Multiple myeloma (MM) is a common malignant tumor, characterized by unlimited proliferation of abnormal plasmocytes in bone marrow. Considering the biological function of B-Lymphocyte stimulator (BLyS) and its receptors in B cell, we examined BLyS and its receptors expression in MM cells. Our studies confirmed that BLyS and its receptors are expressed in MM cells, including KM3, CZ-1, and primary MM cells, playing an important role in the survival and proliferation of MM cells. Additionally, we provide evidence that BLyS protein is located in the MM cell plasma membrane. We also found that IFN-γ and IL-6 can induce BLyS expression on MM cells, while after the treatment of BAY11-7082, an IκB-α phosphorylation inhibitor, IFN-γ induced up regulation of BLyS was completely inhibited, suggesting that nuclear factor κB (NF-κB) might be involved in the mechanism of the regulation of BLyS levels in response to cytokines. Finally, linear correlation analysis of the Lactate Dehydrogenase concentration and beta 2-microglobulin level with BLyS, and expressions of BLyS mRNA in MM patients revealed a significant correlation between them ($P < 0.01$ in all case), showing that BLyS could be a biomarker for the diagnosis and treatment of MM.
complex leading to phosphorylation-induced IkB degradation, the other pathway leads to selective activation of p52: RelB dimers. This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family through selective activation of IKKα by the upstream kinase NIK (5–11). Previous studies have also shown that BLYS can activate NF-κB pathways suggesting that activation of NF-κB family members play a critical role in BLYS signaling pathway in B cells (12–14).

Materials and methods

Human subjects

Thirty-one clinically confirmed MM patients were inpatients of the affiliated hospital of Nantong Medical University, including 14 females and 17 males ranging in age from 40 to 73 yr with a mean of 60 ± 10 yr. And 12 cases of these patients were newly diagnosed and non-treated, 19 cases were treated patients. All of them were in conformity with the MM diagnostic criteria. The main presenting clinical features were bone pain, fatigue, extramedullary plasmacytomas and bacterial infection, renal function impairment (creatinine ≥ 179 micromole/L), and hypercalcemia (serum calcium value greater than or equal to 2.75 mmol/L). And all patients were found to have a monoclonal gammopathy, IgG accounted for 74% of the cases, followed by IgA (23%) and IgM (3%). Thirty healthy adults were used as controls, who were blood donors and normal examiners of Nantong Central Blood Bank, including 14 females and 16 males ranging in age from 38 to 68 yr with a mean of 58 ± 9 yr. Whole blood specimens (3 mL) were obtained from patients with MM, and healthy donors on informed consent and PBMCs were separated by density gradient with Ficoll–Hypaque (Shanghai Huajing Biochemical Reagent CO., Lt., Shanghai Huajing, China). The samples were stored at −70°C until use.

Cell cultures and reagents

Mononuclear cells were isolated from the peripheral blood and bone marrow of three advanced MM patients (one λ light chain type and two κ light chain type) by density gradient centrifugation using lymphocyte isolation solution (Shanghai Biochemical Reagent Co., Lt., Shanghai, China), and then they were cultured in RPMI (HyClone) containing 10% fetal bovine serum (FBS), respectively. The MM cell lines KM3 and CZ-1 were kindly provided by Second Military Medical University, China. Cells were cultured in RPMI (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (GIBCO, San Francisco, CA, USA) supplemented with cytokines (5 ng/mL IFN-γ or 25 ng/mL IL-6) or not. Cell-free supernatants from 3 d cultures in the presence or absence of cytokines were harvested and stored at −20°C. IFN-γ and IL-6 was purchased from Peprotech, Princeton, NJ, USA. The phospho-IκBα inhibitor BAY11-7082 was purchased from eBioscience, San Diego, CA, USA. BLYS monoclonal antibody for Western Blot (RnD System, Minneapolis, MN, USA); NF-κB P65, IκBα (Upstate Biotechnology, Lake Placid, NY, USA). BLYS polyclonal antibody for neutralization of bioactivity (RnD System) and Fluorescein isothiocyanate (FITC) anti-human BLYS, BAFF-R, BCMA, TACI, and FITC-conjugated isotopic control IgG were purchased from eBioscience; cell counting kit-8 (XTT) (Beyotime, Nantong, Jiangsu, China). Ommiscript reverse transcript kit (QIAG, Hildesheim, Germany); pGEM-T vector, E. coli DH5α (E. coli, DH5α) and Trizol (Invitrogen Corporation, Grand Island, NY, USA), DNasel (Takara, Dalian, China); UNG, IPTG, X-gal, and dNTPs plasmid extraction kit (Promega, Madison, WI, USA); capillary tubes (Roche, Mannheim, Germany); DL2000 DNA marker (Dalian TakaRa Bioengineering Co., Ltd., Dalian, China); peptone, yeast extract, and agarose (Shanghai Sheng Gong Bioengineering Co., Ltd., Shanghai, China).

Flow cytometry

Cells (1 × 10⁶) were incubated with 0.1 μg FITC anti-human BLYS, BAFF-R, BCMA, TACI, or FITC-conjugated isotopic control IgG for 30 min at 4°C. Cells were washed, resuspended in phosphate-buffered saline (PBS), and analyzed using a flow cytometry and associated cell QUEST software (Becton Dickinson, Franklin Lakes, NJ, USA). Isotype and fluorochrome controls were done for each sample.

Fluorescence immunocytochemical method

KM3 Cells were fixed in 1 mL 4% paraform for an hour at room temperature and then suspended in 0.01 M PBS. Cells were cytospun onto poly-l-lysine-coated glass slides and air dried. Non-specific protein binding was prevented by blocking the cells with 10% fetal calf serum (FCS) in PBS. Cells were stained with FITC anti-human BLYS away from light overnight at 4°C. After three washes with PBS, the slides were stained with 40 μL Hoechst (5 μg/mL) away from light for 30 min and then washed with PBS 3 times. Coverslips were applied with glycerine. The cells were visualized using an FluoView laser scanning confocal microscope (LEICA, Wetzlar, Germany). Digital images were obtained using the manufacturer’s FLUOVIEW software (LEICA microsystems, Wetzlar, Germany).
Quantitative polymerase chain reaction

For reverse transcription–polymerase chain reaction (RT–PCR), the TRIzol reagent was used to isolate total RNA from MM cells, and DNaseI was used to avoid genomic DNA contamination. And then, RNA (5 μg) was used to perform first-stranded cDNA synthesis by reverse transcription. According to the nucleotide sequences of oligonucleotide BLyS (GenBank Accession number as AY129225), TACI (GenBank Accession number as AF023614), BCMA (GenBank Accession number as BC058291), BAFF-R (GenBank Accession number as AF373846) and β2-microglobulin (β2M) (GenBank Accession number as NM_004048), TaqMan probes, and primers (shown in Table 1) were designed at exon–exon crossing and synthesized by Shanghai Sangon Biological Engineering Technology & Service Company. To quantify BLyS and its receptors mRNA expression, we performed RTQ–PCR on a LightCycler (Roche, Germany). Standard curve was used to determine each expression level, and all readings were within the standard curve. To confirm specificity of PCR products, a melting curve analysis and subsequent inspection of agarose gel electrophoresis were used. The target gene mRNA expression level was presented as a ratio to that of β2M in form of Log value.

Western blot

Proteins in whole cell extracts (15–40 μg of protein, in equal amounts) were resolved by 10–15% SDS-PAGE, transferred to a polyvinylidene difluoride membrane and probed with specific primary antibodies, and treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein was visualized by enhanced chemiluminescence ECL.

XTT proliferation assay

Cells were plated in triplicate at 1.5 × 10⁴ cells per well in 200 μL RPMI 1640 with 10% fetal calf serum and the indicated reagents (20 ng/mL goat IgG or goat polyclonal BLyS antibody) in a 96-well plate and incubated in 5% CO₂ at 37°C. After 24, 48, 72, and 96 h, 20 μL XTT labeling mixture was added to the culture media and incubated 1 h. The samples were measured using a Benchmark microplate reader (BIO-TEK ELx800, Vermont, USA) with 450 nm absorbance wavelength and 650 nm reference wavelength. Each value was calculated as the mean ± SD of triplicate samples.

BLyS ELISA

Specific enzyme-linked immunosorbent assay (ELISA) kits were used for measuring BLyS concentrations in cell culture supernatants (Bender MedSystems, Vienna, Austria), according to the manufacturer’s protocol.

Determination of LDH and beta 2-microglobulin (β2M) concentrations of MM patients and normal controls

Lactate dehydrogenase (LDH) was determined by continuous monitoring assay on the Hitachi-7600-020 auto
Table 2  Mean fluorescence intensity (MFI) of BLyS and its receptors in KM3 (x ± s, n = 30)

<table>
<thead>
<tr>
<th>Group</th>
<th>KM3 cell membrane (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>16.25 ± 4.56</td>
</tr>
<tr>
<td>BLyS</td>
<td>72.62 ± 3.50</td>
</tr>
<tr>
<td>BAFF-R</td>
<td>61.23 ± 5.36</td>
</tr>
<tr>
<td>BCMA</td>
<td>18.16 ± 1.16</td>
</tr>
<tr>
<td>TACI</td>
<td>54.27 ± 2.79</td>
</tr>
</tbody>
</table>

Compared with control IgG group, 1P < 0.01, 2P > 0.05.

Table 3  Mean fluorescence intensity (MFI) of BLyS and its receptors in CZ-1 (x ± s, n = 30)

<table>
<thead>
<tr>
<th>Group</th>
<th>CZ-1 cell membrane (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>15.75 ± 2.56</td>
</tr>
<tr>
<td>BLyS</td>
<td>60.36 ± 1.50</td>
</tr>
<tr>
<td>BAFF-R</td>
<td>43.58 ± 2.36</td>
</tr>
<tr>
<td>BCMA</td>
<td>14.16 ± 2.76</td>
</tr>
<tr>
<td>TACI</td>
<td>37.87 ± 1.83</td>
</tr>
</tbody>
</table>

Compared with control IgG group, 1P < 0.01, 2P > 0.05.

biochemical analyzer (Hitachi Koki Co. Ltd., Tokyo, Japan). And β2M level was analyzed by AxSYM autoimmuno-analyzer (Abbott Laboratories, IL, USA).

**Statistical analysis**

Mean value (x) and standard deviation (SD) were calculated by STATA 8.0 (Computer Resource Center, FL, USA). Related graphs were drawn by computer software. Data comparison was tested by t-test. P-values <0.05 were considered statistically significant.

**Results**

**Expression of BLyS and its receptors on MM cells**

We first wished to gain a better understanding of BLyS and its receptors on MM cells lines. Therefore, we used flow cytometric analyses to determine cell surface expression of BLyS and its receptors. Both KM-3 and CZ-1 cells bind BLyS at high levels, express BAFF-R, TACI, and are deficient for BCMA expression (Tables 2 and 3, Fig. 1A and B). BCMA has been typically found as an intracellular protein, so it is not surprising that the lack of BCMA expression on the cell surface. RT-PCR analysis showed that BLyS and its receptors mRNA are expressed in KM3 and CZ-1 cells (Fig. 1C), and BLyS mRNA is also expressed in patients with MM (Fig. 1D). Western blot analysis further confirmed that BLyS protein is expressed in KM3 and CZ-1 and in representative biopsy-derived MM patient samples (Fig. 1E and F). It also can be seen that BLyS protein is located in the KM3 plasma membrane (Fig. 1G).

**BLyS contributes to MM cell survival and proliferation in vitro**

We next examined the effect of BLyS on MM cell survival. KM3 and CZ-1 were treated with control IgG or BLyS antibody, and effects on cell survival were assessed. Cells cultured in the presence of IgG had greater viability compared to cells cultured in BLyS antibody (Fig. 2).

**Regulation of BLyS expression levels in MM cells in responsive to cytokines**

The aim of the next series of experiments was to investigate whether IFN-γ and IL-6 known to modulate BLyS expression on MM cell. KM3, CZ-1, and primary myeloma cells were treated for 3 d with 5 ng/mL IFN-γ or 25 ng/mL IL-6. IFN-γ and IL-6 were found to be prototype of activating cytokines. Representative results from the western blot are shown in Fig. 3A and B. FACS, ELISA, and RT–PCR results further confirmed that IFN-γ and IL-6 can induce BLyS expression on MM cells; representative results are shown in Table 4, Fig. 3A–D. Several studies have reported that BLyS gene expression and levels of membrane-associated and soluble BLyS were found to be regulated by cytokines, in particular IFN-γ. In MM cells, our data are identical with those reports. While the mechanism underlying the regulation of BLyS expression in response to cytokines is still poorly understood. So, our next experiments wished to gain a better understanding of it. We hypothesized that NF-κB might be involved in it. To evaluate the effects of NF-κB pathways on regulation of BLyS expression in response to cytokines, we first test the toxicity of BAY11-7082 (an IkB-α phosphorylation inhibitor) to MM cells and then the cells treated with BAY11-70829. According to cytotoxicity test, we found that IFN-γ-induced up regulation of BLyS was completely inhibited by BAY11-7082 treatment; representative results are shown (Fig. 3C–E). And Western blot analysis also showed that the expression of IkB-α is substantially
increased after the treatment of BAY11-7082, while the expression of P65 is decreased (Fig. 4). These results suggest that NF-κB pathways are involved in the regulation of BLyS expression. We also found that after the treatment of acenterine, another inhibitor of NF-κB, BLyS expression levels of MM cells themselves were significantly decreased (data not shown).

Correlation of LDH concentration and beta 2-microglobulin level with BLyS protein and expressions of BLyS mRNA

As LDH concentration in MM patient may reflect the tumor-bearing status, predict prognosis, and judge therapeutic outcomes, LDH activity was determined. Based on the concentration of LDH, the MM patients were divided into two groups: elevated LDH group and normal LDH group. BLyS and expression of BLyS mRNA between the two groups were compared (Table 5). It was found that there were significant differences in BLyS and expressions of BLyS mRNA between the two groups. Linear correlation analysis of the LDH concentration with BLyS and expressions of BLyS mRNA in MM patients revealed a significant correlation between them (P < 0.01 in all case, Fig. 6A, B).

Discussion

BLyS is mainly expressed in monocytes, macrophages, dendritic cells, and activated neutrophils (15–18). BCMA and BR3 are expressed by B lymphocytes, while TACI is expressed in B cells and activated T cells. BCMA and TACI can also bind with APRIL, while BAFF-R is a specific receptor of BLyS (19–21).

In this report, we provide evidence that BLyS is expressed by MM cells and is present in the bone marrow of patients with MM. Additionally, we also examined the expression of BLyS receptors in MM cells. We found that both KM-3 and CZ-1 cells express BAFF-R, TACI, and are deficient for BCMA expression on the cell surface. BCMA has been typically found as an intracellular protein(22), so it is not surprising that the lack of BCMA expression on the cell surface. While Novak et al. (23) have reported that ANBL-6, DP-6, KAS-6/1, and JMW cell lines express BLyS receptor BCMA and TACI, deficient for BAFF-R expression, and KP-6 cell line does not express any of BLyS receptors. The finding of variable BLyS receptor expression is of interest, because the mechanism of action of BLyS remains poorly understood, in part because of the complexity introduced by multiple receptors. To gain a better understanding of BLyS and its receptors in the pathogenesis of MM biology, it is necessary to thoroughly examine the expression levels of all three receptors on a large cohort of MM samples. The altered expression of BLyS receptors may be because of different cell types. Various studies have shown that BLyS is a stimulator factor for B-cell homeostasis and survival; we have shown that BLyS protein is located in the KM3 plasma membrane, so we next examined the effect of BLyS on MM cells. We demonstrated that neutralization of BLyS protein by specific BLyS antibody decrease KM3 and CZ-1 cells survival in vitro. Cells cultured in the presence of IgG had greater viability compared to cells cultured in BLyS antibody. Abnormal proliferation of malignant plasmocytes is considered to the central link in the pathogenesis of MM. Some studies showed that BLyS and its receptors had regulatory effects on the proliferation and viability of plasmocytes, and therefore BLyS and its receptors may play some role in the pathogenesis of MM (23–25).

Expression of membrane-bound and soluble BLyS and BLyS mRNA was investigated following cytokine treatment. We used IFN-γ, which has been confirmed to be a stimulator of both membrane-bound and soluble BLyS expression, and we also found that it could enhance BLyS gene expression level. Interestingly, the survival of
MM cells is highly dependent upon the presence of certain growth-promoting environmental factors. One of these MM survival factors is the pleiotropic cytokine IL-6, so we next examined its regulation of BlyS expression level of MM cells; we found that IL-6 could also enhance BlyS gene and protein expression level. The importance of IL-6 as a MM survival factor has been firmly established and anti-IL-6 strategies are under investigation in MM treatment (26, 27). As IL-6 could enhance BlyS gene and protein expression level, our reports further confirmed that anti-IL-6 strategies are meaningful. In addition to the NF-κB site, the IL-6 promoter contains binding sites for AP-1, C/EBP, CREB, and Sp1, all of which can modulate IL-6 promoter activity under cell type- and stimulus-specific conditions. Sarah et al. (28) have also shown that the specific ERK inhibitor U0126 treatment strongly inhibited Sp1 recruitment at the IL-6 promoter and then completely abolished IL-6 transcription. As the effect of U0126 on transcription factor recruitment at the IL-6 promoter and blockade of IL-6 transcription, we propose that these transcriptional factors might be attractive therapeutic targets for the treatment of MM. Meanwhile, the rate of patients with hyperviscosity syndrome is high. We have shown that acenterine decrease the BlyS expression level in MM. Our study confirmed that acenterine not merely has positive

![Figure 3](image)

**Figure 3** Regulation of BlyS expression levels in MM cells in response to cytokines. (A, B) MM Cells were cultured in RPMI(GIBCO) containing 10% fetal bovine serum (FBS) supplemented with cytokines (5 ng/mL IFN-γ or 25 ng/mL IL-6) or not. Western blot analysis of BlyS protein expression after 1, 2, and 3 d, respectively. (C, D) MM cells were cultured for 2 d with or without cytokines. Quantitative PCR was conducted as reported in 'materials and methods'. The amount of released BlyS was determined by ELISA in the medium of KM3 cell cultures, which was conditioned for 2 d. (E) MM Cells were cultured in RPMI containing 10% FBS supplemented with BAY11-7082 alone or in combination with INF-γ after 2 d, Western blot analysis of BlyS protein expression.
functions in controlling and reducing hyperviscosity symptoms when used in MM, it could also contribute to MM cell apoptosis.

Studies (16) have reported that cytokines, such as interleukin 10 (IL-10), IFN-γ, and TNF-α augment BLyS expression in monocytes, macrophages, and dendritic cells, while the mechanism underlying the regulation of BLyS levels in response to cytokines remains to be studied. Many pathogens, growth factors, cytokines, and carcinogens that induce activation of the classic pathway and heterodimer NF-κB1/RELA (p50/p65) have been implicated in promotion and pathogenesis of cancer. Induced activation by cytotoxic agents and constitutive activation of the classic pathway by oncogenic activation

Table 4 Mean fluorescence intensity (MFI) of BLyS in MM cells after the treatment of IFN-γ and IL-6 (x ± s, n = 30)

<table>
<thead>
<tr>
<th>Group</th>
<th>BLyS expression (MFI)</th>
<th>Patient1</th>
<th>Patient2</th>
<th>Patient3</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM3</td>
<td>72.62 ± 3.50</td>
<td>53.28 ± 6.28</td>
<td>69.15 ± 6.57</td>
<td>55.87 ± 7.02</td>
</tr>
<tr>
<td>CZ-1</td>
<td>60.36 ± 1.50</td>
<td>129.15 ± 7.27</td>
<td>121.84 ± 9.32</td>
<td>100.15 ± 9.28</td>
</tr>
<tr>
<td>IFN-γ¹</td>
<td>156.36 ± 9.78</td>
<td>147.46 ± 9.73</td>
<td>139.15 ± 4.08</td>
<td>122.43 ± 7.06</td>
</tr>
<tr>
<td>IL-6²</td>
<td>153.62 ± 7.89</td>
<td>129.15 ± 7.27</td>
<td>121.84 ± 9.32</td>
<td>100.15 ± 9.28</td>
</tr>
</tbody>
</table>

¹²Compared with control group, P < 0.01.

Table 5 The expression levels of BLyS protein and BLyS mRNA in PBMCs in elevated LDH group and normal LDH group

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>LDH (U/L)</th>
<th>BLyS (ng/mL)</th>
<th>BLyS mRNA (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal LDH</td>
<td>20</td>
<td>153 ± 36</td>
<td>5.66 ± 2.82</td>
<td>1.24 ± 0.10</td>
</tr>
<tr>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>11</td>
<td>400 ± 186¹</td>
<td>9.42 ± 2.66¹</td>
<td>1.48 ± 0.10¹</td>
</tr>
<tr>
<td>group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹Compared with normal LDH group.
of upstream receptor and non-receptor tyrosine kinases has been shown to promote therapeutic resistance and cancer cell survival (5–14). So, we hypothesized that NF-κB might be involved in the mechanism of the regulation of BLyS levels in response to cytokines. After the treatment of BAY11-7082, an IkB-α phosphorylation inhibitor, we found that IFN-γ induced up regulation of BLyS was completely inhibited. And more importantly, we found BLyS expression level of MM cells was decreased as the dosage increased. Acenterine, another generally acknowledged inhibitor of NF-κB further confirmed our reports, showing that NF-κB is an important regulator of BLyS expression in response to cytokines. 

LDH concentration is a reflection of the tumor-bearing status, and may be used as a predictor for the prognosis and therapeutic outcomes of the disease. We divided the patients into two groups based on the LDH concentration to observe changes in BLyS and expressions of BLyS mRNA. The results showed that BLyS concentration and expressions of BLyS mRNA were significantly higher than those in normal LDH group. With LDH concentration increasing, BLyS concentration and expressions of BLyS mRNA significantly correlated with the LDH level, suggesting indirectly that BLyS concentration and expressions of BLyS mRNA are related to the prognosis and outcomes of the disease.

β2M, one of the variables was used in predicting MM patient survival. So, we studied correlations of β2M with our target genes. The results showed that BLyS concentration and expressions of BLyS in those high level of β2M patients were significantly higher than those in lower β2M group. With β2M concentration increasing, BLyS concentration and expressions of BLyS mRNA significantly correlated with the β2M level. It also has the implication of BLyS related to the prognosis and outcomes of the disease.

In summary, our findings confirmed that BLyS and its receptors are expressed in MM cells, including KM3 and CZ-1. Additionally, we provide evidence that BLyS protein is located in the KM3 plasma membrane, playing an important role in the survival and proliferation of MM cells. We also found that IFN-γ and IL-6 can induce BLyS expression on MM cells, while after the treatment of BAY11-7082, an IkB-α phosphorylation inhibitor, IFN-γ and IL-6 induced up regulation of BLyS was completely inhibited, suggesting that nuclear factor κB (NF-κB) might be involved in the mechanism of the regulation of BLyS levels in response to cytokines. Finally, we linear correlation analysis of the LDH concentration and β2M-microglobulin (β2M) level with BLyS and expressions of BLyS mRNA in MM patients revealed a significant correlation between them.

References