Characterisation and expression analysis of B-cell activating factor (BAFF) in spiny dogfish (*Squalus acanthias*): Cartilaginous fish BAFF has a unique extra exon that may impact receptor binding

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ABSTRACT

B-cell activating factor (BAFF), also known as tumour necrosis factor (TNF) ligand superfamily member 13B, is an important immune regulator with critical roles in B-cell survival, proliferation, differentiation and immunoglobulin secretion. A BAFF gene has been cloned from spiny dogfish (*Squalus acanthias*) and its expression studied. The dogfish BAFF encodes for an anchored type-II transmembrane protein of 288 aa with a putative furin protease cleavage site and TNF family signature as seen in BAFFs from other species. The identity of dogfish BAFF has also been confirmed by conserved cysteine residues, and phylogenetic tree analysis. The dogfish BAFF gene has an extra exon not seen in teleost fish, birds and mammals that encodes for 29 aa and may impact on receptor binding. The dogfish BAFF is highly expressed in immune tissues, such as spleen, and is up-regulated by PWM in peripheral blood leucocytes, suggesting a potentially important role in the immune system.

1. Introduction

Although cellular precursors of lymphocyte-like cells have recently been identified in jawless fish, the appearance of an adaptive immune system containing immunoglobulins (Igs), T-cell receptors (TCRs) and major histocompatibility complex (MHC) molecules is confined to jawed vertebrates (Flajnik and Kasahara, 2010; Haire et al., 2000). Cartilaginous fish were one of the earliest groups of jawed vertebrates to appear and diverged from a common ancestor with other jawed vertebrates around 500 million years ago. They comprise approximately 700 extant species that include chimeras, sharks, skates and rays.

The adaptive humoral immune system in this lineage was, until recently, considered to be relatively primitive and based solely upon low-affinity IgM responses (Clem et al., 1967; Makela and Litman, 1980). However, it is now known that two additional Ig isotypes, IgW (the shark orthologue of IgD) (Ohta and Flajnik, 2006) and IgNAR (an elasmobranch-specific isotype which does not associate with light chains) (Greenberg et al., 1995) exist and accumulated data has revealed a highly complex, multi-layered humoral response in these species. Shark IgM is produced in two forms, a monomeric (mIgM) and pentameric (pIgM) form (Clem et al., 1967), which do

not interconvert (Small et al., 1970). pIgM provides the 'first line of defence' via a low affinity, high avidity interaction and does not appear to require T-cell help (Dooley and Flajnik, 2005; Rumfelt et al., 2002). In contrast, mIgM and IgNAR seem to be the functional equivalent of mammalian IgG, providing a highly-specific antigen-driven response with all the hallmarks of T-dependency, including affinity maturation and immunological memory (Dooley and Flajnik, 2005). Almost nothing is known about the function of IgW (also previously known as IgNARC, IgX and IgR) in cartilaginous fish (Anderson et al., 1999; Greenberg et al., 1996; Rumfelt et al., 2004; Kobayashi and Tomonaga, 1988), however its unusual expression pattern suggests it may play a role in protecting mucosal surfaces. Cartilaginous fish Ig genes are organised in clusters (rather than the translocon organisation typified in mammals) with each cluster containing all of the components required to generate the full Ig chain (Hinds and Litman, 1986; Rast et al., 1994). There is no isotype switch in elasmobranchs however there is isotype exclusion, with individual B-cells expressing only a single Ig isotype/VH transcript (Eason et al., 2004; Malecek et al., 2008; Rumfelt et al., 2002), indicating that even in species with literally hundreds of Ig clusters, recombination and Ig expression are carefully controlled.

Cartilaginous fish have no bone marrow or lymphatic system but all species studied to date have a thymus, spleen and gut associated lymphoid tissue (GALT), as well as an epigonal organ associated with the gonads (Bodine et al., 2004) and a Leydig organ

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associated with the oesophagus, although the latter is not present in all species. To determine the immunological role of these tissues, studies into the expression of B-cell transcriptional regulators, recombination activating genes, terminal deoxynucleotidyl transferase, TCR and Ig have been carried out. They have shown that the thymus, epigonal and Leydig organs are primary sites of lymphopoiesis (Anderson et al., 2004; Haire et al., 2000; Miracle et al., 2001) and that B-cells are produced in these tissues throughout the life of the animal. In contrast the spleen (and possibly GALT) are sites where adaptive immune responses occur (Miracle et al., 2001; Rumfelt et al., 2002). In the nurse shark, IgM and IgNAR are found expressed at high levels in the spleen, liver, gill and epigonal organ, whereas IgW is expressed predominantly in the spleen and pancreas (Rumfelt et al., 2002).

Despite recent progress in understanding the adaptive immune system of cartilaginous fish, nothing is known about the processes involved in the development, maintenance or proliferation of B-cells. In addition, there is very little known about the regulatory molecules involved in these processes. To date, a few cytokines and their receptors have been cloned from cartilaginous fish, these include interleukin-1β (IL-1β) (Bird et al., 2002), IL-8 (Inoue et al., 2003) and various chemokine receptor genes (Goostrey et al., 2005) but no functional studies have been reported. To better understand humoral immunity in cartilaginous fish, we report the identification of a key cytokine involved in B-cell maturation, B-cell activating factor (BAFF), from the spiny dogfish (Squalus acanthias). BAFF, also known as tumour necrosis factor superfamily member 13b (TNFSF13b), belongs to the TNF ligand family and is critical for survival, proliferation and differentiation of B-cells (Schneider et al., 1999). It is expressed as a homotrimeric type II transmembrane protein on the surface of innate immune cells (e.g. monocytes, dendritic cells and macrophages) and some T cells (Huard et al., 2004; Nardelli et al., 2001; Schneider et al., 1999). However, the C-terminal extracellular domain of the membrane-bound BAFF encodes a furin consensus motif (R–X–R/K–R) which is susceptible to protease cleavage, releasing a soluble, biologically-active cytokine (Nardelli et al., 2001). Soluble BAFF adopts the usual trimeric form of a TNF family ligand, but is unusual in that it can also assemble into an ordered capsid-like structure containing twenty trimers (i.e. a 60-mer) (Liu et al., 2002). BAFF binds preferentially to the BAFF receptor (known as the TNF receptor superfamily member (TNFRSF)-13C) but with much lower affinity relative to TNFRSF-17 (also known as the B cell maturation antigen receptor) and TNFRSF-13B (the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)) (Bossen and Schneider, 2006). The crystal structure of soluble human BAFF reveals an unusually long and flexible D–E loop compared to other TNF superfamily members, with this region being important in receptor binding specificity and the ability to form the capsid-like multimer (Karpusas et al., 2002; Oren et al., 2002). The receptors for BAFF are mainly expressed by B-cells, where ligand binding causes receptor oligomerization (Liu et al., 2003) and enables recruitment of the intercellular signalling molecules of the TNF receptor-associated factors family, eventually leading to NF-κB pathway activation and increased B-cell survival (Gardam et al., 2008).

Although most of the information to date on the structure and function of BAFF has been gained from studies in mammals (Guan et al., 2007a; Shen et al., 2011), more recently a BAFF orthologue has been cloned from a number of bony fish (Ai et al., 2011; Liang et al., 2010) and birds (Guan et al., 2007b; Schneider et al., 2004), with functional studies performed in some species (Ai et al., 2011; Kothlow et al., 2007). This manuscript describes the cloning of a BAFF orthologue from a cartilaginous fish, the spiny dogfish (S. acanthias). The cartilaginous fish BAFF gene shows conservation of a number of functionally important features but contains an extra

exon relative to known genes, with the additional amino acids predicted to impact upon the protein structure. In addition real-time PCR analysis shows BAFF is expressed in a number of spiny dogfish tissues and is up-regulated in peripheral blood leucocytes following incubation with immunostimulants in vitro, providing evidence of its role within the spiny dogfish immune response.

2. Materials and methods

2.1. Spiny dogfish

Wild spiny dogfish were obtained from the North Sea and maintained at the North Atlantic Fisheries College Marine Centre, Shetland, UK. Adult animals were held in large, indoor tanks supplied by flow-through seawater at 5–14 °C. Animals were anaesthetized with MS-222 (0.12–0.16 g/L seawater) prior to any procedure. Blood samples were taken from the caudal vein then centrifuged at ∼300g for 10 min to separate plasma and whole-blood cell pellets. All procedures were conducted in accordance with the UK Home Office 'Animals (Scientific Procedures) Act 1986' and Pfizer corporate policy #507 on animal care and use.

2.2. Total RNA extraction and cDNA preparation

Outwardly healthy, adult spiny dogfish were sacrificed and a selection of immune and non-immune tissues were immediately collected and stored short-term in RNAlater (Invitrogen, UK). Total RNA was extracted by mechanical disruption of the tissues in TRI-zol® Reagent (Invitrogen, UK) and the subsequent addition of 1/5 volume of chloroform. Following centrifugation at 4 °C the separated upper phase, containing the RNA, was transferred to a RNase-free tube and precipitated by the addition of isopropanol. After centrifugation the resultant RNA pellet was washed twice with 75% ethanol, re-centrifuged to pellet the RNA, any remaining ethanol aspirated and the pellet allowed to air dry. The RNA pellets were resuspended in an appropriate volume of RNase-free H_2O and stored at −80 °C for future use. First-strand cDNA for use in rapid amplification of cDNA ends (RACE)-PCR was synthesised from 2 µg of total RNA using bioscript reverse transcriptase (Bioline) with either oligo(dT)$_{16}$ or adaptor-oligo(dT) primer (Table 1) at 42 °C for 1 h, following the manufacturer's instructions.

2.3. Cloning and sequencing

Blast searches using human and Fugu BAFF amino acid (aa) sequences identified a partial BAFF cDNA in the spiny dogfish EST database. To obtain the complete BAFF cDNA sequence, 3′- and 5′-RACE were carried out using first-strand cDNA prepared from spleen and epigonal tissues, using gene-specific primers (Table 1). In 3′ RACE-PCR, cDNA was transcribed from poly (A) mRNA using an adaptor-oligo(dT) primer. PCR was performed with a spiny dogfish BAFF-specific forward primer SaBAFF-F1 and the adaptor primer (Table 1), and further semi-nested with saBAFF-F2 (Table 1) and the adaptor primer under the following conditions: 1 cycle of 94 °C for 5 min; 38 cycles of 94 °C for 1 min, 68 °C for 2.5 min; 1 cycle of 72 °C for 10 min. The 5′ end of BAFF was obtained using a semi-nested RACE-PCR. In 5′ RACE-PCR, cDNA was transcribed from poly(A) mRNA using an oligo (dT) primer, treated with Escherichia coli RNase H (Promega), purified using a PCR purification kit (Qiagen Ltd., UK), and tailed with poly(C) at the 5′ end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed initially with saBAFF-R1 and oligo (dG) primers and further semi-nested with saBAFF-R2, and the oligo (dG) (Table 1). The first round 5′-RACE PCR conditions were: 1 cycle of 94 °C for 5 min; 38 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for

Table 1
Primers used for cloning, expression and gene organization of spiny dogfish BAFF.

Primer	Sequence (5′–3′)	Length	Application
Adaptor-Oligo(dT)	CTCGAGATCGATGCGGCCGC (dT)$_{16}$ VN	37	Cloning (3′-RACE)
Adaptor	CTCGAGATCGATGCGGCCGC	20	Cloning (3′-RACE)
Oligo(dG)	GGGGGGIGGGIIGGGIIG	18	Cloning (5′-RACE)
saBAFF-F1	GATTCTGAGTTTGATAAAGGGAAGTGC	27	Cloning (3′-RACE)
saBAFF-F2	GTACAGTCAGGTGTGGTATAAGG	23	Cloning (3′-RACE)
saBAFF-R1	AGTAGCCAGCCTCTTTGACC	20	Cloning (5′-RACE)
saBAFF-R2	GGCATACACTTCGGACAATC	20	Cloning (5′-RACE)
saBAFF-FLF1	CAATCTTGTTCTGGATGGGTTA	22	Cloning (coding region)
saBAFF-FLR1	AGAAACCACTTTGCCTCTATTGC	23	Cloning (coding region)
saBAFF-gF1	AGTATGCACAATTGACAATCTTGTTCTG	28	Gene walking
saBAFF-gF2	CTCAGCCTTCATCCTCGCTTTAAC	24	Gene walking
saBAFF-gF3	AGCAGTCCTGTGTGCAATTGATTG	25	Gene walking
saBAFF-gF4	ACACGTGACCGAAGACCACTTG	22	Gene walking
saBAFF-gF5	CATGGATTCTGAGTTTGATAAAGGGA	26	Amplifying the last two introns
saBAFF-gF6	GTCAAAGAGGCTGGCTACTTCCT	23	Amplifying the last two introns
saBAFF-gR1	TGCCTTAGAGCAGTTTTATTGCACC	25	Amplifying the last two introns
saBAFF-gR2	GGTATTACAAGTTCAAGCTCATCTTCCAC	29	Amplifying the last two introns
saBAFF-gR3	AGGAAGTAGCCAGCCTCTTTGAC	23	Gene walking
saBAFF-gR4	TCCCTTTATCAAACTCAGAATCCATG	26	Gene walking
saBAFF-gR5	ACACTTCGGACAATCATTCGC	21	Gene walking
saBAFF-gR6	TTTTACAAGTGGTCTTCGGTCACG	24	Gene walking
AP-1	GTAATACGACTCACTATAGGGC	22	Gene walking
AP-2	ACTATAGGGCACGCGTGGT	19	Gene walking
SP6	CATTTAGGTGACACTA	16	Colony PCR
T7	GATATACGACTCACTATAGGG	21	Colony PCR
saBACTIN-RTF1	CATGGTATTGTCACCAACTG	20	Real-time PCR
saBACTIN-RTR1	GTCTCAAACATGATCTGTGTC	21	Real-time PCR
saBAFF-RTF1	CTGAGTTTGATAAAGGGAAGTG	22	Real-time PCR
saBAFF-RTR1	GTATTGTCCTTATACCACACCTGAC	25	Real-time PCR

1 min; and 1 cycle of 72 °C for 10 min. The semi-nested 5′ RACE PCR was the same as for the first round except that the annealing temperature was 52 °C rather than 48 °C.

The 3′ and 5′ RACE-PCR products were ligated into pGEM-T Easy vector (Promega) and transformed into RapidTrans™ E. coli TAM-competent cells (Actif Motif). The colonies containing vectors with inserts were identified through red–white colour selection when grown on MacConkey agar (Sigma–Aldrich). Positive clones were screened by standard colony PCR using the primers SP6 and T7 (Table 1) and plasmid DNA from at least three independent clones were extracted using a Qiagen Miniprep Kit (Qiagen Ltd., UK). The insert size was then confirmed using electrophoresis of the plasmid DNA digested with EcoR I, and plasmids from three independent E. coli colonies sequenced by a commercial company (MWG-Biotech). Finally, the full-length cDNA sequences containing the 5′-untranslated region (UTR) and 3′-UTR were assembled. The coding region of the spiny dogfish sequence was then confirmed by sequencing the PCR products amplified using primers saBAFF-FLF1 and saBAFF-FLR1 (Table 1).

2.4. Sequence analysis

Sequences generated were analyzed for similarity with other known molecules using the FASTA (Mount, 2007a) and BLAST (Mount, 2007b) programs. Direct comparison between cDNA sequences was performed using CLUSTAL W (Larkin et al., 2007) and homology analysis was performed using the MatGat software v2.02 (Campanella et al., 2003). Phylogenetic analysis was also performed on the predicted full-length amino acid sequence of spiny dogfish BAFF along with selected BAFF molecules and other selected TNF superfamily members, using the neighbour-joining method (Tamura et al., 2007) and bootstrapped 10,000 times. The furin cleavage site was predicted using ProP 1.0 Server (Duckert et al., 2004). The protein family signature was analyzed using the PROSITE database of protein families and domains (Sigrist et al., 2010), and transmembrane domain prediction was done using TMHMM Server

v2.0 (Kahsay et al., 2005) and SMART program (Letunic et al., 2009). Finally, the prediction of putative N-glycosylation sites was done using NetNGlyc 1.0 Server (Salim et al., 2003).

2.5. Determination of spiny dogfish BAFF gene organisation

Genomic DNA (gDNA) was extracted using the high salt method described previously (Bird et al., 2002). Blood harvested from the caudal vein was centrifuged at 300g for 10 min to separate plasma and whole-blood cell pellets. 80–100 μl of packed cells from the pellet were used for DNA extraction. The gene organisation of the spiny dogfish BAFF gene was determined using two approaches. Initially, using the known zebrafish gene organisation, the exons within the spiny dogfish sequence were predicted and several sets of forward and reverse primers were designed within these exons, to allow amplification of the introns within the spiny dogfish BAFF gene (Table 1). The last two introns were amplified using nested PCR with saBAFF-gF5 and saBAFF-gR1 in the first round and saBAFF-gF6 and saBAFF-gR2 in the second round. Using this approach we failed to amplify the remaining introns and so gene walking was performed. Genome walking libraries were constructed using a Universal Genome Walker™ kit (BD Clontech) following the manufacturer's instructions. Briefly, the gDNA was digested with one of four restriction enzymes of EcoR V, Pvu II, Stu I and Sma I, respectively, and the blunt ended restricted products ligated into the Genome Walker adapter. A nested PCR was carried out using two BAFF-specific primers designed previously and two adapter primers specific to the library (Table 1). The first-round PCR used the outer adapter primer (AP-1) and a BAFF-specific primer for each reaction (saBAFF-gF1, saBAFF-gR5, saBAFF-gF3 or saBAFF-gR3, Fig. 3) in each library and the second-round used 2 μl of the first round PCR product with the nested adapter primer (AP-2) and a BAFF-specific primer of each set (saBAFF-gF2, saBAFF-gR6, saBAFF-gF4 or saBAFF-gR4). Touchdown PCR was performed using an enzyme mixture of Taq DNA polymerase (Bioline, London, UK) and Pfu DNA polymerase (Promega) at a ratio of 50:1. First-round

PCR amplification was performed using the following two-step cycle parameters: 7 cycles of 25 s at 94 °C, 3 min at 72 °C, 32 cycles of 25 s at 94 °C, 3 min at 68 °C, with a final extension step of 7 min at 68 °C. The second-round was also performed using two-step cycle parameters: 5 cycles of 25 s at 94 °C, 3 min at 72 °C, 20 cycles of 25 s at 94 °C, 3 min at 68 °C, with a final extension step of 7 min at 68 °C.

Gene organisation analysis revealed a seven exon/six intron structure of saBAFF with an extra exon (3a) between exons 3 and 4 of BAFF genes from bony fish, birds and mammals. To confirm whether this extra exon is always present in BAFF mRNA nested PCR was carried out with primers located in exons 3 (saBAFF-gF3, saBAFF-gF4) and 6 (saBAFF-gR1, saBAFF-gR2) using cDNA from multiple tissues as well as from *in vitro* stimulated peripheral blood leucocytes (PBL) as the template. Nested PCR was conducted as the products of first round PCR were weak and we did not want to miss any potential alternative splice products. The PCR products generated were cloned and sequenced.

2.6. Expression of spiny dogfish BAFF in vivo

A range of immune and non-immune tissues were dissected from three outwardly healthy spiny dogfish, and RNA isolated and cDNA produced as described in Section 2.2. After isolation of total RNA, 5 μg was reverse transcribed into cDNA in 20 μl reactions and the resulting cDNA diluted in 80 μl 1×TE buffer (pH 8.0). To evaluate the tissue distribution of spiny dogfish BAFF expression real-time PCR was performed with the primers sa-BAFF-RTF1 and saBAFF-RTR1 (Table 1). The expression level of the spiny dogfish β-actin gene was also determined using the primers saBACTIN-RTF1 and saBACTIN-RTR1 to enable the relative expression of BAFF in the different tissues to be expressed as arbitrary units by normalizing to the expression level of β-actin.

2.7. Expression of spiny dogfish BAFF in vitro

To establish whether spiny dogfish BAFF expression could be induced following stimulation, PBL from three individual spiny dogfish were isolated and treated with either 5 μg/ml of the T-cell mitogen, phytohaemagglutinin (PHA; L1668, Sigma), 10 μg/ml of the B-cell mitogen, pokeweed mitogen (PWM; L9379, Sigma) or 10 μg/ml of the microbial mimic, lipopolysaccharide (LPS; serotype 0127 B8, Sigma). The concentrations and stimulatory period were chosen based upon dose dependency and time-course data sets for splenocytes from another cartilaginous fish (spotted catshark) and bony fish generated previously in our lab (Bird et al., 2002; Wang et al., 2010). In this case blood was collected from the caudal vein of fish using a heparinised syringe and diluted 1:5 in complete Leibovitz (L)-15 medium (Life Technologies, Paisley, UK) adjusted to cartilaginous fish osmolarity, and containing 0.2 M NaCl (Sigma–Aldrich, St. Louis, MO), 0.35 M urea (Sigma–Aldrich), 10% foetal bovine serum (FBS, Life Technologies), 10 U/ml Heparin (Sigma–Aldrich, St. Louis, MO), 100 μg/ml streptomycin (Life Technologies) and 100 U/ml penicillin (Life Technologies). The PBL concentration was determined using a Neubauer counting chamber with trypan blue exclusion and diluted further with L-15 medium (as above) to give a final concentration of 5×10^6 cells/ml. Five millilitres of the diluted blood samples were incubated with one of the three stimulants at 15 °C in 6-well plates for 12 and 24 h. Phosphate buffered saline (PBS), pH 7.2 was used as a negative control for LPS and PHA, whereas 0.9% NaCl was used as a negative control for PWM. Each of the treatments was performed in triplicate with RNA preparation and cDNA synthesis as described in Section 2.2. The fold change was calculated as the average expression level of the stimulated samples divided by that of the negative control samples at the same time point, where the expression of the control samples was defined as 1. Based on initial results that showed PWM was the best stimulator of BAFF expression, we performed experiments examining the effect of PWM concentration on BAFF expression, by stimulation with 0.1, 1, 10 and 100 μg/ml using PBL from three individual dogfish.

2.8. Quantitative real-time PCR

Real-time amplification was performed in 96 well plates using 20 μl reactions containing 4 μl of cDNA template and 16 μl of master mix containing SYBR green I (Invitrogen) as described previously using a Light Cycler 480 real-time PCR machine (Roche) (Wang et al., 2011, 2010). PCR conditions were 95 °C for 10 min, followed by 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Fluorescence outputs were measured and recorded at 80 °C after each cycle for 40 cycles and quantified by comparison with a serial 10-fold dilution of reference samples for each primer pair used. Three reference samples were amplified during each run in order to ensure consistency between PCR runs. PCR primers were designed so that at least one primer in each pair straddled predicted splicing sites and the suitability of each primer pair in real-time PCR assays was tested by conventional PCR using cDNA and gDNA as template. Samples loaded onto an agarose gel stained with ethidium bromide confirmed primer pairs did not amplify a product from gDNA and confirmed a band of the correct size was amplified from cDNA. A negative control (no template) reaction was also performed for each primer pair tested. A melting curve for each PCR reaction was performed between 72 and 94 °C to ensure only a single product had been amplified. Expression levels of BAFF and β-actin using the cDNA prepared above (see Sections 2.6 and 2.7) were determined and the BAFF results normalised to the house keeping gene, β-actin, with the results expressed as fold change compared to the expression level in the unstimulated control cells using the Pfaffl method (Pfaffl, 2001).

2.9. Statistical analysis

The normalized real-time PCR data was calculated relative to the treatment with the lowest expression, which was set to 1, and then transformed using binary logarithms for significance analysis as described previously (Wang et al., 2011). Statistical analysis used the paired-sample *T*-test when comparing the value between control and stimulated samples within the PASW Statistic 18 software package (SPSS Inc., Chicago, Illinois). A ρ value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Sequence retrieval

Blast searches using human and *Fugu* BAFF aa sequences identified a partial BAFF cDNA in the spiny dogfish EST database. This 636 bp partial cDNA (GenBank accession DV496358) was used as the template to design the spiny dogfish BAFF-specific primers for RACE-PCR (Table 1).

3.2. Cloning and sequencing of spiny dogfish BAFF gene

Two RACE-PCR products overlapping with the partial BAFF cDNA were obtained, and when cloned and sequenced were found to contain the full-length spiny dogfish BAFF cDNA. The full-length coding sequence was confirmed by cloning the PCR product obtained with primers saBAFF-FLF and saBAFF-FLR (Table 1). The full-length spiny dogfish BAFF cDNA consists of 1333 bp, with an open reading frame (ORF) of 867 bp, coding for a predicted protein of 288 aa (Fig. 1). The

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   1  GAGAAAGTACAGCTCATGCAAACAGCAGCTGACAGGAAATAGTATGCACAATTGACAATCTTGTTCTGGA    70

  71  TGGGTTATTATACCAGCGTGGCTGAAAAGTGGAATTTGGGATGAAGATGAGAATGTTACAACAAAGGGAG   140
                                                   M  K  M  R  M  L  Q  Q  R  E    10

 141  AGTTTCAATATACAACACAAATGTTTGATCTACTCAGCCTTCATCCTCGCTTTAACAGCTCTTGTGTCGG   210
       S  F  N  I  Q  H  K  C  L  I  Y  S  A  F  I  L  A  L  T  A  L  V  S    33

 211  CTTATGTAGCAGCAGTTGCTCTACACCATGTTCTTGTACTGAAAGCAGAAATCTCATCCATGAAACAGGA   280
       A  Y  V  A  A  V  A  L  H  H  V  L  V  L  K  A  E  I  S  S  M  K  Q  E    57

 281  GTTGGGGACATACAAATTCCAACTGGATCACTTGGAAGCATTGACCAAAGGCCTGTCAACCAACTGGAGC   350
       L  G  T  Y  K  F  Q  L  D  H  L  E  A  L  T  K  G  L  S  T  N  W  S    80

 351  AGTTCAGCAAATCAGGTTAGTGAATTTCTCAAGAAAAGACTACACAGTGGGGAGAAACCGAGAAAAGAAA   420
       S  S  A  N  Q  V  S  E  F  L  K  K  R  L  H  S  G  E  K  P  R  E    103

 421  AAACTAAATTTAGAGGAGGCAGGTCACTTCCTGAACAAGTGCAGCAGTCCTGTGTGCAATTGATTGCCAC   490
       K  T  K  F  R  G  G  R  S  L  P  E  Q  V  Q  Q  S  C  V  Q  L  I  A  T    127

 491  ACGTGACCGAAGACCACTTGTAAAAGCGAATGATTGTCCGAAGTGTATGCCCGGAAGGCCAGAGTCAACC   560
       R  D  R  R  P  L  V  K  A  N  D  C  P  K  C  M  P  G  R  P  E  S  T    150

 561  TGTCGAGTTGAACGAAGATCTGTTTCAAATCAAAACGGTAAACAGGAGACAACTGTCCCATGGATTCTGA   630
       C  R  V  E  R  R  S  V  S  N  Q  N  G  K  Q  E  T  T  V  P  W  I  L    173

 631  GTTTGATAAAGGGAAGTGCTCTCGACAAAAAGGATAACAAGATCTTGGTCAAAGAGGCTGGCTACTTCCT   700
       S  L  I  K  G  S  A  L  D  K  K  D  N  K  I  L  V  K  E  A  G  Y  F  L    197

 701  AGTGTACAGTCAGGTGTGGTATAAGGACAATACTTTTACAATGGGACATTTTATCCAGAGAAAAAAGGCC   770
       V  Y  S  Q  V  W  Y  K  D  N  T  F  T  M  G  H  F  I  Q  R  K  K  A    220

 771  AGCAGTGTTGGCAATGAGCCACGTATTGTAATCCTATTTCGGTGCATCCAAAACATGTCCGCCTGCTGCC   840
       S  S  V  G  N  E  P  R  I  V  I  L  F  R  C  I  Q  N  M  S  A  C  C    243

 841  CAAATAATTCTTGCTTTACTGCTGGTATTGCAAAACTTGAAGTGGAAGATGAGCTTGAACTTGTAATACC   910
       P  N  N  S  C  F  T  A  G  I  A  K  L  E  V  E  D  E  L  E  L  V  I  P    267

 911  CCGAATTCAAGCACAAATTGCATTAAATGGTGATGGGACATTTTTTGGTGCAATAAAACTGCTCTAACTG   980
       R  I  Q  A  Q  I  A  L  N  G  D  G  T  F  F  G  A  I  K  L  L  -    288

 981  TTGGCTAAGGCAATAGAGGCAAAGTGGTTTCTGTCATTTAAAGAAAAAGAGAGAGGAATTTAAAGAAGAG   1050
1051  CCCTGTCAGTATCAACTTCCTTTTTGGTTTGGGGTGATGGACATATGGTTGTGAACCATTTCTATTTCAG   1120
1121  AAAATCAAATCGAGTTTCTTTCTCTCTGTGCAAACAAGAAATCAAAAGGAACAACTGAAAATAGAATTGC   1190
1191  TAAAATGGTTTTCATAATTTTTTGGATGTGTATGATGCCTTATGATTTTAACTGTGATGCCTCAACAATT   1260
1261  GTTTCCTTTTTAAGATAGTTTCACACAATAAACAAAACAGATGATAAGAACTGGGAAAAAAAAAAAAAAA   1330
1331  AAA                                                                    1333
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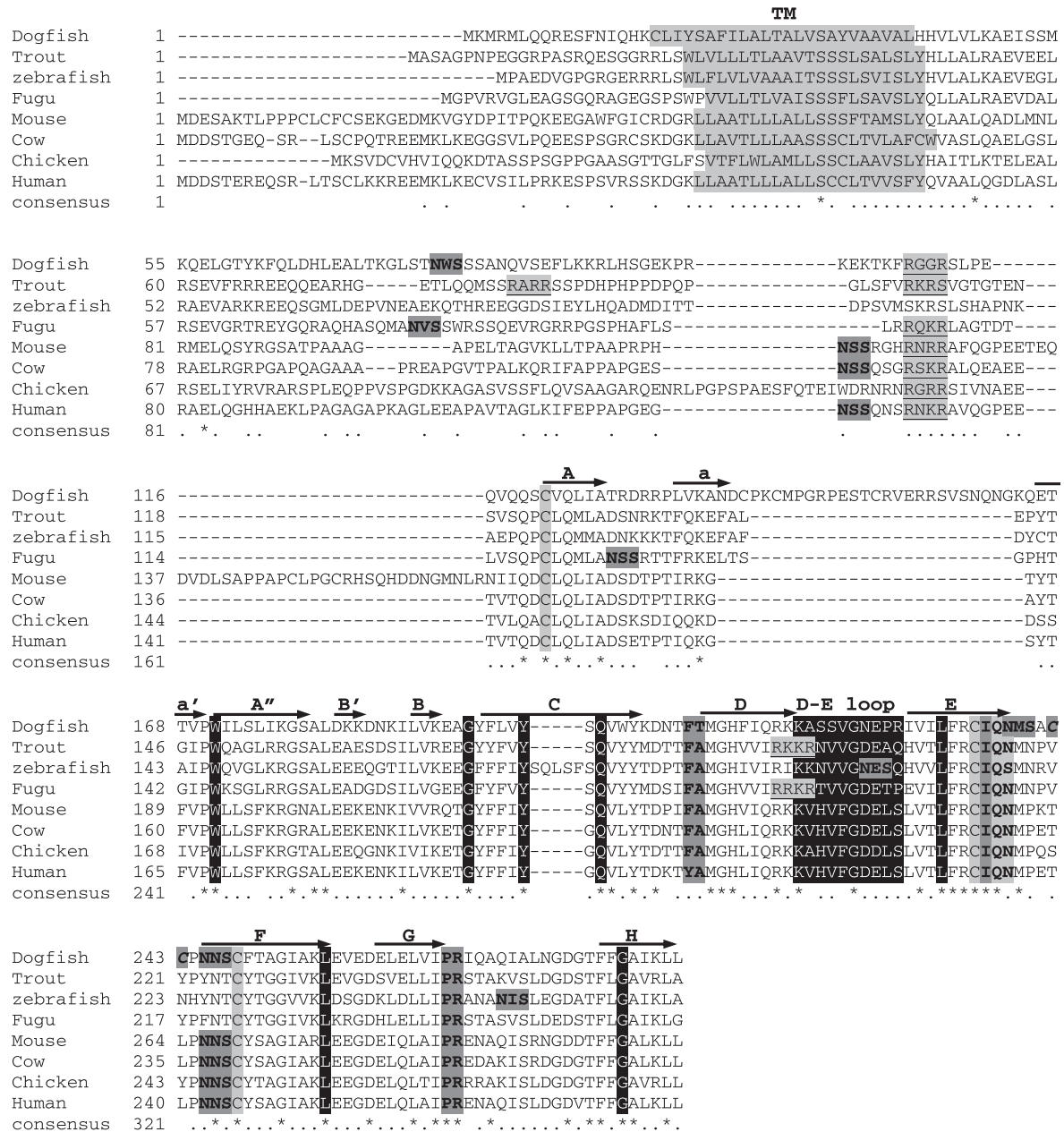
Fig. 1. Nucleotide and deduced amino acid (aa) sequence of spiny dogfish BAFF cDNA. The start and stop codons are boxed and in bold and the polyadenylation signal (AATAAA) is in bold and underlined. The predicted transmembrane domain is highlighted in light grey, the TNF family profile region in dark grey. The putative glycoslylation sites and predicted furin cut site (RGGR) are boxed. The exon boundaries are indicated with black arrows. In the 3′-UTR the mRNA instability motifs (ATTTA) are underlined. The BAFF transcript is 1333 bp, with an ORF of 867 bp that encodes a protein of 288 aa.

cDNA also contains a 5′-UTR of 110 nucleotides and a 3′-UTR of 356 nucleotides. The 5′-UTR contains two ATGs with in-frame stops before the authentic ORF. The 3′-UTR contains two mRNA instability motifs (ATTTA) and a polyadenylation signal (AATAAA) 23 bp upstream of a 18 bp poly (A) tail. Multiple alignments with other known BAFF aa sequences revealed areas of high aa conservation in all species examined, particularly at the C-terminal end of the protein but also that spiny dogfish BAFF carries a species-specific insertion (of 24 aa relative to bony fish and 28 aa relative to chicken/human BAFF) in the region between the a and a′ β-strands (Fig. 2). The predicted spiny dogfish BAFF protein contains the TNF family profile, located between Ser158 to Leu287, and a transmembrane domain (LIYSAFILALTALVSAYVAAVAL) confirming that, as in other vertebrates, spiny dogfish BAFF is expressed as an anchored type II transmembrane protein. Three potential N-glycosylation sites were also predicted, at position 78 (NWS), 238 (NMS) and 245 (NNS), with the latter conserved in humans, cow and chicken (Fig. 2). A predicted furin cleavage site was also present (RGGR at aa 108–111), similar to

the one found in other species except in zebrafish. Spiny dogfish BAFF showed ∼31–38% aa identity and ∼42–60% aa similarity to BAFF from other species (Table 2). However, there was an insertion of 28 aa relative to chicken/human BAFF between β-strands a and a′ (Fig. 2). Phylogenetic tree analysis of the BAFF sequences with three closely related TNF superfamily members (APRIL, TNF-α and CD40L) revealed that the spiny dogfish BAFF sequence grouped with BAFF molecules from other species and was separate from other TNF superfamily members (Fig. 3), further confirming the dogfish sequence is indeed a BAFF molecule.

3.3. Spiny dogfish BAFF gene organisation

Five products were obtained using nested PCR and the gene walking approach with gene-specific primers (Fig. 4A). Nested PCR with forward primers saBAFF-gF5 and saBAFF-gF6 and reverse primers saBAFF-gR1 and saBAFF-gR2 gave a product (P5) that covered the last two introns. The rest of the gene organisation

```
                                                                        TM
Dogfish     1  -------------------------MKMRMLQQRESFNIQHKCLIYSAFILALTALVSAYVAAVALHHVLVLKAEISSM
Trout       1  -------------------MASAGPNPEGGRPASRQESGGRRLSWLVLLLTLAAVTSSSLSALSLYHLLALRAEVEEL
zebrafish   1  ---------------------------MPAEDVGPGRGERRRLSWLFLVLVAAAITSSSLSVISLYHVLALKAEVEGL
Fugu        1  ------------------------MGPVRVGLEAGSGQRAGEGSPSWPVVLLTLVAISSSFLSAVSLYQLLALRAEVDAL
Mouse       1  MDESAKTLPPPCLCFCSEKGEDMKVGYDPITPQKEEGAWFGICRDGRLLAATLLLALLSSSFTAMSLYQLAALQADLMNL
Cow         1  MDDSTGEQ-SR--LSCPQTREEMKLKEGGSVLPQEESPSGRCSKDGKLLAVTLLLAASSSCLTVLAFCWVASLQAELGSL
Chicken     1  -------------MKSVDCVHVIQQKDTASSPSGPPGAASGTTGLFSVTFLWLAMLLSSCLAAVSLYHAITLKTELEAL
Human       1  MDDSTEREQSR-LTSCLKKREEMKLKECVSILPRKESPSVRSSKDGKLLAATLLLALLSCCLTVVSFYQVAALQGDLASL
consensus   1                       .     .    .    .   .  .. .......  *. .............*..... .

Dogfish    55  KQELGTYKFQLDHLEALTKGLSTNWSSSANQVSEFLKKRLHSGEKPR-------------KEKTKFRGGRSLPE------
Trout      60  RSEVFRRREEQQEARHG-----ETLQQMSSRARRSSPDHPHPPDPQP--------------GLSFVRKRSVGTGTEN---
zebrafish  52  RAEVARKREEQSGMLDEPVNEAEKQTHREEGGDSIEYLHQADMDITT--------------DPSVMSKRSLSHAPNK---
Fugu       57  RSEVGRTREYGQRAQHASQMANVSSWRSSQEVRGRRPGSPHAFLS------------------LRRQKRLAGTDT----
Mouse      81  RMELQSYRGSATPAAAG--------APELTAGVKLLTPAAPRPH----------------NSSRGHRNRRAFQGPEETEQ
Cow        78  RAELRGRPGAPQAGAAA---PREAPGVTPALKQRIFAPPAPGES---------------NSSQSGRSKRALQEAEE---
Chicken    67  RSELIYRVRARSPLEQPPVSPGDKKAGASVSSFLQVSAAGARQENRLPGPSPAESFQTEIWDRNRNRGRRSIVNAEE---
Human      80  RAELQGHHAEKLPAGAGAPKAGLEEAPAVTAGLKIFEPPAPGEG---------------NSSQNSRNKRAVQGPEE---
consensus  81  . *.  ..     ..       .       .        .   .                  ..     . ....... ..

                                                    A              a
Dogfish   116  -------------------------QVQQSCVQLIATRDRRPLVKANDCPKCMPGRPESTCRVERRSVSNQNGKQET
Trout     118  ----------------------SVSQPCLQMLADSNRKTFQKEFAL-----------------------EPYT
zebrafish 115  ----------------------AEPQPCLQMMADNKKKTFQKEFAF-----------------------DYCT
Fugu      114  ----------------------LVSQPCLQMLANSSRTTFRKELTS-----------------------GPHT
Mouse     137  DVDLSAPPAPCLPGCRHSQHDDNGMNLRNIIQDCLQLIADSDTPTIRKG-------------------------TYT
Cow       136  ----------------------TVTQDCLQLIADSDTPTIRKG-------------------------AYT
Chicken   144  ----------------------TVLQACLQLIADSKSDIQQKD-------------------------DSS
Human     141  ----------------------TVTQDCLQLIADSETPTIQKG-------------------------SYT
consensus 161                             ...* *.*..*...  ...*                              ..

               a'    A"     B'    B           C              D     D-E loop      E
Dogfish   168  TVPWILSLIKGSALDKKDNKILVKEAGYFLVY-----SQVWYKDNTFTMGHFIQRKKASSVGNEPRIVILFRCIQNMSAC
Trout     146  GIPWQAGLRRGSALEAESDSILVREEGYYFVY-----SQVYYMDTTFAMGHVVIRKKRNVVGDEAQHVTLFRCIQNMNPV
zebrafish 143  AIPWQVGLKRGSALEEEQGTILVKEEGFFFIYSQLSFSQVYYTDPTFAMGHIVIRIKKNVVGNESQHVVLFRCIQSMNRV
Fugu      142  GIPWKSGLRRGSALEADGDSILVGEEGFYFVY-----SQVYYMDSIFAMGHVVIRRKRTVVGDETPEVILFRCIQNMNPV
Mouse     189  FVPWLLSFKRGNALEEKENKIVVRQTGYFFIY-----SQVLYTDPIFAMGHVIQRKKVHVFGDELSLVTLFRCIQNMPKT
Cow       160  FVPWLLSFKRGRALEEKENKILVKETGYFFIY-----GQVLYTDNTFAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPET
Chicken   168  IVPWLLSFKRGTALEEQGNKIVIKETGYFFIY-----GQVLYTDTTFAMGHLIQRKKAHVFGDDLSLVTLFRCIQNMPQS
Human     165  FVPWLLSFKRGSALEEKENKILVKETGYFFIY-----GQVLYTDKTYAMGHLIQRKKVHVFGDELSLVTLFRCIQNMPET
consensus 241  .**... ..*.**.......*.....*....          .**.*.* ...***...*.*  *......*.*******.. .

                    F          G                H
Dogfish   243  CPNNSCFTAGIAKLEVEDELELVIPRIQAQIALNGDGTFFGAIKLL
Trout     221  YPYNTCYTGGIVKLEVGDSVELLIPRSTAKVSLDGDSTFLGAVRLA
zebrafish 223  NHYNTCYTGGVVKLDSGDKLDLLIPRANANISLEGDATFLGAIKLA
Fugu      217  YPFNTCYTGGIVKLKRGDHLELLIPRSTASVSLDEDSTFLGAIKLG
Mouse     264  LPNNSCYSAGIARLEEGDEIQLAIPRENAQISRNGDDTFFGALKLL
Cow       235  LPNNSCYSAGIAKLEEGDELQLAIPREDAKISRDGDGTFFGALKLL
Chicken   243  YPNNSCYTAGIAKLEEGDELQLTIPRRRAKISLDGDGTFFGAVRLL
Human     240  LPNNSCYSAGIAKLEEGDELQLAIPRENAQISLDGDVTFFGALKLL
consensus 321  ..*.*...*...*....*....*.*** .*.......*.**.**..*.
```

Fig. 2. Multiple alignment of spiny dogfish BAFF with human, chicken, cow, mouse, *Fugu*, zebrafish, and trout using ClustalW v2.1. The transmembrane domain (TMD) is shaded in light grey, and furin cleavage site is underlined and highlighted in light grey. Potential *N*-glycosylation sites N–X–[S/T] are in bold and shaded in dark grey. The conserved cysteines of BAFF are highlighted in light grey, the dogfish-specific cysteine pair is in bold italics and highlighted in dark grey and the conserved residues which are part of the mammalian TNF family profile are in white letters and highlighted in black. Individual β-strands (A–H), relating to the human sequence, are indicated by arrows on the top of spiny dogfish sequence. The long D–E loop, characteristic of BAFF, is in white letters and highlighted in black. Residues shown to interact with BAFF-R in the human molecule are in bold and highlighted in dark grey and the residues which form the metal binding site are shown in bold and highlighted in light grey. The accession numbers of the sequences used are detailed in Fig. 3.

was obtained using gene walking. Nested PCR with primers sa-BAFF-gF1 and saBAFF-gF2 and gene walking adapter primers AP1 and AP2 gave a 2354 bp product (P1) that covers the first intron and a partial sequence of the second intron. Nested PCR with primers saBAFF-gF3 and saBAFF-gF4 and gene walking adapter primers AP1 and AP2 gave a 2483 bp product (P3) that amplified the third intron and a partial sequence of the fourth intron in the 3′ direction. Nested PCR with primers saBAFF-gR5 and saBAFF-gR6 and gene walking adapter primers AP1 and AP2 gave a 2015 bp product (P2) that covers a partial sequence of the second intron in the 5′ direction. Lastly, primers saBAFF-gR3 and saBAFF-gR4 and gene walking adapter primers AP1 and AP2 gave a 2721 bp product

(P4) that covers a partial sequence of the fourth intron in the 5′ direction. Once the products were assembled, the final gene organization of spiny dogfish BAFF was found to consist of seven exons and six introns (Fig. 4B), with all exon–intron splice junction sequences following the GT–AG rule. The spiny dogfish gene organisation is different to human, chicken and the bony fish zebrafish and *Fugu*, which each have only six exons. Introns two and four in the dogfish could not be fully sequenced due to their large size. The size of exon three and the last exon (coding region only for the last exon) were highly conserved across species. It was also apparent that the entire insertion of 28 aa between the a and a′ strands was encoded by the extra 3a exon in the spiny dogfish (Figs. 2 and

Table 2
Amino acid identities and similarities of known BAFF molecules within vertebrates.[a]

	Dogfish	Zebrafish	Trout	Salmon	Fugu	Chicken	Mouse	Cow	Human
Dogfish		30.6	33.9	33.3	35.1	**38.6**[b]	33.3	36.8	37.9
Zebrafish	52.4		54.3	54.0	46.5	36.8	34.2	36.9	37.3
Trout	55.9	71.6		**98.1**[b]	63.1	41.1	37.9	38.9	37.9
Salmon	54.5	71.6	**98.5**[b]		63.1	42.3	38.2	40.0	37.2
Fugu	42.1	46.5	52.7	53.0		28.1	28.6	30.0	28.8
Chicken	**60.4**[b]	55.2	58.0	58.7	54.9		45.5	51.8	50.5
Mouse	54.7	53.1	52.4	52.4	49.8	57.6		57.7	60.4
Cow	56.3	56.1	56.1	56.8	54.6	66.7	70.9		78.4
Human	60.1	56.5	55.1	56.1	52.6	68.4	73.8	85.6	

[a] The top triangle shows % aa identity, bottom triangle shows % aa similarity.
[b] The highest identity and the highest similarity are in bold.
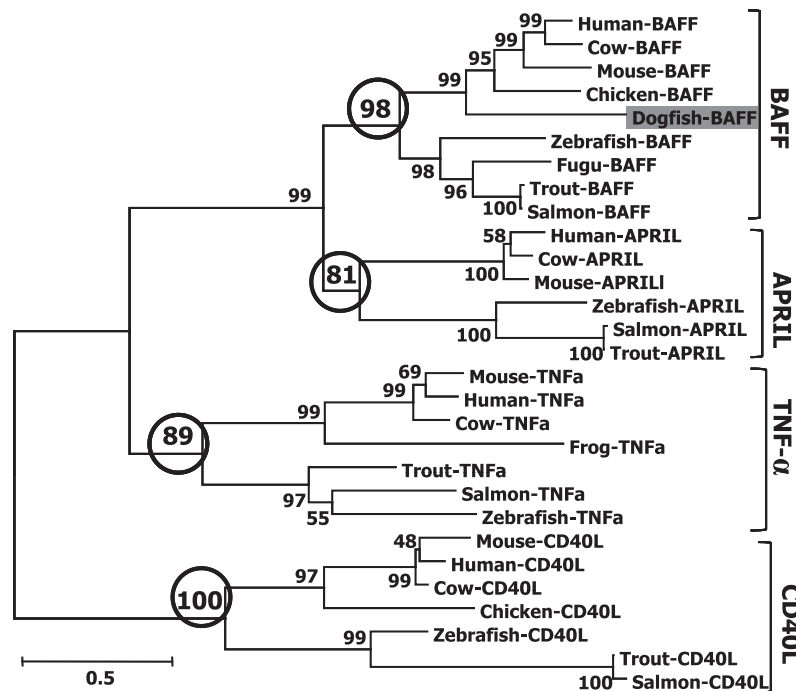
Fig. 3. Unrooted phylogenetic tree showing the relationship between spiny dogfish BAFF and BAFF/TNF superfamily members of other species. This tree was constructed by the 'neighbour-joining' method using the MEGA5 program based on a CLUSTALW multiple alignment, and was bootstrapped 10,000 times. All bootstrap values are shown and the spiny dogfish BAFF is shaded in grey. The accession numbers of the sequences used for the phylogenetic analysis are as follows: chicken BAFF [NP_989658] and CD40L [Q9I8D8]; mouse BAFF [NP_296371], APRIL [Q9D777], TNFα, [AAI37721] and CD40L [NP_035746]; human BAFF [CAG46617], APRIL [O75888], TNFα [NP_000585] and CD40L [NP_000065]; cow BAFF [NP_001107978], APRIL [O3ZBM0], TNFa [Q06599] and CD40L [P51749]; zebrafish BAFF [NP_001107062], APRIL [NP_001161936], TNFα, [NP_998024] and CD40L [ENSDARP00000098917]; trout BAFF [ABC84582], APRIL [NP_001118143], TNFα [NP_001117829] and CD40L [NP_001118138]; salmon BAFF [ACI33633], APRIL [NP_001135076], TNFa [ABG91800] and CD40L [NP_001140007]; frog TNFa [NP_001108250] and *Fugu* BAFF [AEB69781].

4). Taken in isolation the sequence for this exon returned no significant hits when subject to BLAST analysis against teleost and tetrapod BAFF genomic sequences. To confirm whether this extra exon 3a is present in all mRNA or could be spliced out nested PCR was conducted using primers located in exons 3 and 6. A band with the expected size for the full length product was amplified from all tissue samples as well as from *in vitro* stimulated PBL (data not shown) (Fig. 4C). However, a weak band smaller than the expected size was obtained from three of the tissues (epigonal, gut and liver). No products were obtained with genomic DNA as the template (because of the large intron 3). Sequence analysis revealed that the smaller product was spiny dogfish BAFF lacking the 110 bp encoded by exon 4. This result suggests that exon 3a is present in all spiny dogfish BAFF mRNA and that there is low level expression of an alternative splice form which skips exon 4.

By mapping the spiny dogfish residues on the human structure we anticipate the 28 aa insertion causes a considerable extension of the loop between the a and a′ β-sheets (Fig. 2). This loop is located near the functionally important DE-loop, and has been shown to border the receptor binding groove (Oren et al., 2002). We also observed high structural conservation of those residues which in the human molecule have been shown to be important for the BAFF/BAFF receptor interaction (Fig. 2). The extra cysteine pair found in spiny dogfish, but not the other species examined, mapped to the middle of the EF-loop, also located adjacent to the receptor binding region of BAFF. Interestingly, it is at the base of this loop that the (phylogentically conserved) metal binding site residues are found; in the BAFF trimer these residues link adjacent monomers via the co-ordination of magnesium ions and thus play an important role in stabilising the trimer (Oren et al., 2002). The presence of the double-cys at the tip of this loop in spiny dogfish suggests that it may also play a role in stabilizing the trimer through the formation of intermolecular disulphide bonds.
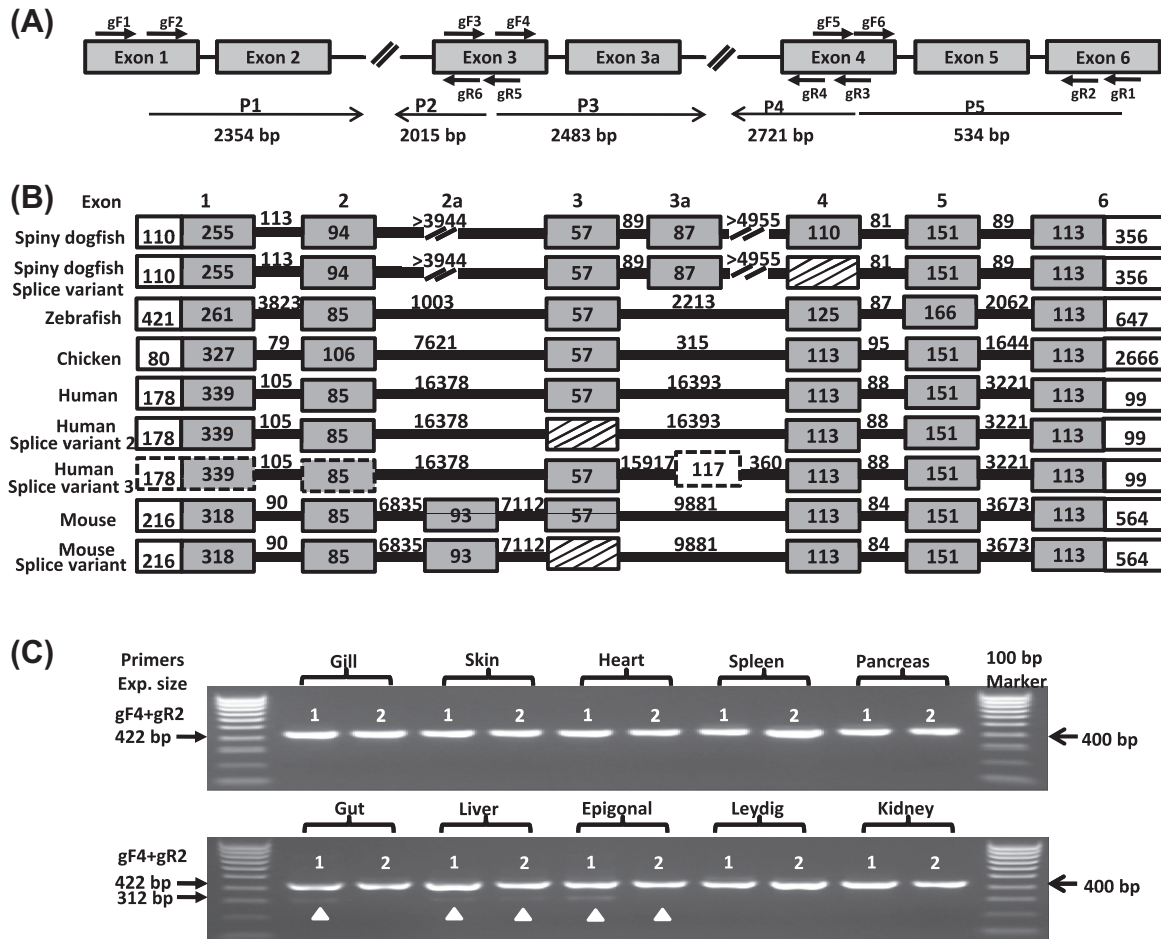
Fig. 4. Analysis of BAFF gene organisation in spiny dogfish. (A) Primer positions and the five PCR products obtained (P1–5) from spiny dogfish genomic DNA. Not drawn to scale. The primers and PCR products obtained using gene walking are indicated by arrow. The exons are presented as grey boxes and introns as black lines. (B) Comparison of the gene organisation and intron/exon sizes between spiny dogfish, zebrafish, chicken, human and mouse BAFF genes. Grey boxes indicate the coding regions, whilst white boxes indicate the known or predicted untranslated regions. The numbers are the nucleotides associated with the exons and introns. The striped boxes represent the skipped exons in the various splice forms. The grey boxes with a dashed outline are presumed exons whilst the white box with dashed outline represents the cryptic exon (3a) of human BAFF (based upon Smirnova et al., 2008). The zebrafish BAFF gene organization is derived from NC_007120 (based on Zv9) and mRNA sequence NM_001113590. The BAFF genomic DNA sequences of chicken, human and mouse are obtained from ENSGALG00000016852, ENSG00000102524 and ENSMUSG00000031497, respectively. (C) Nested PCR products were amplified from tissue cDNA using primers located at exon 3 (saBAFFgF3, saBAFFgF4) and exon 6 (saBAFFgR1, saBAFFgR2). The expected band of 422 bp corresponding to the primer pairs of saBAFFgF4 and saBAFFgR2 was amplified. A weak band of ∼312 bp (white arrowhead) was amplified from epigonal, gut and liver. Similar products have been amplified with the cDNA samples from *in vitro* stimulated peripheral blood leucocytes but no products were obtained from genomic DNA (data not shown). The PCR products from two animals are shown.

3.4. In vivo tissue distribution of spiny dogfish BAFF

Constitutive mRNA expression of the BAFF gene was detected in all tissues examined, with the weakest expression seen in blood (Fig. 5). The pancreas was found to express the highest level of BAFF, followed by the spleen, heart, skin and gill tissues. The Leydig organ, kidney and gut all showed a low level of BAFF expression (Fig. 5).

### 3.5. Expression of spiny dogfish BAFF in blood cells incubated with immunostimulants

Incubation of spiny dogfish blood cells with PHA and LPS for 12 and 24 h had little effect on BAFF expression, as assessed by real-time PCR (Fig. 6A). However, after 24 h of incubation with PWM, BAFF levels had increased ∼4-fold above the control (Fig. 6A). To investigate this response further, blood cells from three dogfish were stimulated with various concentrations of PWM (0.1, 1, 10 and 100 µg/ml) for 12 and 24 h (Fig. 6B). Low constitutive expression was seen in the negative control, with a marked increase in

BAFF transcript level detected after stimulation with 1, 10 and 100 µg/ml PWM after incubation for 24 h ($0.001 < \rho < 0.05$) in a dose-dependent manner (Fig. 6B). Cell counts indicated that there was no difference in live white cell numbers between PWM treated and untreated samples at either 12 or 24 h (data not shown) confirming the increase in BAFF transcript levels was indeed due to an up-regulation of its expression and not due to PWM-induced cell proliferation and/or survival.

4. Discussion

In mammals, the cytokine BAFF is required for B-cell maturation and maintenance (Rolink et al., 2002; Tribouley et al., 1999) and is therefore a good candidate to begin to study B-cell development in non-mammalian vertebrates. Most research to date has focused upon mammalian and avian BAFF (Ettinger et al., 2007; Gilbert et al., 2006; Khare et al., 2000; Schneider et al., 2004) but more recently BAFF has been characterised in bony fish (Ai et al., 2011; Liang et al., 2010). In this work, we have identified and characterized the full-length spiny dogfish BAFF mRNA. In addition, the
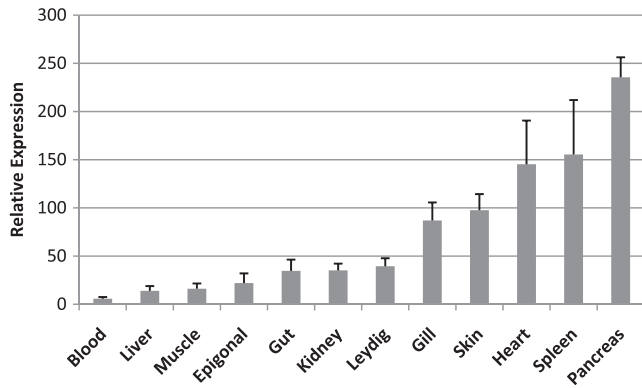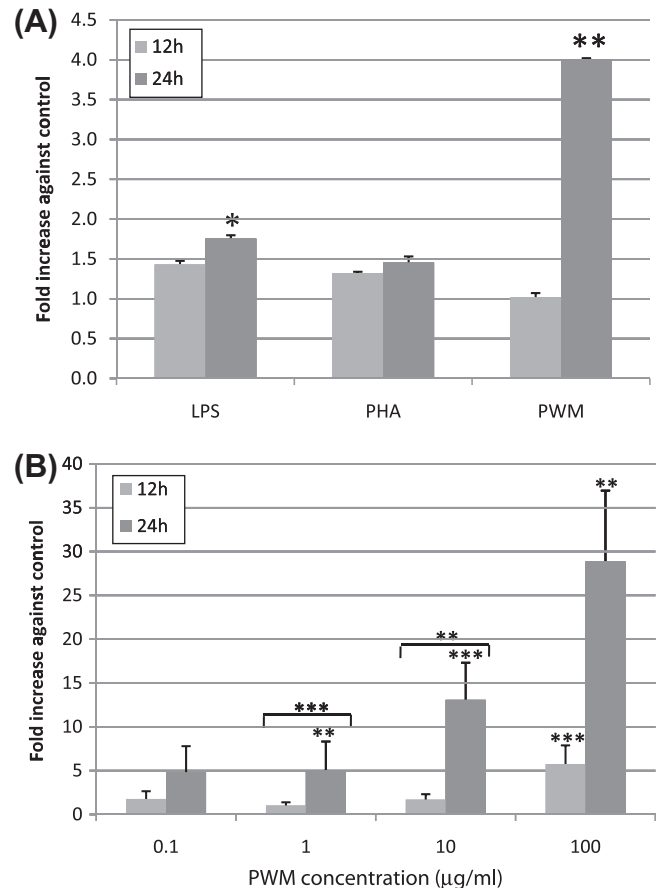
Fig. 5. Constitutive expression of the spiny dogfish BAFF transcript. BAFF expression was determined within selected tissues taken from three individual fish using real-time PCR. The transcript level was first calculated using a serial dilution of reference samples in the same run. The relative expression level of the BAFF transcript was then expressed as arbitrary units normalized against the expression level of β-actin. The results are presented as averages + standard error.

Fig. 6. Modulation of spiny dogfish BAFF expression *in vitro*. (A) Blood cells were incubated with LPS (10 μg/ml), PHA (5 μg/ml) or PWM (10 μg/ml) at 15 °C for 12 or 24 h. The expression of BAFF and β-actin were detected using real-time PCR. The expression of BAFF was normalized to that of β-actin and expressed as a fold change relative to mock-treated samples (PBS treated cells for LPS and PHA, 0.9% NaCl treated cells for PWM), which were defined as 1 at the same time point. (B) Blood cells incubated with different concentrations of PWM. Fresh blood cells were stimulated with 0, 0.1, 1, 10 and 100 μg/ml of PWM at 15 °C for 12 and 24 h, and the expression of BAFF and the house keeping gene β-actin detected by real-time PCR. The relative expression of BAFF was normalized to that of β-actin, and then expressed as a fold change relative to the control samples, which were defined as 1 at the same time point. All results are presented as averages + standard error of three fish. Asterisks indicate significant differences ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

genomic organisation of the spiny dogfish BAFF gene has been determined and its tissue expression and modulation in peripheral leucocytes has been investigated using real-time PCR.

The cDNA sequence of spiny dogfish BAFF was 1333 bp with an ORF of 867 bp, encoding for a polypeptide of 288 aa (Fig. 1). Comparison with BAFF from other vertebrate species shows that many of the structurally and functionally important features are conserved in spiny dogfish BAFF. For example, the presence of a transmembrane domain and furin-like cleavage motif indicate spiny dogfish BAFF, like BAFF in other species, is expressed as a typical type II transmembrane protein which is proteolytically cleaved to release the biologically active soluble protein (Nardelli et al., 2001). Interestingly, this cleavage site is not conserved in BAFF from all bony fish (Ai et al., 2011; Liang et al., 2010). The spiny dogfish soluble domain shows high (∼60%) similarity to those of human and chicken (Bossen and Schneider, 2006).

In all species examined the mature (soluble) portion of BAFF encodes three cysteine residues (one each in the A, E and F strands) however in addition to these conserved cysteines spiny dogfish carries three more in the insertion between the a and a′ β-strands and a further pair of cysteines in the EF-loop (Fig. 2). Spiny dogfish BAFF also has two additional (potential) N-linked glycosylation sites in addition to the one also found in β-strand F of human, cow and chicken.

A high degree of gene synteny was observed around the BAFF gene in humans and bony fish (*Fugu, Tetraodon* and stickleback), suggesting that the genes around BAFF have been highly conserved during vertebrate evolution (data not shown). Unfortunately, with only an incomplete cartilaginous fish genome available at present, it remains to be determined if this is also the case in this ancient vertebrate group. However, when compared to bony fish, birds and human, the spiny dogfish BAFF gene was found to have an extra exon (seven rather than six exons) located between the 3rd and 4th exon. A similar situation is observed in murine BAFF which also has an extra exon (2a), located between the 2nd and 3th exons (Fig. 4B). This dogfish BAFF extra exon is present in all the BAFF transcripts sequenced in this study (Fig. 4C). Comparison of the genomic and mRNA sequences show that this additional exon encodes the insertion between the a–a′ strand in spiny dogfish (Fig. 2). To explore this finding further we searched the elephant shark (*Callorhinchus milii*, a chimera) genomic database with spiny dogfish BAFF and found a single scaffold (http://esharkgenome.imcb.a-star.edu.sg/; scaffold AAVX01314814) which contained both exon 3 and the additional (shark-specific) exon 3a.

To confirm this finding we cloned BAFF from a second cartilaginous fish species (unpublished data) and found this also contained the additional exon which was expressed in all tissues examined. These findings suggest that either the cartilaginous fish acquired an extra exon very early in their evolution (before the elasmobranch (sharks, skates and rays) – holocephali (chimera) split over 350 MYA) or that this exon was present in the proto-BAFF gene and is generally not utilized (or has been lost) in other lineages. Interestingly, there is a single report of a human BAFF splice isoform that is expressed at low levels in both stimulated and unstimulated human PBMCs and which utilizes a cryptic exon also found in the intron between exons 3 and 4 (Smirnova et al., 2008). Unfortunately the sequence of the new human variant was not determined in the published study and close examination of intron 3 by ourselves did not reveal any ORF with sequence similarity to shark exon 3a. However, in light of this information, future studies are certainly warranted to determine the structural and biological significance of this 'additional' exon.

Whilst we were unable to model the structure of spiny dogfish BAFF, through mapping on the human structure we anticipate this

insertion causes a considerable extension of the loop between the a and a′ β-sheets in spiny dogfish. This loop is located near the functionally important DE-loop, and has been shown to border the receptor binding groove in the soluble BAFF trimer (Kim et al., 2003; Oren et al., 2002). Although the impact (if any) of this insertion upon the function of elasmobranch BAFF has yet to be tested it will be interesting to see if elasmobranch BAFF has a different receptor binding specificity profile compared to BAFF from other vertebrate lineages.

It was interesting to find low level expression of an alternative spice form (lacking exon 4) of spiny dogfish BAFF in some of the tissues from the animals examined. An alternative splice form, lacking exon 3 and called ΔBAFF, has been found in both humans and mice. The ΔBAFF isoform is receptor non-binding but can multimerize with full length BAFF thereby blocking its activity (Gavin et al., 2005, 2003). Our discovery of an alternative splice form in spiny dogfish, combined with preliminary evidence that under certain stimulatory conditions the ratio of expression of the two dogfish BAFF splice forms can change (data not shown), suggests a sensitive transcriptional mechanism which may act to regulate the co-stimulatory activity of BAFF on shark B cells.

When the constitutive expression of spiny dogfish BAFF was investigated using real-time PCR our results showed that BAFF is highly expressed in spleen, heart, skin and gill (Fig. 5); whilst spleen and gill are known immune tissues, to our knowledge an immunological role for skin has never been shown in this lineage. We also cannot explain why BAFF expression should be so high in dogfish heart tissue, especially when it is so low in blood. Interestingly, the highest level of BAFF expression was observed in dogfish pancreas, fitting with accumulating evidence that this organ has an immune function in cartilaginous fish. Studies in the nurse shark show that IgW (the shark IgD orthologue) is also expressed at very high levels in the pancreas (Rumfelt et al., 2004). Combined with the high expression of BAFF it suggests the pancreas may act to protect the mucosal surfaces of the shark gut, perhaps by constitutively secreting IgW into the gut lumen.

Whilst there are many studies on the role of BAFF in promoting B-cell survival and differentiation in mammals, very few studies have examined the effect of immunostimulants upon BAFF expression (Huard et al., 2004; Moon et al., 2006; Schneider et al., 1999). LPS, PHA and PWM are widely used to stimulate leucocytes in vitro however in this study we found that LPS and PHA had little effect in vitro upon BAFF expression in shark peripheral blood cells. Expression was markedly (p < 0.01) up-regulated following PWM stimulation. Whilst the lack of effect of PHA (a T cell stimulant) on BAFF expression was not wholly unexpected, the results for LPS were surprising; LPS is a potent B-cell mitogen in mammals and has also been shown to stimulate cytokine production in bony fish (Holland et al., 2010; Honma et al., 2005). However, bacteria are routinely cultured from the blood and tissues of healthy sharks (Mylniczenko et al., 2007) and preliminary experiments in nurse sharks showed no discernable increase in Ig production following in vivo administration of LPS (Dooley and Flajnik., unpublished data). Taken together with the new BAFF data this suggests cartilaginous fish may respond poorly to LPS, perhaps as a result of their high bacterial load.

Although PWM is known to activate B-cells, macrophages, monocytes and T-cells (Kehrer et al., 1998; Kucharzik et al., 1997; Smith and Goldman, 1972) to date there is no published data on its effect upon BAFF expression. Our results clearly show that PWM stimulation significantly up-regulates spiny dogfish BAFF expression in blood leucocytes after 12–24 h (dependent upon the concentration used) and that the largest effect was seen using 100 μg/ml. This suggests that BAFF may be important in the activation of B-cells in cartilaginous fish, although this requires further investigation. The role of BAFF on B-cells in bony fish has been investigated by producing the recombinant protein, where in Fugu and zebrafish it promotes the survival of fish splenocytes (Ai et al., 2011; Liang et al., 2010). The future generation of recombinant spiny dogfish BAFF will allow us to study its precise role in B-cell development and maturation in cartilaginous fish.

Acknowledgment

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