© Indian Academy of Sciences

RESEARCH ARTICLE

Transgenic overexpression of BAFF regulates the expression of immune-related genes in zebrafish, *Danio rerio*

LI ZHANG[1], CHAO LIU[1], XIN ZHOU[2], YING XIE[1], LIBO SU[1], QI GENG[1], BINGHUI LIU[3] and SHUFENG LIU[1]*

[1]*Hebei Key Lab of Laboratory Animal Science, Hebei Medical University, Shijiazhuang 050017, People's Republic of China*
[2]*Department of Medical Genetics, Tongji Medical College; Huazhong University of Science and Technology, Hubei 430030, People's Republic of China*
[3]*Second Hospital of Hebei Medical University, Shijiazhuang, Hebei 050000, People's Republic of China*

Abstract

The B-cell activating factor (BAFF) is a member of tumour necrosis factor (TNF) superfamily that specifically regulates B lymphocyte proliferation and survival. Excess BAFF leads to overproduction of antibodies for secretion, anti-dsDNA antibodies and a lupus-like syndrome in mice. To investigate whether transgenic overexpression of the zebrafish BAFF leads to immunoglobulin changes and/or early maturing of the immune system, a Tol2-GFP-2A-BAFF/His recombinant plasmid was constructed by inserting a 2A peptide between the green fluorescent protein (GFP) and BAFF sequences. Functional GFP and BAFF proteins were expressed separately and confirmed in HeLa cells. The relative expression of immune-related genes (IgLC-1, IgLC-2, IgLC-3, IgD, IgM and IL-4), early lymphoid markers (Ikaros, Rag-1 and TCRAC), and the protooncogene Bcl-2 were evaluated by quantitative polymerase chain reaction (PCR) in F0 founder of transgenic zebrafish juveniles and adults. Ectopic expression of BAFF in adults was confirmed using Western blots and was shown to upregulate IgLC-1, IgLC-2, IgD, IgM, IgZ/T, Ikaros, Rag-1, TCRAC, IL-4 and Bcl-2 expression in juveniles on day 21 and IgLC-1, IgLC-2, IgD, IgM, IgZ/T, Rag-1, TCRAC and Bcl-2 expression in zebrafish three months postfertilization. The relative titers of specific IgM against *Edwardsiella tarda* WED were assessed using modified enzyme-linked immunosorbent assay (ELISA) with the whole body homogenate of zebrafish and demonstrated a significant increase in BAFF-transgenic group. Therefore, our findings provided novel insight into further exploration of modulating adaptive immunity and studying autoimmune diseases caused by regulating BAFF.

[Zhang L., Liu C., Zhou X., Xie Y., Su L., Geng Q., Liu B. and Liu S. 2016 Transgenic overexpression of BAFF regulates the expression of immune-related genes in zebrafish, *Danio rerio*. *J. Genet.* **95**, 751–760]

Introduction

Adaptive immunity is a highly specialized and tightly regulated process, involving the interaction of both lymphocytes and immune-relevant molecules, including T-cell receptor (TCR), B-cell receptor (BCR), recombination activating genes (Rags), immunoglobulins (Igs) and the major histocompatibility complex (MHC). Zebrafish (*Danio rerio*) are an established attractive model for studying ontogenetic development of the immune system (Hansen and Zapata 1998; Trede and Zon 1998; Willett *et al.* 1999; Trede *et al.* 2001). The factors Rag1, TCR alpha constant domain (TCRAC), immunoglobulin light chain (IgLC), and immunoglobulins are marker genes for early manifestation of the adaptive immune system. They displayed similar trends in expression patterns, the levels were relatively low at early stages and then rose distinctly (Lam *et al.* 2004).

BAFF, also known as Blys, TALL-1 and TNFSF13b, is a member of the TNF super family (Moisini and Davidson 2009). BAFF is predominantly produced by innate immune cells, such as neutrophils, macrophages, monocytes, dendritic cells (DCs) and follicular DCs (FDCs), and it plays a major role in the survival, proliferation and differentiation of B-cells (Bossen *et al.* 2008). Overexpression of BAFF in transgenic mice results in abnormal B cell activation and secretion of a variety of pathogenic autoantibodies (Mackay *et al.* 1999; Khan *et al.* 2013). The phenotype is reminiscent of certain human autoimmune disorders. Recent evidence has demonstrated that constitutive overexpression of BAFF in mice that are not prone to autoimmunity induces the development of elevated circulating autoantibody titers and immune complex-mediated nephritis. BAFF antagonists in animal models could effectively remove B cells, delay the

*For correspondence. E-mail: liushf@126.com.
Li Zhang and Chao Liu contributed equally to this work.

Keywords. zebrafish; adaptive immunity; B-cell activating factor; transgenic; gene expression.

occurrence of kidney disease and prolong survival (Stohl 2013). All these results suggest that dysregulation of BAFF expression may be a critical element in the chain of events leading to autoimmunity. However, little is known about the impact of BAFF overexpression in zebrafish due to lack of corresponding antibodies in zebrafish, for e.g. antiDNA autoantibodies and rheumatoid factors.

In this study, we established BAFF-overexpression juveniles and zebrafish, and detected the expression of nine immune-related genes (IgLC-1, IgLC-2, IgLC-3, IgD, IgM, IgZ/T, Rag-1, TCRAC and IL-4) and the protooncogene Bcl-2 in both juveniles and zebrafish spleen using real-time qPCR. These genes are suitable markers for the maturation of the immune system. Ikaros, which encodes a transcription factor, is used as an early lymphoid marker; Rag-1 is a good marker for maturing lymphocytes, while TCRAC and three IgLC isotypes genes, encode part of the antigen receptors of matured T and B lymphocytes, respectively. The mRNA levels of the eight immune-related genes (IgLC-1, IgLC-2, IgD, IgM, IgZ/T, Rag-1, TCRAC and IL-4) and the protooncogene Bcl-2 were significantly increased in juveniles, while those of seven immune-related genes (IgLC-1, IgLC-2, IgD, IgM, IgZ/T, Rag-1 and TCRAC) and Bcl-2 were also markedly increased in transgenic adults. Therefore, we collected evidence that BAFF overexpression affects immune-related gene expression and promotes cell proliferation in spleen. Our findings provided novel insight into further exploration of modulating adaptive immunity and studying autoimmune diseases caused by regulating BAFF.

Materials and methods

Zebrafish husbandry

The AB strains of zebrafish were kindly provided by Anming Meng (Tsinghua University). Adult fish were housed in a recirculating rack system (aquatic habitats) at 28°C with a 14 h / 10 h light/dark cycle and fed Zeigler adult zebrafish diet and brine shrimp according to the conditions in our system (Li *et al.* 2014). Fertilized eggs were obtained by natural mating, incubated at the same temperature and light conditions as adults. The animal research was approved by the Heibei Medical University Committee on the use and care of laboratory animals.

Construction of plasmid Tol2-GFP-2A-BAFF/His

The Tol2 transposon system, which includes pT2AL200R150G (Tol2) and pCS2^{+}-TP, was shown to be active from fish to mammals with high efficiency for germline transmission (Kawakami *et al.* 1998; Kawakami and Shima 1999; Kawakami 2007). The vectors were gifted by Prof. Kawakami, Division of Molecular and Developmental Biology, National Institute of Genetics, Mishima, Shizuoka, Japan. The pT2AL200R150G (Tol2) vector contains a green fluorescent protein (GFP) expression cassette composed of the Xenopus EF1 enhancer/promoter for ubiquitous expression, the rabbit globin intron, the EGFP gene, and the SV40 polyA (figure 1a). The empty Tol2 vector was used as a control (figure 1a). To ensure that the GFP and BAFF proteins were expressed separately and were functional, we used porcine teschovirus-1 2A (P2A) combined with Tol2 to construct the expression vector Tol2-GFP-2A-BAFF/His. A 2A peptide was a good candidate to replace IRES due to its small size and high cleavage efficiency between the genes upstream and downstream of the 2A peptide (Rothwell *et al.* 2010; Kim *et al.* 2011). Sequences and primers were synthesized (Invitrogen, Carlsbad, USA), and 2A was inserted between *Eco*RI and *Bsp*EI sites of pEGFP-C1. This new plasmid was named pEGFP-C1-2A. The 2A sequences were as follows:

2AForward: 5′-CCGGAGGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAGAACCCTGGACCTG-3′;

2AReverse: 5′-AATTCAGGTCCAGGGTTCTCCTCCACGTCTCCAGCCTGCTTCAGCAGGCTGAAGTTAGTAGCTCCGCTTCCT-3′.

Polymerase chain reaction (PCR) was used to isolate a fragment of BAFF cDNA. RNA was extracted from the zebrafish spleen of a wild-type AB. The cDNA fragments were generated by reverse transcription (Fermentas, Waltham, USA). Primers for cloning are mentioned below. The underlined sequences represent the *Eco*RI and *Bam*HI sites.

BAFFForward: GCGAATTCATGCCTGCTGAAGATGTT

BAFFAReverse: GCGGATCCATGGTGATGGTGATGATGGGCCAGTTTTATTGC

The primers amplified a 792-nucleotide fragment which was cloned into pEGFP-C1-2A and the new plasmid was named pEGFP-C1-2A-BAFF/His. A GFP-2A-BAFF/His fragment was cloned by InFusion primers. The uppercase letters in the primer sequences were designed from the Tol2 backbone, while the lowercase letters were from the pEGFP-C1-2A-BAFF/His backbone.

Tol2-2A-C1AForward: 5′-CGACGGATCCACCGGTcgccaccatggtgagca-3′;

Tol2-2A-C1AReverse: 5′-CCGCTTTACTTGTACAatggtgatggtgatgatgggcca-3′

This fragment was ligated to the Tol2 vector digested by *Age*I and *Bsr*GI (Clontech, Heidelberg, Germany). This newly constructed plasmid was named Tol2-GFP-2A-BAFF/His and was used to overexpress BAFF in zebrafish after a microinjection.

Microinjection and detection of GFP expression

Transgenic zebrafish were generated with microinjections of one-cell-stage fertilized embryos from AB* zebrafish with a 1 nL mixture of Tol2-GFP-2A-BAFF/His (25 ng/μL or 50 ng/μL) and transposase RNA (100 ng/μL) or 1 nL of Tol2 (25 ng/μL or 50 ng/μL) and transposase RNA (100 ng/μL) as a control. The transposase was a transcript obtained using a mMESSAGE mMACHINE SP6 kit (Abi-ambion, Foster City, USA). Injected embryos were screened for green

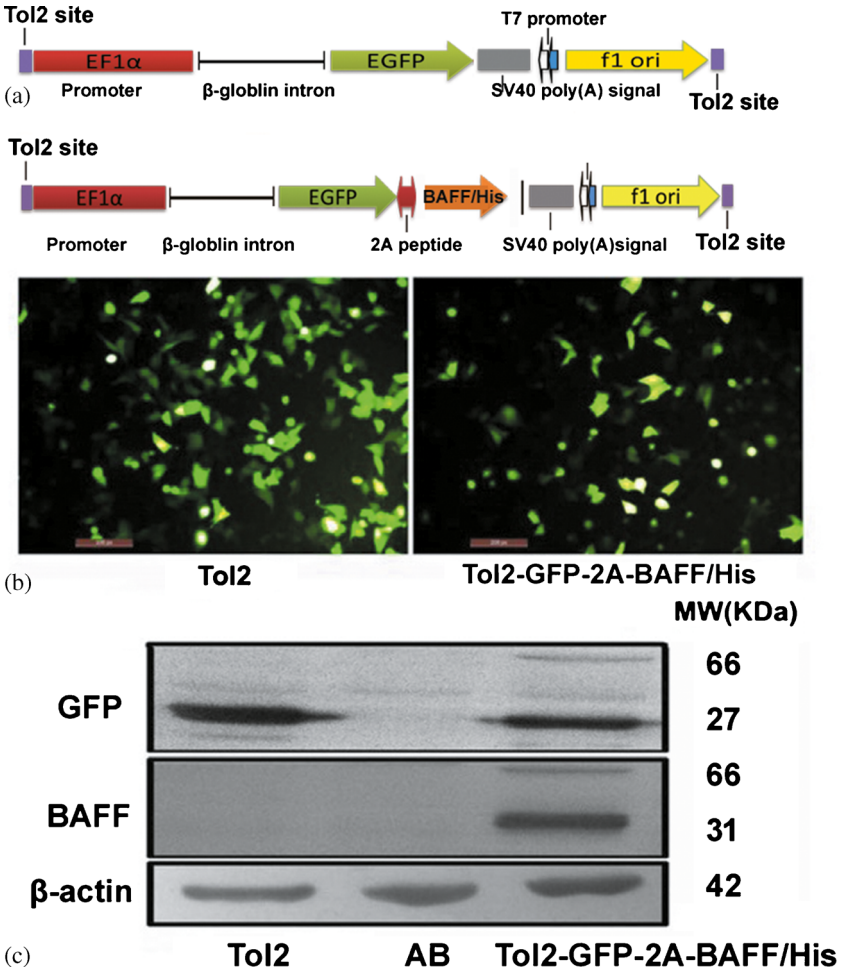


Figure 1. Construction of the Tol2-GFP-2A-BAFF/His plasmid and identification of GFP and BAFF expressions by Western blotting. (a) Components of the Tol2-GFP-2A-BAFF/His plasmid; (b) GFP expression in HeLa cells transfected with Tol2 and Tol2-GFP-2A-BAFF/His under a fluorescence microscope with blue excitation light; (c) Identification of GFP and BAFF expression in HeLa cells using Western blot.

fluorescence after 24-h postfertilization, and fluorescent images were captured using a Leica DM3000B microscope. Embryos and juveniles were randomly selected for photos. Finally, eight GFP-positive founder fish were raised to adulthood and three of them were used to generate a stable transgenic zebrafish line (Tol2-GFP-2A-BAFF/His). All three F0 founders were raised to adults and crossed with the wild type. Few adults had offspring that survived for 48 h due to the high death rate at the embryonic stage. Three F0 transgenic zebrafish (g1, g2 and g7) were used for the following experiments. Survival rate was calculated by survival embryos/total embryos; GFP positive rate: GFP positive embryos/survival embryos.

Western blotting

Spleens in g1, g2 and g7 were crushed in a mortar filled with liquid nitrogen for Western blot analysis. The ground tissues or HeLa cells were prepared by incubating in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (10 μL/mL) (Beyotime, China) for 30 min on ice. Total protein concentrations were determined using the Bradford protein assay (Biorad, Hercules, USA). Cell lysates (100 μg total proteins per sample) were subjected to SDS-PAGE and then transferred to a PVDF membrane. The membrane was incubated for 1 h in blocking buffer, incubated in the primary antibody 4°C overnight and then washed twice with blocking buffer without milk. The membrane was then incubated with the appropriate secondary antibody for 1 h at room temperature. After washing with blocking buffer, the protein bands were visualized using a DAB kit (Boster, China). Images of the blots were captured using a BioDoc-it^{TM} Imaging System (UVP, Upland, USA). The antiGFP antibody was purchased from PIERCE (1:1000, Pierce, USA), and anti-His antibodies (1:500, CST, USA), anti-β actin antibodies (1:2000, Beyotime, China), mouse and rabbit HRP-conjugated secondary antibodies (1:2000, Santa Cruz, USA) were also used.

Cell culture and transfection

For the transfection experiment, HeLa cells were plated in a 3.5-cm plate to achieve 80–90% confluence within 24 h in culture medium without penicillin/streptomycin. The cells were transfected with Lipofectamine 2000 (Invitrogen) according to the instructions with a transfection reagent (mL): DNA (mg) ratio of 3:1. Cell lysis was performed 48-h posttransfection for Western blotting.

Reverse transcription and quantitative RT-PCR

The whole bodies of three GFP-positive juveniles at day 21 were selected for experiment each time. Due to the limited number of the transgenic adults, spleen from each adult was collected for three intraclass repetitions for each group. Tissues were crushed in a mortar filled with liquid nitrogen and stored at −80°C in TRIzol reagent (Invitrogen). Total RNA was isolated from the thawed cells by Trizol method according to manufacturer's instructions and the pellet was resuspended in RNA secure solution (Ambion, St Austin, USA) and was run on a 1% agarose gel containing ethidium bromide to visualize integrity of 28S and 18S bands. The resulting RNA was digested by RNase-free DNaseI (Fermentas) to remove genomic DNA contamination prior to reverse transcription with the first-strand cDNA Synthesis kit (Fermentas, Maryland, USA).

Two μg of total RNA was reverse transcribed to cDNA with the first-strand cDNA Synthesis kit (Fermentas) with 5× MMLV reaction buffer, 2.5 μL oligodT primer, 125 μM dNTP and 100 u MMLV reverse transcriptase for 90 min at 37°C and the cDNA then used for SYBR Green PCR assays in a CFX Connect RT-PCR detection system (Biorad, Hercules, USA). All genes were amplified using SYBR Premix Ex Taq (Takara, Dalian, China). The PCR condition consisted of initial denaturation step of 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and 58°C for 30 s. Primers used in this study are provided in table 1. The dissociation curves were analysed at the end of PCR to confirm the identity of the PCR products. The levels of mRNA were measured using the $2^{-\Delta\Delta Ct}$ method and normalized to the reference gene, β-actin.

Vaccination and sampling

An *E. tarda* strain was purchased from the network of Microbial query, Beijing, China. The live attenuated vaccine strain WED was constructed according to the previous work (Xiao *et al.* 2011). Both Tol2 and Tol2-BAFF transgenic zebrafish were immunized with WED (5 μL fish, 2×10^7 cfu mL^{-1}). After immunization by intramuscular injection, the fish were maintained in aerated free flowing freshwater for 30 days. Due to the limitation of F0 numbers, WED injection was performed with a parallel group for sampling with one fish per group. The experiments were performed on three fish for replicates.

The whole body of zebrafish was collected and homogenated at 30 days after exposed to *E. tarda* WED. The supernatant was collected after centrifugation at 50000× g for 60 min

Table 1. Oligonucleotide primers used in this study.

Targeted transcript	Primer (5′–3′)	T_m	Amplicon size (bp)	Location
β-actinF	CCGTGACATCAAGGAGAAGCT	60	201	NM_181601.4
β-actinR	TCGTGGATACCGCAAGATTCC	60		
lgLC-1-F	ACTGGAGGCTGGTCTGGAAG	61	135	XM_003201349.2
lgLC-1-R	GCTGCTCAGTGAGAGTCAGG	60		
lgLC-2-F	TCGGTTACCGAAGGCGTCTT	61	160	XM_005168483.1
lgLC-2-R	TGATGCTTGCTCTGCTGGTT	60		
lgLC-3-F	GTTCCTGACCAGTGCAGAGA	59	131	XM_002666963.3
lgLC-3-R	CCTGATCACCTCCAGCATGA	59		
IgD-F	GACACATTAGCCCATCAGCA	58	156	BX510335
IgD-R	CTGGAGAGCAGCAAAAGGAT	58		
IgM-F	GAAGCCTCCAATTCTGTTGG	57	147	BX510335
IgM-R	CCGGGCTAAACACATGAAG	56		
IgZ/T-F	CACCCAGCATTCTACAGCAA	58	152	AY643750
IgZ/T-R	GAACCAAACTCAGGGTTGGA	58		
Ikaros-F	CAAACAGCTGAAGGCAATGG	58	156	NM_130986.1
Ikaros-R	ACGCTCCTCATTGTTGCTCT	60		
Rag-1-F	CGACGTGAGGCTCTATTGAA	57	144	XM_005162976.1
Rag-1-R	GGAGGATAGAAGGTCAGCAA	56		
Pten-F	CAAGGGTGAGCGAGGCACGG	61	218	NM_200708.2
Pten-R	CGGCTGGAAAGCCCATGGCA	61		
Akt1-F	TGCACTCCGAAAGAAACGTG	59	197	NM_001281801.1
Akt1-R	CGCACGACCGTAATCATTGT	59		
NFκB-F	TCTTTCGCGACAGGCGATTA	59	169	XM_005170256.2
NFκB-R	CCGAACAGTGTCTTTGGCCT	60		

Referenced the primer sequeces from Lam *et al.* (2004).

at 4°C, divided into several aliquots and stored at −20°C until use according to the previous method (Holbech *et al.* 2001).

Specific antibody detection

Antibody levels in supernatant of whole body homogenate against *E. tarda* WED was determined using a modified enzyme-linked immunosorbent assay (ELISA) method (Yang *et al.* 2013). After incubation for 10 min, H_2SO_4 (2 M, 50 μL) as a stop solution was added into each well and the intensity of ELISA reading was determined using the OD at 450 nm with a microplate reader. The relative amount of IgM against *E. tarda* was represented as OD_{450} (Tol2-BAFF)/OD_{450} (Tol2).

Statistics

Differences among groups were analysed using Student's *t*-test. Data are shown as the means ± standard error. Statistical significance was accepted when $P < 0.05$.

Results

BAFF cloning and plasmid construction

The ORF 807-bp coding sequence of the zebrafish BAFF was obtained by RT-PCR amplification (GenBank accession number FJ587513). It encoded a protein of 269 amino acids with a calculated molecular mass of ~29.902 kDa. The transmembrane form can be cleaved from the membrane, generating a soluble protein fragment with a molecular weight of 18 kDa. The cloned BAFF sequence shares 100% similarity with the amino acid sequence in NCBI (data not shown).

In the recombinant plasmid Tol2-GFP-2A-BAFF/His, BAFF/His was located downstream of GFP. BAFF/His and GFP were in the same ORF but were isolated by the 2A peptide. The BAFF/His fragment were integrated into the Tol2 plasmid by InFusion driven by the EF1α promoter. The recombinant plasmid was named Tol2-GFP-2A-BAFF/His. The Tol2 empty plasmid containing only GFP was driven by the EF1α promoter and used as a control (figure 1a). To identify BAFF expression *in vitro*, Tol2 and Tol2-GFP-2A-BAFF/His were transiently transfected into HeLa cells separately; both expressed strong green fluorescence under blue light (figure 1b). GFP expression was distributed in both cytoplasm and nuclei, regardless of Tol2 or Tol2-GFP-2A-BAFF/His transfection in HeLa cells. The Western blot results confirmed that GFP (27 kDa), BAFF/His (32 kDa) and GFP-2A-BAFF/His fusions (66 kDa) were all expressed in Tol2-GFP-2A-BAFF/His transfected cells, which suggested that the GFP-BAFF/His fusion protein was cleaved at the 2A peptide site in HeLa cells (figure 1c).

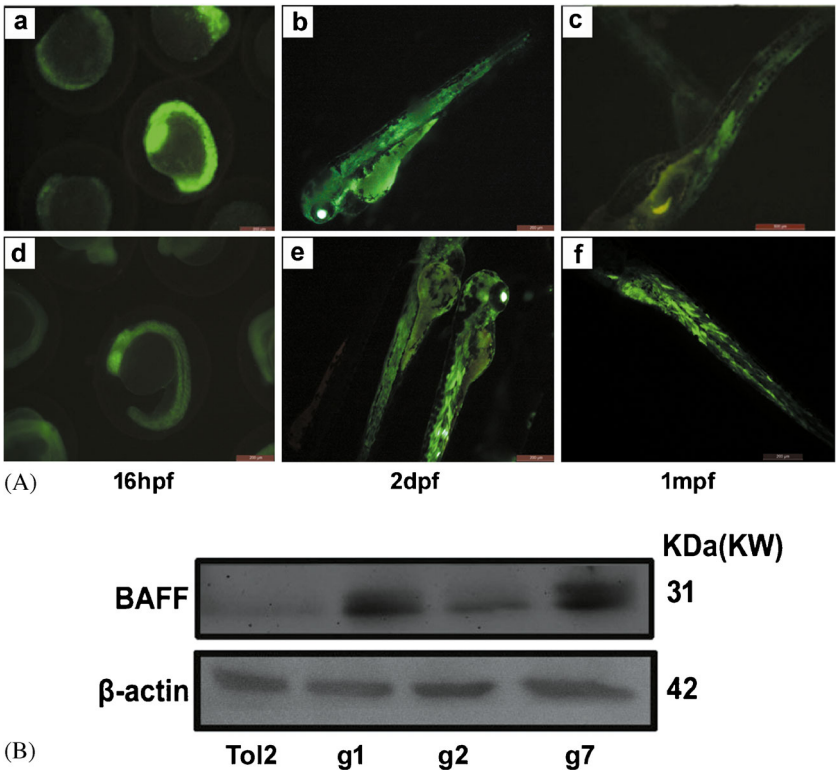


Figure 2. Identification of GFP and BAFF expression in Tol2-GFP-2A-BAFF/His transgenic zebrafish. (A) GFP expression in transgenic zebrafish at different developing times. Scale bar represents 200 μm. (a–c) Tol2 transgenic embryo and juveniles; (d–f): Tol2-GFP-2A-BAFF/His transgenic embryo and juveniles. (B) Western blot results for BAFF expression. GFP-2A-BAFF/His encoded a protein with 536 amino acids and a calculated molecular mass of ~66 kDa. The two bands represent the two membrane forms of BAFF with molecular weights of ~31 kDa.

Generation of Tol2-GFP-2A-BAFF/His F0 transgenic zebrafish

The Tol2-GFP-2A-BAFF/His plasmid and transposase were injected into one-cell embryos. Three founders were selected and named group 2, group 3 and group 8 (g1, g2 and g7). GFP expression was confirmed only in embryos and juveniles (figure 2A). As juveniles developed, the fluorescence intensity under the microscope was gradually weakened due to the cover of thick skin and scales. However, the Western blot results confirmed strong BAFF expression in g1, g2 and g7 in spleens (figure 2B). It was common for both g1 and g7 to have two bands at 31 kDa identifying the two membrane forms of BAFF. Due to the relatively low expression of BAFF, g3 demonstrated only one weak band at 31 kDa.

Survival rate and developmental abnormality in injected embryos

To exclude the damage caused by injection manipulation and DNA concentration, the survival rate was examined for the embryos injected with different concentrations of Tol2 and Tol2-BAFF groups (tables 2 and 3). It was found that the transgenic embryos at 16 hpf (hours postfertilization) showed traits of prematurity with a distinct tail and a yolk extension elongating away from the yolk (figure 2A, d). We believe this prematurity was attributed to BAFF overexpression because a similar developmental abnormalities did not occur in the control group, i.e. embryos injected with Tol2 and transposase RNA. The survival rates after injection with low and high concentration of Tol2-GFP-2A-BAFF/His were 27.5% and 15.33% separately. However, the rates of GFP-positive embryos were 27.88% and 63.13%. Although most of the embryos died after injection, the survival rate was still higher in the group with concentration of 25 ng/μL Tol2-GFP-2A-BAFF/His. But the GFP positive rate was higher in the group with concentration of 50 ng/μL, which indicated that high injected concentration was prone to high mortality rate and GFP positive rate. The same situation also applied to the Tol2 group except for the higher survival rate and GFP positive rate compared to the Tol2-BAFF group. The embryos with a premature development only existed in Tol2-BAFF transgenic group which inferred the high mortality rate and early-maturing for embryos was attributed to BAFF overexpression but not a very general developmental abnormal.

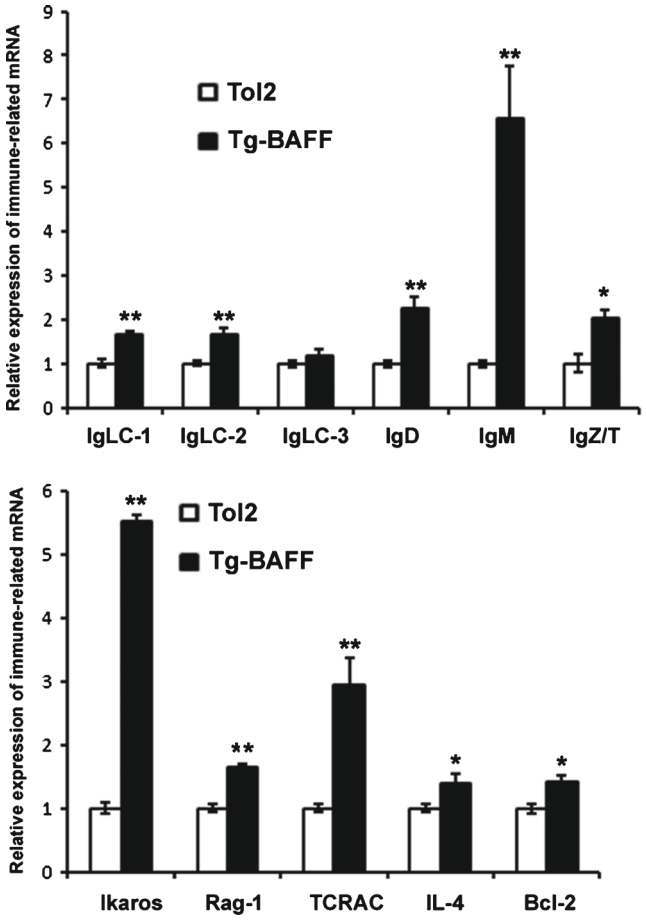


Figure 3. Real-time qPCR results for the relative expression of immune-related genes in transgenic juveniles at 21 dpf. Control: transgenic juveniles with Tol2; Tg-BAFF: transgenic juveniles with Tol2-GFP-2A-BAFF/His. Deviation bars represent the standard errors. After normalization to β-actin, Student's *t*-test was used to analyse the differences compared to the control.

Table 2. Developmental rate of transgenic embryos injected with 25 ng/μL concentration of DNA.

Group	Replicate	Survival rate (%)	GFP positive embryo (%)	Early development embryo	Total
AB	3	88 ± 5.04	0 ± 0	0	50
Tol2 (25 ng/μL)	3	81.5 ± 3.12	63.02 ± 5.24	0	200
Tol2-BAFF (25 ng/μL)	3	27.5 ± 3.02	27.88 ± 9.52	7 ± 1.58	200

Table 3. Developmental rate of transgenic embryos injected with 50 ng/μL concentration of DNA.

Group	Replicate	Survival rate (%)	GFP positive embryo (%)	Early development embryo	Total
AB	3	81.33 ± 3.06	0 ± 0	0	50
Tol2 (50 ng/μL)	3	66.17 ± 3.01	84.70 ± 10.31	0	200
Tol2-BAFF (50 ng/μL)	3	15.33 ± 4.16	63.13 ± 13.68	4 ± 0.58	200

Immune-related gene expression was activated by BAFF overexpression in F0 juveniles

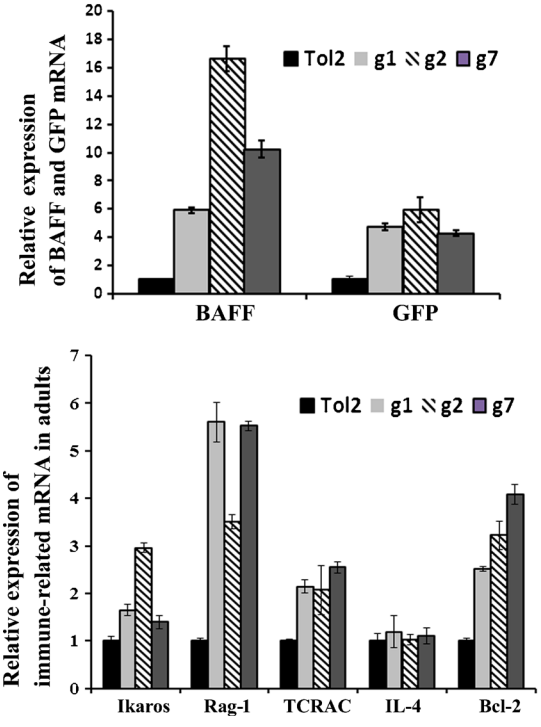
The whole body of juveniles at 21 dpf with GFP expression were collected for qPCR because B cells were not present until 2–3 weeks postfertilization (wpf) (Page *et al*. 2013). The qPCR results demonstrated that IgLC-1, IgLC-2, IgD, IgM and IgZ/T relative expression was significantly increased to 1.7-fold, 1.7-fold, 2.3-fold, 6.6-fold and 2.0-fold, respectively. Ikaros, Rag-1, TCRAC, IL-4 and Bcl-2 were significantly increased to 5.5-fold, 1.6-fold, 3.0-fold, 1.2-fold and 1.4-fold, respectively; IgLC-3 expression did not significantly change (figure 3).

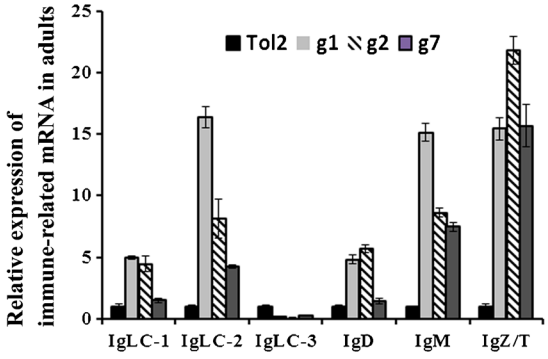
Immune-related gene expression was activated by BAFF overexpression in F0 adults

The relative expression of immune-related genes was also evaluated by qPCR in transgenic zebrafish (figure 4). The RNAs were isolated from three tails of 3 mpf (month postfertilization) F0 transgenic zebrafish g1, g2 and g7, demonstrating that BAFF expression was significantly expressed by 59-fold, 166-fold and 102-fold, respectively. One stable transgenic line harbouring the Tol2 empty vector was selected as the control. Endogenous IgLC-1 expression in g1, g2 and g7 was increased by 4.9-fold, 4.5-fold, 1.5-fold, respectively; IgLC-2 expression was increased by 16.3-fold, 8.1-fold and 4.2-fold, respectively. However, IgLC-3 expression was significantly reduced to 0.1-fold, 0.1-fold and 0.3-fold, respectively. Similar results for other immune-related components were also demonstrated in these three groups. In the g1, IgD, IgM and IgZ/T expression was significantly increased to 4.8-fold, 15.1-fold and 15.4-fold, respectively. In g2, IgD, IgM and IgZ/T expression was significantly increased to 5.7-fold, 8.6-fold and 7.5-fold, respectively; in g7, the relative expression was significantly increased to 15.4-fold, 21.8-fold and 15.7-fold, respectively. The relative expression levels of Ikaros, Rag-1, TCRAC and Bcl-2 were also significantly activated in all three groups; however, IL-4 expression did not show a significant change (figure 4). Besides, the expression of signalling pathway-related genes Pten, Akt1 and NF-κB were also detected by qPCR in transgenic adults. The results demonstrated that NF-κB significantly upregulated but the Pten and Akt1 did not change, which indicate BAFF overexpression is not related to PTEN/PI3K/AKT pathway (figure 5).

Specific antibody formation in supernatant of whole body homogenate

Due to the importance of BAFF for antibody responses, the secondary immune responses were examined after *E. tarda* WED stimulation. Because of the limited number of F0 generation, the relative titers of specific IgM against *E. tarda* WED were assessed using modified ELISA with the whole body homogenate of zebrafish at 30 days after immune stimulation. The results demonstrated that the amount of specific





	IgLC-1	IgLC-2	IgLC-3	IgD	IgM	IgZ/T
AB	1.00±0.2	1.00±0.15	1.00±0.13	1.00±0.15	1.00±0.05	1.00±0.16
g1	4.97±0.17**	16.34±0.85 **	0.13±0.04 **	4.81±0.38 **	15.12±0.72 **	15.43±0.88 **
g2	4.48±0.64 **	8.14±1.57 **	0.11±0.03**	5.67±0.29 **	8.64±0.37 **	21.81±1.15 **
g7	1.51±0.18 *	4.23±0.14 **	0.27±0.03 **	1.45±0.21 *	7.46±0.36 **	15.68±1.69 **

	Ikaros	Rag-1	TCRAC	IL-4	Bcl-2
AB	1.00±0.1	1.00±0.05	1.00±0.03	1.00±0.15	1.00±0.05
g1	1.65±0.12 *	5.6±0.42 **	2.14±0.14 **	1.19±0.34	2.52±0.04 **
g2	2.95±0.1 **	3.5±0.15 **	2.07±0.51 **	1.02±0.11	3.22±0.30 **
g7	1.39±0.14 *	5.53±0.1 **	2.55±0.12 **	1.11±0.17	4.09±0.21 **

Figure 4. Real-time qPCR results for the relative expression of immune-related genes in transgenic adults. Control: Tol2 transgenic zebrafish at 3 mpf; g1, g2, and g7 are F0 transgenic zebrafish harbouring Tol2-GFP-2A-BAFF/His. The data in the tables represent the mean fold ± SEM. *Significantly different at $P < 0.05$ between the BAFF overexpression group and the control group. **Extremely significant difference at $P < 0.01$.

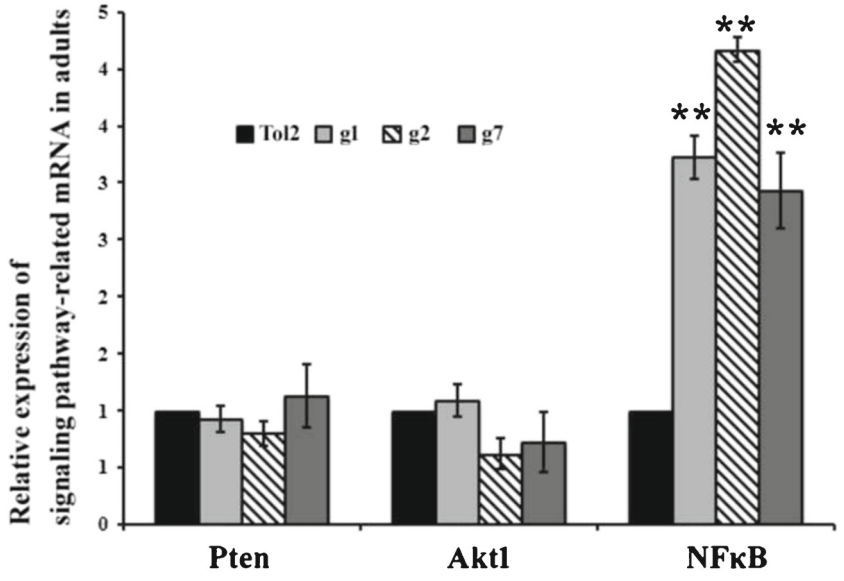


Figure 5. Real-time qPCR results for the relative expression of signalling-related genes in transgenic adults. Control: Tol2 transgenic zebrafish at 3 mpf; g1, g2, and g7 are F0 transgenic zebrafish harbouring Tol2-GFP-2A-BAFF/His.

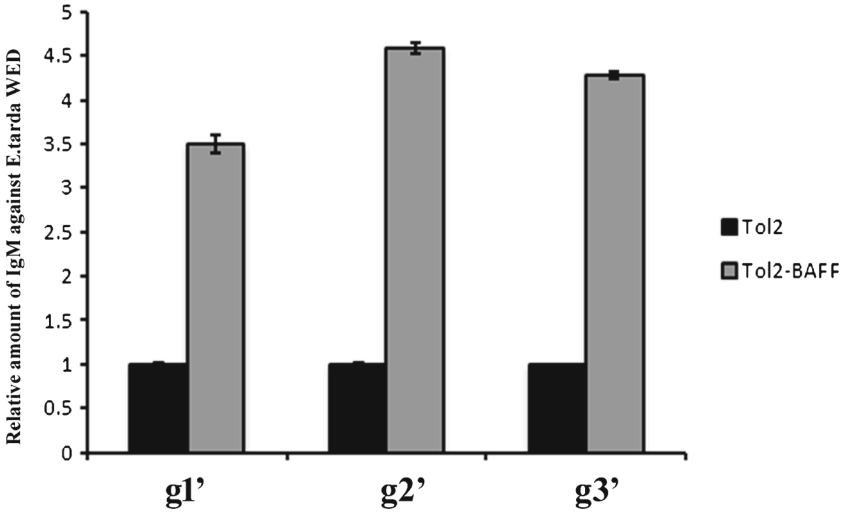


Figure 6. Relative amount of IgM against *E. tarda* WED using modified ELISA. g1′, g2′, g3′ were all F0 generation harbouring Tol2-GFP-2A-BAFF/His after *E. tarda* simulation. Three zebrafish derived from Tol2 injection were randomly selected as controls. The vertical axis represents the relative amount of IgM and is calculated by $OD450_{(Tol2\text{-}BAFF)}/OD450_{(Tol2)}$.

antibody increased by 3–4.5 folds in Tol2-BAFF group, compared to the Tol2 group, which were consistent with the qPCR results (figure 6). Thus, we speculated BAFF overexpression could not only induce primary responses of antibody genes but also could stimulate the specific antibody expression *in vivo*.

Discussion

Previous studies showed that transgenic mice with BAFF have increased number of mature B and effector T cells and developed autoimmune-like manifestations, such as the presence of high levels of rheumatoid factors, circulating immune complexes, antiDNA autoantibodies and immunoglobulin deposition in kidneys (Stohl *et al.* 2005; Thorn *et al.* 2010). Therefore, the upregulation of immunoglobulins, including IgG and IgM, is an obvious manifestation of autoimmunity disease. Zebrafish were selected as a model due to their small body size, rapid growth and development, similarity to mammalian genes and advantages as a model in immunity (Traver *et al.* 2003; Trede *et al.* 2004; Lieschke and Currie 2007; Iwanami 2014).

In this study, early embryos were injected with Tol2-GFP-2A-BAFF/His, and the qPCR results demonstrated that most of the immunoglobulins (IgLC-1, IgLC-2, IgD, IgM, and IgZ/T) were significantly increased in juveniles, which indicated that BAFF overexpression affects early serologic autoimmunity. However, IgLC-3 did not change in our study. The mRNAs of IgLC-1, IgLC-2 and IgLC-3 were reported to be detected at low levels by 3 dpf, and their expression levels increased steadily to the adult range between 4 and 6 wpf. We speculated that the different expression of IgLC-1, IgLC-2 and IgLC-3 in transgenic fish might be caused by the diverse expression of immunoglobulins during different developmental stages which requires further study. Rag-1 encodes a protein involved in the genomic rearrangement of the TCR and immunoglobulin (Ig) loci and is a suitable marker for maturing lymphocytes (Willett *et al.* 1997). We found that BAFF overexpression not only affects Rag-1 expression in juveniles but also highly expressed in adults, which indicated that BAFF upregulated immunoglobulins dependent on genomic rearrangement from Rag-1. The Ikaros encodes a transcription factor in mice that is essential for the correct differentiation of B and T lymphocytes (Willett *et al.* 2001; Schorpp *et al.* 2006). In our study, Ikaros expression was detected at 21 dpf and significantly increased in adults that were supposed to be expressed at low levels according to previous reports. As we know, the production of anti-dsDNA in systemic lupus erythematosus (SLE) is also a T cell-dependent antigen-driven process in which anti-dsDNA production coincides with concurrent B and T cell activation during the development of the active disease (Danilova *et al.* 2004). Thus, Ikaros activation might reflect indirectly not only B-cell overexpansion and maturation but also T-cell activation induced by BAFF overexpression. Subsequently, the significantly high expression of T-cell antigen receptor alpha chain constant region (TCRAC) confirmed T cell expansion and early signs of thymic involution (Lam *et al.* 2002). Bcl-2 is a protooncogene, and its high expression indicated that cell expansion occurred not only in juveniles but also in adults (Prudent *et al.* 2015). IL-4 is a cytokine known to activate B cells and augment their proliferation and induces Th2-mediated immune responses by upregulating the expression of MHC class II, CD23, and IL-4R on B cells (Hu *et al.* 2010). Considering the function of these genes, their high expression is indirectly reflected in B cell overexpansion and maturation.

According to previous reports, BAFF overexpression, even when present, may not necessarily drive disease in some SLE patients. Constitutive overexpression of BAFF in autoimmune-resistant mice drives only some aspects of SLE. This may help explain the great heterogeneity of the clinical response to BAFF antagonists in SLE. Similarly, this situation may also exist in zebrafish. In adult zebrafish, the qPCR results were consistent with those in juveniles. The expression levels of IgLC-1, IgLC-2, IgD, IgM and IgZ/T were all significantly increased, but not proportionally with the level of BAFF overexpression, which indicated BAFF overexpression only induced immunoglobulin expression to some extent and BAFF is not the only determinant for inducing autoimmune diseases. We also detected the expression of signalling pathway-related genes, PTEN, PI3K and NF-κB, since BAFF is reported to be an activator of the noncanonical NF-κB pathway, which provides critical survival signals during B cell maturation and contributes to B cell proliferation. The results demonstrated that NF-κB significantly upregulated, but the PTEN and Akt1 did not change, which confirmed BAFF overexpression could activate NF-κB signalling pathway, but not related to the PTEN/PI3K/AKT pathway.

Even though all F0 founders had GFP-positive offspring by crossing with wild-type AB, none of the F_1 embryos could survive for 48 h. Our preliminary experiments suggested it was more workable to study on F0 BAFF-overexpression zebrafish or constructed the BAFF-overexpression transgenic line induced by tissue-specific promoter, due to the low survival rate of F_1 generation. To exclude great functional consequences that the variable expression levels among F0 generations, juveniles with GFP expressed ubiquitously in the whole body were confirmed under microscopy and strictly selected for qPCR at 21 dpf. Spleen as one of the most important lymphoid organs was isolated for qPCR in F0 adults and the amount of BAFF overexpression in spleens were firstly confirmed to increase by 6-fold, 16-fold and 10-fold. The discordances of the BAFF expression reflected the mosaic expression of BAFF in the spleens of F0 founders which might be a feedback mechanism for survival. That is why eight F0 zebrafish were confirmed by the ubiquitous GFP expression at 1 mpf and only three of them still had GFP expression in spleens at 3 mpf. All the results above suggested that BAFF is vital factors for embryonic development and ectopic BAFF expression might lead to early death of embryos.

Zebrafish embryos and juveniles can be applied for the generation of transgenic animals with relative convenience, which makes them an extremely versatile model for immune system developmental studies. Our study is the first report of BAFF overexpression in zebrafish and represents the great effects of BAFF on zebrafish immune-related gene expression. Although BAFF plays a vital role in embryonic development where high expression is prone to cause death, the surviving adults revealed similarities with mammalians in the upregulation of immune-related genes which provides a system for studying zebrafish autoimmunity.

Acknowledgements

This work was supported by the Construction Programme of Hebei Scientific Conditions (13967607D, 12965519D and 12966121D) and National Natural Science Foundation of Hebei province (C2012206096).

References

Bossen C., Cachero T. G., Tardivel A., Ingold K., Willen L., Dobles M. *et al.* 2008 TACI, unlike BAFF-R, is solely activated by

oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood* **111**, 1004–1012.
Danilova N., Hohman V. S., Sacher F., Ota T., Willett C. E. and Steiner L. A. 2004 T cells and the thymus in developing zebrafish. *Dev. Comp. Immunol.* **28**, 755–767.
Hansen J. D. and Zapata A. G. 1998 Lymphocyte development in fish and amphibians. *Immunol. Rev.* **166**, 199–220.
Holbech H., Andersen L., Petersen G. I., Korsgaard B., Pedersen K. L. and Bjerregaard P. 2001 Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **130**, 119–131.
Hu Y. L., Xiang L. X. and Shao J. Z. 2010 Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: implications for a distinct B cell receptor in lower vertebrates. *Mol. Immunol.* **47**, 738–746.
Iwanami N. 2014 Zebrafish as a model for understanding the evolution of the vertebrate immune system and human primary immunodeficiency. *Exp. Hematol.* **42**, 697–706.
Kawakami K. 2007 Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol.* **8** (suppl 1), S7.
Kawakami K. and Shima A. 1999 Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. *Gene* **240**, 239–244.
Kawakami K., Koga A., Hori H. and Shima A. 1998 Excision of the tol2 transposable element of the medaka fish, *Oryzias latipes*, in zebrafish, *Danio rerio*. *Gene* **225**, 17–22.
Khan W. N., Wright J. A., Kleiman E., Boucher J. C., Castro I. and Clark E. S. 2013 B-lymphocyte tolerance and effector function in immunity and autoimmunity. *Immunol. Res.* **57**, 335–353.
Kim J. H., Lee S. R., Li L. H., Park H. J., Park J. H., Lee K. Y. *et al.* 2011 High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* **6**, e18556.
Lam S. H., Chua H. L., Gong Z., Wen Z., Lam T. J. and Sin Y. M. 2002 Morphologic transformation of the thymus in developing zebrafish. *Dev. Dyn.* **225**, 87–94.
Lam S. H., Chua H. L., Gong Z., Lam T. J. and Sin Y. M. 2004 Development and maturation of the immune system in zebrafish, *Danio rerio*: a gene expression profiling, in situ hybridization and immunological study. *Dev. Comp. Immunol.* **28**, 9–28.
Li Y., Wang J., Xie Y., Liu S. and Tian Y. 2014 Pattern of change in histone 3 lysine 9 acetylation and histone deacetylases in development of zebrafish embryo. *J. Genet.* **93**, 539–544.
Lieschke G. J. and Currie P. D. 2007 Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* **8**, 353–367.
Mackay F., Woodcock S. A., Lawton P., Ambrose C., Baetscher M., Schneider P. *et al.* 1999 Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* **190**, 1697–1710.
Moisini I. and Davidson A. 2009 BAFF: a local and systemic target in autoimmune diseases. *Clin. Exp. Immunol.* **158**, 155–163.
Page D. M., Wittamer V., Bertrand J. Y., Lewis K. L., Pratt D. N., Delgado N. *et al.* 2013 An evolutionarily conserved program of B-cell development and activation in zebrafish. *Blood* **122**, e1–e11.
Prudent J., Popgeorgiev N., Bonneau B. and Gillet G. 2015 Bcl-2 proteins, cell migration and embryonic development: lessons from zebrafish. *Cell Death Dis.* **6**, e1910.
Rothwell D. G., Crossley R., Bridgeman J. S., Sheard V., Zhang Y., Sharp T. V. *et al.* 2010 Functional expression of secreted proteins from a bicistronic retroviral cassette based on foot-and-mouth disease virus 2A can be position dependent. *Hum. Gene Ther.* **21**, 1631–1637.
Schorpp M., Bialecki M., Diekhoff D., Walderich B., Odenthal J., Maischein H. M. *et al.* 2006 Conserved functions of Ikaros in vertebrate lymphocyte development: genetic evidence for distinct larval and adult phases of T cell development and two lineages of B cells in zebrafish. *J. Immunol.* **177**, 2463–2476.
Stohl W. 2013 Future prospects in biologic therapy for systemic lupus erythematosus. *Nat. Rev. Rheumatol.* **9**, 705–720.
Stohl W., Xu D., Kim K. S., Koss M. N., Jorgensen T. N., Deocharan B. *et al.* 2005 BAFF overexpression and accelerated glomerular disease in mice with an incomplete genetic predisposition to systemic lupus erythematosus. *Arthritis Rheum.* **52**, 2080–2091.
Thorn M., Lewis R. H., Mumbey-Wafula A., Kantrowitz S. and Spatz L. A. 2010 BAFF overexpression promotes anti-dsDNA B-cell maturation and antibody secretion. *Cell. Immunol.* **261**, 9–22.
Traver D., Herbomel P., Patton E. E., Murphey R. D., Yoder J. A., Litman G. W. *et al.* 2003 The zebrafish as a model organism to study development of the immune system. *Adv. Immunol.* **81**, 253–330.
Trede N. S. and Zon L. I. 1998 Development of T-cells during fish embryogenesis. *Dev. Comp. Immunol.* **22**, 253–263.
Trede N. S., Zapata A. and Zon L. I. 2001 Fishing for lymphoid genes. *Trends Immunol.* **22**, 302–307.
Trede N. S., Langenau D. M., Traver D., Look A. T. and Zon L. I. 2004 The use of zebrafish to understand immunity. *Immunity* **20**, 367–379.
Willett C. E., Zapata A. G., Hopkins N. and Steiner L. A. 1997 Expression of zebrafish rag genes during early development identifies the thymus. *Dev. Biol.* **182**, 331–341.
Willett C. E., Cortes A., Zuasti A. and Zapata A. G. 1999 Early hematopoiesis and developing lymphoid organs in the zebrafish. *Dev. Dyn.* **214**, 323–336.
Willett C. E., Kawasaki H., Amemiya C. T., Lin S. and Steiner L. A. 2001 Ikaros expression as a marker for lymphoid progenitors during zebrafish development. *Dev. Dyn.* **222**, 694–698.
Xiao J., Chen T., Wang Q., Liu Q., Wang X., Lv Y. *et al.* 2011 Search for live attenuated vaccine candidate against edwardsiellosis by mutating virulence-related genes of fish pathogen Edwardsiella tarda. *Lett. Appl. Microbiol.* **53**, 430–437.
Yang D., Liu Q., Ni C., Li S., Wu H., Wang Q. *et al.* 2013 Gene expression profiling in live attenuated Edwardsiella tarda vaccine immunized and challenged zebrafish: insights into the basic mechanisms of protection seen in immunized fish. *Dev. Comp. Immunol.* **40**, 132–141.

Received 12 November 2015, in revised form 14 January 2016; accepted 3 February 2016
Unedited version published online: 10 February 2016
Final version published online: 20 September 2016

Corresponding editor: INDRAJIT NANDA