1 **Molecular structure, distribution, and immunology function of**
2 **TNFSF13B (BAFF) in Nile tilapia (*Oreochromis niloticus*)**

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21 **Abstract:**

22 B cell-activating factor (BAFF) is a member of the tumor necrosis factor (TNF)
23 family and plays roles in B cell survival and maturation. In this study, the full-length

cDNA of Nile tilapia (*Oreochromis niloticus*) BAFF (tBAFF) was amplified from the spleen by reverse transcription PCR (RT-PCR). The open reading frame of this cDNA encodes a protein of 261 amino acids containing a predicted transmembrane domain and a furin protease cleavage site, similar to mammalian, avian, and reptile BAFF. Real-time quantitative PCR (qPCR) analysis revealed that tBAFF is present in various tissues and is predominantly expressed in the spleen.

The predicted three-dimensional (3D) structure of the Nile tilapia (*Oreochromis niloticus*) soluble BAFF (tsBAFF) monomer was determined by (3D) structure modeling monomeranalyzed by (3D) structure mouse counterpart. Both tsBAFF and EGFP/tsBAFF were efficiently expressed in *Escherichia coli* BL21 (DE3), as confirmed by SDS-PAGE and Western blot analysis.

After purification, the EGFP/tsBAFF fusion protein showed a fluorescence spectrum similar to that of EGFP. Laser scanning confocal microscopy showed that EGFP/tsBAFF bound to its receptor. *In vitro*, tsBAFF promoted the proliferation of Nile tilapia and mouse splenic B cells together with/without a priming agent (*Staphylococcus aureus* Cowan 1, SAC) or anti-mouse IgM. Furthermore, tsBAFF showed a similar proliferation-stimulating effect on mouse B cells compared to msBAFF. These findings indicate that tsBAFF plays an important role in the proliferation of Nile tilapia B cells and has functional cross-reactivity among Nile tilapia and mammals. Therefore, BAFF may represent a useful factor for enhancing immunological efficacy in animals.

Key words: BAFF, Cell proliferation, Immune evolution, *in vitro* expression,
Molecular structure, Nile tilapia (*Oreochromis niloticus*)

1. Introduction

B- cell-activating factor (BAFF), also designated as BLyS, THANK, TNFSF13B, and zTNF4, is considered an important member of the tumor necrosis factor (TNF) superfamily that plays a major role in B cell homeostasis, survival, and proliferation [1-4]. BAFF is a type II transmembrane protein that is found either anchored to the cell surface or released in its soluble form after cleavage by furin-like proteases [5]. Both forms have biological activity. BAFF is produced mainly by innate immune cells, such as monocytes, dendritic cells (DCs), follicular DCs (FDCs), and some T- cells [6]. Crystal structure analysis of soluble BAFF (sBAFF) has shown that it has an unusually long D–E loop (“flap”) compared to other TNF family members, which forms a region that may be important for receptor binding and a virus-like assembly process [7, 8].

Under physiological conditions, trimeric BAFF regulates the proliferation and function of T and B lymphocytes by binding to three different receptors: BCMA, TACI, and BAFF-R [9-15]. Only BAFF-R is bound specifically by BAFF, while APRIL can also bind BCMA and TACI [10]. BAFF-R and BCMA are expressed exclusively in B cells [14, 16-18]. BCMA is important for long-term plasma cell

biology and antigen presentation [19]. TACI exerts negative control over BAFF-mediated B cell survival [20, 21], while BAFF-R may be the principal receptor responsible for BAFF-mediated function [14].

The production of BAFF by innate immune cells and some lymphocytes plays a central role in immune responses. BAFF plays an important role not only in B cell survival and tolerance, but also in B cell maturation and diversification, including IgG and IgA class switching [1, 22, 23]. In addition, it is now clear that BAFF can modulate T cell function *in vitro* and *in vivo* [24-26]. Overexpression of BAFF is associated with the pathogenesis and progression of many types of autoimmune disorders in humans, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS) [27-29]. These activities suggest the potential for BAFF or its antagonists to be developed as protein drugs targeting immune deficiency disorders or autoimmune diseases [30].

BAFF genes have been identified and characterized in mammals, birds, reptiles, and fishes. There have been few studies on immune regulation as well as on the development of B lymphocytes in Nile tilapia (*Oreochromis niloticus*). The present study was performed to clone the Nile tilapia BAFF (tBAFF) full-length cDNA, investigate its expression profile, produce bioactive tBAFF (the functional soluble form of tBAFF, aa106–260 [tsBAFF]) in a prokaryotic expression system, and determine the bioactivity of recombinant tsBAFF. This is the first report on the *in vitro* characterization of Nile tilapia BAFF, and the results raise the possibility that tsBAFF may be useful as an immunological factor to enhance immunological efficacy

in Nile tilapia.

2. Materials and methods

2.1. Animal and cell preparations

Healthy Nile tilapia (300 – 500 g) were purchased from Red-Sun Breed Co. Ltd. (Jiangsu, China) and maintained at 28°C. Nile tilapia splenic lymphocytes were separated by density centrifugation on Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ). Mouse B cells were isolated from the spleen using B cell-specific antibodies B220 (CD45R) coupled to magnetic beads (Miltenyi Biotec, Auburn, CA). Mouse B cells were maintained in RPMI-1640 medium with 100 U/ml penicillin/streptomycin (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) at 37°C in a CO₂ incubator, and Nile tilapia spleen cells were maintained in RPMI-1640 medium containing penicillin/streptomycin (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) at 28°C in a CO₂ incubator.

The use of all laboratory animals in the present study was approved by the Scientific Ethics Committee of Nanjing Normal University.

2.2. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Various tissues from Nile tilapia, including the heart, spleen, liver, gill, kidney, brain, and intestine, were collected, immediately snap frozen in liquid nitrogen, and stored at –80°C until total RNA extraction. Total RNA was extracted using TRIzol

reagent (Invitrogen, Carlsbad, CA) according to the standard protocol. RNA quality was assessed by electrophoresis on 1% agarose gels. First-strand cDNA was synthesized from 1 µg of RNA isolated from tissues using a PrimeScript[®] 1st Strand cDNA Synthesis Kit (Takara, Kyoto, Japan) according to the manufacturer's protocol.

RT-PCR was performed using degenerate primers tBAFFP-1 and tBAFFP-2 (Table 1), designed according to the regions of high homology among the sequences of *Lateolabrax japonicus* (JF812967), *Epinephelus awoara* (JX087434), *Oncorhynchus mykiss* (ABC84582), *Danio rerio* (FJ587513), *Homo sapiens* (AAD25356), and *Mus musculus* (AF119383) (Table 2). Amplification was as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 90 s, with a final extension step at 72°C for 7 min. The full-length cDNA sequence of tBAFF (786 bp) was then cloned into the pMD19-T vector (Takara) and sequenced (Invitrogen, Beijing, China).

2.3. Bioinformatics analyses and predicted three-dimensional structure of tsBAFF

Nucleotide and amino acid sequence data were analyzed to determine their identity to other sequences using the GenBank database. Sequence homology was determined using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Real-time quantification PCR primer pairs were designed manually based on Primer 3 Input (<http://frodo.wi.mit.edu/primer3/>). The deduced amino acid sequences were analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>), and the

predicted transmembrane domain was identified based on the program SOUSI (<http://www.enzim.hu/hmmtop/index.html>). Functional domains were analyzed by SMART (<http://smart.embl-heidelberg.de/>). The tBAFF amino acid sequence was deduced using DNASTar software. The percent identity was calculated between tBAFF and the known BAFF sequences using the MatGAT program with default parameters. Multiple alignments to tBAFF were performed with ClustalW (<http://www.ebi.ac.uk/clustalw/>). Comparison between sequences was performed using ClustalW 1.83 software, with minor optimizations made by hand. Percent identity was calculated using the FASTA program. A phylogenetic tree was constructed based on the proportion of amino acid differences (p-distance) and complete gap deletion by the neighbor-joining method using MEGA 4.0 software. The tsBAFF protein structure was predicted using I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER>), an internet service for protein structure and function predictions. Three-dimensional (3D) models were built based on multiple-threading alignments using LOMETS and iterative TASSER simulations; function insights were then derived by matching the predicted models with protein function databases. Critical Assessment of Structure Prediction (CASP) experiments were designed to obtain an objective assessment using the state-of-the-art method in the field and I-TASSER, which was ranked as the best method in the server section of the recent 7th CASP experiment [31-34].

2.4. qPCR analysis of gene expression

Real-time quantitative PCR was performed on a MyiQ Single-Color Real-Time qPCR Detection System. To evaluate the tissue distribution of tBAFF, one-step RT-PCR was performed on total RNA from different tissues using tBAFF-F and tBAFF-R primers (Table 1). The expected product size was 153 bp. PCR conditions were as follows: 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and then 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The endogenously expressed Nile tilapia glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA (NC_022209.1) was used as an internal control. The primer pair Nile tilapia GAPDH-F and GAPDH-R (Table 1) was used to amplify a 160-bp fragment of Nile tilapia GAPDH cDNA according to the same protocol. The cDNA template was replaced with DEPC water as a negative control.

2.5. Construction of tsBAFF and EGFP/tsBAFF protein expression vectors

The DNA fragment encoding the extracellular region of tsBAFF (aa 106 – 260), also known as the soluble form of tBAFF (tsBAFF), was amplified by PCR. The DNA encoding the tsBAFF domain was generated by PCR using the primers tBAFF1 and tBAFF2 (Table 1), which included unique *Bam*HI and *Hind*III restriction sites, respectively. The resulting PCR product was cloned into the pET28a expression vector (Novagen, San Diego, CA), forming a sequence encoding a fusion protein of tsBAFF with an NH₂-terminal His-6 tag. This recombinant plasmid was sequenced on both strands and termed pET28a-tsBAFF. Based on the EGFP and tsBAFF sequences and using the pET-28a vector and overlap method for PCR, we designed four primers

(Table 1) for PCR. The EGFP gene was cloned by PCR using primers pET-EGFP and EGFP-Linker with an extra *NdeI* recognition site at its 5' terminus (underlined). PCR was performed for 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Primers Linker-tsBAFF and tsBAFF-pET were used to amplify the DNA sequence of tsBAFF, which contains a *HindIII* recognition site at its 3' terminus (underlined). PCR was performed for 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s. After the first round of PCR using P1/P2 and P3/P4 primers, the two products were gel-purified and subjected to a second round of PCR using the pET-EGFP and tsBAFF-pET primers to obtain a fragment encoding EGFP/tsBAFF, which contained the linker sequence (Gly₄Ser)₃ between the two domains. Amplification was performed for 30 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 52°C, and extension for 90 s at 72°C (Fig. 6A).

The resulting PCR products of tsBAFF and EGFP/tsBAFF were digested with *Bam*HI and *Hind*III or *Nde*I and *Hind*III, respectively, and ligated into the pET-28a plasmid at the corresponding restriction sites. The ligation mixtures were transformed into *Escherichia coli* DH5α competent cells for propagation of the recombinant plasmids. The recombinant plasmids, pET28a-tsBAFF and pET28a-EGFP/tsBAFF, were confirmed by restriction endonuclease digestion and sequencing.

2.6. Expression, purification of recombinant pET28a-tsBAFF and pET28a-EGFP/tsBAFF, and Western blot analysis

Recombinant plasmids pET28a-tsBAFF and pET28a-EGFP/tsBAFF were

transformed into competent *E. coli* BL21 (DE3) (Novagen) cells to express the fusion protein. Bacteria were cultured in Luria Broth (LB) medium for kanamycin resistance with vigorous shaking (220 rpm) at 37°C to an optical density at 600 nm (OD₆₀₀) ≈ 0.6. To efficiently obtain soluble tsBAFF and EGFP/tsBAFF proteins, an induction scheme was established as follows: final isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, 0.2 mM; induction temperature, 16°C; total induction hours, 16 h; shaking speed, 100 rpm. After induction, bacteria were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C. Pellets were sonicated on ice for a 10-s pulse with an intervening 10-s pause until cells were completely lysed. Lysates were centrifuged at 12,000 × *g* for 30 min at 4°C. Then, supernatants were collected and applied to a nickel column to purify histidine-tagged proteins under native conditions following the manufacturer-tagged protein.

Concentrations of purified tsBAFF and EGFP/tsBAFF were determined spectroscopically at 595 nm using a protein assay kit based on the Bradford reagent. Purified fusion proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels. Proteins were detected by staining with Coomassie brilliant blue R-250 or blotted onto nitrocellulose membranes (Amersham, Bucks, UK). Membranes were blocked for 2 h at 37°C with Tris-buffered saline Tween-20 (TBST) (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20, pH 8.0) containing 5% skim milk. After washing five times with TBS, blots were incubated with an anti-His6-tag monoclonal antibody (Novagen) at 37°C. After washing three times for 5 min with TBST, the membranes were incubated with horseradish

peroxidase (HRP)-conjugated goat anti-mouse IgG in TBST containing 5% skim milk for 2 h at 37°C. After washing, TMB (Promega) was added to the corresponding reaction for color development. Final confirmation was performed using ESI-mass spectrometry and matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry (MS) [35].

2.7. Measurement of fluorescence spectra

Fluorescence spectra of EGFP/tsBAFF and EGFP (5µM) were measured using an F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Emission spectra were measured by excitation at 488 nm, and excitation spectra were measured at 509 nm [33].

2.8. Laser scanning confocal microscopy analysis

Mouse splenic B cells (5×10^5 cells/ml) in staining buffer (PBS with 2% FBS) were incubated for 1 h at 37°C with/without EGFP/tsBAFF (10 µg/ml). After incubation, B cells were washed three times with PBS (pH 7.2). Free EGFP (10 µg/ml) was used as a control. Images were captured with an LSM 510 confocal microscope with a cooled MicroMax CCD camera.

2.9. MTT analysis

Purified Nile tilapia and mouse splenic B cells (adjusted to 10^5 cell/ml) were cultured in RPMI-1640 (Gibco-BRL) containing 10% FCS and 100 U/ml

penicillin/streptomycin (Gibco-BRL) in triplicate in 96-well flat-bottomed plates. After 4 h in culture, purified Nile tilapia B cells were treated with the indicated concentrations of tsBAFF alone or together with a priming agent (*Staphylococcus aureus* Cowan 1 [SAC], 1:300,000 dilution of stock).

Mouse splenic B cells were treated with the indicated concentrations of tsBAFF or EGFP/tsBAFF alone or together with 2 µg/ml anti-mouse IgM (Sigma, St. Louis, MO) as a priming agent. msBAFF (10µg/ml) was used as a positive control. PBS was added to cells alone as a control. After incubation at 37°C in a humidified atmosphere of 5% CO₂ for 48 h, 20 µl of MTT (5 mg/ml) was added to each well and allowed to react for 4 h. After centrifugation at 1,000 rpm for 10 min, the supernatant of each well containing medium and unreduced MTT was carefully removed. Then, 150 µl of dimethyl Sulfoxide (DMSO) was added to each well to dissolve formazan crystals.

Plates were shaken gently for a few minutes until a purple color appeared. Optical density (OD) values were measured by spectrophotometry at 450 nm using an EL×800 Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT).

2.10. Flow cytometric analysis

Mouse splenic lymphocytes (5×10^6 cell/ml) were cultured for 48 h in medium containing 10 µg/ml tsBAFF, and PBS was used as a control. Discrimination of dead cells for *in vitro* survival assays was performed by staining with propidium iodide (PI 2.5 µg/ml). Flow cytometric analysis was performed (FACScan; Becton Dickinson, Heidelberg, Germany) using Cell Quest software.

2.11. Statistical analysis

All statistical tests were performed in triplicate. Data are expressed as the means $\pm$ standard error (SE). Statistical analysis was performed using Student's *t*-test (STATISTICA; StatSoft Inc., Tulsa, OK) on a personal computer. One-way analysis of variance (ANOVA) was used to examine the significance of differences. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. cDNA cloning and identification of the Nile tilapia BAFF gene

The open reading frame (ORF) 786-bp coding sequence of Nile tilapia BAFF was obtained by RT-PCR amplification and deposited into GenBank under accession number KF741364. This ORF encoded a protein of 261 amino acids with a 156-aa mature peptide. The molecular weight of tsBAFF was approximately 17.436 kDa, and the isoelectric point was 5.71. The cDNA sequence of tBAFF and deduced amino acid sequence are shown in Fig. 1.

SMART online tool analysis of tBAFF protein functional domains is shown in Fig. 2. The results indicated that tBAFF had a typical TNF member domain (Fig. 2A), a transmembrane region domain (aa 28 – 50 s), and TNF domains (aa 106 – 260 s) (Fig. 2B). Analysis of the primary structure indicated that tBAFF contains a putative furin protease cleavage site (R–R–R–R) corresponding to other identified BAFF molecules and the conserved long D–E loop, known as the “flap,” as it appears in

human BAFF, which is unique to BAFF in the TNF family and is suggested to function in receptor binding and inter-trimer interactions.

3.2.Nile tilapia sequence comparison of tBAFF, phylogenetic analysis, and 3D structure of tsBAFF

BLAST searches indicated sequence identities of 62.88%, 62.36%, 30.83%, and 32.07% at the amino acid level between tsBAFF and *L. japonicas* BAFF (ljBAFF), *E. awoara* BAFF (eaBAFF), *H. sapiens* BAFF (hBAFF), and *M. musculus* BAFF (mBAFF), respectively, which were markedly higher than those for most other known cytokines. Multiple alignments generated using ClustalW are shown in Fig. 3 and Table 2. A phylogenetic tree constructed using the full-length amino acid sequences of BAFF proteins showed that tBAFF clusters together with avian sequences and away from other vertebrate BAFF proteins (Fig. 4B). We predicted the 3D structures of *L. japonicas* soluble BAFF (ljsBAFF), *E. awoara* soluble BAFF (easBAFF), *O. mykiss* soluble BAFF (omsBAFF), *human* soluble (hsBAFF), *M. musculus* soluble (msBAFF), and tsBAFF (Fig. 4A) using the I-TASSER server. The predicted 3D structures revealed that Nile tilapia BAFF was very similar to its counterparts, further suggesting that Nile tilapia BAFF has similar biological functions as ljsBAFF, easBAFF, and omsBAFF *in vivo*. Crystal structure analysis of the functional soluble part of Nile tilapia BAFF (tsBAFF) indicated that it has an unusually long D–E loop (also called the “flap”) compared to other TNF family members, such as yellow grouper BAFF, which forms a region that may be important for receptor binding and

virus-like assembly.

3.3. Tissue expression analysis of Nile tilapia

The physiological function of a protein may be determined by analyzing its tissue distribution. The constitutive mRNA expression of Nile tilapia BAFF in different tissues was analyzed by real-time PCR normalized relative to GAPDH levels. A dissociation curve showing a single peak at the expected melting temperature suggested specific amplification. Expression was determined as fold increased $2^{-\Delta\Delta Ct}$ level relative to the tissue with the lowest expression (brain) set to 1.

The Nile tilapia BAFF gene was expressed in all tissues examined, with high mRNA levels observed in the spleen, followed by the kidney, gill, liver, intestine, and heart (Fig. 5), indicating that BAFF plays an important role in the immune system of Nile tilapia.

3.4. *In vitro* expression and purification of tsBAFF and EGFP/tsBAFF proteins

For tsBAFF and EGFP/tsBAFF protein expression, pET28a-tsBAFF and pET28a-EGFP/tsBAFF plasmids were transformed into *E. coli* BL21 (DE3). The His₆-tagged tsBAFF (17.327 kDa) and EGFP/tsBAFF (44.959 kDa) were then efficiently expressed and purified under optimal conditions. Soluble recombinant proteins were successfully purified using Ni-nitrilotriacetic acid resin. On SDS-PAGE analysis, after IPTG induction, bands of 17.436 kDa (Fig. 6B, lane 2) and 44.959 kDa (Fig. 6C, lane 3) were consistent with the expected sizes of the respective fusion

products. The identities of the purified proteins were determined using an anti-His6 monoclonal antibody (Novagen). Western blot analysis indicated that bacterially expressed proteins showed predominant bands with estimated molecular weights of 17.327 kDa (Fig.6B, lane 4) and 44.959 kDa (Fig. 6C, lane 5). Finally, the purified tsBAFF (Fig. 7) and EGFP/tsBAFF proteins were passed through a 0.22 μ m filter and stored at -80°C until needed.

3.5. Fluorescence activity

When enhanced green fluorescent protein (EGFP) is fused to the N-terminus of overexpressed globular proteins, overexpression in soluble form allows EGFP to fold correctly. To confirm correct folding of the EGFP domain, the purified soluble EGFP/tsBAFF was tested for its fluorescent activity with comparison to free EGFP. As determined by measurement of fluorescence spectra, the fluorescent properties of EGFP/tsBAFF were similar to those of free EGFP, with excitation maximum at 488 nm and emission maximum at 508 nm.

However, the fluorescence intensity was weaker than that of free EGFP (Fig. 8A). These observations indicated no shift in excitation or emission spectra of the EGFP/tsBAFF fusion protein compared to free EGFP. The decrease in fluorescence in the chimera may be explained by formation of the fluorophore but quenching of the fluorescence by tsBAFF, which may absorb the inducing or induced radiation.

3.6. Recombinant EGFP/tsBAFF binds to mouse splenic B cells

BAFF can bind three receptors of the TNF receptor family: TACI, BCMA, and BAFF-R. TACI is expressed on B cells and activated T cells, whereas BAFF-R and BCMA are expressed mainly or exclusively on B cells [36]. The major BAFF receptor, BAFF-R protein, is present in the cell nucleus in addition to its integral presence in the plasma membrane and cytoplasm [37]. The binding activity of EGFP/tsBAFF was confirmed by laser scanning confocal microscopy (LSCM) using mouse splenic lymphocytes, as shown in Fig. 9. Nile tilapia BAFF bound to its major receptor (BAFF-R) mainly in the nucleus and cytoplasm of B cells. This result was consistent with recent research. EGFP or tsBAFF protein only was used as a negative control.

3.7. tsBAFF and EGFP/tsBAFF may promote the survival or proliferation of Nile tilapia or mouse splenic B cells

In vitro, human sBAFF co-stimulates B cell proliferation in response to anti-IgM as a priming agent, suggesting that tsBAFF may also have similar bioactivities on Nile tilapia splenic B cells. An MTT assay was performed to verify whether tsBAFF also functions as a co-stimulator to promote proliferation of Nile tilapia splenic B cells. Freshly purified Nile tilapia splenic B cells were co-stimulated with purified tsBAFF proteins at the indicated concentrations together with the priming agent SAC (1:300,000 dilution of stock). As shown in Fig. 10A, tsBAFF demonstrated strong saturable activity to co-stimulate B cell proliferation. The observed proliferation of Nile tilapia B cells was entirely dependent on the presence of the priming agent, SAC, indicating that tsBAFF also functions as a co-stimulator in B cell proliferation (Fig.

10A). Mouse splenic B cells were cultured with varying concentrations of tsBAFF together with the priming agent, anti-mouse IgM. As shown in Fig. 10B, after stimulation for 48 h, we observed a dose-dependent response to tsBAFF/anti-IgM treatment. The viability of B cells in cultures treated with 6 – 12 $\mu\text{g/ml}$ tsBAFF was 1- to 2-fold higher than that in control cultures with PBS, while that in cultures co-stimulated with tsBAFF and anti-IgM at the same dose was double that in cultures stimulated with anti-IgM, and even higher than that in the culture treated with tsBAFF alone. In the negative control group, anti-IgM did not show any proliferation-promoting effect on B cells. As shown in Fig. 10C, treatment with 10 $\mu\text{g/ml}$ msBAFF significantly stimulated mouse splenic B cell proliferation in contrast to 10 $\mu\text{g/ml}$ tsBAFF with or without anti-mouse IgM ($P < 0.01$ for treatment with tsBAFF or msBAFF alone and $P < 0.05$ for tsBAFF or msBAFF together with anti-mouse IgM). As shown in Fig. 10D, EGFP/tsBAFF promoted the proliferation of B cells alone and, together with anti-IgM, co-stimulated the B cell proliferation, which is similar to the results observed with tsBAFF. The MTT results indicated that a dose of 10 $\mu\text{l/ml}$ tsBAFF or EGFP/tsBAFF had the optimal effect on cell activation. Flow cytometric analysis showed that, after 48 h, although cell death occurred rapidly, tsBAFF and EGFP/tsBAFF markedly prolonged the survival of splenic B cells in culture (Fig. 11).

These results show that tsBAFF can promote the proliferation of mouse splenic B cells, which is partially dependent on the presence of anti-IgM, indicating that tsBAFF also functions as a co-stimulator of B cell proliferation, similar to human

sBAFF.

4. Discussion

BAFF is a potent survival factor for B cells and plays a central role in B cell maturation and survival, as well as T cell activation. There is increasing evidence that BAFF influences many important biological traits, including immune recognition and autoimmune diseases. Therefore, the BAFF gene is a good candidate for analyzing evolution and studying different mechanisms related to the vertebrate immune system.

This report presented the isolation and characterization of the Nile tilapia BAFF gene. Phylogenetic tree analysis based on the amino acid sequence alignment indicated the relationships of BAFF among 20 species and suggested a different evolutionary direction (Fig. 4B). Mammalian BAFF is processed by a furin-like protease after a characteristic sequence motif, which results in the release of biologically active soluble protein. A putative furin cleavage site (R-R-R-R) is located near the amino terminus of the tBAFF protein, suggesting that it may be processed similarly. Moreover, despite some differences at the amino acid level, the predicted 3D structure of tsBAFF monomers very similar to its counterparts, and the important structural amino acids are conserved in the same spatial positions (Fig. 4A), further suggesting that Nile tilapia BAFF may have similar biological functions to human BAFF *in vivo*. In particular, tsBAFF protein has a conserved long D-E loop, known as the “flap,” which is also observed in human BAFF. Previous studies indicated that the unique flap region of soluble BAFF mediates trimers

oligomerization, leading to remarkable virus-like assembly. Real-time quantitative PCR (qPCR) analysis indicated that the expression pattern of the Nile tilapia BAFF gene has been well-conserved throughout evolution (Fig. 5).

To study the activity of the Nile tilapia BAFF protein, we expressed the soluble mature form in *E. coli* with the pET28a plasmid. Soluble tsBAFF proteins were produced under hypothermic conditions to enhance the expression of the soluble form. Western blot analysis of tsBAFF purified from *E. coli* revealed a prominent band at 17.436 kDa (Fig. 6B, lane 4), indicating expression of the recombinant protein in its soluble form. To examine the receptor-binding activity, we also constructed a fusion protein consisting of enhanced green fluorescent protein (EGFP) and tsBAFF. Our results indicated that EGFP/tsBAFF exhibited the activities of both domains, which not only bound to the receptor-positive cell lines but also co-stimulated the proliferation of B cells *in vitro*.

MTT assays and flow cytometric analysis were performed to investigate whether tsBAFF could induce Nile tilapia and mouse B cell survival or proliferation. tsBAFF and SAC co-stimulated the proliferation of Nile tilapia B cells in a dose-dependent manner; tsBAFF and anti-mouse IgM also co-stimulated the proliferation of mouse B cells in a dose-dependent manner (Fig. 10A, 10B). EGFP/tsBAFF showed similar effects. These findings correspond well with a recent study suggesting that the BAFF–BAFF-R interaction is indispensable for B cell survival and maturation in the spleen [22]. The proliferative signal mediated by BAFF is a consequence of more cells surviving to enter the cell cycle, rather than the actual co-stimulation of B cells

[38].

In summary, we report the cloning and characteristics of the BAFF gene from Nile tilapia. Moreover, we analyzed the structure of the protein based on this sequence, demonstrated its bioactivity, and examined the expression of putative BAFF receptors.

Our results also suggest that tsBAFF plays an important role in the survival and proliferation of Nile tilapia B cells, and that there is functional cross-reactivity between mammalian BAFF and Nile tilapia BAFF. These results will facilitate further immune-modulating experiments with vaccination strategies in Nile tilapia. Evidence for a role of BAFF may be useful for understanding antibacterial immunity in fish, and suggest that tBAFF plays a crucial role in survival and proliferation of mouse B cells. There is likely functional cross-reactivity between human and Nile tilapia BAFF because of its high degree of conservation throughout evolution, which may make it useful as a model of human disease.

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Figure Legends

Fig. 1. Nucleotide and deduced amino acid sequences of tBAFF cDNA (GenBank accession number: KF741364). The stop codon (TGA) is indicated by an asterisk (*). The entire deduced amino acid sequence is depicted in single-letter code beneath the corresponding nucleotide sequence. Furin cleavage sites are indicated by gray shading.

Fig. 2. SMART online tool analysis of tBAFF protein functional domains. The results revealed a typical structure of a TNF member domain. The transmembrane domain (aa 28 – 50 s) is shown in blue, and the TNF domain (aa 106 – 260 s) is shown in pink.

Fig. 3. Amino acid sequence alignment was performed using ClustalW on BAFF from the following species: Japanese sea perch (*Lateolabrax japonicus*, JF812967), yellow grouper (*Epinephelus awoara*, JX087434), human (*Homo sapiens*, AY129225), and mouse (*Mus musculus*, AF119383). Amino acids that are identical among all sequences are indicated by asterisks, whereas those with high or low similarity are indicated by colons and periods, respectively. The furin cleavage site is boxed and

gray shading represents the three conserved cysteine residues. The double line above
BAFF sequences indicates the conserved long D–E loop, known as the “flap.”

Fig. 4. (A) Homology modeling of the tsBAFF monomer (aa 114 – 260) based on the
human structure (aa 134 – 285). Cartoon and sphere displays of structure models are
shown. β -strands are shown in yellow, α -helices in red, turns in blue, and other
residues in white. The unusually long D–E loop is designated as the “flap.” (B)
Phylogenetic tree showing the relationship between tsBAFF amino acid sequences
with other identified BAFF sequences. The tree was constructed by the
neighbor-joining method using ClustalW and MEGA 4.0, and was bootstrapped 1,000
times. Accession numbers of BAFF sequences obtained from GenBank are shown in
Table 2. (For interpretation of the colors in this figure legend, the reader is referred to
the web version of this article.)

Fig. 5. Expression of the Nile tilapia BAFF gene in various tissues. Mean mRNA
levels in seven tissues were analyzed by real-time qPCR. Data are $2^{-\Delta\Delta Ct}$ levels
calculated relative to the tissue with the lowest expression (brain) set to 1, normalized
relative to GAPDH mRNA levels. Vertical bars represent the means $\pm$ S.E. ($n = 3$).

Fig. 6. (A) Schematic representation of the construction of the recombinant vector,
pET28a-EGFP/tsBAFF. Arrows represent the primers used. The plasmid
pET28a-EGFP/tsBAFF contains *NdeI* and *HindIII* ligation sites and expresses a

His₆-tag fusion protein. T7 and His-tag indicate the T7 promoter and the His₆-tag, respectively. SDS-PAGE analysis of recombinant tsBAFF and EGFP/tsBAFF fusion proteins expressed in *E. coli* BL21 (DE3). The molecular weight marker is shown in the left lane. (B) Lane 1 indicates cell lysates from bacteria transformed with empty pET28a under IPTG induction. Lane 2 represents cell lysates from bacteria transformed with pET28a-tsBAFF under IPTG (0.2 mM) induction at 16°C for 16 h. Lane 3 shows the fusion protein purified by immobilized metal affinity chromatography. Lane 4 shows the Western blot analysis of tsBAFF using mAb against His₆-tag. (C) Lane 1 shows pure EGFP protein. Lane 2 shows cell lysates from bacteria transformed with pET28a-EGFP/tsBAFF without IPTG induction. Lane 3 represents cell lysates from bacteria transformed with pET28a-EGFP/tsBAFF under IPTG induction. Lane 4 shows the EGFP/tsBAFF fusion protein purified by immobilized metal affinity chromatography. Lane 5 shows the Western blot analysis of purified EGFP/tsBAFF using mAb against His₆-tag.

Fig.7. The molecular mass of recombinant tsBAFF is 17,327.007 Da.

Fig.8. Fluorescence spectra of free EGFP and EGFP/tsBAFF. Emission spectra were measured with excitation at 488 nm over the range 500-600 nm. Excitation spectra were measured with emission at 507 nm over the range 300-500 nm. Maximum emission wavelengths of free EGFP and EGFP/tsBAFF were both at 507 nm, and maximum excitation wavelengths were both at 488 nm.

640

641 **Fig. 9.** Confocal laser microscopy images of EGFP/tsBAFF binding to mouse splenic
642 B cells. Images were captured using a CCD camera with identical settings below the
643 saturation limits. (A) Mouse splenic B cells cultured with EGFP/tsBAFF showed
644 fluorescent signals. (B) Mouse splenic B cells cultured with EGFP did not show
645 fluorescent signals. (a, d) Images with transmitted light (phase contrast). (b, e) Same
646 images as in (a, d) with EGFP fluorescence (green) following excitation at 488 nm.
647 (c, f) Images merged with (a, d) and (b, e). (For interpretation of color in this figure
648 legend, the reader is referred to the web version of the article.)

649

650 **Fig. 10.** (A) *In vitro* tsBAFF co-stimulates the proliferation of Nile tilapia splenic B
651 cells in the presence of SAC. Cells cultured with the indicated doses of tsBAFF +
652 SAC (1:300,000 dilution of stock) were used as positive controls. Values were
653 expressed as the means $\pm$ S.E. (B) tsBAFF promotes the proliferation of mouse
654 splenic B cells in culture. In addition, tsBAFF and anti-mouse IgM co-stimulate the
655 proliferation of mouse B cells *in vitro*. Freshly isolated mouse splenic B cells were
656 cultured with the indicated doses of tsBAFF with/without anti-IgM. Anti-IgM was
657 used as a control protein. Three independent experiments were performed. Values are
658 expressed as the means $\pm$ S.E. (C) Effects of tsBAFF protein compared to msBAFF
659 on mouse splenic B cell proliferation *in vitro*. Freshly isolated mouse splenic B cells
660 were cultured with/without 2 μ g/ml anti-mouse IgM with 10 μ g/ml tsBAFF or
661 msBAFF for 72 h. Cell growth was then evaluated by the MTT assay. Values are

expressed as the means $\pm$ S.E. $*P < 0.05$, $**P < 0.01$. (D) EGFP/tsBAFF promotes the proliferation of mouse splenic B cells in culture. In addition, EGFP/tsBAFF and anti-mouse IgM co-stimulated the proliferation of mouse B cells *in vitro*. Freshly isolated splenic B cells were cultured with the indicated doses of EGFP/tsBAFF with/without anti-IgM. Anti-IgM was used as a control protein. Three independent experiments were performed. Values were expressed as the means $\pm$ S.E.

Fig. 11. tsBAFF promotes the survival of mouse splenic B cells in culture. Fresh isolated B cells were cultured for 48 h with 10 μ g/ml tsBAFF or with PBS as a control, and then analyzed by flow cytometry. To justify the cut-off between live and dead cells upon PI staining, the original flow cytometry data including the percentages of dead cells with each treatment are attached.

1 ATGGGTCCTGCAATGGCAGTTTTGGCAGGTCTTAAGCCTGGCTCTGGACAAGGGGATAGT
 1 M G P A M A V L A G L K P G S G Q G D S
 61 GAAAGAATGCTCTCCTGGCCAAATTTCTGTTGATGCTGGCCGCTTTCAGCTCATCTTT
 21 E R M L S W P I F L L M L A A F S S S F
 121 CTTTCAGCTTTGTCTCTGTACCAGCTGGTAGCTCTCAGAGCTGAAATAGAGCAACTTCAA
 41 L S A L S L Y Q L V A L R A E I E Q L Q
 181 TCAGATGTCTCTTGTGGGAGAGAAGAGGGACAACCCAGCGCAGACAGATTTTCAGGTGGAG
 61 S D V S C G R E E G Q P S A D R F Q V E
 241 ATTCCAAACAACATCAAAGATGTTGCTCGCCCAACCCGAGTGTCACCTGTCTTCATCCGA
 81 I P N N I K D V A R Q P E C P P V F I R
 301 ACACGACGAAGGAGAACGGTGTCTGGAGCAGAAGTGTTAATTTCTCAGCCTTTCTGCAG
 101 T R R R T V S G A E V L I S Q P F L Q
 361 ATGCTGGCAAACGACAGCAGAAAAAACTACGTGAAAGAAATTTTCAGCAGATACGTACACA
 121 M L A N D S R K N Y V K E I S A D T Y T
 421 GGATCCCTTGGCAGCCTGGGTTAAAGAGAGGCACTGCCCTGGAGCCCGACGGTGACCGC
 141 G I P W Q P G L K R G T A L E P D G D R
 481 ATTTTAGTCCAAGAGGAGGGCTTCTATTATGTGTACAGTCAGGTCTTCTACAGGGACAAC
 161 I L V Q E E G F Y Y V Y S Q V F Y R D N
 541 ACCTTTGCAATGGGTACAGTGGTGTTCGGTGGAAGAGAAACGTTGTTGAAACGAGCCT
 181 T F A M G H V V F R W K R N V V G N E P
 601 CAGTATGTGTCCCTGTTCCGCTGCATCCAAAACATGAACCCTGTCCAGTCGTTCAACACC
 201 Q Y V S L F R C I Q N M N P V Q S F N T
 661 TGCTACACAGGAGGTATTGTGAAGCTGGAGGCTGGGGACTACTTGGAGCTTCTGATCCCC
 221 C Y T G G I V K L E A G D Y L E L L I P
 721 CGGGCCACAGCAGACGTGTCCCTGAGTGGAGATTCCACCTTTCTGGGTGCTCTCAAACTG
 241 R A T A D V S L S G D S T F L G A L K L
 781 GCTTAA
 261 A *

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Name	Start	End	E-value
transmembrane region	28	50	N/A
TNF	117	260	0.176

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Table 1.

Primer sequences used in this study (F: forward and R: reverse).

Primer	Nucleotide sequence(5'-3')	Amplification
tBAFF-F	ACGGTGACCGCATTTTAGTC	Real-time PCR
tBAFF-R	AGCGGAACAGGGACACATAC	Real-time PCR
GAPDH-F	CCAGGTGGTGTCTCTGATT	Real-time PCR control
GAPDH-R	GTGCATGTACAGCAGCAGGT	Real-time PCR control
tBAFF1	ATGGGTCCTGCAATGGCAGT	Full-length gene
tBAFF2	TTAAGCCAGTTTGAGAGCA	Full-length gene
tsBAFF1	<u>CGCGGATCC</u> ACGGTGTCTGGAGCAGAAG (<i>Bam</i> HI)	Expression of tsBAFF
tsBAFF2	<u>CCCAAGCTT</u> TTAAGCCAGTTTGAGAGCACCC (<i>Hind</i> III)	Expression of tsBAFF
pET-GFP	<u>GGAATTCCATATG</u> GTGAGCAAGGGCGAGGAGCTG (<i>Nde</i> I)	Expression of EGFP/tsBAFF
EGFP-Linker	GCTGCCACCTCCACCCTTGACAGCTCGTC	Expression of EGFP/tsBAFF
Linker-tsBAFF	GGTGGAGGTGGCAGCACGGTGTCTGGAGCA	Expression of EGFP/tsBAFF
tsBAFF-pET	<u>CCCAAGCTT</u> TTAAGCCAGTTTGAGAGCACCC ((<i>Hind</i> III))	Expression of EGFP/tsBAFF

Table 2.

Amino acid identity comparison of the *Oreochromis niloticus* BAFF protein with other known BAFF proteins.

Species	Common name	Accession no.	Identity(%)
<i>Oreochromis niloticus</i>	Nile Tilapia	KF741364	100.0
<i>Lateolabrax japonicus</i>	Japanese sea perch	JF812967	62.88
<i>Epinephelus awoara</i>	Yellow grouper	JX087434	62.36
<i>Takifugu obscurus</i>	Mefugu	HQ830264	58.49
<i>Oncorhynchus mykiss</i>	Rainbow trout	ABC84582	56.39
<i>Danio rerio</i>	Zebrafish	ACR19336	45.65
<i>Columba livia</i>	Dove	EU334145	35.27
<i>Gallus gallus</i>	Chicken	AY263378	34.93
<i>Capra hircus</i>	Goat	FJ793192	34.86
<i>Elaphurus davidianus</i>	Père David's deer	JF441054	34.86
<i>Bos taurus</i>	Cattle	EU213012	34.86
<i>Alligator sinensis</i>	Chinese alligator	GU126733	34.71
<i>Anser cygnoides</i>	Chinese goose	DQ874394	32.88
<i>Anas platyrhynchos</i>	Duck	DQ445092	32.53
<i>Mus musculus</i>	Mouse	AF119383	32.07
<i>Chiloscyllium plagiosum</i>	White-spotted bamboo shark	HQ893880.1	31.27
<i>Homo sapiens</i>	Human	AAD25356	30.83
<i>Xenopus laevis</i>	African clawed frog	KC207689	30.26