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A role of both NF-κB pathways in expression and transcription regulation of BAFF-R gene in multiple myeloma cells

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Abstract B-lymphocyte stimulator (BAFF) is a recently recognized member of the tumor necrosis factor ligand family (TNF) and a potent cell-survival factor expressed in many hematopoietic cells. BAFF regulates B-cell survival, differentiation, and proliferation by binding to three TNF receptors: TACI, BCMA, and BAFF-R. The mechanism involved in BAFF-R gene expression and regulation remains elusive. In this study, we examined BAFF-R gene expression, function, and regulation in multiple myeloma (KM3) cells. It was found that BAFF–BAFF-R induced cell survival by activating NF-κB1 pathway and NF-κB2 pathway. It was also found that NF-κB was an important transcription factor involved in regulating BAFF-R expression through one NF-κB binding site in the BAFF-R promoter, suggesting that inhibiting NF-κB could decrease the expression of BAFF-R mRNA and protein, and promote activity of BAFF-R gene. Our findings indicate that both NF-κB pathways are involved in the regulation of BAFF-R gene and the NF-κB-binding site of BAFF-R may be a new therapeutic target in this disease.

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Keywords B-lymphocyte stimulator (BAFF) · B-lymphocyte stimulator receptor (BAFF-R) · Multiple myeloma · NF-κB pathway

Introduction

B-lymphocyte stimulator (BAFF), also known as BAFF, TALL-1, THANK, zTNF4, and TNFSF13B, exists as a homotrimer in hematopoietic cells. BAFF belongs to the tumor necrosis factor (TNF) ligand family, playing crucial roles in B-cell homeostasis, tolerance, and malignancy [1–3]. Three BAFF receptors have been identified: BCMA (B-cell maturation antigen), TACI (transmembrane activator, calcium modulator, and cyclophilin ligand interactor), and BAFF-R/BR3 (BAFF receptor/BLyS receptor 3), of which BAFF-R is specific for BAFF and mediates most BAFF-elicited B cell survival signals [4].

BAFF is expressed in various cell types, including dendritic cells, monocytes, macrophages, activated T cells, neutrophils, and antigen-presenting cells [1–3]. While BAFF is necessary for B cell survival, its over expression may lead to immunologic derangement [5], such as in systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, and autoimmune thyroid disease. Several studies [6–8] have shown that malignant B cells from patients with non-Hodgkin lymphoma, chronic lymphocytic leukemia, and MM also express abnormal levels of BAFF protein, thus, protecting malignant B cells from spontaneous apoptosis. The mechanism may be that BAFF binding to the three receptors could activate the NF-κB pathway to promote growth, differentiation, and maturation of the cells [9].

Nuclear factor-κB (NF-κB) is a family of important transcription factors that regulate B-cell development, maintenance, and stimulation [10, 11]. B-cell receptor

signaling and some TNF cytokine signaling induce NF-κB activation by means of the NF-κB1 pathway, where phosphorylation of NF-κB inhibitor IκB by IκB kinase (IKK) complex allows cytoplasmic protein p65 and p50 to translocate to the nucleus when their nuclear localization sequence (NLS) is exposed. Alternatively, NF-κB also can be activated through the NF-κB2 pathway, in which the NF-κB2 precursor p100 is proteolytically cleaved to activate p52 through NF-κB-inducing kinase (NIK) and IKKα [12–14]. Activated p50, p65, and p52 proteins are able to translocate into the nucleus, where it binds to a specific consensus sequence in the DNA [13], and subsequently activates NF-κB-regulated genes [15].

Constitutive NF-κB activation is a molecular "signature," which has been reported in several malignant B-cell types [16]. Studies [17] suggest that MM cells may constitutively activate NF-κB, and seemingly enhance myeloma cell survival. However, the relative contribution of each NF-κB pathway to MM cell survival has not been described. Although it is known that NF-κB and BAFF-R are important components of cellular pathways that promote MM cell survival and proliferation, the mechanism underlying their interactions in MM cells remains unclear. In this study, we examined the role of each NF-κB pathway in BAFF–BAFF-R induced MM cell survival and the mechanism underlying NF-κB and BAFF-R interactions.

Methods and methods

Materials

Materials used in this study were pGL3-Basic, pGL3-Control, psv-β-gal vectors, β-gal assay kit, and luciferase assay system (Promega, Beijing, China); PCR reagents (TaKaRa, Dalian, China); inhibitor of NF-κB BAY11-7082 (Invitrogen Diego, CA, USA); WST-1 Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Nantong, JS, China); Trizol (Invitrogen Corporation, Grand Island, NY, USA); BAFF-R polyclonal antibody (R&D Systems, Minneapolis, MN); NF-κB p50, p52, p65, and IκB-α (BioLegend, San Diego, CA); nuclear and cytoplasmic extraction reagents (Pirece Biotechnology, Rockford, IL, USA); and recombinant human BAFF (rhBAFF) (R&D Systems, Minneapolis, MN).

Cell culture

MM cell line KM3 cells (Second Military Medical University, Shanghai, China) were cultured in RPMI-1640 (Gibco, Rockville, MD) supplemented with 15% fetal bovine serum (FBS, Gibco) and penicillin–streptomycin–glutamine (culture media) in 5% CO_2 in air at 37°C. Cells were supplemented with or without stimuli.

RNA extraction and RT-PCR analysis

The Trizol reagent was employed to isolate total RNA from cells. RNA purity was determined by the OD_{260}/OD_{280} ratio. Total RNA was prepared using the protocol from First-Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Total RNA (5 μg) was employed to perform first-strand cDNA synthesis by reverse transcription. These sequences of the BAFF-R primers were previously described [18] as follows: 5′-TGGGTCTGGTGAGCTGGA-3′ (forward) and 5′-CCGGAGACAGAATGATGACCTT-3′ (reverse). The sense and antisense primers of β_2M are 5′-CTATCCAGCG TACTCCAA-3′ and 5′-GCAGGCATACTCATCTTTT-3′. The polymerase chain reaction conditions were 94°C for 20 s, 58°C for 40 s, and 72°C for 40 s for 32 cycles. The expected PCR products were 120 bp for BAFF-R and 242 bp for β_2M. The PCR products were analyzed on 2% agarose gels by ethidium bromide staining. Expression of the message level was measured with an ABI PRISM 7500 Sequence Detection System and normalized to β_2M mRNA level.

Flow cytometry

To analyze the surface membrane expression of BAFF-R, KM3 cells (1×10^6) were collected and washed with phosphate-buffered saline (PBS), and incubated with 20 μl FITC anti-human BAFF-R or isotype control antibody for 20 min at room temperature. Cells that were not incubated with FITC anti-human BAFF-R served as the negative control. Cells were washed once with PBS and re-suspended in PBS, analyzed by flow cytometry, and associated cell QUEST software (Becton–Dickinson, Franklin Lakes, NJ, USA). Isotype and fluorochrome controls were done for each experiment as the basic control.

WST-1 cell viability assay

Cell viability was measured by modified MTT assay. In brief, 4×10^3 cells were cultured in a flat-bottomed 96-well plate in a final volume of 200 μl/well culture medium. Then, KM3 cells were administered with 100 μg/ml goat IgG or goat polyclonal BAFF-R antibody for 48 h. 20 μl WST-1(Beyotime, Haimen, China) was added to each well and cells were cultured for additional 3 h in a humidified atmosphere. The samples were measured using a Benchmark microplate reader (BIO-TEK Elx800) at 450 nm absorbance wavelength. The result was represented through three separate experiments.

Immunoblot analysis

The method of protein extraction and Western blot analysis have been described elsewhere [19]. In brief, cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate) containing complete protease inhibitor cocktail (Pirece Biotechnology, IL, USA) and placed on ice for 30 min. The supernatant was the whole cell protein, which was collected and concentrated by centrifugation at 15,000×*g* for 5 min at 4°C. Nuclear and cytosolic extracts were prepared according to the manufacturer's instruction (Pirece Biotechnology, IL, USA) or the steps in the literature [20]. Lysates were normalized for total protein (30 μg) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–15% gradient gels; Bio-Rad, Hercules, CA) and immunoblot assay. The blots were incubated with secondary antibodies conjugated with horseradish peroxidase, and then prepared for enhanced chemiluminescence (ECL) detection and subsequent autoradiography with Super RX film (Beyotime, JS, China). Defined sections of the film were scanned for density measurement and analyzed using the image software from the National Institutes of Health (Bethesda, MD).

TUNEL assay of apoptotic cells

TdT-mediated dUTP nick end labeling (TUNEL) assay was performed with One Step TUNEL Apoptosis Kit (Beyotime, JS, China). KM3 cells (2×10^6) were collected and washed with PBS two times. The cell suspension was put onto poly-L-lysine-coated glass slides, fixed, permeabilized, and incubated with TUNEL reaction mixture at 37°C for 1 h as described in the manufacturer's protocol. The cells were analyzed by fluorescence microscopy.

Transfection, luciferase and beta-galactosidase (*β*-gal) assays

The KM3 cells were plated in 6-well dishes at 2×10^6 per well in 3 ml RPMI 1640 with 15% fetal bovine serum and incubated at 37°C before transfection. One of the BAFF-R promoter-luciferase constructs and psv-*β*-gal vector were cotransfected into each well using 3 μl lipofectamine DMRIE-C reagent according to the manufacturer's instruction. The latter plasmid served as an internal control for transfection efficiency. 4 μg DNA/well at a ratio of 4:1 for the experiment versus psv-*β*-gal was used for each transfection. After 48 h, cells were harvested and washed with 1 × PBS. Cell lysates were prepared in Reporter Lysis Buffer (Promega). Luciferase activity was measured by luminometry according to the manufacturer's instruction by using the kit from Promega. *β*-gal activity was determined by absorbance at 420 nm. The promoter activity was expressed as a ratio of luciferase to *β*-gal activity (relative luciferase activity) in each sample. In the same experiment, each transfection was performed in triplicate, and meanwhile pGL3-Basic plasmid was transfected as the negative control, and pGL3-control plasmid as the positive control.

BAFF-R ELISA

A sandwich ELISA was employed to quantitate sBAFF-R protein levels in cell culture supernatants. The experiment was done according to the manufacturer's instruction. Plates were read at 450 nm absorbance using a Benchmark microplate reader (BIO-TEK Elx800). A standard curve was created for each plate. Every parameter was measured five times and the results were averaged.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay (EMSA) was employed to detect the activity of NF-κB. Nuclear proteins were detected in the same way as the Immunoblot analysis. A 20-mer double-stranded BAFF-R promoter oligonucleotide (5′-AGAAAGGGGGGCCCCAGGCG-3′) (the putative NF-κB sites were underlined) and site-directed mutagenesis of the BAFF-R promoter oligonucleotide (5′-AGAAA GGTTGGCCCCAGGCG-3′) (the mutation sites were underlined) were labeled with biotin. Corresponding unlabeled oligonucleotide probes were used for competition experiments. Nuclear protein extract (4 mg) was incubated with the antibodies to p65 and p52 for 20 min at room temperature, followed by addition of the probes, and incubation at room temperature for 30 min. Samples were loaded on a 6.5% nondenaturing acrylamide gel, run at 180 V for 1.5 h by using 0.25 TBE buffer, and electroblotted to a nylon membrane. After incubation in blocking buffer for 15 min at room temperature, the membrane was incubated with streptavidin-HRP conjugate for 30 min at room temperature. The membrane was washed and visualized with ECL, and then autoradiographed with Super RX film (Beyotime, JS, China).

Immunofluorescence microscopy

KM3 cells (1×10^6) were collected, washed with PBS, fixed by 8% paraformaldehyde at room temperature for 40 min, and centrifuged at 1,000 rpm for 5 min at 4°C. The supernatant was discarded. Then, 50 μl PBS was employed to suspend cells and the cell suspension was put onto poly-L-lysine-coated glass slides and dried. All cells were blocked with the same 10% normal serum blocking solution-species as the secondary antibody. The glass slides

were then incubated overnight at 4°C with primary antibodies against BAFF-R. On the next day, FITC conjugated (1:1000, Jackson lab) secondary antibodies were added in dark room and incubated for 2–3 h at 4°C. After three washes in PBS, cells were mounted onto slides with glycerol containing 1,4-diazabicyclo (2,2,2) octane (DABCO), and observed under a Leica fluorescence microscope (Germany).

Results

Expression of BAFF-R in MM cells

To observe whether BAFF-R was expressed in KM3 cells, total RNA was extracted from KM3 cells with or without rhBAFF treatment and reverse-transcribed for PCR analysis using primers for the human BAFF-R gene. It was found that BAFF-R mRNA was expressed in KM3 cells, and this expression was increased significantly within 24 h of stimulation with rhBAFF. β_2M level was measured as control (Fig. 1a). Localization of BAFF-R protein was then determined by immunofluorescence microscopy. It was found that BAFF-R protein was located in the plasmamembrane of KM3 cells (Fig. 1b). To see whether this increase at the RNA level was correlated with an increase in the amount of BAFF-R protein, membrane-bound BAFF-R protein was detected on KM3 cell surfaces by flow cytometry after treatment of rhBAFF for 48 h (Fig. 1c). ELISA was employed to quantitate sBAFF-R protein levels in cell culture supernatants (Fig. 1d). It was found that rhBAFF increased BAFF-R mRNA and protein expression in KM3 cells.

BAFF-R expression contributes to MM cell survival and growth in vitro

To determine whether BAFF-R protein was involved in KM3 cell survival, KM3 cells were treated with control IgG or BAFF-R antibody, and the effect on MM cell survival was assessed. The growth of KM3 cells was inhibited when BAFF-R bioactivity was blocked by specific BAFF-R antibody in 48 h as compared with the control IgG (Fig. 2a). To determine whether BAFF-R antibody inhibited cell growth due to cell death, BAFF-R antibody-treated cells were subjected to TUNEL assays to detect apoptosis. Based on the result of WST-1, 15 μg/ml was chosen as the final concentration of BAFF-R antibody. As shown in Fig. 2b, there were significantly more apoptotic (green fluorescent) cells after treatment with BAFF-R antibody (right panel) as compared with the control IgG (left panel). Western blot analysis of the Bcl-2 family survival proteins also showed that the expression of Bcl-2 protein was down-regulated by approximately 20% and the expression of Bax protein was up-regulated by approximately 40% after inhibition of BAFF-R expression (Fig. 2c). BAFF-R antibody induced apoptosis of KM3. These results suggested that BAFF-R enhanced in the survival and growth of KM3 cells.

Effects of rhBAFF and BAFF-R on NF-κB signaling pathways in MM cells

NF-κB pathway activation involves processing of subsequent translocation of p50, p65, and p52 to the nucleus. Western blot in this study showed that rhBAFF induced translocation of the proteins of p50, p65, and p52 to the nucleus in KM3 cells (Fig. 3a), suggesting that the NF-κB pathway was activated. We next determined whether NF-κB activation by rhBAFF would lead to degradation of IκB-α protein. It was found that rhBAFF treatment lowered the levels of IκBα expression (Fig. 3b), suggesting that rhBAFF could induce the NF-κB1 and NF-κB2 pathways in KM3 cells.

To verify the selective capacity of BAFF-R in activating the NF-κB pathway, KM3 cells were cultured with rhBAFF and increasing concentrations of anti-BAFF-R antibody. Anti-BAFF-R at 15 μg/ml greatly inhibited BAFF-induced translocation of the proteins of p50, p65, and p52 (Fig. 4a). Anti-BAFF-R also inhibited degradation of IκB-α expression (Fig. 4b). These data indicated that BAFF-R activated the NF-κB1 and NF-κB2 pathways in KM3 cells.

Transcription factor NF-κB binds to BAFF-R promoter

To determine whether BAFF-R was targeted by NF-κB, BAFF-R promoter gene probes were synthesized. To assess the binding activity of NF-κB in BAFF-R promoter, EMSA was performed using the nuclear extract from KM3 cells and specific antibodies to NF-κB family members. Knowing that p65 and p52 proteins are the principle members of the NF-κB family, we chose them to test the transcription factor binding on the BAFF-R gene promoter. The p65 and p52 proteins were found in the NF-κB complex binding to the BAFF-R promoter (Fig. 5). The results showed that BAFF-R gene promoter contained a binding site for the NF-κB transcription factor.

NF-κB inhibitor blocks activation of the NF-κB pathway and the activity of BAFF-R promoter

The KM3 cells were pre-incubated with or without varying concentrations of BAY11-7082 (the specific NF-κB inhibitor) for 48 h. First, survival of KM3 cells was measured by WST-1 assay. Viability of KM3 cells cultured

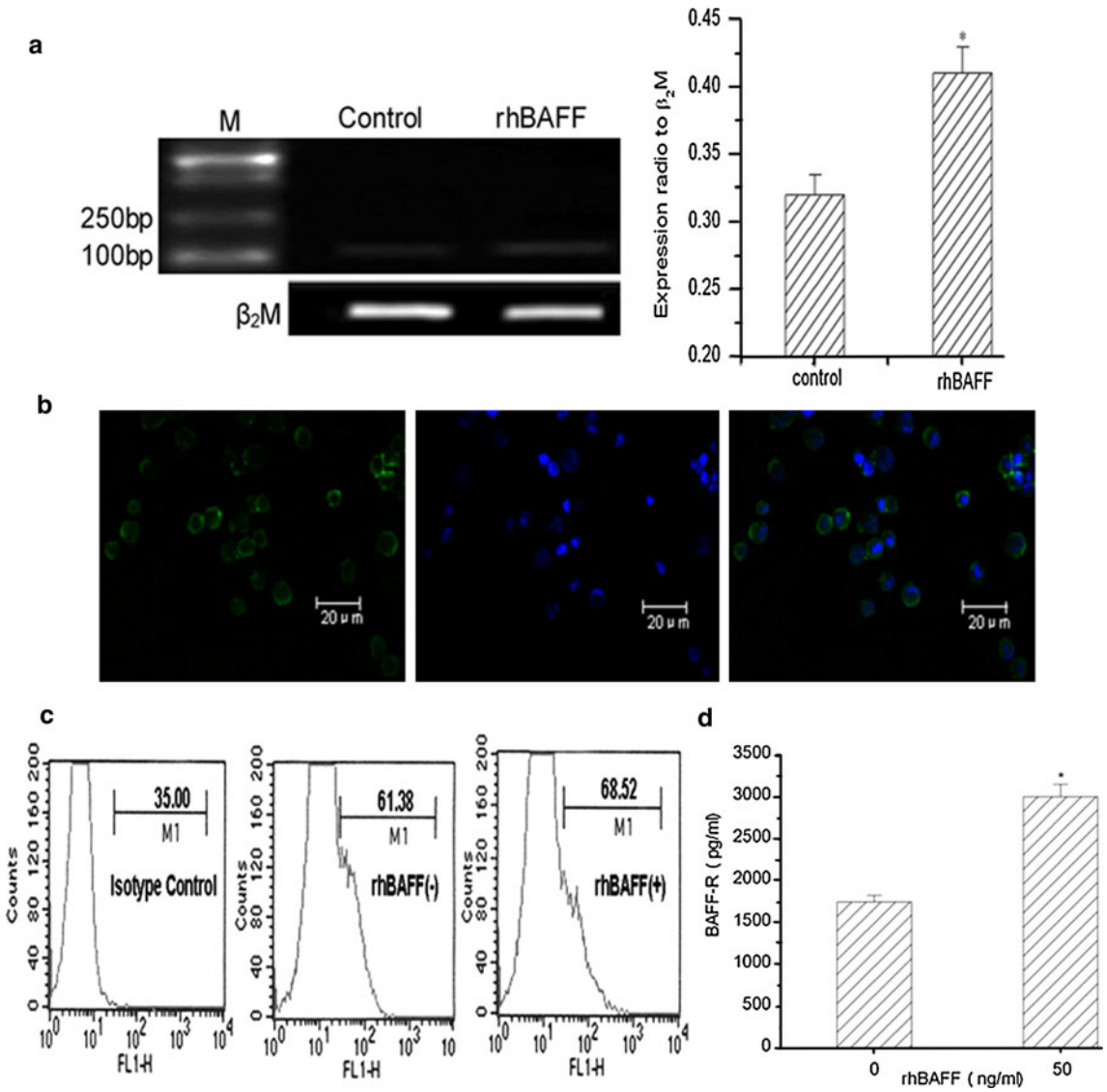

Fig. 1 The expression of BAFF-R in MM cells. **a** KM3 cells were cultured with or without rhBAFF (50 ng/ml) for 24 h. BAFF-R mRNA and β_2M mRNA were cloned by RT-PCR (*compared with control, $P < 0.05$). **b** Immunofluorescence microscopic analysis of BAFF-R protein localization in KM3 cells. BAFF-R protein was stained for FITC fluorescence (the *left graph*), the nuclear marker for hochest fluorescence (the *middle graph*), and the *right graph* was merged by the *left* and *middle graphs*. **c** KM3 cells were cultured with or without rhBAFF (50 ng/ml) for 48 h, then examined by flow cytometry for surface expression of BAFF-R after labeling with FITC anti-human BAFF-R primary antibody or isotype control antibody. **d** ELISA detected the soluble form of BAFF-R in KM3 cells. *compared with negative control, $P < 0.05$

with BAY11-7082 was decreased significantly compared with that of cells cultured in the medium alone (Fig. 6a). Second, total RNA was extracted from these cells, and RT-PCR was performed by using the human BAFF-R primers. As shown in Fig. 6b, BAY11-7082 abrogated the BAFF-R gene expression. To see whether this increase in the amount of RNA level was correlated with increased BAFF-R protein, cells and supernatants were harvested, and sBAFF-R protein levels were quantitated by ELISA (Fig. 6c). Cell extracts were then subjected to SDS-PAGE, and Western blot was performed using the BAFF-R antibody. It was found that BAFF-R protein decreased significantly in a dosage-dependent manner after BAY11-7082 treatment (Fig. 6d), indicating that the NF-κB pathway played a critical role in the regulation of BAFF-R and survival of KM3 cells. Then, luciferase assay was performed to evaluate transcriptional activity of the BAFF-R promoter. A region of the gene containing the 5′ promoter region of the human BAFF-R gene was fused to the luciferase reporter gene, and the promoter-reporter construct was transfected in KM3 cells. Relative luciferase activity was assayed in cells treated with or without BAY11-7082. As shown in Fig. 7, BAY11-7082 treatment resulted in a significant decrease in the amount of

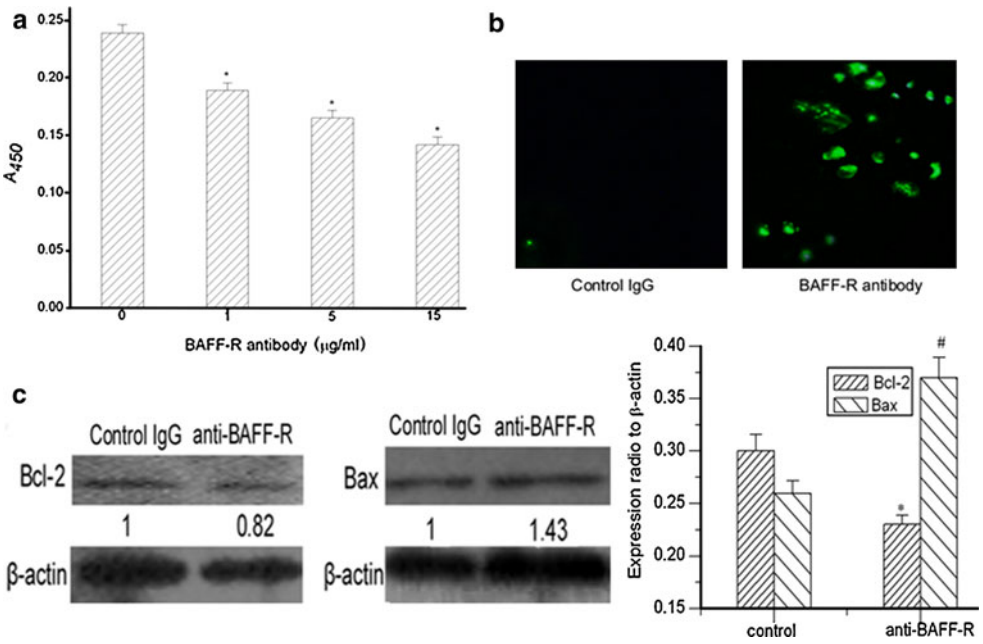

Fig. 2 BAFF-R expression contributes to MM cell survival and growth in vitro. **a** WST-1 proliferation assay of KM3 cells treated with control IgG or different concentrations of BAFF-R antibody. *Compared with negative control, $P < 0.05$. **b** TUNEL analysis of KM3 cells treated with control IgG or BAFF-R antibody. Free DNA fragments in apoptotic cells were labeled with FITC fluorescence. **c** KM3 cells were cultured with control IgG or BAFF-R antibody (15 μg/ml). Immunoblot analysis the expression of Bcl-2 and Bax. Bcl-2 and Bax levels were normalized to those of β-actin. (Statistical differences compared with the controls were given as $^{*}P < 0.05$, $^{\#}P < 0.05$)

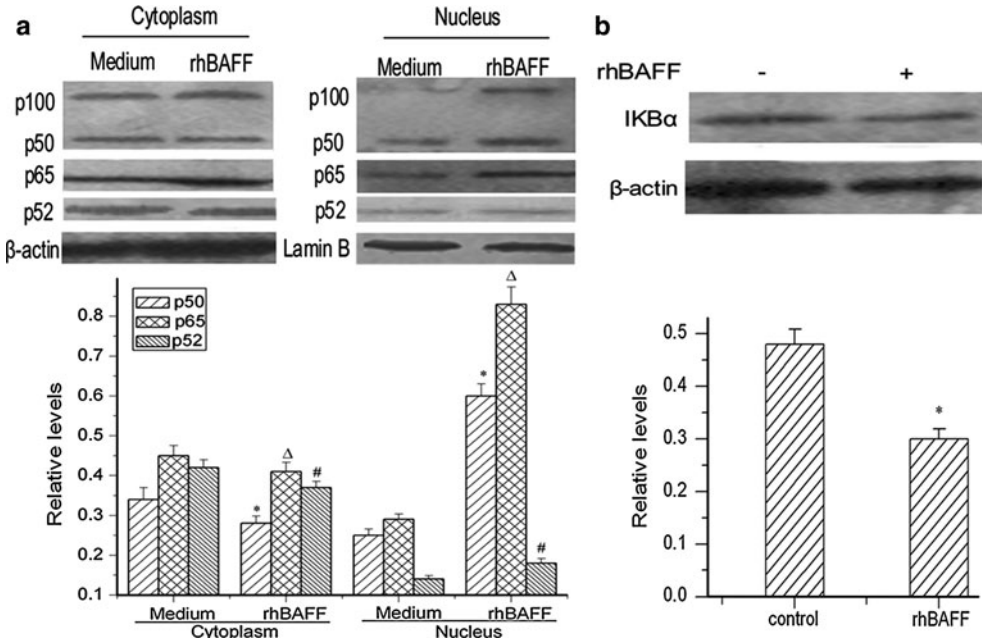

Fig. 3 Effects of rhBAFF on the NF-κB signaling pathway in MM cells. **a** Immunoblot analyzes with anti-p100/p50 or anti-p65 or anti-p52 antibodies. The equal loading in each lane was evaluated by stripping the blot and probing it with antibodies specific to β-actin (for cytoplasmic extracts) or Lamin B (for nuclear extracts). Translocation of p50, p65, p52 proteins to the nucleus was seen in KM3 cells treated with rhBAFF (50 ng/ml) for 48 h. (Statistical differences compared with the controls were given as $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{\#}P < 0.05$). **b** Immunoblot analysis with anti-IκBα antibodies. Degradation of IκBα protein was observed in KM3 cells treated with rhBAFF. (*compared with control, $P < 0.05$)

luciferase activity when compared with untreated cells. These results indicated that the putative NF-κB site on the BAFF-R promoter was in fact a true NF-κB-binding site, further suggesting that NF-κB pathway was indeed involved in the observed up-regulation of expression of the BAFF-R gene via transcriptional event by means of one NF-κB site on the BAFF-R gene promoter.

Discussion

B cell-activating factor is a member of the TNF ligand family and can promote maturation and survival of B-lymphocytes at least in the early stages of cell-cycle progression. Some studies [21–23] reported that BAFF binds with three receptors (TACI, BCMA, and BAFF-R),

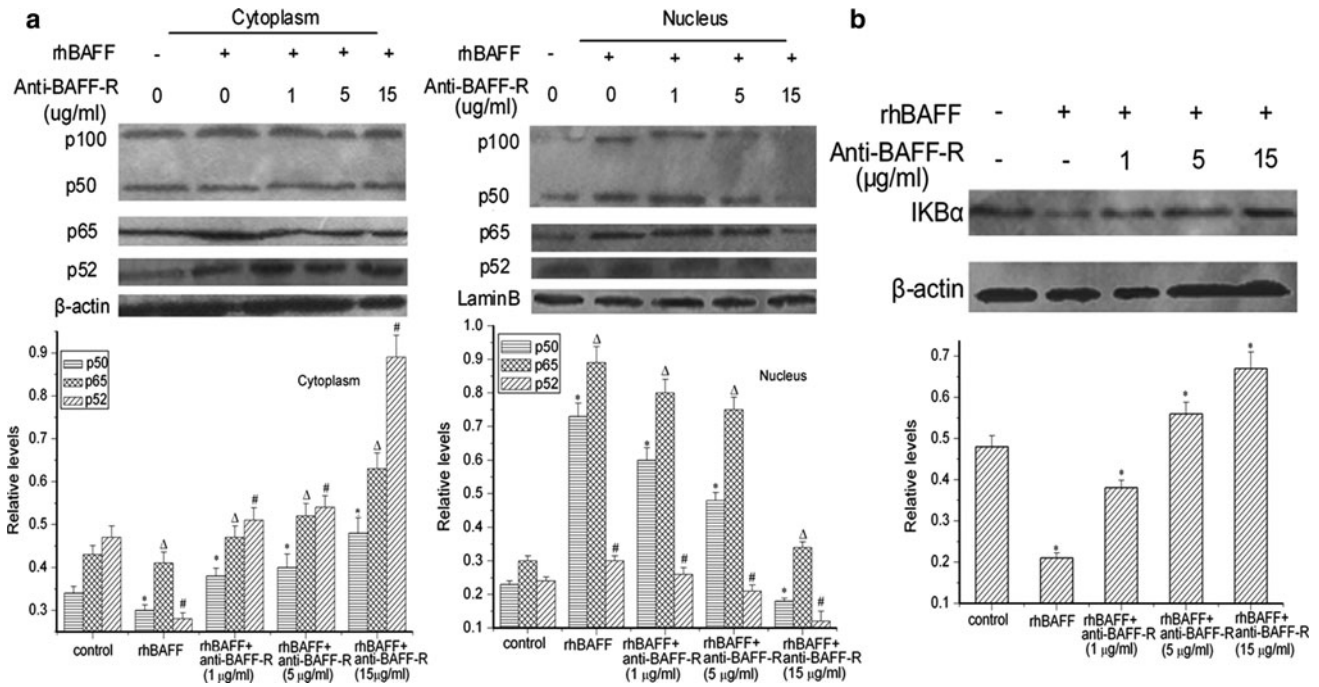

Fig. 4 Effects of BAFF-R on the NF-κB signaling pathway in MM cells. **a** KM3 cells were cultured for 48 h with or without rhBAFF (50 ng/ml) and anti-BAFF-R at the indicated concentrations. Immunoblot analyzes the translocation of p50, p65, and p52 proteins to the nucleus. Anti-BAFF-R at 15 μg/ml inhibited nuclear translocation of p50, p65, and p52 proteins induced by rhBAFF. (Statistical differences compared with the controls were given as $^{*}P < 0.05$, $^{\triangle}P < 0.05$, $^{\#}P < 0.05$). **b** Immunoblot analysis with anti-IκBα antibodies. KM3 cells were cultured with or without rhBAFF (50 ng/ml) for 120 min. Anti-BAFF-R inhibited the degradation of IκBα protein by rhBAFF. (*compared with control, $P < 0.05$)

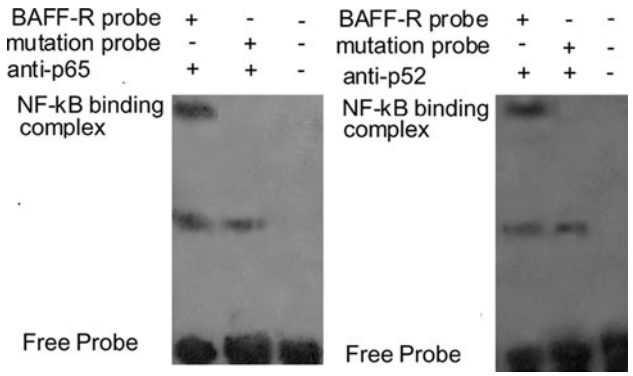

Fig. 5 Transcription factor NF-κB binds to the BAFF-R promoter. EMSA analysis NF-κB binding to the BAFF-R promoter. Nuclear extracts from KM3 cells were incubated with BAFF-R-NF-κB binding site oligonucleotides. BAFF-R-NF-κB cold probe, BAFF-R-NF-κB probe and antibody to p65 and antibody to p52 were added to the binding reaction mixture

and BAFF signaling/function is predominantly by means of high-affinity binding of BAFF to BAFF-R. Other studies [24, 25] also demonstrated that the BAFF ligand could activate the NF-κB pathway via BAFF-R binding. However, there is little knowledge about transcriptional regulation of BAFF-R in MM. Therefore, our experiment was to verify the selective capacity of BAFF-R in activating each NF-κB pathway and to understand the regulation of the BAFF-R gene.

Various studies [26, 27] also have shown that inhibiting BAFF through the use of decoy receptors or blocking antibodies could reverse the apoptotic process in malignant B cells. However, whether constitutive BAFF–BAFF-R expression is directly involved in MM cell survival remains unclear. It was found in the present study that BAFF-R protein was constitutively expressed in MM cell line (KM3). We demonstrated not only that cell surface bound BAFF-R protein was present in KM3 cell line but also that rhBAFF increased BAFF-R mRNA and protein expression of KM3 cells (Fig. 1). We also confirmed that neutralization of BAFF-R protein by specific BAFF-R antibody decreased KM3 cell survival in vitro (Fig. 2a, b). In addition, specific BAFF-R antibody changed the expression of Bcl-2 family survival proteins, thus, reducing the proliferation and apoptosis of KM3 cells (Fig. 2c). It is well known that NF-κB regulates the expression of several antiapoptotic proteins, including Bcl-2 and Bcl-xL, and we considered that BAFF-R decreased the expression of Bcl-2 via the NF-κB pathway. Figure 4a, b demonstrated that signaling through BAFF-R was necessary and sufficient to activate the NF-κB1 and NF-κB2 pathways in KM3 cells, at least under the in vitro culture conditions employed in

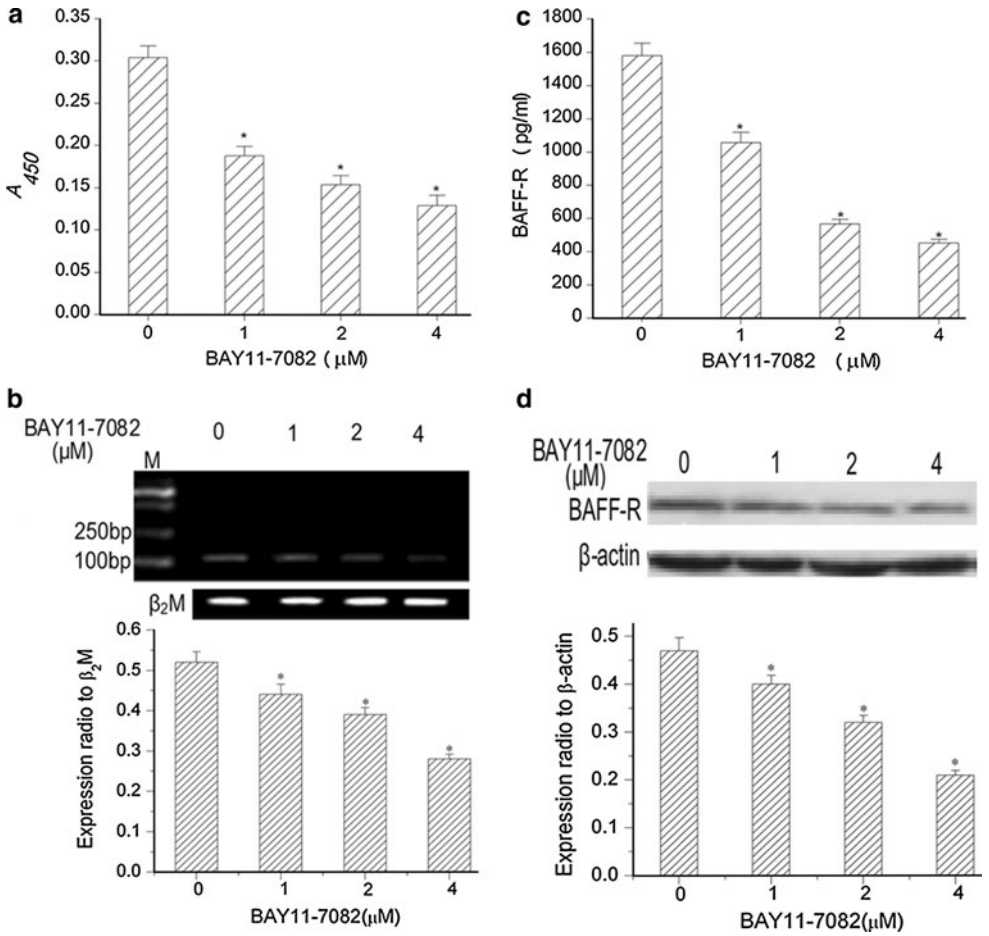

Fig. 6 NF-κB inhibitor blocks activation of the NF-κB pathway. **a** KM3 cells were either unstimulated (*first pillar*) or stimulated with different concentrations of BAY11-7082 for 48 h. Then WST-1 tested cell proliferation. The capacity of cell survival was decreased by BAY11-7082 treatment. *compared with negative control, $P < 0.05$. **b** RT-PCR of the BAFF-R and β_2M genes in KM3 cells. Cells were either unstimulated or stimulated with varying concentrations of BAY11-7082 for 48 h (*compared with control, $P < 0.05$). **c** KM3 cells were stimulated with different concentrations of BAY11-7082 for 48 h and assessment of secreted soluble BAFF-R levels by ELISA. Data shown represent mean ± SD of 5 separate experiments. *compared with negative control, $P < 0.05$. **d** Immunoblot of BAFF-R membrane bound protein in KM3 cells. Cells were harvested and either untreated or treated with different concentrations of BAY11-7082. Whole-cell extracts were prepared and Western blots were incubated with the BAFF-R antibody at 4°C for 10 h

this study. Our findings indicated that BAFF–BAFF-R induced KM3 cell survival through activation of similar survival pathways in normal B cells treated with rhBAFF protein in vitro [28]. Our findings also indicated that BAFF–BAFF-R supported KM3 cell survival by means of the activation of the NF-κB1 and NF-κB2 pathways. In addition, NF-κB was an important transcription factor involved in regulating BAFF-R expression through one NF-κB binding site in the BAFF-R promoter (Fig. 5). We deduce that BAFF–BAFF-R directly induce KM3 cell survival at the transcriptional level.

NF-κB inhibitor could induce apoptosis of neoplastic cells in myeloma, lymphoma, and myeloid leukemia cells [29, 30]. Specifically, we chose BAY11-7082 to block the NF-κB pathway, and found that the viability of KM3 cells cultured with BAY11-7082 was lower than that of the cells cultured in the medium alone, and the higher the concentration of BAY11-7082 the better the effect of the inhibition (Fig. 6a), suggesting that the NF-κB pathway was constitutively activated in KM3 cells. In addition, BAY11-7082 decreased the expression of BAFF-R mRNA and protein (Fig. 6b–d), implying that the antiapoptotic effect of BAFF-R on MM cells was highly dependent on the activation of the NF-κB pathway. Conceivably, strategies that can block MM cell signaling pathways induced by BAFF-R may disrupt the protective effect of BAFF–BAFF-R on MM cells and prove effective in the treatment of this disease. The drugs that inhibit BAFF and NF-κB activation and overcome conventional drugs resistance were employed in preclinical and early clinical trials [31], but

these novel drugs also have other multiple biologic actions, and the benefit of specifically targeting NF-κB in novel MM therapeutics has not yet been defined.

Most previous experiments [17, 18] focused on the BAFF-R gene downstream, and little has been known about the BAFF-R gene upstream. In this study, we found one NF-κB binding site in the BAFF-R promoter that was constitutively bound by activated NF-κB in KM3 cells, indicating that this putative NF-κB-binding site was indeed bound by NF-κB proteins and was a functional NF-κB site (Fig. 5). Indeed, examination of the 5′ region of the BAFF-R gene promoter indicated the presence of NF-κB binding sites, which may help in seeking other transcription factors such as AP-1, Sp1, and Oct-2. Clarification of the role of these transcription factors in the BAFF-R promoter region would help understand the regulation of the BAFF-R gene. Furthermore, the potential for the cooperative binding of these transcription factors and NF-κB binding and its synergistic effect in the activation of transcription would be of great importance to further elucidate the mode of regulation of this gene. Next, using a promoter–reporter construct approach, we showed that the promoter activity of BAFF-R gene was down-regulated by the NF-κB inhibitor-BAY11-7082, and that this down-regulation was mediated at the transcriptional level by NF-κB (Fig. 7). We speculated that BAY11-7082 inhibited the translocation of p52 and p65 proteins to the nucleus, leading to a decrease in the amount of p52 and p65 bound to NF-κB-binding site in BAFF-R, or BAY11-7082 directly inhibited the promoter activity of BAFF-R gene, which resulted in the loss of capability of NF-κB DNA binding activity. These data imply that the antiapoptotic effect of BAFF/BAFF-R on MM cells is highly dependent on the activation of the NF-KB1 and NF-KB2 pathways, and the putative NF-κB-binding site in BAFF-R is indeed a functional NF-κB site. As such, we speculate that the NF-κB-binding site of BAFF-R may be a new therapeutic target in this disease.

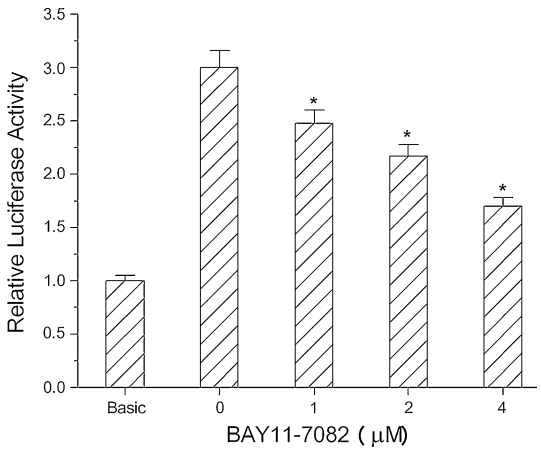

Fig. 7 NF-κB inhibitor decreases the activity of BAFF-R promoter. KM3 cells were transiently transfected with the BAFF-R luciferase promoter construct pGL3 along with β-gal vector. At 24 h after transfection, cells were treated with BAY11-7082 for another 48 h and harvested 72 h after transfection to detect the promoter activity by luciferase assay. Values were normalized to β-gal vector activity to correct for transfection efficiency. The results shown represent mean ± SD of 5 different experiments.*$P < 0.05$ compared with transfected cells untreated with BAY11-7082

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