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Construction and Function of Two Cys146-Mutants with High Activity, Derived from Recombinant Human Soluble B Lymphocyte Stimulator

Guangyu Chen, Shanyun Peng, Minji Zou, Honglian Xu, Donggang Xu and Jiaxi Wang*
Laboratory of Molecular Genetics, Beijing Institute of Basic Medical Sciences, Beijing, China 100850

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B lymphocyte stimulator (BLyS) is a novel member of tumor necrosis factor (TNF) ligand family that is important in B cell maturation and survival. Previous studies were almost related to the function or mechanism of its wild type. Here, we constructed two site-directed mutants of the recombinant human soluble BLyS, the BY-A and BY-V, and found that BY-V ranked the highest whenever in the process of promoting proliferation of B lymphocytes *in vitro* or stimulating total serum IgG and IgM secretion *in vivo*. Besides, assays for the biological responses of human leukemic cell lines to BLyS, BY-A and BY-V demonstrated that they could suppress the proliferation of Raji cells but promote the growth of THP-1. The discovery of BY-V with high activity will help come to a conclusion that the mutation of Cys146 to Val146 might improve the biological activity of BLyS.

Key words: B cell proliferation, BLyS, cytokine, immunoglobulin secretion, mutation.

B lymphocyte stimulator (BLyS) (*1*), also identified independently as TALL-1, BAFF, THANK, TNFS20 and zTNF4 (*2–6*), is a new member of an ever growing TNF superfamily, which can promote expansion and differentiation of B cell population, leading to increases in serum immunoglobulin levels. It is a type II transmembrane protein, which can be expressed as a surface-membrane-bound molecule or secreted from cells as a soluble ligand (*4*).

Former studies indicate that both the membrane and soluble forms of BLyS are biologically active in promoting proliferation of B cells treated with anti-IgM *in vitro* (*1*, *3*). Animals injected with soluble recombinant BLyS exhibit the disrupted splenic T and B cell zones with increased B cell counts and immunoglobulin levels (*1*). BLyS overexpression in mice results in severe autoimmune lupus-like characteristics with high levels of rheumatoid factors, proteinuria, anti-DNA autoantibodies and increased numbers of B and effector T cells (*7–11*). Recent studies on BLyS gene knockout further reveal that BLyS is required for normal B lymphocyte development (*10*, *12*, *13*). Taken together, such results demonstrate that BLyS plays a critical role in regulating B cell immune responses (*14*).

The identification of soluble BLyS may have therapeutic applications in B cell-related disease states such as single IgA, IgG, and IgM deficiencies, common variable immunodeficiency (CVID) and other panhypogammaglobulinemias. In such conditions, abnormal B cell function leads to global or specific hypoimmunoglobulinemia and/or decreased secretory immunoglobulin levels. In many of these disease states, the treatment of choice is parenteral administration of immunoglobulin. However, B cell expansion and differentiation by means of BLyS treatment could provide a unique alternative to conventional therapy of parenteral immunoglobulin (*15*, *16*).

Previous studies on BLyS were almost limited to its wild type. If BLyS mutant of higher activity but little side-effect can be developed, we are sure, they will assist in the development of a new BLyS substitute. Bodmer *et al.* (*17*) systemically compared the extracellular protein sequences of 16 members of TNF superfamiy. BLyS is found to be remarkable in that it contains an unpaired Cys residue (Cys146) at the corresponding position where some other members have about 37.5% (6/16) Ala or 37.5% (6/16) Val. Furthermore, BLyS has a potential to inhibit apoptosis (*18*) but the other TNF members can almost promote apoptosis of tumor cells. Such phenomenon is just a coincidence? Is Cys146 indispensable to the normal function of BLyS or a crucial site for the functional differences between BLyS and other members? Can the substitution of Ala or Val for Cys146 increase the activity of BLyS? Here, we constructed two site-directed mutants of BLyS and fortunately obtained the mutant BY-V with high activity thereinto through functional identification *in vitro* and *in vivo*. Different responses of human leukemic cell lines to BLyS, the two mutants and TNF-a were also compared.

MATERIALS AND METHODS

Antibodies—Goat F $(ab')_2$ anti-human IgM was from Southern Biotechnology Associates Inc (Birmingham, USA). For enzyme-linked immunosorbent assay (ELISA), the following materials were used: mouse IgG, IgM, IgA, goat anti-mouse HRP-IgG, goat anti-mouse HRP-IgM, goat anti-mouse HRP-IgA (all from Sigma, St. Louis, MO, USA); goat anti-mouse IgG, goat anti-mouse IgM, goat anti-mouse IgA (Beijing Zhongshan Biotechnology Inc, P.R. China); and goat anti-human BLyS from Scant Cruz

*To whom correspondence should be addressed. Tel: +86-10-66931322, Fax: +86-10-68213039, E-mail: wangjx20000@sina.com

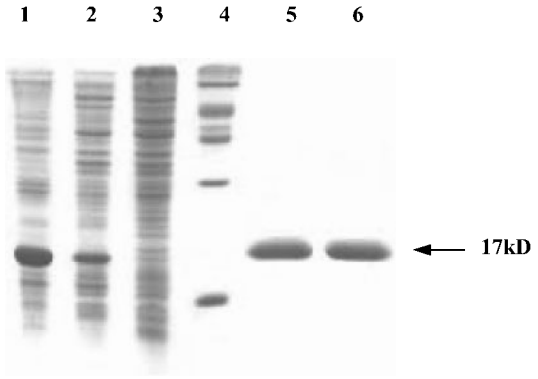

Fig. 1. **The recombinant mutant BY-V protein analysed on 15% SDS-PAGE before/after purification.** The recombinant BY-V protein was found expressed in the form of inclusion body. The inclusion bodies were washed in 1.5% (v/v) tween-20 and 2 M urea solution, denatured and solublized in the PB buffer containing 8 M urea, then purified by a Sephacryl-200 gel filtration. inclusion body of pBV*BY-V* before being loaded onto a Sephacryl-200 gel filtration column (lane 1); whole cell lysates of *E. coli* DH5α containing pBV*BY-V* before being sonicated (lane 2); whole cell lysates of *E.coli* DH5α containing pBV220 (lane 3); mid-range protein molecular weight markers (lane 4); purified BY-V protein under reduced conditions (lanes 5 and 6), there seemed no other bands being visible to the naked-eye, which showed comparatively higher purifty of the recombinant proteins.

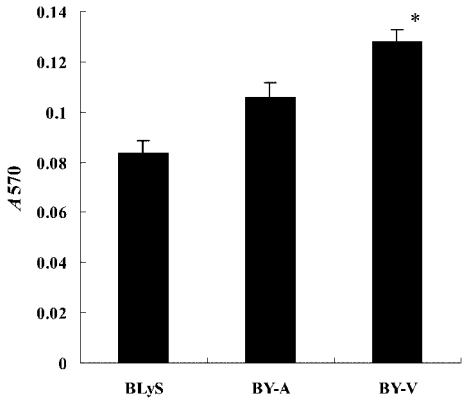

Fig. 2. **Effect of three recombinant proteins on B cell proliferation and survival *in vitro*.** B lymphocytes (2.5×10^4) isolated from human peripheral blood leukocytes were cultured with/without 30 mg/liter of goat F (ab′) 2 anti-human IgM and/or the indicated amount of each recombinant protein for 72 h at 37°C, 5% CO_2. Cell growth was then evaluated by MTT Assay. *Statistically significant differences ($p < 0.05$) between BY-V and BLyS, which means the mutant BY-V possesses higher stimulating capacity of B cell proliferation and survival than that of BLyS.

biotechnology (Scant Cruz, CA). For flow cytometry analyses, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD23 antobodies were from eBioscience (San Diego, CA, USA).

Plasmids Construction—The full length cDNA encoding BLyS was amplified by RT-PCR from total RNA of human peripheral blood leukocytes and inserted into the pBV220, a thermo-sensitive expression vector, constructing recombinant plasmid pBV*BLyS*. pBV*BLyS* was used as a template to introduce site-directed mutations at position 146 (Cys146) by two-step PCR. The mutations introduced were substitution of Ala for Cys and Val for Cys. Then, *BY-A* (Cys146→Ala146) and *BY-V* (Cys146→Val146) were inserted into the pBV220 to form pBV*BY-A* and pBV*BY-V* expression plasmids.

The plasmids above were confirmed by standard techniques (*19*).

Protein Purification—Three recombinant plasmids mentioned above were transformed into *Escherichia coli* DH5α. Cells of *E.coli* DH5α carrying pBV*BLyS*, pBV*BY-A* or pBV*BY-V* were harvested by centrifugation (10,000 × *g*, 10 min). After suspension in 50 mM sodium phosphate buffer (PB) (pH 7.4), they were sonicated and centrifuged at 10,000 × *g* for 15 min to remove cell debris. Recombinant proteins were purified from the inclusion bodies according to the following steps.

Step 1: The inclusion bodies were washed once in 1.5% (v/v) tween-20 and twice in 2 M urea solution (50 mM PB, 100 mM NaCl, 2 mM EDTA and 10 mM DTT, pH 7.4), respectively. Then, the precipitate containing the recombinant proteins was gained by centrifugation at 10 000 × *g* for 15 min and then dissolved in denaturing buffer (8 M urea, 50 mM PB, 100 mM NaCl, 2 mM EDTA and 10 mM DTT, pH 7.4) to be solubilized for correct protein folding.

Step 2: The crude protein solution was loaded onto a Sephacryl-200 gel filtration column (BioRad Molecular Bioscience Group, CA, USA) pre-equilibrated with the denaturing buffer same to step 1 and eluted with the same solution.The purified fractions were collected.

Step 3: The purity of the three purified proteins was analysed by 15% (w/v) SDS-PAGE and identified by N-terminal amino acid sequencing.

Step 4: The purified proteins were enclosed in dialytic bags and immersed in renaturing buffer (50 mM PB, 100 mM NaCl, 0.1 mM GSSG, 1 mM GSH, 0.1% PEG4000, pH 7.4), then they were gradually refolded and renatured at 4–7°C by discontinuously replacing fresh PB.

Cells—Human peripheral blood B lymphocytes were isolated by density gradient centrifugation of EDTA-treated peripheral blood of health donors over Ficoll-Paque™ (Amersham Pharmacia Biotech, Buckinghamshire) and then passed over a 70-μm nylon cell strainer (Falcon, Becton Dickinson), and erythrocytes were thawed by osmotic lysis with red blood cell lysis solution (8.34 mg/ml ammonium chloride, 0.84 mg/ml sodium bicarbonate and 1 mM EDTA, pH 8.0). The isolated B cells were cultured at 37°C, 5% CO_2 in RPMI 1640 supplemented with 10% selected fetal calf serum and 100 U/ml penicillin/streptomycin (Gibco BRL), then used for B cell proliferation assay.

Human leukemic cell lines, such as Jurkat, HL-60, Raji, THP-1, U937, and MOLT-4, were all preserved by our lab and cultured at a density of 1.2×10^5 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin, then used for cytotoxicity experiments.

MTT Assays—For B cell proliferation assay, the B cells isolated by the method above were adjusted to 10^5 cells/ml and cultured in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Gibco BRL) in triplicate in 96-well flat-bottom plates with/without 30 mg/liter of goat F $(ab')_2$ anti-human IgM (Southern Biotechnology Associates Inc.) and/or the indicated amount

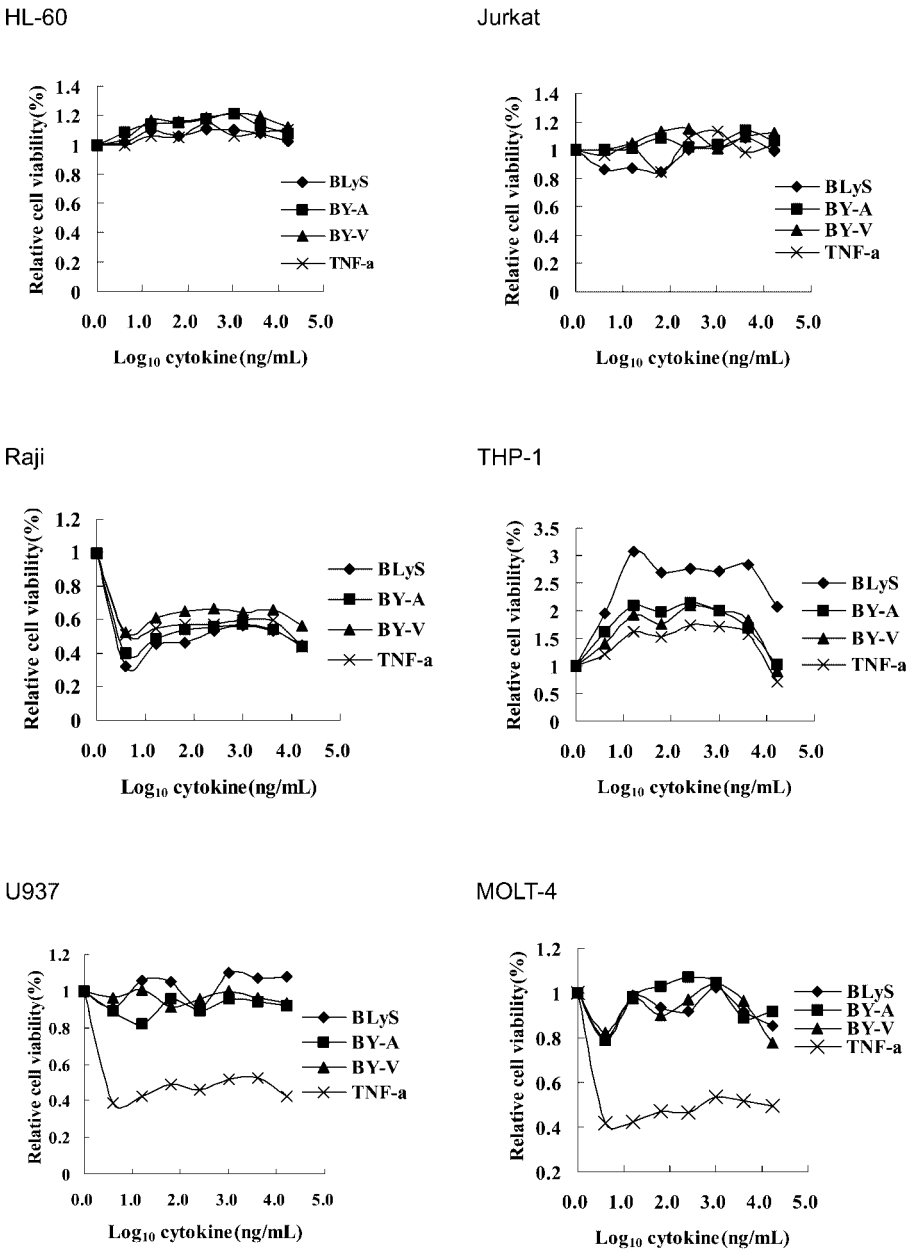

Fig. 3. **Dose-dependent cytotoxic effects of the recombinant proteins on human leukemic cell lines.** The six strains of cell lines (1.2×10^5/ml) were cultured in 96-well flat-bottom plates and/or indicated amount of each recombinant protein or TNF-a used as positive control for 72 h at 37°C, 5% CO_2. Cell viability was determined as absorbance (A_{570}) values. It was found that HL-60 and Jurkat cells could withstand the cytotoxity of the four proteins but B tumor cell line Raji could not. However, the four proteins could specifically stimulate the proliferation of THP-1 in a given range of protein density. For the U937 and MOLT-4, BLyS, BY-A and BY-V could not display cytotoxic effect on their proliferation but TNF-a could.

of each recombinant protein for a period of 72 h at 37°C, 5% CO_2. Cell proliferation was measured by MTT method using a Bio-Rad model 550 microplate reader (BioRad Molecular Bioscience Group, A).

For biological responses of leukemic cell lines to recombinant proteins, the six strains of cell line were cultured in triplicate in 96-well flat-bottom plates and/or indicated amount of each recombinant protein or TNF-a used as positive control for a period of 72 h at 37°C, 5% CO_2. Cell viability was determined as above.

Enzyme-Linked Immunosorbent Assay (ELISA)—Forty female BALB/c mice of 6 weeks old were randomized into 4 groups of 10 animals each. Mice were given BLyS, BY-A , BY-V or PB by injection via the tail vein at a dose of 3.0 mg/kg for 10 days (experimental day 1–10, inclusive). On day 0, 3, 8, 10 of the study and on the 15th day after the last protein or PB injection, blood was collected from the tail vein and placed into serum separator tubes, respectively, allowed to clot for 2 h at 4°C, and then centrifuged at 12,000 rpm for 10 min at room temperature. Serum was separated, frozen at –20°C, and later analysed for IgG, IgM, IgA and the type of anti-recombinant protein Ig.

Flow Cytometry Analyses—Spleens were taken out of 16 female BALB/c mice randomized into 4 groups injected daily via the tail vein with 3.0 mg/kg BLyS, BY-A, BY-V or PB for 4 days. On day 5 of the study, animals were killed by breaking neck. Spleens were taken out, weighed, and prepared for analysis by FACS. Splenic tissue was sieved through a 100-mm nylon mesh. The resultant splenocytes were gently washed by RPMI 1640 with 10% FBS, and erythrocytes were lysed by red blood cell lysis solution. Purified splenocytes were pelleted and resuspended to a density of 10^6 cells/ml in FACS buffer (d-PBS with 0.1% BSA and 0.1% sodium azide). One hun-

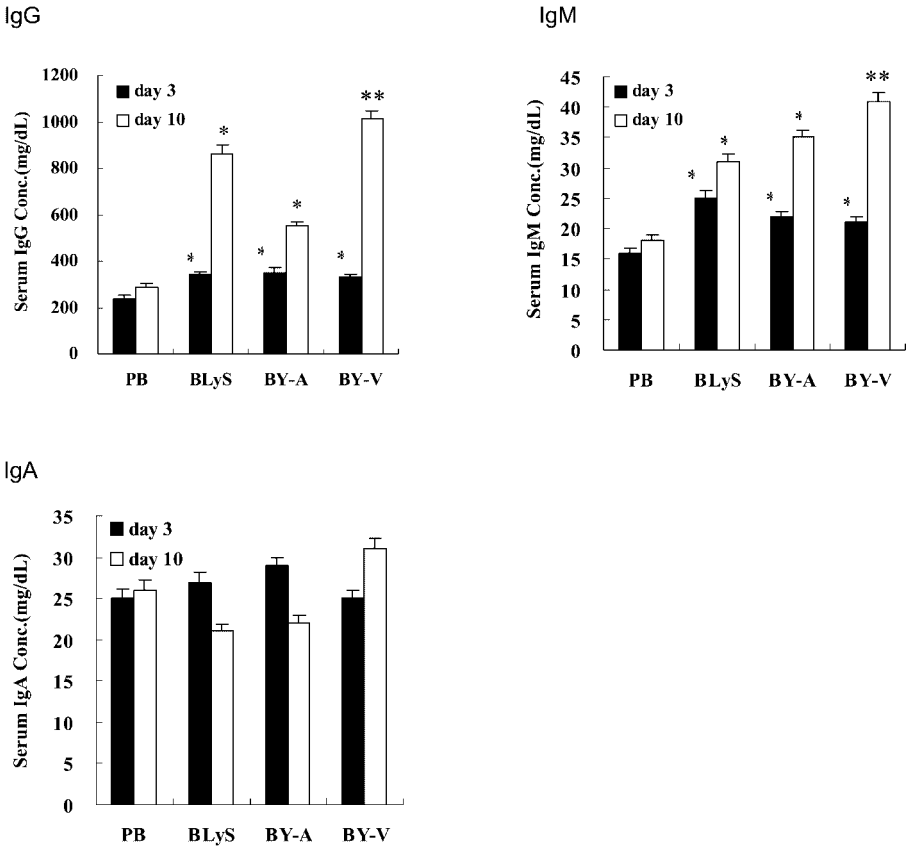

Fig. 4. **Effects of i.v. BLyS and its two mutants administration on serum immuoglobulins after 10 days of dosing from mice euthanized 11 days after the beginning of the three proteins or control treatment.** Mice were injected BLyS, BY-A , BY-V or PB via the tail vein at a dose of 3.0 mg/kg for 10 days (experimental day 1–10, inclusive). On day 3 and 10 of the study, blood was collected from the tail vein and serum was separated, and later analysed by ELISA for total serum IgG, IgM and IgA levels. *Statistically significant differences ($p < 0.05$) between three proteins and control. **Statistically significant differences ($p < 0.05$) between BY-V and BLyS or BY-A. Administration of BLyS, BY-A and BY-V to mice for 10 days resulted in marked increases in IgG and IgM levels, compared with that of 3 days of dosing. There was significant difference among the three protein groups and the mutant BY-V was the best in stimulating such two kinds of Ig secretion, for BLyS and BY-A, it was observed that BLyS could mainly promote serum IgG secretion but the effect of the latter led to elevated IgM levels. As to serum IgA, no detectable increase was found.

dred microliters of cell suspension were incubated for 30 to 60 min on ice with FITC-conjugated anti-mouse CD23 (eBioscience). Cell suspensions were pelleted and then analyzed on a FACScan with CellQuest software (BD Biosciences, San Jose, CA) for the relative percentage of $CD23^+$ splenocytes.

Statistical Analysis—Statistical significance of differences was determined using the LSD post hoc test.

RESULTS

Cloning, Expression and Purification of BLyS and Its Mutants—The BLyS cDNA was amplified, purified and inserted into pBV220 to form expression vector pBV-*BLyS*. *BY-A* and *BY-V* were amplified by overlap PCR from pBV*BLyS* and formed pBV*BY-A* and pBV*BY-V*. The three expression plasmids were transformed into *E. coli* DH5α and it was found that the three recombinant proteins were highly expressed as inclusion bodies. Purified recombinant proteins were obtained and their purity was over 95% (Fig. 1). Similar results have been published in our previous work (*20*).

In addition, the recombinant proteins were confirmed by N-terminal amino acid sequencing (Data not shown).

Activity Test of BLyS and ItsTwo Mutants in vitro—Compared with the control, no detectable B lymphocyte proliferation was observed when anti-IgM was only added, but the higher concentration of the recombinant proteins the more B lymphocytes. After a series of preliminary test we observed that the activity of three proteins was the most significant at the dose of 1 μg/ml (*20*). Then, we compared their activity several times at such concentration with the result that BY-V stimulated proliferation of B lymphocytes markedly better than the wild type (Fig. 2).

Different Biological Responses of Human Leukemic Cell Lines to Recombinant Proteins—Assay for biological responses of human leukemic cell lines such as HL-60, Jurkat, Raji and THP-1 to the three proteins demonstrated the same results as the positive TNF-a control (Fig. 3). HL-60 and Jurkat cells could resist the cytotoxity of four proteins but B tumor cell line Raji displayed very high sensitivity, however, THP-1 was shown to proliferate in a given range of protein density.

As to the cell line U937 and MOLT-4, BLyS, BY-A and BY-V could not inhibit their proliferation, which was very different from the effects of TNF-a on them (Fig. 3).

Administered Recombinant Protein-Induced Changes in Serum Immnoglobulins and The Type of Anti-Recombinant Protein Ig—Compared with the PB group as blank control, the three proteins could stimulate the production of total serum IgG and IgM, but not detectable IgA secretion (Fig. 4).

As for total serum IgG and IgM secretion, mice of the three protein groups received dose of 3.0 mg/kg for 3 days displayed obvious increases. Statistical tests indicated that there was not much difference in IgG production among the various protein groups, but the level of IgM was higher in BLyS group than that of the mutant ones. Systemic administration of BLyS, BY-A and BY-V to mice for 10 days resulted in marked increases in IgG and IgM secretion, compared with that of 3 days of dosing, statistical tests showed that there was significant difference among the three protein groups and BY-V ranked the

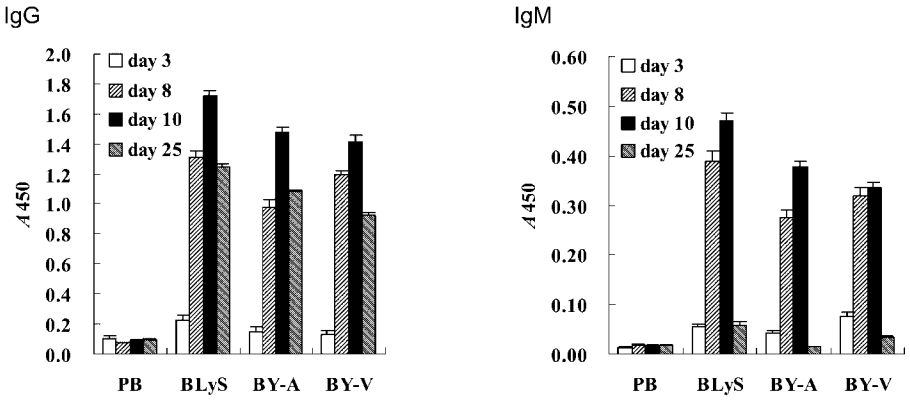

Fig. 5. **Effects of i.v. BLyS and its two mutants or control administrations on the IgG and IgM levels of anti-BLyS.** Mice received injections of 3.0 mg/kg of BLyS, BY-A , BY-V or PB via the tail vein of for 10 days (experimental day 1–10, inclusive). On day 0, 3, 8, 10 of the study and on the 15th day after the last protein or PB injection, blood was collected from the tail vein and serum was separated, and then analysed by ELISA for the type of anti-BLyS Igs. The levels of such two Igs were determined as absorbance (A_{450}) values. IgG and IgM were found to be the major types. In the period of 10 days' dosing the change for the levels of anti-protein IgG and IgM displayed time-dependent relationship with the levels of such two Igs ranking the highest on the 10 day of dosing and returning to basal level two weeks after the last dosing.

highest in the process of inducing such two type of immunoglobulin secretion, which was consistent with the result of B cells survival and proliferation assay. Taken together, it was found that 5 times for IgG and 2 times for IgM secretion increased, respectively, in BY-V group. With regard to BLyS and BY-A, it was observed that BLyS could mainly promote serum IgG secretion but the effect of the latter led to elevated IgM levels.

Compared with the PB group, administration of the recombinant proteins could not improve the total serum IgA levels whatever 3 or 10 days of dosing.

ELISA for Igs against recombinant protein in mice indicated that IgG and IgM were the major types. In the period of 10 days' dosing the change of anti-protein IgG and IgM displayed time-dependent relationship with the levels of such two immuoglobulins ranking the highest on the 10th day of dosing and returning to basal level two weeks after the last dosing (Fig. 5).

Effects of The Recombinant Proteins on Spleen Weight and Splenic Cell Populations—In a separate study, mice treated i.v. with 3.0 mg/kg/day BLyS, BY-A or BY-V for 4 days and euthanized on day 5 showed a 28 to 68% increase in spleen weight (Table 1). Statistical test indicated that there were significant differences between the proteins and PB group ($p < 0.05$).

FACS analyses of the splenocytes stimulated with the recombinant proteins and PB control revealed a general trend toward recombinant protein-induced expansion and differentiation in more developmentally mature splenic B cells ($CD23^+$) compared with time-matched PB controls. The relative percentage of $CD23^+$ splenocytes in different groups was as follows: 31% for PB control, 42% for BLyS, 36% for BY-A and 41% for BY-V group (Fig. 6).

Table 1. **Effects of BLyS and its mutants administration on spleen weight of BALB/c mice.**

Group	Number of mice	Average weight of spleen (mg) (X ± S)	*P* value
P B	4	89.95 ± 3.72	
BLyS	4	150.8 ± 26.28	BLyS:PB < 0.05
BY-A	4	115.05 ± 5.79	BY-A:PB < 0.05
BY-V	4	118.85 ± 2.90	BY-V:PB < 0.05

DISCUSSION

BLyS, a B cell survival factor, is being developed to increase the production of endogenous immunoglobulins in patients with hypogammaglobulinemia, such as CVID and IgA deficiency syndrome. So far, researchers have studied its wild type and there are not any reports about the derivatives of high activity but little side-effect of

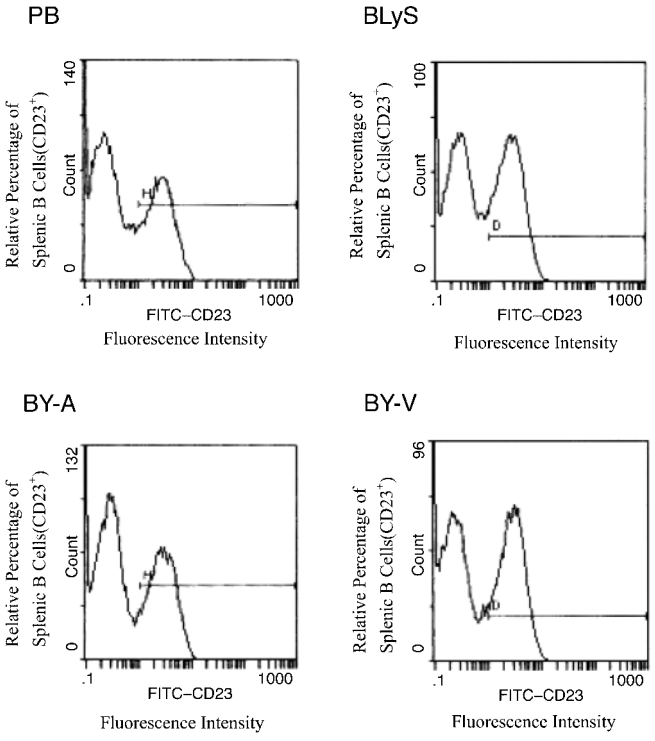

Fig. 6. **FACS analysis of splenic cells of BALB/c mice treated with control or BLyS or its two mutants for 4 days, performing necropsy on day 5.** BALB/c mice were injected daily via the tail vein with 3.0 mg/kg BLyS, BY-A, BY-V or PB for 4 days. On day 5 of the study, animals were killed and spleens were taken out. Then splenocytes were isolated, washed and the relative percentage of splenic B cells ($CD23^+$) was analysed by flow cytometry with an FITC-antibodies against CD23. The relative percentage of $CD23^+$ splenocytes in different groups was 31% for PB group, 42% for BLyS group, 36% for BY-A and 41% for BY-V group, respectively.

BLyS. We think if such molecules could be exploited, they would aid the development of a new kind of clinical BLyS substitute.

There are three Cys in BLyS, that is Cys146, Cys232 and Cys245. The latter two form inter-chain disulphur bond and Cys146 is free. In the course of the denaturation and renaturation of recombinant protein, Cys146 might be oxidized and linked to any one of Cys232 and Cys245, probably leading to incorrect refolding and resulting in inactive BLyS. Mutation of Cys146 can avoid formation of wrong disulphur bond and incorrect refolding. On the other hand, it seemes that Ala146 and Val146 is the result of evolutional selection in the TNF family, so the mutation of Cys146 to either of them might be a good choice to get the potential BLyS derivatives of higher activity. In this research, we specifically mutated Cys146 to Ala and Val, aiming to get the BLyS mutants with higher activity. Statistical test for B cell proliferation assay indicated that BY-V could promote the proliferation of B cells remarkably better than the wild type of BLyS, hence we inferred that Val146 was substituted for Cys146 might benefit to increase the bioactivity of BLyS.

The bioactivity of the three proteins trended identically, all reaching the highest activity at 1 μg/ml. Therefore, we drew a conclusion that the recombinant proteins could promote proliferation of B lymphocytes best at 1 μg/ml.

As members of TNF superfamily, BLyS and TNF-a should function similarly in some ways so that the cytotoxic effect on leukemic cell lines of the three proteins and TNF-a was compared, and it was observed different results directed against different cell lines. The reason why makes the differences of the effect on the six cell lines between the two kinds of TNF family members, BLyS and TNF-a, still need to be elucidated.

To further understand the functional change of the mutants, we compared the abilities stimulating the expansion of B lymphocytes and immunoglobulin secretion *in vivo*. We found that BLyS, BY-A and BY-V administered to mice resulted in elevated total serum IgG and IgM. The serum immunoglobulin level increases following the protein administration were dependent on the duration of dosing as the increases in immunoglobulins were substantially greater after 10 days of dosing compared with just 3 days of dosing. It should be noted that, since dosing was not continued after 10 consecutive days, the maximum possible increases in the levels of serum immunoglobulins at a particular dose was not known. Besides**,** in consistence with the result of B cell survival assay, BY-V was the best in stimulating IgG and IgM secretion after dosing for 10 days. Nevertheless, with regard to the levels of mucous immunoglobulin IgA, no change was detected.

Administration of the recombinant proteins also resulted in a trend toward expansion and differentiation (relative number and progression to a more mature form) of splenic B cell population. This effect was observed in mice killed after 4 days of dosing even when only a single dose of the three proteins was administered. The rapidity of the splenic B cell response, compared with the delayed increase in the concentration of serum immunoglobulins, suggests that the mechanism for the recombinant proteins-induced increases in serum immunoglobulins is mediated by B-cell expansion/differentiation rather than stimulation of pre-existing plasma cell Ig secretion (*1*, *3*).

In conclusion, administration of the three proteins promoted expansion and differentiation of B cell population, leading to increases in total serum IgG and IgM levels. B cell expansion and differentiation by means of the protein treatment demonstrated that BLyS and its mutants may offer enhanced host immunological protection in treating certain hypogammaglobulinemias without the need for intravenous immunoglobulin G, and could provide a useful means of monitoring the efficacy of them in the clinical practice.

Determining the type of Ig anti-recombinant protein in mice showed that there was the highest concentration of free recombinant proteins after dosing for 10 days and then returned to baseline by at least 2 weeks after the cessation of dosing. Moreover, using each recombinant protein as coating substance for ELISA, we always found that the A_{450} values in the BLyS group were higher than those of the mutant ones, which might indicate that the mutations of BLyS increased the affinity between the proteins and their receptors on the target cell membrane or the clearance of the mutants.

In a word, we identified the bioactivity of BLyS, BY-A and BY-V *in vitro* and *in vivo*, and concluded that BY-V behaved best in stimulating expansion and differentiation of B lymphocytes and immunoglobulin secretion, therefore, we inferred that the mutation of Cys146 to Val146 could improve the biological activity of BLyS. The reason for that might be reduction of the probability for wrong refolding of disulphur bond, and the appropriate mutation of Cys146 to Val146 in terms of systematic evolution.

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