hsBAFF-upregulated intracellular free Ca^{2+} homeostasis regulates ERK1/2 activity and cell proliferation in *in vitro* B cells

Jun Qing Liang[1,2], Wei Zhang[1,2], Ling Wen[1,2], Wei Gao[1], Shuang Quan Zhang[1], Long Chen[1,2*]

[1]College of Life Sciences, Nanjing Normal University, Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, and [2]Jiangsu Engineering Research Center for Bio-medical Function Materials, Nanjing 210046, P. R. China

*** Address correspondence to:**

Long. Chen, Ph.D.

College of Life Sciences, Nanjing Normal University

1 Wenyuan Road, Chixia District, Nanjing 210046

Jiangsu, P. R. China

Tel: 86-25-85891797 (office)

Fax: 86-25-85891526

Email: lchen@njnu.edu.cn (L. Chen)

Short title:

hsBAFF-upregulated $[Ca^{2+}]_i$ regulates B cell proliferation in by ERK1/2

Summary

We studied hsBAFF activity in *in vitro* mouse splenic B cells. hsBAFF effects on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) 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 μg/ml hsBAFF resulted in significantly higher $[Ca^{2+}]_i$ levels in a dose-dependent fashion at 12 and 24 h, respectively ($p<0.05$ or $p<0.01$ *vs.* control). Further, we noticed that 2.5 μg/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), revealing that hsBAFF may promote B cell survival by direct upregulation of $[Ca^{2+}]_i$ physiological homeostasis contributing to prevention of $[Ca^{2+}]_i$ dysfunction. Using immunocytochemistry and Western blot analysis, we found that the activation of ERK1/2 due to hsBAFF was triggered by a $[Ca^{2+}]_i$-dependent pathway, leading to elevation of B cell proliferation.. This is supported by the findings that BAPTA/AM, an intracellular Ca^{2+} chelator, 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 $[Ca^{2+}]_i$ 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

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 in cleaved 152-amino acid soluble forms (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 (DCs). 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 key roles 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 a plethora of fundamental cellular processes such as endocytosis and exocytosis, excitation, fertilization, as well as 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 refolded human soluble BAFF (hsBAFF)-administered mice (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; 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 regulates 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 r/min 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 of >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 μg/ml) and five treatment groups with hsBAFF (0.5, 1, 2, 3, 5 μg/ml, respectively) with a

triplicate of each group. The cultures were maintained for 72 h at 37°C with 5% CO_2 humidfied 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 spectrophotomety 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 μg/ml hsBAFF in the presence or absence of BAPTA/AM (20 μM), or with/without 2 μg/ml hsBAFF following pretreatment with 5 μM 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 μM Tg in the presence or absence of hsBAFF (2.5 μg/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 use as an intracellular free Ca^{2+} fluorescent probe for analysis of $[Ca^{2+}]_i$ in hsBAFF -treated B cells under laser scanning confocal microscope (LSCM). In short, 20 μl B cell suspensions, from a normal control group and three hsBAFF-treated groups (1,

2.5, 5 μg/ml, respectively) at 12 and 24 h post experiment, were collected and loaded with 20 μl of 30 μM Fluo-3/AM for 30 min at 37°C, and then washed 3 times with D-Hank's solution to remove the extracellular Fluo-3/AM as described (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×, 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 μg/ml) in the presence or absence of BAPTA/AM (20 μM). BAPTA/AM was administered 1h 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. After that, 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 and centrifuged at 13,000 r/min 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, 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 values ± standard error (mean ± S.E.). 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 effect of an appropriate concentration of hsBAFF on proliferation in B cells. As shown in Fig. 1, treatment with 0.5-5 μg/ml hsBAFF significantly stimulated B cell proliferation in a concentration-dependent manner ($p<0.05$ for 1 mg/kg and $p<0.01$ for 2, 3 and 5 μg/ml hsBAFF *vs.* control, respectively). We also observed that *in vitro* proliferation effect between standard hBAFF-treated and refolded hsBAFF-treated cells with the final concentration of 2 μg/ml was similar (Fig. 1), indicating that refolded hsBAFF is biologically active in a validly induced B cell proliferation.

Fig. 1

$[Ca^{2+}]_i$ fluorescence visualizations and intensities of administered hsBAFF in murine splenic B cells

Because the main intracellular signaling events that induce an increase of $[Ca^{2+}]_i$ are practically shared by all cell types of the immune system, we therefore tested whether hsBAFF promoted B cell proliferation by regulation of $[Ca^{2+}]_i$ signaling. As demonstrated in Fig. 2A, treatment of B cells with 1-5 μg/ml hsBAFF resulted in significantly stronger fluorescent intensities in manifestation of $[Ca^{2+}]_i$ status in a dose-dependent fashion at 12 and 24 h compared to control group, respectively. Fig. 2B clearly showed an significantly higher $[Ca^{2+}]_i$ levels in hsBAFF-treated B cells

than in control cells at 12 and 24 h ($p<0.05$ or $p<0.01$), suggesting that hsBAFF may regulate B cell function by direct upregulation of $[Ca^{2+}]_i$ signaling.

Fig. 2A, B

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

To understand whether hsBAFF-upregulated $[Ca^{2+}]_i$ signaling exhibited potent action as a physiological homeostasis in B cells, the isolated B cells were treated with/without hsBAFF (2.5 μg/ml) in the presence or absence of Tg (0.5 μM), a endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, for 48 h. As shown in Fig. 3, 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.

Fig. 3

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 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 Fig. 4A, the immunocytochemical labeling for phosphorylation of ERK1/2 was very dense by the indicated different colour 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 μg/ml) for indicated different time, and then the calcium chelator BAPTA/AM (20μM) was administered 1h 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 texted 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.

Fig. 4A, B, C, D

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 an increased tendency of $[Ca^{2+}]_i$ at 6 h ($p>0.05$ *vs.* control), but treatment of the cells with the same dosage of hsBAFF didn't instantaneously elicit the change of $[Ca^{2+}]_i$ in time course under LSCM (data not shown). This suggests that hsBAFF may promote higher but delayed $[Ca^{2+}]_i$ levels in B cells. Further, we noticed that hsBAFF-treated cells were significantly more resistant to thapsigargin (Tg)-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 regulation of $[Ca^{2+}]_i$ homeostasis. It has previously been demonstrated that Tg is toxic to a variety of cell types, which specifically inhibits endoplasmic reticulum Ca^{2+}-ATPase, transiently increases the level of $[Ca^{2+}]_i$ 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. These data clearly indicate that the large increase in $[Ca^{2+}]_i$ stimulated by Tg is 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 $[Ca^{2+}]_i$ 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, DCs, 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 best-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 to sustained activation of MEK/ERK signaling (Craxton *et al*. 2005). We recently demonstrated that a significant $[Ca^{2+}]_i$ 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, our results clearly showed hsBAFF promoted the phospho-ERK1/2 expression in cell nucleus at 24 h after hsBAFF stimulation, however, the 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. Collectively, the 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.

Acknowledgements

This work was supported by the National Natural Science Funds, P.R. China (No. 30670264), and the funding from Jiangsu Engineering Research Center for Bio-medical Function Materials, P.R. China. We thank Dr. C.Y. Zhang for the help and technical support in LSCM analysis.

Reference

BERRIDGE MJ, LIPP P, BOOTMAN MD: The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* **1**: 11-21, 2000.

CANCRO MP: The BLyS family of ligands and receptors: an archetype for niche-specific homeostatic regulation. *Immunol Rev* **202**: 237-249, 2004.

CAO P, MEI JJ, DIAO ZY, ZHANG S: Expression, refolding, and characterization of human soluble BAFF synthesized in Escherichia coli. *Protein Expr Purif* **41**: 199-206, 2005.

CARAFOLI E: Calcium signaling: a tale for all seasons. *Proc Natl Acad Sci U S A* **99**: 1115-1122, 2002.

CHAO TS, BYRON KL, LEE KM, VILLEREAL M, ROSNER MR: Activation of MAP kinases by calcium-dependent and calcium-independent pathways. Stimulation by thapsigargin and epidermal growth factor. *J Biol Chem* **267**: 19876-19883, 1992.

CHU VT, ENGHARD P, RIEMEKASTEN G, BEREK C: In vitro and in vivo activation induces BAFF and APRIL expression in B cells. *J Immunol* **179**: 5947-5957, 2007.

CRAXTON A, DRAVES KE, GRUPPI A, CLARK EA: BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway. *The Journal of experimental medicine* **202**: 1363-1374, 2005.

DE MARCHI G, DE VITA S, FABRIS M, SCOTT CA, FERRACCIOLI G: Systemic connective tissue disease complicated by Castleman's disease: report of a case and review of the literature. *Haematologica* **89**: ECR03, 2004.

IRITANI BM, FORBUSH KA, FARRAR MA, PERLMUTTER RM: Control of B cell development by Ras-mediated activation of Raf. *EMBO J* **16**: 7019-7031, 1997.

KANEKO Y, TSUKAMOTO A: Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells. *Cancer lett* **79**: 147-155, 1994.

KASS GE, ORRENIUS S: Calcium signaling and cytotoxicity. *Environ Health Perspect* **107 Suppl 1**: 25-35, 1999.

MACKAY F, SCHNEIDER P, RENNERT P, BROWNING J: BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* **21**: 231-264, 2003.

MEYER-BAHLBURG A, ANDREWS SF, YU KO, PORCELLI SA, RAWLINGS DJ: Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. *J Exp Med* **205**: 155-168, 2008.

MOORE PA, BELVEDERE O, ORR A, PIERI K, LAFLEUR DW, FENG P, SOPPET D, CHARTERS M, GENTZ R, PARMELEE D, LI Y, GALPERINA O, GIRI J, ROSCHKE V, NARDELLI B, CARRELL J, SOSNOVTSEVA S, GREENFIELDW, RUBEN SM, OLSEN HS, FIKES J, HILBERT DM: BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* **285**:260-263, 1999.

MUKHOPADHYAY A, NI J, ZHAI Y, YU G.L, AGGARWAL BB: Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase. *J Biol Chem* **274**: 15978-15981, 1999.

PARTISETI M, LE DEIST F, HIVROZC, FISCHER A, KORN H, CHOQUET D: The calcium current activated by T cell receptor and store depletion in human lymphocytes is absent in a primary immunodeficiency. *J Biol Chem* **269**: 32327-32335, 1994.

RUBINFELD H, SEGER R: The ERK cascade: a prototype of MAPK signaling. *Mol Biotechnol* **31**: 151-174, 2005.

SAWE N, STEINBERG G, ZHAO H: Dual roles of the MAPK/ERK1/2 cell signaling pathway after stroke. *J Neurosci Res* **86**: 1659-1669, 2008.

SCHNEIDER P, MACKAY F, STEINER V, HOFMANN K, BODMER JL, HOLLER N, AMBROSE C, LAWTON P, BIXLER S, ACHA-ORBEA H, VALMORI D, ROMERO P, WERNER-FAVRE C, ZUBLER RH, BROWNING JL, TSCHOPP J: BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* **189**: 1747-1756, 1999.

SHU HB, HU WH, JOHNSON H: TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *J Leukoc Biol* **65**: 680-683, 1999.

WANG SS, CHEN L, XIA SK: Cadmium is acutely toxic for murine hepatocytes: effects on intracellular free Ca^{2+} homeostasis. *Physiol Res* **56**: 193-201, 2007.

YANG XM, SHAN XX, CHEN L, CAO P, ZHANG SQ: Effects of splenic B lymphocyte proliferation and response and intracellular Ca^{2+} of hsBAFF in mice. *Chin J Appl physiol* **24**: 478-481, 2007 (in Chinese).

YOON S, SEGER R: The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* **24**: 21-44, 2006.

Legends for figures

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 ± S.E. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ (student's t-test). hsBAFF treatment group *vs.* control group.

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

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 μM Tg in the presence or absence of hsBAFF (2.5 μg/ml) for 48 h. Cellular viability was assessed using an MTT assay. Results are presented as mean ± S.E. (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.

Fig.4. hsBAFF-upregulated $[Ca^{2+}]_i$ activated ERK1/2 signaling contributing to cell proliferation in murine splenic B cells. (A): Purified mouse splenic B cells were treated with/without hsBAFF (2.5 μg/ml) and/or BAPTA/AM (20 μM) for 24 h. The BAPTA/AM was administered 1h before harvest. Then an immunocytochemistry was conducted to detect the phospho-ERK1/2 expression in cell nucleus for normal control cells (the left), hsBAFF-treated cells (the middle), and hsBAFF plus BAPTA/AM-treated cells (the right). **(B):** B cells treated with/without hsBAFF (2.5 μg/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 ± S.E. (n=3). $^{*}p<0.05$ (student's t-test). hsBAFF treatment group *vs.* control group, BAPTA treatment group or U0126 treatment group, or hsBAFF plus U0126 treatment group *vs.* hsBAFF treatment group.

Figures

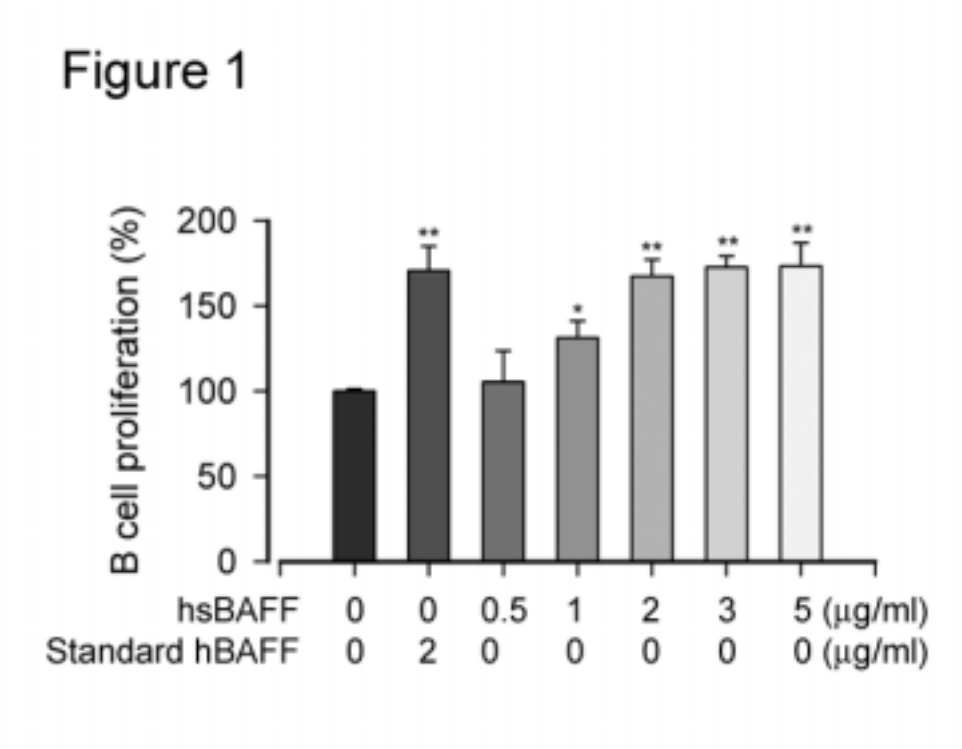


Figure 1
B cell proliferation (%)
200
150
100
50
0
**
**
*
**
**
**
hsBAFF 0 0 0.5 1 2 3 5 (µg/ml)
Standard hBAFF 0 2 0 0 0 0 0 (µg/ml)

Figure 2

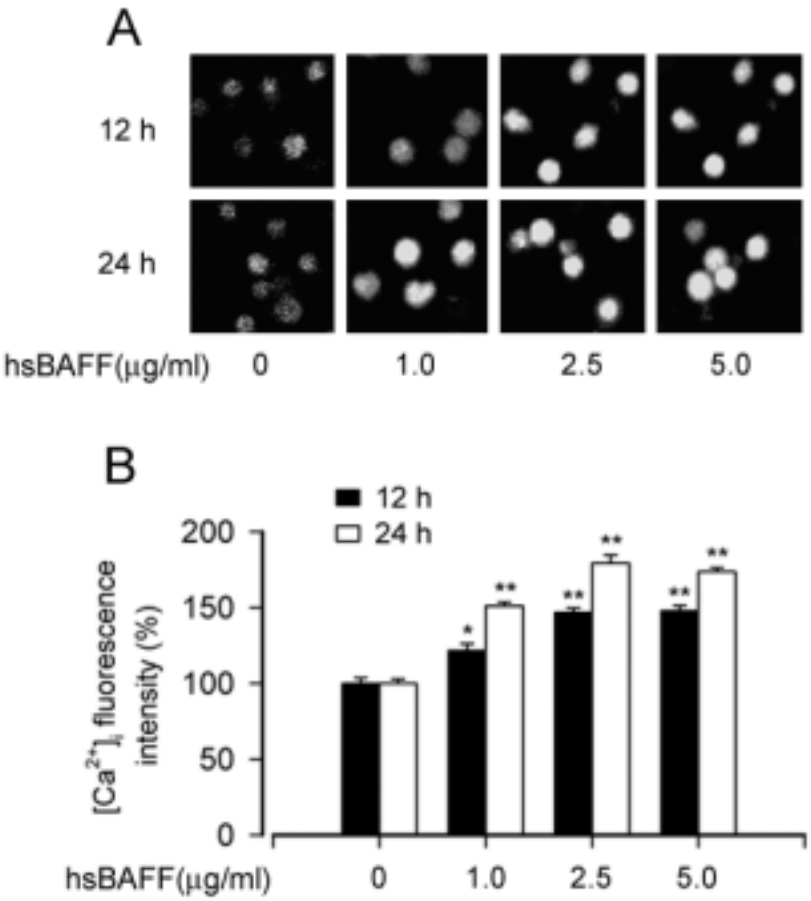

A
12 h
24 h
hsBAFF(μg/ml) 0 1.0 2.5 5.0
B
12 h
24 h
[Ca2+]i fluorescence intensity (%)
200
150
100
50
0
*
**
hsBAFF(μg/ml) 0 1.0 2.5 5.0

Figure 3

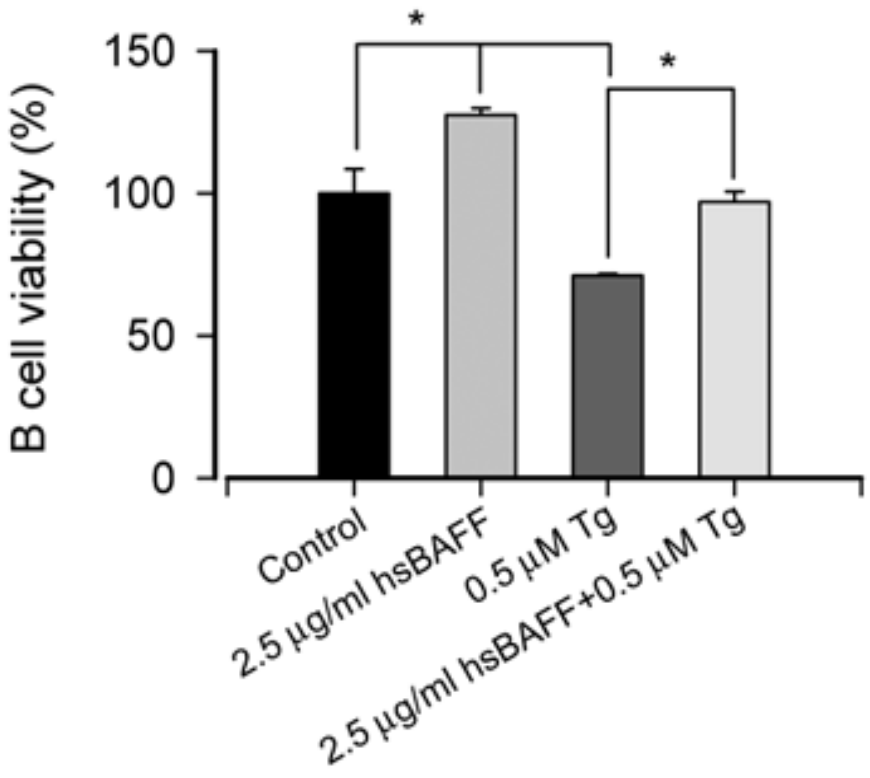
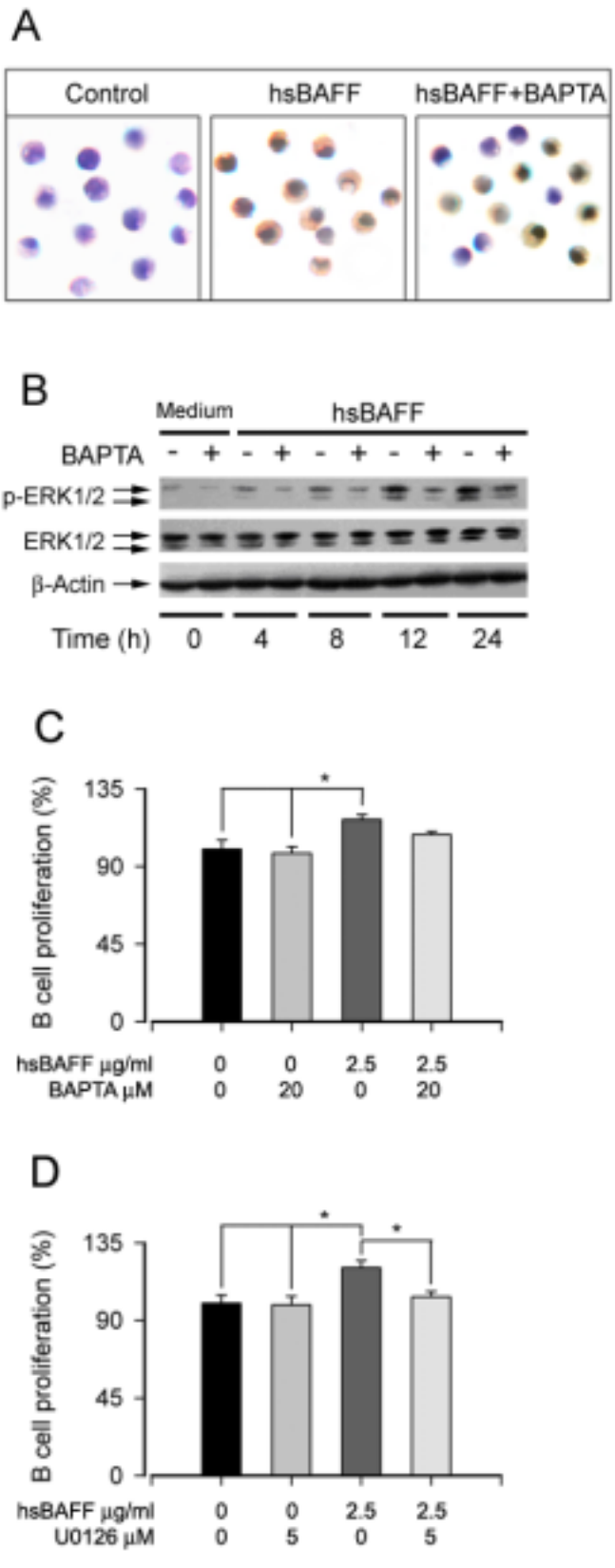

B cell viability (%)
150
100
50
0
*
*
Control
2.5 µg/ml hsBAFF
0.5 µM Tg
2.5 µg/ml hsBAFF+0.5 µM Tg

Figure 4


A
Control
hsBAFF
hsBAFF+BAPTA
B
Medium
hsBAFF
BAPTA - + - + - + - + - +
p-ERK1/2
ERK1/2
β-Actin
Time (h) 0 4 8 12 24
C
B cell proliferation (%)
135
90
45
0
*
hsBAFF μg/ml 0 0 2.5 2.5
BAPTA μM 0 20 0 20
D
B cell proliferation (%)
135
90
45
0
*
*
hsBAFF μg/ml 0 0 2.5 2.5
U0126 μM 0 5 0 5