that BLyS may participate in a variety of systemic immune-based disorders. Serum BLyS levels correlated positively with (1) anti-double-stranded DNA antibody titers among patients with SLE, (2) rheumatoid factor titers among seropositive patients with rheumatoid arthritis, and (3) the levels of autoantibodies among subjects with Sjögren's syndrome (3, 35). BLyS also protects tumor cells (e.g., B-cell chronic lymphocytic leukemia cells) from apoptosis and thereby enhances their survival (36). Thus, interruption of the BLyS pathway is a candidate for therapeutic targeting of those diseases. Treatment of a mouse model of SLE with a BLyS protein antagonist has been shown to ameliorate disease progression and enhance survival (37). On the other hand, our data showing that BLyS can induce CSR in human B cells independent of CD40 stimulation suggests that pharmacologic administration of BLyS might enhance CSR and Ig production in CD40L- or CD40-deficient patients. *In vitro*, BLyS induced normal quantities of IgM from B lymphocytes of individuals with common variable immunodeficiency that is characterized by the inability to generate adequate serum Ig despite normal or slightly depressed peripheral B, T, and myeloid cell populations (38). Overall, these *in vitro* and *in vivo* studies of BLyS and the analysis of BLyS signaling reinforces the idea of BLyS being a potential therapeutic modality and also the idea of BLyS and its signaling pathway being potential targets for drug interventions.

Conflict of Interest Statement: T.Y. has no declared conflicts of interest; K.Z. has no declared conflicts of interest; A.Y. has no declared conflicts of interest; D.Z. has no declared conflicts of interest; and A.S. has no declared conflicts of interest.

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