

hsBAFF-Upregulated Intracellular Free Ca^{2+} Homeostasis Regulates ERK1/2 Activity and Cell Proliferation in B cells in Vitro

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Summary

We studied hsBAFF activity in *in vitro* mouse splenic B cells. hsBAFF effects on intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were assayed, using a laser scanning confocal microscope with fluorescent probe, Fluo-3/AM. We showed that treatment of B cells with 0.5-5 $\mu\text{g/ml}$ hsBAFF resulted in significantly higher $[\text{Ca}^{2+}]_i$ levels in a dose-dependent fashion at 12 and 24 h, respectively ($p < 0.05$ or $p < 0.01$ vs. control). Furthermore, we noticed that 2.5 $\mu\text{g/ml}$ hsBAFF-treated cells were significantly resistant to decrease of cellular viability induced by thapsigargin (Tg), an endoplasmic reticulum (ER) Ca^{2+} -ATPase inhibitor ($p < 0.05$ hsBAFF plus Tg group vs. Tg group). Thus hsBAFF may promote B cell survival by direct upregulation of $[\text{Ca}^{2+}]_i$ physiological homeostasis contributing to prevention of $[\text{Ca}^{2+}]_i$ dysfunction. Using immunocytochemistry and Western blot analysis, we found that the activation of ERK1/2 due to hsBAFF was triggered by a $[\text{Ca}^{2+}]_i$ -dependent pathway, leading to elevation of B cell proliferation. This is supported by the findings that intracellular Ca^{2+} chelator BAPTA/AM attenuated phosphorylated ERK1/2 expression and cell proliferation in hsBAFF-stimulated B cells. hsBAFF-stimulated B cell proliferation was obviously reduced by mitogen extracellular kinase 1/2 (MEK1/2, upstream of ERK1/2) inhibitor U0126. Taken together, the main finding of this study is that hsBAFF elicits higher but homeostatic $[\text{Ca}^{2+}]_i$ levels, which regulates ERK1/2 activity and cell proliferation in *in vitro* B cells.

Key words

Refolded human soluble BAFF (hsBAFF) • B cell • Calcium ion • Extracellular signal-regulated kinase 1/2

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Introduction

The TNF family ligand B cell activating factor belonging to the TNF family (BAFF), also known as BLyS, TALL-1, THANK, zTNF4, and TNFSF13b, is synthesized as a 285-amino acid type II transmembrane protein and exists in membrane and is cleaved to 152-amino acid soluble form (Moore *et al.* 1999, Mukhopadhyay *et al.* 1999, Schneider *et al.* 1999, Shu *et al.* 1999). BAFF is produced by a variety of cell types such as macrophages/monocytes and dendritic cells. Binding of BAFF to three TNF family receptors, including BAFF-R (BR3), B cell maturation antigen (BCMA), and transmembrane activator and cyclophilin ligand interactor (TACI), is required for generation and maintenance of mature B cells (Mackay *et al.* 2003, Cancro 2004, De Marchi *et al.* 2004, Craxton *et al.* 2005). Therefore, it plays a key role in peripheral B cell survival, maturation, and differentiation (Chu *et al.* 2007, Meyer-Bahlburg *et al.* 2008). However, the underlying mechanism(s) by which BAFF promotes B cell proliferation and response remains to be defined.

It is well known that calcium ion (Ca^{2+}) is involved in numerous fundamental cellular processes such as endocytosis and exocytosis, excitation, fertilization, as well as in regulation of cell fate

determination, i.e. proliferation, differentiation, or apoptosis (Berridge *et al.* 2000, Carafoli 2002). Cellular proliferation, differentiation and development in response to growth factors or mitogens are mainly involved in the activation of extracellular signal-regulated protein kinase (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family (Rubinfeld and Seger 2005, Yoon and Seger 2006). Previous data have demonstrated that ERK/MAPK signaling pathway is sensitive to changes in intracellular Ca^{2+} levels (Chao *et al.* 1992). The activation of MEK and ERK in B cells is dependent on calcium flux (Iritani *et al.* 1997). A recent report exhibited that BAFF regulates B cell survival by promoting Bim down-regulation *via* sustained activation of ERK signaling (Craxton *et al.* 2005). We recently demonstrated that a significant $[Ca^{2+}]_i$ upregulation exists in splenic B lymphocytes of mice administered refolded human soluble BAFF (hsBAFF) (Yang *et al.* 2007). Whether BAFF regulates ERK signaling and cell proliferation by upregulating $[Ca^{2+}]_i$ signaling in B cells is largely unknown.

Here we demonstrate that hsBAFF promotes *in vitro* B cell proliferation and response by direct upregulation of $[Ca^{2+}]_i$ physiological homeostasis and prevention of $[Ca^{2+}]_i$ dysfunction. The activation of ERK1/2 due to hsBAFF is triggered by a $[Ca^{2+}]_i$ -dependent pathway, leading to elevation of B cell proliferation. This is supported by the findings that i) BAPTA/AM, an intracellular Ca^{2+} chelator, attenuated phosphorylated ERK1/2 expression as well as cell proliferation in hsBAFF-stimulated B cells, and ii) hsBAFF-stimulated B cell proliferation was obviously reduced by MEK1/2 (upstream of ERK1/2) inhibitor U0126. These findings indicate that hsBAFF elicits higher but homeostatic $[Ca^{2+}]_i$ levels, which regulate ERK1/2 activity and cell proliferation in *in vitro* B cells.

Materials and Methods

Reagents

Anti-CD19 magnetic fluorobeads-B was purchased from One Lambda (Canoga Park, CA, USA). RPMI 1640 medium was from Gibco (Rockville, MD, USA). Neonatal cattle serum (NCS) was from Hangzhou Sijiqing Co. (Hangzhou, China), Refolded human soluble BAFF (hsBAFF) was a gift from Dr Shuangquan Zhang (College of Life Sciences, Nanjing Normal University, Nanjing, China) (Cao *et al.* 2005). Standard Human BAFF (hBAFF) was from PeproTech EC Ltd. (London,

UK). Thapsigargin (Tg) and 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM) was purchased from Calbiochem Co. (San Diego, CA, USA). Protease inhibitor cocktail, paraformaldehyde, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and Mayor's hematoxylin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mitogen extracellular kinase 1/2 (MEK1/2, upstream of ERK1/2) inhibitor U0126 was obtained from LC Laboratories (Woburn, MA, USA). Fluo-3/AM and Con A were supplied by Fluka Co. (Buchs, SG, Switzerland). The ethylene glycol-bis (β -amino ethyl ether)-N,N,N'-tetraacetic acid (EGTA), hydroxyethyl piperazine ethanesulfonic acid (HEPES) and L-glutamine were from Amresco Co., USA. Poly-L-lysine (PLL) was from Bio Basic Inc. (Markham Ontario, Canada). Primary antibodies for β -actin, ERK1/2 and phospho-ERK1/2 (Thr202/Tyr204) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. DAB kit, HRP-goat anti-rabbit IgG were from Wuhan Boster Bio-technology (Wuhan, Hubei, China). Coomassie brilliant blue assay kit was from Nanjing Jiangcheng Bioengineering Institute (Nanjing, Jiangsu, China). Other chemicals were purchased from local commercial sources and were of analytical grade quality.

Isolation of splenic cells

ICR mice, each weighing 20-25 g, were obtained from Experiment Animal Center of Nanjing Medical University, Nanjing, China. The animals were sacrificed *via* cervical dislocation to collect spleens under sterile conditions. Spleen was minced with dissecting scissors into pieces, followed by further grinding and filtering through sterilized nylon membranes and washed in Hank's solution (pH 7.2) *via* two centrifugations at 1500 rpm for 5 min after erythrocytes were dissolved with 0.85 % amchlor solution. After that, the isolated cells are fresh splenic cells.

Assay for B cell proliferation and Tg-induced B cell viability

B cells (>97 % purity) were isolated from splenic cell suspensions using anti-CD19 magnetic fluorobeads as described (Moore *et al.* 1999, Schneider *et al.* 1999), then resuspended and diluted to 1×10^5 cells/ml and cultured in RPMI 1640 medium containing 10 % NCS and 100 U/ml penicillin/streptomycin. The isolated cells were seeded at 0.1 ml/well in 96-well flat-bottomed

plates and randomly divided into a normal control group, a treatment group with standard hBAFF (2 μ g/ml) and five treatment groups with hsBAFF (0.5, 1, 2, 3, 5 μ g/ml, respectively) with a triplicate of each group. The cultures were maintained for 72 h at 37 °C with 5 % CO₂ humidified air. Then, each well was added 0.01 ml (5 mg/ml) of MTT reagent and incubated for 4 h. After the incubation, the incubation precipitates were dissolved with 0.1 ml of SDS. The optical density (OD) values were measured by spectrophotometry at 570 nm using an ELx800 Microplate Reader (Bio-Tek Instruments, Inc. Winooski, Vermont, USA).

To test the effect of BAPTA/AM or U0126 on hsBAFF-stimulated B cells proliferation, purified mouse splenic B cells (1×10^4 /well) were treated with/without 2 μ g/ml hsBAFF in the presence or absence of BAPTA/AM (20 μ M), or with/without 2 μ g/ml hsBAFF following pretreatment with 5 μ M MEK1/2 (upstream of ERK1/2) inhibitor U0126 for 30 min, respectively. Then, B cells proliferation was evaluated using an MTT assay as described above.

For Tg-induced B cell viability assay, purified mouse splenic B cells (2×10^4 /well) were seeded in 96-well flat-bottomed plates and treated with/without 0.5 μ M Tg in the presence or absence of hsBAFF (2.5 μ g/ml) for 48 h. Then, an MTT assay was carried out for cell viability assay.

Analysis for $[Ca^{2+}]_i$ using laser scanning confocal microscope

The effect of hsBAFF on $[Ca^{2+}]_i$ in B cells was evaluated. Fluo-3/AM was chosen to be used as an intracellular free Ca²⁺ fluorescent probe for analysis of $[Ca^{2+}]_i$ in hsBAFF-treated B cells under laser scanning confocal microscope (LSCM). In short, 20 μ l B cell suspensions, from a normal control group and three hsBAFF-treated groups (1, 2.5, 5 μ g/ml, respectively) at 12 and 24 h post experiment, were collected and loaded with 20 μ l of 30 μ M Fluo-3/AM for 30 min at 37 °C, and then washed three times with D-Hank's solution to remove the extracellular Fluo-3/AM (Wang *et al.* 2007). To determine the $[Ca^{2+}]_i$ distribution in B cells, LSCM analysis was performed with a Bio-Rad MRC 1024 laser scanning confocal imaging system (Bio-Rad, Cambridge, MA). The parameters of LSCM were set up to the magnification 40 \times , the excited light 488 nm, the emission light 522/35 nm, the pinhole 10-40 nm and the power 30 %. After that, B cell $[Ca^{2+}]_i$ levels with green fluorescence were represented with fluorescent intensity

(FI). Total images were scanned with each experiment and the data were stored in disks for analysis.

Immunocytochemistry

Purified mouse splenic B cells were seeded at a density of 1×10^6 cells/well in 6-well plate containing a PLL-coated glass coverslip per well. Next day, cells were treated with hsBAFF (2.5 μ g/ml) in the presence or absence of BAPTA/AM (20 μ M). BAPTA/AM was administered one hour before harvest. After treatment for 24 h, cells were fixed with 4 % paraformaldehyde prepared in PBS for 2 h at 4 °C. The cells were washed three times with PBS, and then treated with 0.1 % Triton X-100. Thereafter coverslips containing cells were incubated with normal goat serum for 1 h at 37 °C to block non-specific binding and were incubated with phospho-ERK1/2 antibody (1:50) overnight at 4 °C, then with HRP-goat anti-rabbit IgG (1:500) used as the secondary antibody for 1 h at room temperature. Immunoreactive staining was visualized by using DAB kit and Mayor's hematoxylin. Slides were then dehydrated through graded concentrations of alcohol, cleared in xylene and coverslipped. Slides for negative control were treated identically except for the absence of primary antibody. No positive immunostaining was found in any controls (data not shown).

Western blot analysis

After treatment, cells were briefly washed with cold PBS. On ice, cells were lysed in the assay buffer [50 mmol/l Tris, pH 7.2; 150 mmol/l NaCl; 1 % sodium deoxycholate; 0.1 % sodium dodecyl sulfate (SDS); 1 % Triton X-100; 10 mmol/l NaF; 1 mmol/l Na₃VO₄; protease inhibitor cocktail (1:1000, Sigma)]. Lysates were sonicated for 10 s and centrifuged at 13000 rpm for 10 min at 4 °C. Protein concentration was determined by Coomassie brilliant blue assay with bovine serum albumin as standard (Nanjing Jiangcheng Bioengineering Institute, China). Equivalent amounts of protein were separated on 12 % SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with PBS containing 0.05 % Tween 20 and 5 % non-fat dry milk to block non-specific binding and were incubated with primary antibodies, then with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized by using Renaissance chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA). To check the amount of protein loaded, the immunoblots were treated with stripping solution (62.5 mM Tris buffer,

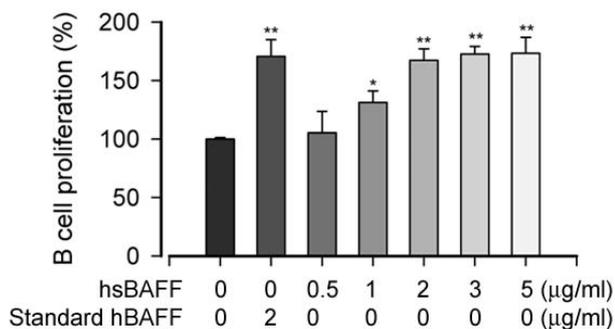


Fig. 1. hsBAFF severely promotes *in vitro* murine splenic B cell proliferation. B cell of splenic cells from healthy mice were isolated by anti-CD19 magnetic fluorobeads. B cell proliferation was evaluated using an MTT assay after treatment with hsBAFF and standard hBAFF for the indicated concentration, respectively. Results are presented as mean \pm S.E.M. (n=3). * p <0.05, ** p <0.01 (Student's *t*-test). hsBAFF treatment group vs. control group.

pH 6.7, containing 2% SDS and 100 mM β -mercaptoethanol) for 30 min at 50 °C and incubated with mouse monoclonal anti- β -actin antibody (Santa Cruz) followed by horseradish peroxidase-coupled goat anti-mouse IgG (Pierce).

Statistical analysis

Results were expressed as mean \pm S.E.M. Statistical analysis was performed by Student's *t*-test (STATISTICA, Statsoft Inc, Tulsa, OK). A level of P <0.05 was considered to be significant.

Results

hsBAFF severely promotes in vitro murine splenic B cell proliferation

Purified mouse splenic B cells were chosen as a model to study the effect of an appropriate concentration of hsBAFF on proliferation in B cells. The treatment with 0.5-5 μ g/ml hsBAFF significantly stimulated B cell proliferation in a concentration-dependent manner (p <0.05 for 1 μ g/ml and p <0.01 for 2, 3 and 5 μ g/ml hsBAFF vs. control, respectively). We also observed that *in vitro* proliferation effect was similar in standard hBAFF-treated and in refolded hsBAFF-treated cells with the final concentration of 2 μ g/ml (Fig. 1), indicating that refolded hsBAFF is biologically active in a validly induced B cell proliferation.

[Ca²⁺]_i fluorescence visualizations and intensities of administered hsBAFF in murine splenic B cells

Because the main intracellular signaling events

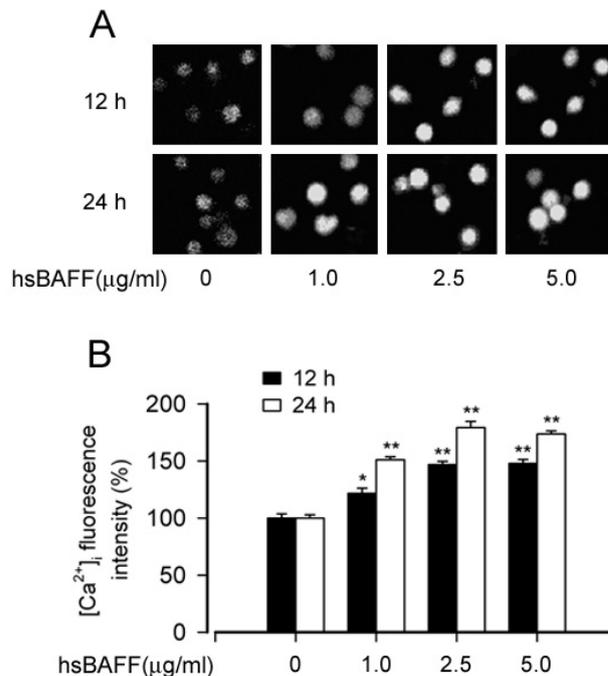


Fig. 2. [Ca²⁺]_i fluorescence visualizations and intensities of administered hsBAFF in *in vitro* murine splenic B cells. **(A)** Significantly stronger [Ca²⁺]_i fluorescent visualizations of purified mouse splenic B cells with hsBAFF for the indicated concentration at 12 and 24 h post treatment, were recorded, using a LSCM with fluorescent probe, Fluo-3/AM. **(B)** Changes of [Ca²⁺]_i fluorescence intensities in hsBAFF-treated B cells. Results are presented as mean \pm S.E.M. (n=6). * p <0.05, ** p <0.01 (Student's *t*-test). hsBAFF treatment groups vs. control group.

that induce an increase of [Ca²⁺]_i are practically shared by all cell types of the immune system, we therefore tested whether hsBAFF promoted B cell proliferation by regulation of [Ca²⁺]_i signaling. As demonstrated in Figure 2A, treatment of B cells with 1-5 μ g/ml hsBAFF resulted in significantly stronger [Ca²⁺]_i fluorescent intensities in a dose-dependent fashion at 12 and 24 h compared to control group, respectively. Figure 2B clearly showed significantly higher [Ca²⁺]_i levels in hsBAFF-treated B cells than in control cells at 12 and 24 h (p <0.05 and p <0.01, respectively), suggesting that hsBAFF may regulate B cell function by direct upregulation of [Ca²⁺]_i signaling.

hsBAFF effectively prevented the decreased viability of Tg-induced murine splenic B cells

To understand whether hsBAFF-upregulated [Ca²⁺]_i signaling exhibited potent action in physiological homeostasis of B cells, the isolated B cells were treated with/without hsBAFF (2.5 μ g/ml) in the presence or absence of Tg (0.5 μ M), an endoplasmic reticulum (ER) Ca²⁺-ATPase inhibitor, for 48 h. As shown in Figure 3,

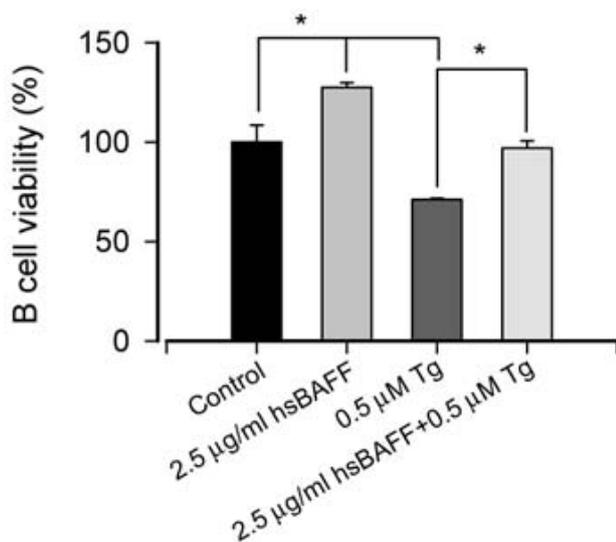


Fig. 3. hsBAFF effectively prevented the decreased viability of thapsigargin-induced *in vitro* murine splenic B cells. Purified mouse splenic B cells were treated with/without 0.5 μ M Tg in the presence or absence of hsBAFF (2.5 μ g/ml) for 48 h. Cellular viability was assessed using an MTT assay. Results are presented as mean \pm S.E.M. (n=6). * p <0.05 (Student's t-test). hsBAFF treatment group or Tg treatment group vs. control group, or hsBAFF plus Tg co-treatment group vs. Tg treatment group.

Tg-induced viability in B cells significantly decreased (p <0.05 vs. control). However, the effect was obviously rescued by hsBAFF (p <0.05 hsBAFF plus Tg group vs. Tg group), suggesting that hsBAFF may play an effective role in upregulating $[Ca^{2+}]_i$ physiological homeostasis and preventing $[Ca^{2+}]_i$ dysfunction.

hsBAFF-upregulated $[Ca^{2+}]_i$ activated ERK1/2 signaling contributing to cell proliferation in murine splenic B cells

Previous data have demonstrated that ERK/MAPK signaling pathway is sensitive to changes in intracellular Ca^{2+} levels (Chao *et al.* 1992). The activation of ERK in B cells is dependent on calcium flux (Iritani *et al.* 1997). We therefore hypothesized that the elevation of $[Ca^{2+}]_i$ in hsBAFF-stimulated B cells may be closely implicated in ERK signaling, leading to proliferation and response of B cells. As shown in Figure 4A, the immunocytochemical labeling for phosphorylation of ERK1/2 was very dense by the indicated different color and staining intensity in hsBAFF-stimulated B cell nucleus, whereas treatment with BAPTA/AM, an intracellular Ca^{2+} chelator, significantly attenuated phospho-ERK1/2 expression in hsBAFF-stimulated B cells, revealing that activation of ERK1/2 due to hsBAFF may be triggered by a $[Ca^{2+}]_i$ -dependent pathway.

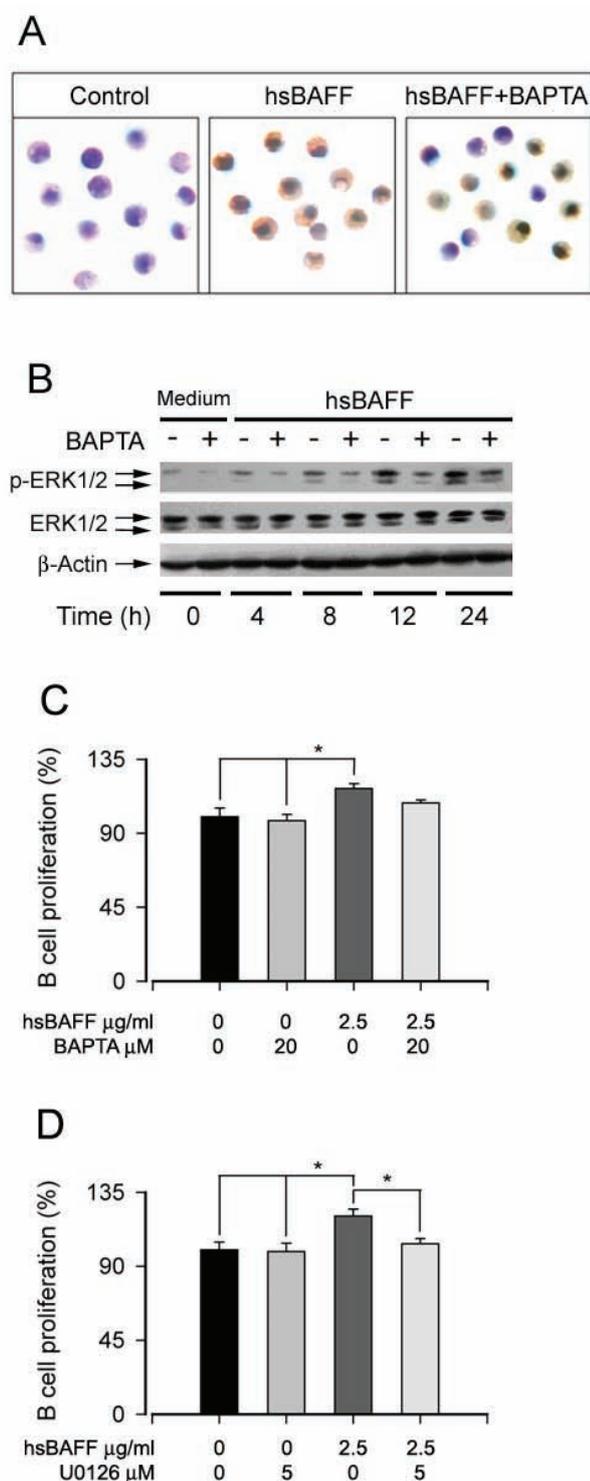
To dissect the mechanism, we next pretreated the cells with/without hsBAFF (2.5 μ g/ml) for indicated

different time, and then the calcium chelator BAPTA/AM (20 μ M) was administered one hour before harvest. Western blot assay showed that an evident increase of phosphorylated ERK1/2 due to hsBAFF appeared at 4 h and sustained until 24 h, but the loss of hsBAFF-increased $[Ca^{2+}]_i$ level due to calcium chelator BAPTA/AM resulted in an obvious reduction of ERK1/2 phosphorylation at every time point for 24 h (Fig. 4B), clearly suggesting that hsBAFF-induced $[Ca^{2+}]_i$ upregulation is closely associated with activation of ERK1/2 signaling.

Since hsBAFF promoted B cell proliferation by upregulation of $[Ca^{2+}]_i$ signaling (Fig. 1), we next tested whether hsBAFF-induced B cell proliferation depends on the ERK1/2 pathway *via* upregulated $[Ca^{2+}]_i$ signaling. Our observations showed that the elevated B cell proliferation due to hsBAFF was indeed attenuated by BAPTA/AM (Fig. 4C), and obviously reduced by MEK1/2 (upstream of ERK1/2) inhibitor U0126 for the indicated concentration (p <0.05 hsBAFF plus U0126 group vs. hsBAFF group) (Fig. 4D). Taken together, these findings reveal that hsBAFF-upregulated $[Ca^{2+}]_i$ promotes cell proliferation by activation of ERK1/2 signaling in *in vitro* B cells.

Discussion

Our results suggest that hsBAFF-mediated upregulation of $[Ca^{2+}]_i$ homeostasis is a key mechanism by which hsBAFF promotes B cell proliferation and response. The finding that hsBAFF affects B cell fate *via* its regulation of $[Ca^{2+}]_i$ is consistent with the striking similarity observed in B cells of hsBAFF-administered mice (Yang *et al.* 2007). In the current study, our group showed that administration of hsBAFF resulted in significantly higher $[Ca^{2+}]_i$ levels in *in vitro* murine splenic B cells at 12 and 24 h (p <0.05 or p <0.01 vs. control). Additionally, we also observed that there was a tendency to increased $[Ca^{2+}]_i$ at 6 h, but treatment of the cells with the same dosage of hsBAFF did not instantaneously elicit the changes of $[Ca^{2+}]_i$ under laser scanning confocal microscope (data not shown). This suggests that hsBAFF may promote higher but delayed $[Ca^{2+}]_i$ levels in B cells. Furthermore, we noticed that hsBAFF-treated cells were significantly more resistant to thapsigargin-induced decrease of cellular viability (p <0.05 hsBAFF plus Tg group vs. Tg group), revealing that hsBAFF may protect B cells from Tg-induced cell toxicity through the regulation of $[Ca^{2+}]_i$ homeostasis. It



has previously been demonstrated that Tg is toxic to a variety of cell types, because it specifically inhibits endoplasmic reticulum Ca^{2+} -ATPase, transiently increases the level of $[\text{Ca}^{2+}]_i$ and subsequently induces chromatin condensation, nuclear fragmentation, and internucleosomal DNA cleavage in the cells. These alterations are followed by the loss of plasma membrane integrity and by cell death. Available data clearly indicate that the large increase in $[\text{Ca}^{2+}]_i$ stimulated by Tg is

Fig. 4. hsBAFF-upregulated $[\text{Ca}^{2+}]_i$ activated ERK1/2 signaling contributes to cell proliferation in murine splenic B cells. **(A)** Purified mouse splenic B cells were treated with/without hsBAFF (2.5 $\mu\text{g/ml}$) and/or BAPTA/AM (20 μM) for 24 h. The BAPTA/AM was administered 1 h before harvest. Then an immunocytochemistry was conducted to detect the phospho-ERK1/2 expression in cell nucleus for normal control cells (left panel), hsBAFF-treated cells (middle panel), and hsBAFF plus BAPTA/AM-treated cells (right panel). **(B)** B cells treated with/without hsBAFF (2.5 $\mu\text{g/ml}$) in the presence or absence of BAPTA/AM (20 μM) for the indicated time were harvested and total lysates were subjected to Western blot analysis using indicated antibodies against the indicated proteins. The blots were probed for β -actin as a loading control. Similar results were observed in at least three independent experiments. **(C-D)** B cells proliferation was evaluated using an MTT assay after treatment with/without hsBAFF in the presence or absence of BAPTA/AM, or with/without hsBAFF following pretreatment with 5 μM mitogen extracellular kinase 1/2 (upstream of ERK1/2) inhibitor U0126 for 30 min, respectively. Results are presented as mean \pm S.E.M. ($n=3$). * $p<0.05$ (Student's t-test). hsBAFF treatment group vs. control group, BAPTA treatment group vs. U0126 treatment group, or hsBAFF plus U0126 treatment group vs. hsBAFF treatment group.

primarily responsible for its toxicity (Kaneko and Tsukamoto 1994, Kass and Orrenius 1999). Thus, we tentatively conclude hsBAFF may protect B cells from Tg-induced B cell death by sustaining homeostatic $[\text{Ca}^{2+}]_i$ levels. Undoubtedly, more studies are needed to address this issue.

The Ca^{2+} signals have been described in various cells of the immune system, including T and B cells, NK cells, mast cells, dendritic cells, monocytes and macrophages, in which they contribute to the cells' activation, effector functions, gene expression, or differentiation (Partiseti *et al.* 1994). ERK1/2, one of the well-characterized members of MAPK family, is also mainly associated with cellular proliferation, differentiation and development (Rubinfeld and Seger 2005, Yoon and Seger 2006). It is phosphorylated and activated through a three-tiered MEK mode *via* cell surface receptors stimulated by growth factors or cytokines (Sawe *et al.* 2008). Previous data have demonstrated that ERK/MAPK signaling pathway is sensitive to changes in intracellular Ca^{2+} levels (Chao *et al.* 1992). The activation of MEK and ERK in B cells is dependent on calcium flux (Iritani *et al.* 1997). Recent reports exhibit that BAFF-regulated B cell survival is implicated in sustained activation of MEK/ERK signaling (Craxton *et al.* 2005). We recently demonstrated that a significant $[\text{Ca}^{2+}]_i$ upregulation exists in splenic B cells of hsBAFF-administered mice (Yang *et al.* 2007). However, the molecular link between Ca^{2+} signaling and ERK activation was largely unknown. Thus, in our studies, to better understand the role and significance of hsBAFF-

induced $[Ca^{2+}]_i$ signaling in living murine splenic B cells, we investigated whether hsBAFF-induced Ca^{2+} signaling is implicated to ERK1/2 activity contributing to B cell proliferation. Using immunocytochemistry and Western blot analysis, we clearly showed that hsBAFF promoted the phospho-ERK1/2 expression in cell nucleus at 24 h after hsBAFF stimulation, but this effect was obviously inhibited by the calcium chelator BAPTA/AM (Fig. 4A). We also observed that an evident increase of phosphorylated ERK1/2 due to hsBAFF appeared at 4 h and sustained until 24 h (Fig. 4B), which is consistent with previous findings showing that BAFF promoted a delayed but sustained phosphorylation of ERK1/2 that was only evident at 4 h after BAFF stimulation (Craxton *et al.* 2005). However, the phosphorylated ERK1/2 signaling was potently blocked by BAPTA/AM at every time point for 24 h (Fig. 4B). Especially, we also observed that the elevated B cell proliferation due to hsBAFF was attenuated by BAPTA/AM, and obviously

reduced by MEK1/2 (upstream of ERK1/2) inhibitor. All these findings support our hypothesis that hsBAFF-induced $[Ca^{2+}]_i$ upregulation may link ERK1/2 activation contributing to cell proliferation in *in vitro* B cells.

In conclusion, the main finding of this study is that hsBAFF elicits higher but homeostatic $[Ca^{2+}]_i$ levels, which regulates ERK1/2 activity and cell proliferation in *in vitro* B cells.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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