

Ancient *BCMA*-like Genes Herald B Cell Regulation in Lampreys

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The TNF superfamily ligands BAFF and APRIL interact with three receptors, BAFFR, BCMA, and TACI, to play discrete and crucial roles in regulating B cell selection and homeostasis in mammals. The interactions between these ligands and receptors are both specific and redundant: BAFFR binds BAFF, whereas BCMA and TACI bind to either BAFF or APRIL. In a previous phylogenetic inquiry, we identified and characterized a *BAFF*-like gene in lampreys, which, with hagfish, are the only extant jawless vertebrates, both of which have B-like and T-like lymphocytes. To gain insight into lymphocyte regulation in jawless vertebrates, in this study we identified two *BCMA*-like genes in lampreys, *BCMAL1* and *BCMAL2*, which were found to be preferentially expressed by B-like lymphocytes. In vitro analyses indicated that the lamprey BAFF-like protein can bind to a BCMA-like receptor Ig fusion protein and to both *BCMAL1*- and *BCMAL2*-transfected cells. Discriminating regulatory roles for the two BCMA-like molecules are suggested by their differential expression before and after activation of the B-like lymphocytes in lampreys. Our composite results imply that BAFF-based mechanisms for B cell regulation evolved before the divergence of jawed and jawless vertebrates. *The Journal of Immunology*, 2019, 203: 2909–2916.

Members of the TNF superfamily (TNFSF) and their receptors (TNFSFR) orchestrate a wide range of immune system functions and other biological processes in mammals. The closely related TNFSF ligands B cell activating factor (BAFF; also known as BLyS and TNFSF13b) and a proliferation-inducing ligand (APRIL; TNFSF13) are key regulators of B lymphocyte activation, selection, and survival (reviewed in Refs. 1, 2). BAFF and APRIL influence B cells at various stages of differentiation via interaction with three different receptors (3–6). Both BAFF and APRIL bind to the B cell maturation Ag (BCMA) and transmembrane activator and CAML interactor (TACI) cell-surface receptors (7);

BAFF also binds uniquely to the BAFF receptor (BAFFR; also known as BR3), another TNFSFR family member (6, 8–10). Whereas BAFF binds more avidly to BAFFR, APRIL has higher binding affinities for BCMA and TACI (reviewed in Ref. 11). The cellular expression patterns of BAFFR, BCMA, and TACI also differ; BAFFR and TACI are both expressed on pre-immune B cells and are differentially downregulated during activation and plasma cell differentiation, whereas BCMA is expressed solely among plasma cells (12). BAFFR interactions with BAFF mediate the survival of preimmune B cells and, thus, regulate the steady-state numbers of these populations in mammals (13).

The extant jawless vertebrates (agnatha), lampreys and hagfish, have an alternative adaptive immune system featuring B-like and T-like lymphocytes, but they use entirely different types of receptors for Ag recognition (14–18). Instead of the Ig domain-based BCRs and TCRs (TCR $\alpha\beta$ and TCR $\gamma\delta$) that jawed vertebrates (gnathostomes) use, the jawless vertebrates use three types of variable lymphocyte receptors (*VLRA*, *VLRB*, and *VLRC*) containing leucine-rich repeat sequences for Ag recognition (14, 15, 19–26). *VLRB* is expressed by B-like lymphocytes, whereas *VLRA* and *VLRC* are expressed by T-like lymphocytes with characteristics akin to the TCR $\alpha\beta$ and TCR $\gamma\delta$ populations of T cells in jawed vertebrates (18, 26, 27).

In a prior phylogenetic analysis of TNFSF members, we identified a lamprey BAFF-like (BAFFL) gene and demonstrated its expression by T-like, B-like, and innate immune cells of this ancient jawless vertebrate representative (28). The lamprey BAFFL protein exhibits amino acid sequence and surface electric charge similarities with both BAFF and APRIL (28, 29). In this study, we have identified and characterized two lamprey *TNFSFR* genes that have sequence similarity to mammalian BCMA. The two *BCMA*-like genes are predominantly expressed by *VLRB*⁺ B cell-like lymphocytes, and both can bind the lamprey BAFFL molecule. Our findings indicate that this set of ligands and receptors had already evolved in a common ancestor of jawed and jawless vertebrates and suggest they play regulatory roles for *VLRB*⁺ B cell-like lymphocytes.

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Abbreviations used in this article: APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor; BAFFL, BAFF-like; BAFFR, BAFF receptor; BCMA, B cell maturation Ag; *BCMAL1*, *BCMA*-like 1; *BCMAL2*, *BCMA*-like 2; CRD, cysteine-rich domain; EDAR, ectodysplasin A receptor; HA, hemagglutinin; hEDA, human ectodysplasin A; ML, maximum likelihood; NJ, neighbor joining; PDB, Protein Data Bank; qPCR, quantitative real-time PCR; TACI, transmembrane activator and CAML interactor; TN, triple negative; TNFSF, TNF superfamily; TNFSFR, TNF receptor; TRAF, TNFR associated factor.

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Materials and Methods

Cloning

Lamprey BAFF-associated receptors BCMA-like 1 (BCMAL1) and BCMA-like 2 (BCMAL2) were found by a TBLASTN search of the *Petromyzon marinus* (sea lamprey, genome assembly Pmarinus_7.0) and *Lethenteron japonicum* (Japanese lamprey, genome assembly LetJap1.0) genome sequences. Retrieved sequences from Japanese lamprey were used primarily for sequence comparison. Sea lamprey was used as a source of cDNA and was the species in which gene expression was analyzed. For the amplification, the following primers were used with KOD polymerase (TOYOBO): 1) BCMAL1, *P. marinus* BCMAL1-forward, 5'-ATGGAGACGTCTCGGGAGCCGGC-3', and *P. marinus* BCMAL1-reverse, 5'-TACCCCTACAACAAGCTCAGATGGGCTT-3'; and 2) BCMAL2, *P. marinus* BCMAL2-forward, 5'-ATGACCTCGCACTGCCCCGGCT-3', and *P. marinus* BCMAL2-reverse, 5'-TCAAGTGTAGTTTGTGACCAT-TTGGGAAG-3'. After adding adenine to the 3' ends, the amplified DNA was ligated into the pGEM-Teasy vector (Promega).

Phylogenetic and bioinformatics analyses

Full-length amino acid sequences of BAFFR, BCMA, and TACI from human (NP_443177, CAA82690, and NP_036584) and mouse (NP_082351, XP_006522052, and NP_067324) were downloaded from the National Center for Biotechnology Information database. To retrieve their orthologous sequences in other vertebrate and invertebrate chordate species, we performed a TBLASTN search for matches with *E* value $<10^{-5}$ against genome sequence databases as well as cDNA databases (when available) from ENSEMBL (<http://useast.ensembl.org/index.html>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The hypothetical translations of retrieved sequences were aligned using the MUSCLE and ClustalW programs (30, 31). Signal peptide cleavage sites were predicted with SignalP 4.0 (32). Protein domain prediction was performed using the SMART database (33).

After sequences were aligned and configured for highest accuracy, phylogenetic trees were constructed by neighbor-joining (NJ) and maximum-likelihood (ML) methods, implemented in MEGA5.0 (34, 35). The evolutionary distances were computed by the Jones–Taylor–Thornton matrix-based method (36). Reliability of internal branches was evaluated using the bootstrapping method (1000 bootstrap replicates).

Homology modeling

The quaternary structures of sea lamprey BCMAL1 and BCMAL2 were modeled by SWISS-MODEL (<http://swissmodel.expasy.org/>), based on the crystal structure of human BCMA (Protein Data Bank [PDB]: 4zfo) as a modeling template. The electrostatic potential was calculated by the Adaptive Poisson–Boltzmann Solver in CueMol2 (<http://www.cuemol.org/en/>). The percent identity at particular amino acid sites was calculated by Jalview (37).

Sorting of VLRA⁺, VLRB⁺, VLRC⁺, and VLR[−] lymphocytes

Leukocyte isolation from blood and tissues and staining for flow cytometry were conducted as described (18). Briefly, leukocytes from blood and tissues were stained with anti-VLRA rabbit polyclonal serum (R110), anti-VLRB mouse mAb (4C4), and biotinylated anti-VLRC mouse mAbs (3A5) and with matched secondary reagents. Staining and washes were in 0.67× PBS with 1% BSA. Flow cytometric analysis was performed on an Accuri C6 flow cytometer (BD Biosciences). VLRA⁺, VLRB⁺, VLRC⁺, and VLR triple-negative (TN) cells in the lymphocyte gate were sorted on a BD FACSAria II (BD Biosciences) for quantitative real-time PCR (qPCR) analysis. The purity of the sorted cells was >95%. Lampreys were injected intracoelomically with 25 µg of PHA (Sigma) as described previously (17, 18).

Expression analysis by qPCR

Total RNAs from tissues (skin, kidneys, intestine, gills, and blood) from lamprey larvae and purified VLRA⁺, VLRB⁺, VLRC⁺, and TN lymphocytes were extracted using RNeasy kits with on-column DNA digestion by DNase I (QIAGEN). First-strand cDNA was synthesized with random hexamer primers by Superscript III (Invitrogen). qPCR was performed using SYBR Green on a 7900HT ABI Prism thermocycler (Applied Biosystems). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The values for both BCMAL1 and BCMAL2 genes were normalized to the expression of β -Actin.

Transfection

293T cells were cultured in DMEM (Sigma) containing 5% FBS at 37°C and 5% CO₂. Lamprey BAFFL ligand and human ectodysplasin A (hEDA) were ligated into the pDisplay vector (Thermo Fisher Scientific), fused with N-terminal hemagglutinin (HA) tag (pDisplay-HA-BAFFL and pDisplay-HA-hEDA). Lamprey BCMAL1 and BCMAL2 receptors are also ligated into the pDisplay vector, fused with N-terminal FLAG tag (pDisplay-FLAG-BCMAL1 and pDisplay-FLAG-BCMAL2). The extracellular domain of lamprey BCMA-like receptors were also fused with human IgG Fc tag at the C terminus and ligated in pIRES puro vector (pIRES puro-FLAG-BCMAL1-Fc and pIRES puro-FLAG-BCMAL2-Fc). The expression vectors were transfected to 293T cells using polyethylenimine. After 3 d incubation, the culture medium and transfected cells were harvested for further assay.

Protein analysis by gel electrophoresis

The procedure of Blue Native PAGE was described previously (38). Briefly, the culture medium of pDisplay-HA-BAFFL-transfected 293T cells was mixed with 5% glycerol and 0.01% Ponceau S and then applied on 10% native PAGE gel. Mouse IgG (~160 kDa), BSA (66 kDa), and OVA (43 kDa) were electrophoresed as size markers. The separated proteins were transferred to a nitrocellulose membrane by conventional semidry blotting. The HA-tagged lamprey BAFFL protein was reacted with mouse anti-HA mAb (clone 2-2.2.14; Thermo Fisher Scientific). After washing with PBS containing 0.1% Tween 20, the blot was reacted with HRP-conjugating goat anti-mouse IgG (SouthernBiotech). After washing with PBS containing 0.1% Tween 20, the HA-BAFFL was detected using SuperSignal West Pico (Thermo Fisher Scientific) on an Image Station 4000MM Pro (Kodak).

Flow cytometry

To detect ligand–receptor interaction, 293T cells transfected with the pDisplay-FLAG-BCMA-like receptor construct were incubated with HA-BAFFL containing DMEM for 30 min. After washing with PBS, the cells were reacted with a rabbit anti-FLAG polyclonal Ab (Sigma) and a mouse anti-HA mAb (clone 2-2.2.14; Thermo Fisher Scientific). PE-conjugated goat anti-mouse IgG (SouthernBiotech) and Alexa 488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) were used for detection. The stained cells were analyzed using a BD Accuri C6 cytometer and Cytobank (<https://community.cytobank.org/>).

Immunoprecipitation

The secreted HA-BAFFL protein and BCMAL-Fc proteins from transfected 293T cells were purified using anti-HA agarose (Thermo Fisher Scientific) and Protein G Sepharose 4 Fast Flow (GE Healthcare) following the manufacturers' protocols. One microgram of purified receptor and ligand proteins was incubated at 4°C in 100 µl of PBS with gentle mixing overnight. Thirty microliters of protein G slurry was added and then incubated for 1 h at 4°C with gentle mixing. After washing the protein G beads with PBS, the proteins were denatured with 2× SDS sample buffer, 0.1 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 12% 2-ME, and bromophenol blue. The protocols for SDS-PAGE and Western blotting were described previously (39). The Abs for detecting HA-BAFFL proteins were the same as those used for Western blotting after Blue Native PAGE. The secreted HA-BAFFL, HA-hEDA, and FLAG-BCMAL-Fc proteins from transfected 293T cells were also mixed with anti-HA agarose (Pierce) for pull-down assay.

Results

Identification of two BCMA-like genes in lampreys

When human and mouse BAFFR, BCMA, and TACI amino acid sequences were used in a similarity search against sea lamprey and Japanese lamprey genomic sequences, we found two significant partial matches in each lamprey species. By extension of the complementary hit regions in the genome sequences, we could identify two potential open reading frames using the hypothetical translation of retrieved genomic sequences in both the sea lamprey and Japanese lamprey. The two predicted BAFF-interacting *TNFSFR* homologs in sea lamprey were cloned and sequenced from blood cell cDNA. These two genes, sequence1 and sequence2, which are found in the same scaffold (PIZI01000003.1) of the reference sea lamprey genome, face in head-to-head transcriptional directions and encode proteins of 179 aa and 152 aa, respectively. Whereas the

putative BAFF-interactive TNFSFR members in sea lamprey exhibit only ~40% aa sequence similarity with each other, sequence1 and sequence2 exhibit 89 and 96% similarity with their orthologous Japanese lamprey counterparts, respectively.

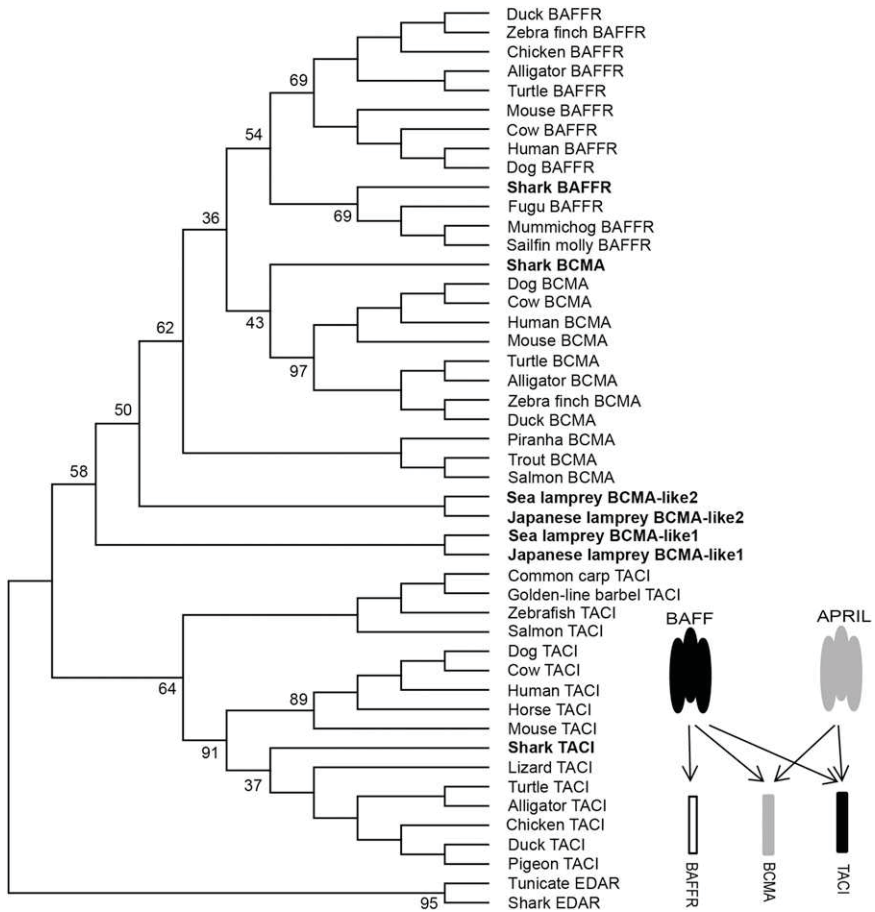
When we conducted a comprehensive sequence comparison of this pair of BAFF-interactive TNFSFR-homologous sequences in lampreys with currently identified BAFFR, BCMA, and TACI sequences of representative jawed vertebrate species using NJ and ML methods, the resultant tree topologies were found to be very similar (Fig. 1, Supplemental Fig. 1). Two major clusters are evident in both trees. All of the TACI sequences form one phylogenetic cluster, whereas the BAFFR, BCMA, and lamprey TNFSFR homologs are in the other cluster. In the latter cluster, BAFFR sequences are separated from heterogeneous BCMA sequences, and the lamprey TNFSFR homologs are closest to BCMA, although the bootstrap support is low (Fig. 1, Supplemental Fig. 1). We therefore designate sequence1 as BCMAL1 and sequence2 as BCMAL2. In this phylogenetic analysis, we also identified orthologs of mammalian BAFFR, BCMA, and TACI in the elephant shark genome sequence. Note that the phylogenetic trees are rooted with the closely related ectodysplasin A receptor (EDAR) sequences, which, like the TACI sequences, contain multiple cysteine-rich domains (CRDs).

Among the three types of BAFF-interactive receptors, the TACI sequences in jawed vertebrates contain two complete CRDs, whereas BCMA sequences contain only one complete CRD (Fig. 2, Supplemental Fig. 2A, 2B). Each CRD domain of TACI and BCMA contains six conserved cysteine residues, which could allow formation of three disulfide bonds. In contrast, BAFFR sequences contain a partial CRD that could allow a single disulfide bond (Fig. 2, Supplemental Fig. 2C). Like the

BCMA sequences in jawed vertebrates, the BAFF-interactive TNFSFR-homologous sequences in both sea lamprey and Japanese lamprey possess one complete CRD with six cysteine residues, which potentially could contribute to three disulfide bonds (Fig. 2). In mammalian BCMA, a tyrosine (Y13 in human), an aspartic acid (D15 in human), and a leucine (L17 in human) are located between the first and second cysteine residues of the CRD, and these participate in BAFF and APRIL binding (11). Notably, those residues are also conserved in the lamprey BCMA-like sequences, BCMAL1 and BCMAL2 (Fig. 2). Additionally, both sea lamprey and Japanese lamprey BCMAL1 sequences have a conserved leucine (L26 in humans) and arginine (R27 in humans) immediately upstream of the fourth cysteine residue in the CRD (Fig. 2), which favors APRIL binding in mammals (40).

The sequence identity of BAFFR, BCMA, and TACI is relatively high in their CRDs, transmembrane domains, and a short stretch of the TNFR-associated factor (TRAF)-binding region located close to the C terminus. Members of the TRAF family of signaling adaptor proteins participate in downstream signaling via a consensus TRAF-binding motif (11, 41, 42). A putative TRAF-binding motif, PVQDT, which is homologous to the experimentally determined TRAF-binding motif present in human CD40 (PVQET), mouse Cardif (PVQDT), and human OX40 (PIQEE), was identified in the cytoplasmic domains of the lamprey BCMAL1 (Fig. 3A). However, a slightly divergent homologous sequence, PVSET, in which the consensus glutamine residue (amide group containing polar amino acid) was substituted by the hydroxyl group containing polar amino acid serine, was found in the C-terminal region of lamprey BCMAL2 (Fig. 3A). In the human BCMA and BAFFR, the glutamine residue is also substituted by methionine and proline, respectively. Among the

FIGURE 1. Phylogenetic relationship among BAFF-interactive receptors. The phylogenetic tree is constructed by the NJ method. BCMA-like sequences in lampreys and BAFFR, BCMA, and TACI sequences in elephant shark are highlighted in bold letters. Bootstrap supports in interior branches are shown. The tree is rooted by EDAR sequences from vase tunicates (*C. intestinalis*) and elephant shark (*Callorhynchus milii*). A cartoon depicting receptor–ligand interactions in mammals is shown in the lower right corner.



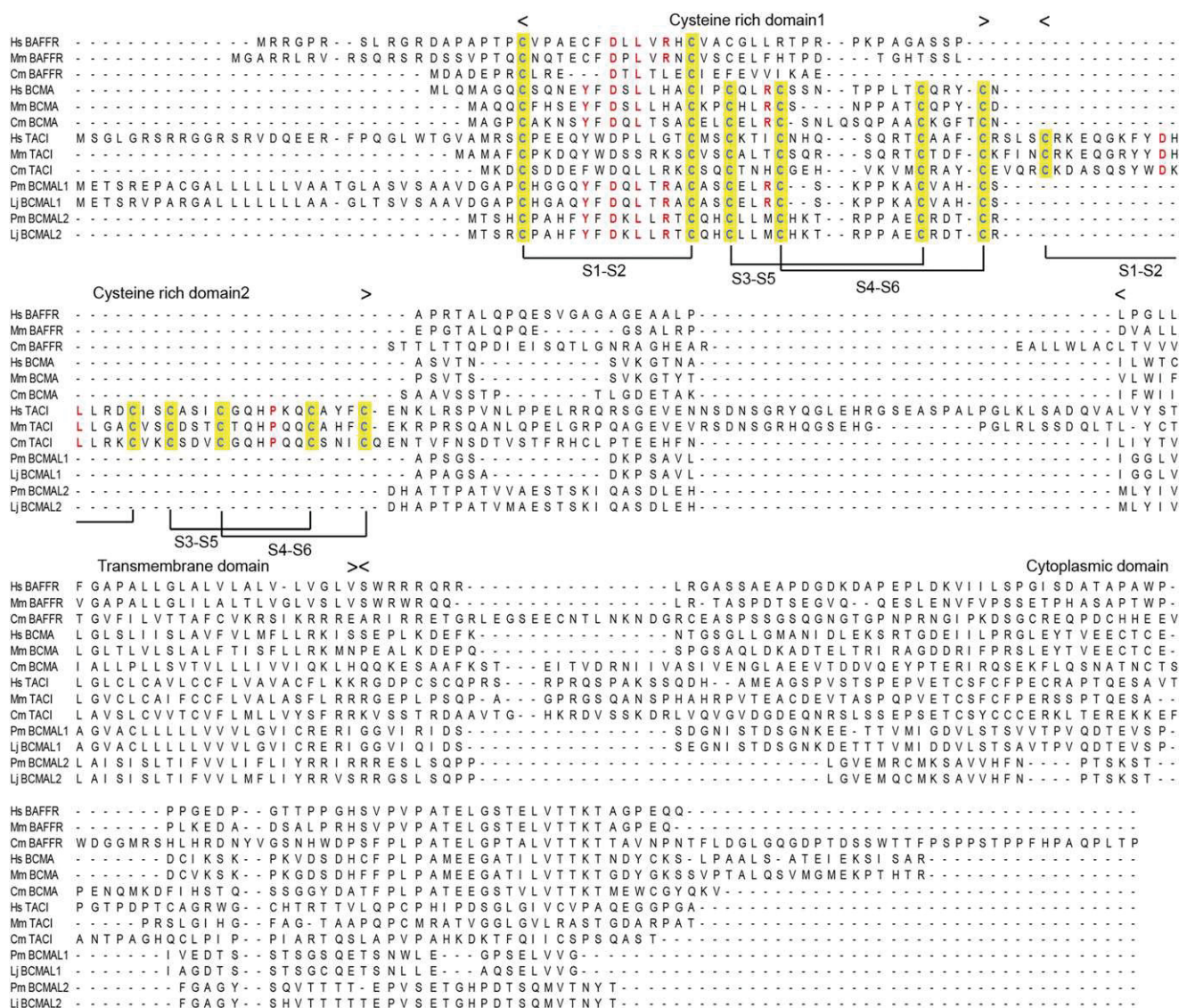


FIGURE 2. Alignment of BAFF-interacting receptors. The location of CRDs (CRD1 and CRD2), transmembrane domain, and cytoplasmic domain are shown above the alignment. Conserved cysteine residues in CRD1 and CRD2 are highlighted in yellow, and residues involved in mammalian BAFF and/or APRIL binding are shown in red. The formation of predicted disulfide bonds (S1–S2, S3–S5, and S4–S6) is indicated by brackets. Cm, *C. milii* (shark); Hs, *Homo sapiens* (human); Lj, *L. japonicum* (Japanese lamprey); Mm, *Mus musculus* (mouse); Pm, *P. marinus* (sea lamprey).

TRAF family members, TRAF1, TRAF2, TRAF3, and TRAF5 interact with similar motifs within the cytoplasmic region of the receptors (a major consensus sequence, [P/S/A/T]x[Q/E]E, where x is any amino acid) (41–43). In some instances, atypical TRAF-binding motifs are also found (44, 45). For example, the atypical TRAF-binding motif (PVPAT) of BAFFR specifically interacts with TRAF3, but the modification of PVPAT to the TRAF-binding PVQET motif of CD40 allows the modified BAFFR to interact with both TRAF2 and TRAF3 (44, 45). When we searched for TRAFs in sea lamprey and Japanese lamprey genome sequences using query sequences of human TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, and TRAF6 (accession numbers: NP_005649, NP_066961, NP_663777, NP_004286, NP_001306136, and NP_004611) and a hagfish TRAF3 (accession number: BAG85182), we found two TRAF3 homologs (TRAF3a and TRAF3b) and a TRAF6 homolog in both lamprey species (Fig. 3B). In the sea lamprey genome, TRAF3a, TRAF3b, and TRAF6 are located in scaffolds PIZI01000029.1, PIZI01000036.1, and PIZI01000002.1, respectively. In the Japanese lamprey, they are also distributed in three different scaffolds (scaffolds KE993941, KE993702, and KE993865).

There is ~63% sequence similarity between these two TRAF3 homologs in lampreys. The differences in the putative TRAF-binding motifs in lamprey BCMA1 and BCMA2 and the presence of multiple TRAFs in lampreys open the possibility that BCMA1 and BCMA2 have different signaling functions through the interaction with different TRAFs.

We could not find BAFFR, BCMA, and TACI homologs in the reference genomes of the lower chordate representatives vase tunicate (*Ciona intestinalis*), colonial star tunicate (*Botryllus schlosseri*), and amphioxus (*Branchiostoma floridae*). Previously, we reported that BAFF and APRIL homologs are absent in invertebrate chordates (28). Altogether, our observations are consistent with the conjecture that the homologs of BAFF and BAFF-interacting receptors coevolved in vertebrates.

Homology modeling of lamprey BCMA-like receptors

To evaluate the concordance and divergence in the three-dimensional structures for the BCMA-like proteins in lampreys and BAFF-interacting proteins in humans, we conducted homology modeling. When we estimated surface electrostatic charge by the

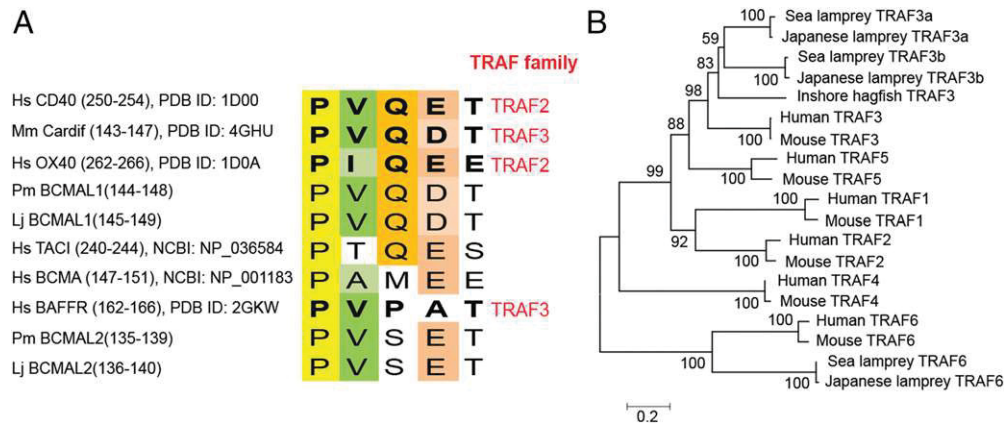


FIGURE 3. TRAFs and potential TRAF-binding motifs in lamprey BCMAL1 and BCMAL2. **(A)** Putative TRAF-binding motif in the cytoplasmic domain of BCMAL1 and BCMAL2 in lampreys. The typical consensus TRAF-binding motif is shown in dark color shades, and the substitution with similar amino acids is shown in lighter shades. As modes of interaction between TRAF family members and TNFRs have been well studied for TRAF2 and TRAF3, we used TRAF2- and TRAF3-binding motifs as representatives (shown in bold letters, PDB identification number is given). Positions of amino acid residues are shown in parentheses. **(B)** Phylogenetic relationship of human, mouse, hagfish, and lamprey TRAFs. The unrooted phylogenetic tree is constructed by NJ method. Bootstrap supports in all branches are shown. Hs, *H. sapiens*; Lj, *L. japonicum*; Mm, *M. musculus*; Pm, *P. marinus*.

Adaptive Poisson-Boltzmann Solver, charged residues were accumulated around the ligand binding surface in human BAFFR and TACI. In contrast, human BCMA has only relatively moderate charges on the ligand binding surface (Fig. 4). We constructed three-dimensional models of the lamprey BCMA-like receptors to estimate the surface electrostatic charge. The surface charge distributions predicted by these models suggest that both lamprey BCMA-like receptors have relatively moderate charge on the surface corresponding to the ligand binding site in the human BAFFR. The sequence comparison in the ligand recognition site suggests that lamprey BCMA-like receptors have conserved features that could favor designation as either BAFF or APRIL types of ligands (Supplemental Fig. 3). Both lamprey BCMA-like receptors have a tyrosine corresponding to the Tyr¹³ residue in human BCMA, which is required to bind mammalian APRIL. In contrast, the lamprey BCMA-like receptors retain the conserved Arg³⁰ residue found in human BAFFR that preferentially binds mammalian BAFF. The lamprey BCMAL2 shares a hydrophobic residue (Met) in the corresponding site for Leu³⁸ in human BAFFR, which preferentially binds BAFF rather than APRIL (40). In our previous study, we noted that the lamprey BAFFL protein possesses important amino acid similarities to both mammalian BAFF and APRIL (28). These conserved features suggest that both BCMA-like proteins could interact with the lamprey BAFFL protein.

Receptor-ligand interaction

To examine the potential for ligand-receptor interactions between the lamprey BAFFL protein and BCMA-like receptors, a flow cytometry analysis was performed using purified soluble lamprey BAFFL protein and recombinant forms of the two lamprey BCMA-like receptors that were expressed on 293T cells. The soluble BAFFL protein was bound by BCMAL1- or BCMAL2-expressing 293T cells, thereby indicating that BCMAL1 and BCMAL2 can each bind the lamprey BAFFL protein (Fig. 5). Immunoprecipitation analysis was also performed using the recombinant BAFFL protein and soluble recombinant forms of the two BCMA-like receptors. The size of the recombinant HA-tagged BAFFL proteins was estimated in native PAGE to be ~78 kDa, which is about four times the expected monomer size of 18 kDa (Supplemental Fig. 4). This size shifting could be explained by spontaneous homo-multimer formation of lamprey

BAFFL protein, akin to the homo-trimer formation by human BAFF (46, 47). We confirmed that soluble lamprey BAFFL protein was coprecipitated by proteins formed by the extracellular domains of the BCMAL1 and BCMAL2 receptor proteins fused with an Ig Fc domain (Supplemental Fig. 4). We also confirmed that the FLAG-tagged extracellular domains of the BCMAL1 and BCMAL2 receptor proteins were coprecipitated by the lamprey BAFFL protein but not by hEDA (Supplemental Fig. 4).

Cellular expression patterns of the lamprey BCMA-like genes

Like the jawed vertebrates, jawless vertebrates have discrete hematopoietic tissues that contain B-like cells (VLRB⁺), T-like cells (VLRA⁺ and VLRC⁺), innate lymphocytes, and other types of hematopoietic cells including thrombocytes and erythroid, monocytic, and granulocytic cells (17, 18). Hence, we performed

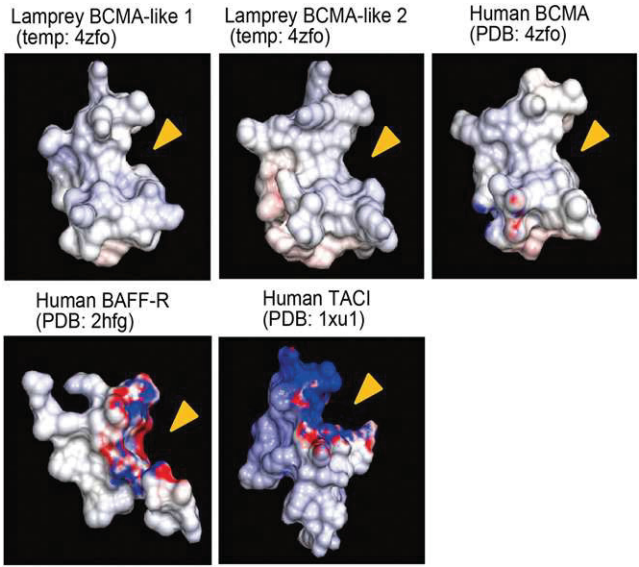


FIGURE 4. The protein structure and surface electrostatic potential of BAFF-related receptors. The structures for two lamprey BCMA-like receptors, BCMAL1 and BCMAL2, were predicted by SWISS-MODEL, based on the crystal structure of human BCMA (PDB: 4zfo). The blue and red colors represent positive and negative surface electrostatic potential, respectively. Yellow arrowhead indicates the ligand binding site.

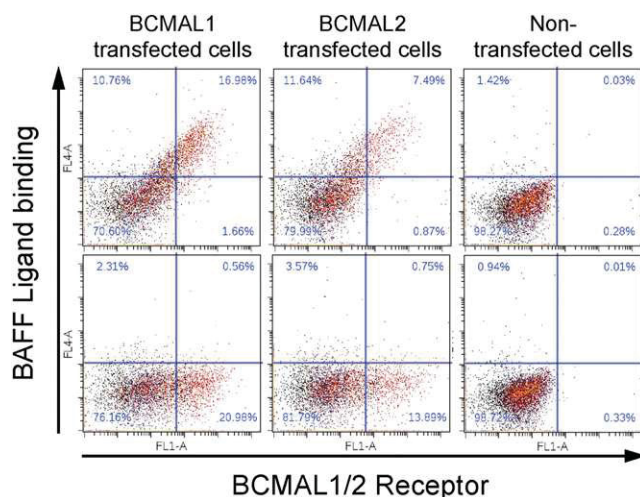


FIGURE 5. Interaction between the lamprey BCMA-like receptor proteins (BCMAL1 and BCMAL2) and the lamprey BAFFL ligand. The purified soluble HA-tagged BAFFL protein was incubated with 293T cells (top right panel) and 293T cell transfects expressing FLAG-BCMAL1 (top left panel) or FLAG-BCMAL2 (top middle panel). Binding was evaluated by flow cytometry. The receptors and the ligand were detected by anti-FLAG and anti-HA Abs, respectively. The data without the HA-BAFFL ligand are shown in the bottom panels. Two flow cytometry experiments were overlaid in each panel (red and black dots). Red set, Cells stained with both primary and secondary Abs. Black set, Cells stained with secondary Ab only. The experiments were repeated three times with similar results.

a qPCR analysis to examine the tissue expression profiles of transcripts encoding the two *BCMA*-like genes in lampreys. Our qPCR analysis indicated that both *BCMA*-like genes are highly expressed by circulating WBCs and, to a lesser extent, by cells obtained from intestine, gill region, and skin tissue samples (Fig. 6A). When the different lymphocyte-like populations in lamprey blood and other tissues were examined, preferential expression of both *BCMA*-like genes was observed for the VLRB⁺ B cell-like population and, to a lesser extent, for the VLRA⁺ and VLRC⁺ T cell-like populations (Fig. 6B). Significant expression of both *BCMA*-like genes was also observed in the TN lymphocyte population. These expression profiles for *BCMAL1* and *BCMAL2* suggest their potential for contribution to the regulation of B-like cells in lampreys. When the expression profiles of *BCMAL1* and *BCMAL2* were examined for VLRB⁺ cells derived from naive nonstimulated and PHA-stimulated lampreys, we found that *BCMAL1* was preferentially expressed by VLRB⁺ cells of naive animals, whereas *BCMAL2* was preferentially expressed by PHA-stimulated animals (Fig. 6C).

Discussion

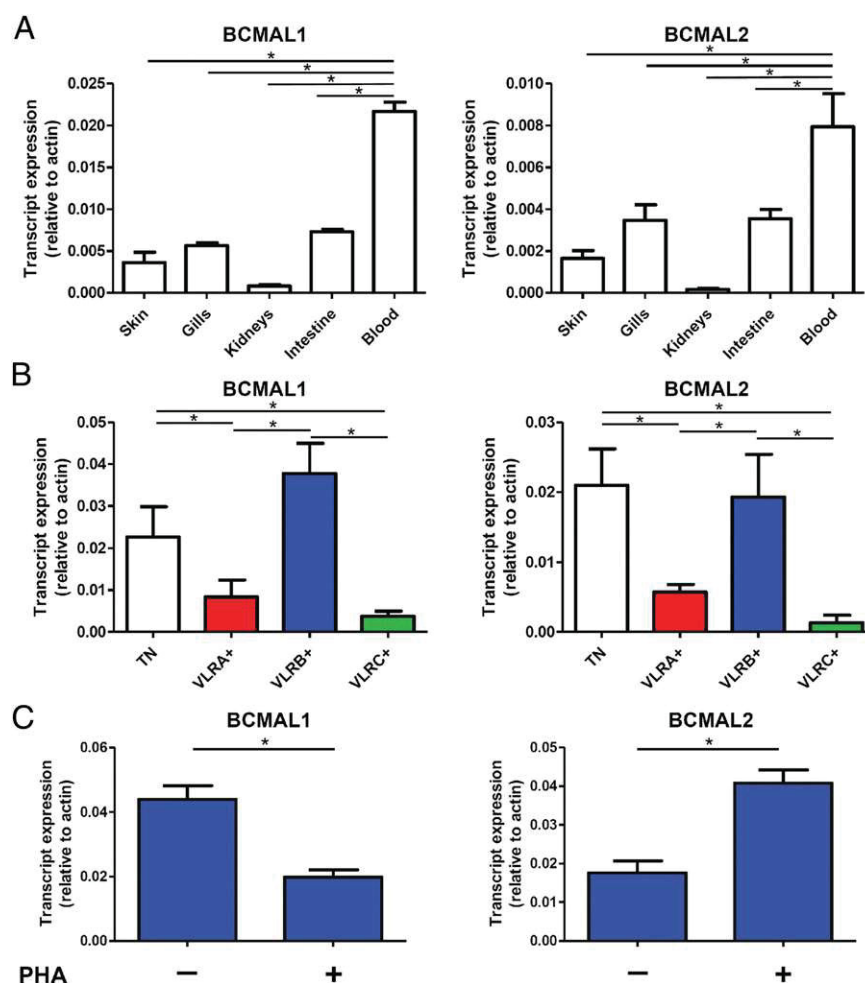
Lampreys offer an important model organism with links to the early evolution of vertebrates and in which both B-like and T-like lymphocytes are present (17, 18). In considering the immunological roles of BAFF, APRIL, and their receptors in the B cell arm of the immune system, we examined the available genome sequences of diverse vertebrates, including lamprey and elephant shark, and the invertebrate chordates amphioxus and tunicates. Previously, we identified and characterized a single *BAFF*-like gene in lamprey (28). The lamprey BAFFL protein is a bipolar TNF with both a negatively charged BAFFL side and a positively charged APRIL-like side (28). Like mammalian BAFF and APRIL, this *BAFF*-like relative is expressed in a wide range of tissues and by a variety of cell types.

In the current study, we identified two BCMA-like receptors in lampreys, BCMAL1 and BCMAL2, and used this information to reconstruct a plausible evolutionary scenario for the emergence of this B cell signaling system. Our in vitro binding assays indicate that both BCMA-like receptors are capable of interaction with the lamprey BAFFL protein. The search for *BAFF* or *APRIL*-like orthologs (28) and *BAFFR*, *BCMA*, and *TACI* sequences in invertebrate chordates like vase tunicates (*C. intestinalis*), colonial star tunicate (*B. schlosseri*), and amphioxus (*B. floridae*) was fruitless. BAFF/APRIL and their cognate receptors thus appear to be vertebrate lineage innovations.

The mammalian BAFFR, BCMA, and TACI proteins are type III transmembrane receptors characterized by an extracellular ligand binding site at the N terminus, a transmembrane domain, and the C-terminal cytoplasmic domain that is responsible for intracellular signaling (48). The binding of BAFF or APRIL by these receptors occurs in the fairly conserved CRD located within the extracellular region (49). TNFRs typically have multiple CRDs (50), but BCMA receptors in all of the jawed vertebrates, including the cartilaginous fish, have only one CRD, and the BAFFR family members have only a partial CRD (Fig. 2, Supplemental Fig. 2). In contrast, the TACI sequences in all jawed vertebrates contain two CRDs (Fig. 2, Supplemental Fig. 2). In humans, the first CRD of TACI has been shown to have a much weaker affinity than the second CRD for BAFF and APRIL binding (40). Even a transcript variant of the human TACI sequence, which lacks the first CRD, binds as efficiently as the native TACI with two CRDs (40). These findings suggest that one CRD is sufficient for interaction with BAFF and APRIL. Like BCMA in jawed vertebrates, both of the BCMA-like receptors in lampreys possess one CRD. Moreover, phylogenetic analyses for whole sequences and the CRDs, as well as homology modeling, support the conclusion that both lamprey receptors are close relatives of the BCMA receptors of jawed vertebrates. Both NJ and ML trees for the entire sequences of BAFF-interacting receptors reveal two main clusters, one of which includes all TACI sequences and the other of which contains BAFFR, BCMA, and lamprey BCMA-like sequences (Fig. 1, Supplemental Fig. 1). In view of the multiple CRDs in other TNFRs, including the closely related EDAR, an evolutionary scenario is suggested that a *TACI*-like organization is ancestral to the BAFF-interacting receptor genes and the evolution of *BCMA*-like genes involved the loss of one CRD. A parsimonious interpretation is that the common ancestor of jawed and jawless vertebrates should have both *TACI*-like and *BCMA*-like genes; a *TACI*-like gene was lost later in jawless vertebrates, and the *BAFFR*-like gene in jawed vertebrates evolved by gene duplication and further loss of cysteine residues in the single CRD. The presence of two *BCMA*-like genes in sea lampreys and Japanese lampreys also indicates that a duplication event occurred before the divergence of these two lamprey species. Overall, the evolutionary scenario indicates that CRD loss and degeneration occurred during the evolution of BAFF-interacting receptors.

The cytoplasmic domains of BAFFR, BCMA, and TACI exhibit only a short stretch of similarity, which includes the respective TRAF-binding sites (11). Despite poor conservation within the C-terminal region of the two BCMA-like receptors in lampreys, our analysis indicates that both proteins have a putative TRAF-binding motif in this region, thereby supporting their role in downstream signaling (Fig. 3). However, these potential TRAF-binding regions differ from each other. Lampreys also possess multiple TRAF genes that may interact differentially with the lamprey BCMA-like proteins. Of note in this regard, mammalian

FIGURE 6. Expression of two *BCMA*-like genes in lampreys. **(A)** Tissue distribution of *BCMAL1* and *BCMAL2*. Relative expression levels of *BCMAL1* and *BCMAL2* transcripts were determined by real-time PCR analysis of skin, gills, kidneys, intestine, and WBCs of sea lamprey larvae ($n = 3$ larvae). **(B)** *BCMAL1* and *BCMAL2* expression by sorted VLRA⁺, VLRB⁺, VLRC⁺, and TN lymphocyte populations ($n = 3$ larvae) in lampreys. **(C)** The expression of *BCMAL1* and *BCMAL2* by VLRB⁺ cells in naive and PHA-stimulated lampreys ($n = 3$). Relative expression levels of two *BCMA*-like genes were determined by real-time PCR (mRNA abundance relative to that of β -Actin). Error bars represent mean $\pm$ SEM from three independent experiments. Statistical significance was calculated using a two-tailed unpaired t test. $*p < 0.05$.



BCMA may bind to several TRAFs (51, 52). These features raise the possibility that there are differences in the downstream signaling pathways for the two lamprey BCMA-like proteins. Parenthetically, we still lack a complete molecular understanding of the signaling events of BAFF-interacting receptors in mammals.

Our evaluation of the two *BCMA*-like genes in sea lampreys indicates that both have similar expression patterns: they are highly expressed in peripheral blood cells and, to a lesser extent, in cells derived from the intestine, gills, and skin. Among the different lymphocyte populations, both lamprey *BCMA*-like genes are most highly expressed in the VLRB⁺ B cell-like lymphocytes, consistent with the largely B-lineage-skewed expression of BAFFR and BCMA in mammals (53–55). The expression profiles for *BCMAL1* and *BCMAL2* suggest their equivalent potential for contributing to the regulation of lamprey B-like cells. When we compared their individual expression levels in VLRB⁺ cells in naive versus PHA-stimulated lampreys, we found significantly higher expression of *BCMAL1* in naive animals and lower expression in VLRB⁺ cells of the PHA-stimulated animals. The opposite expression pattern was observed for *BCMAL2*, for which the expression level is increased in VLRB⁺ cells from the PHA-stimulated animals. This result suggests that *BCMAL1* and *BCMAL2* could regulate different stages of lamprey B-like cell differentiation, reminiscent of the differential roles of BAFFR and BCMA in naive versus plasma cells in mammals.

Homology modeling of BCMAL1 and BCMAL2 revealed that both have moderate charge distribution on their putative ligand binding surfaces and conservation of amino acid residues (Fig. 4,

Supplemental Fig. 3) typically involved in the interaction with both BAFF and APRIL (11). Our previous study suggests that the lamprey BAFFL ligand has both BAFF and APRIL characteristics in terms of conserved amino acids and surface electrostatic charge (28). Our in vitro analysis also indicates that, like mammalian BAFF, the lamprey BAFFL protein forms homo-multimers and can bind with both BCMAL1 and BCMAL2. We hypothesize, therefore, that both BCMA-like receptors and the BAFFL ligand in lampreys were selected evolutionarily for their receptor–ligand interaction.

In conclusion, the preferential expression of both BCMA-like receptors in the VLRB⁺ B cell-like lymphocytes and their interaction with the BAFFL protein in lampreys may reflect an ancient mechanism for BAFF-mediated regulation of B-like lymphocytes. The present results also suggest that the interaction between a BAFFL ligand and its receptors evolved in a common ancestor of jawed and jawless vertebrates prior to the emergence of the specific interacting receptor BAFFR in jawed vertebrates. Further studies concerning mechanisms of regulation of VLRB-producing B-like lymphocytes by the interaction between lamprey BAFFL ligand and BCMA-like receptors may facilitate the development of methods to maintain lamprey B-like cells ex vivo.

Acknowledgments

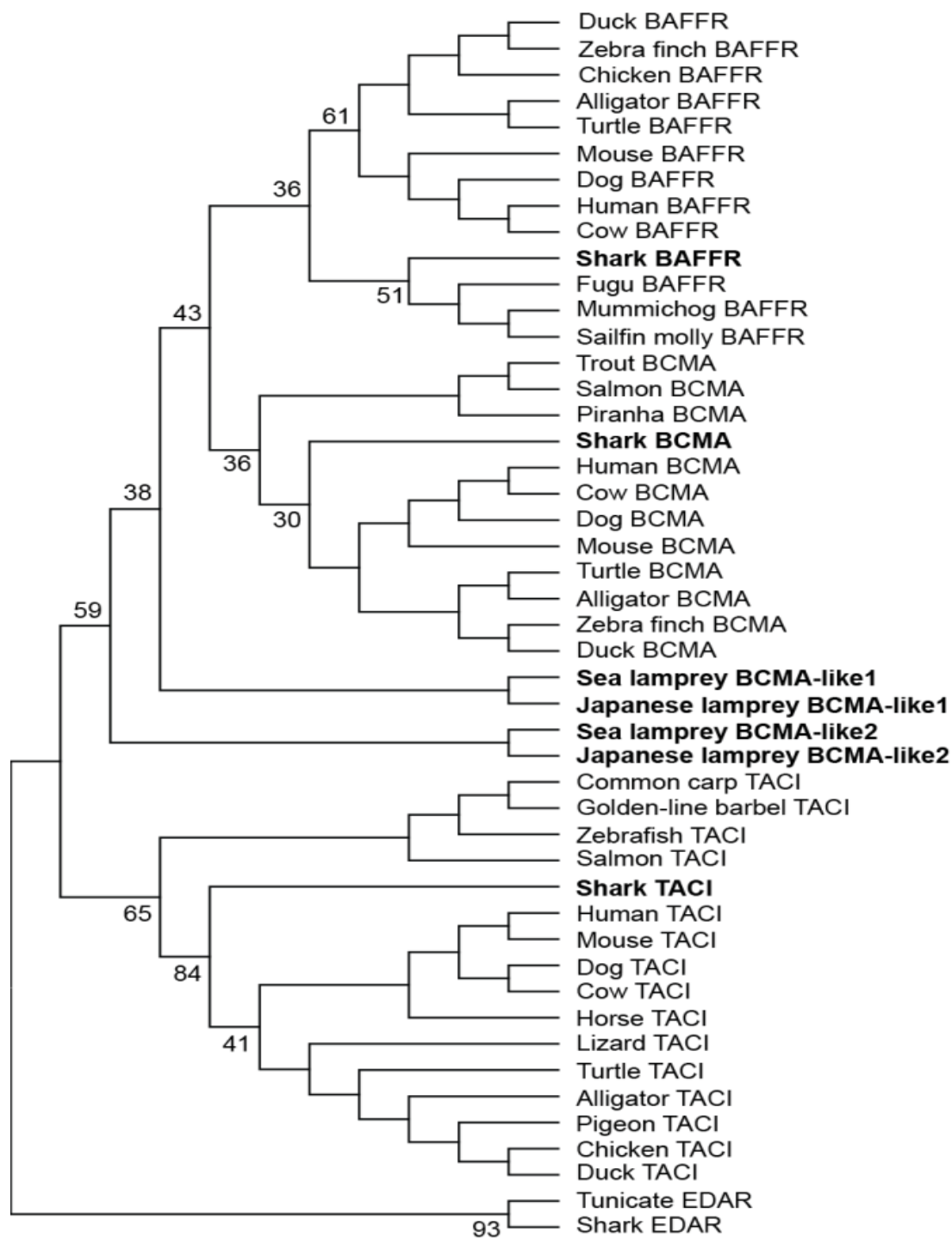
We thank Katherine Augustus for technical support.

Disclosures

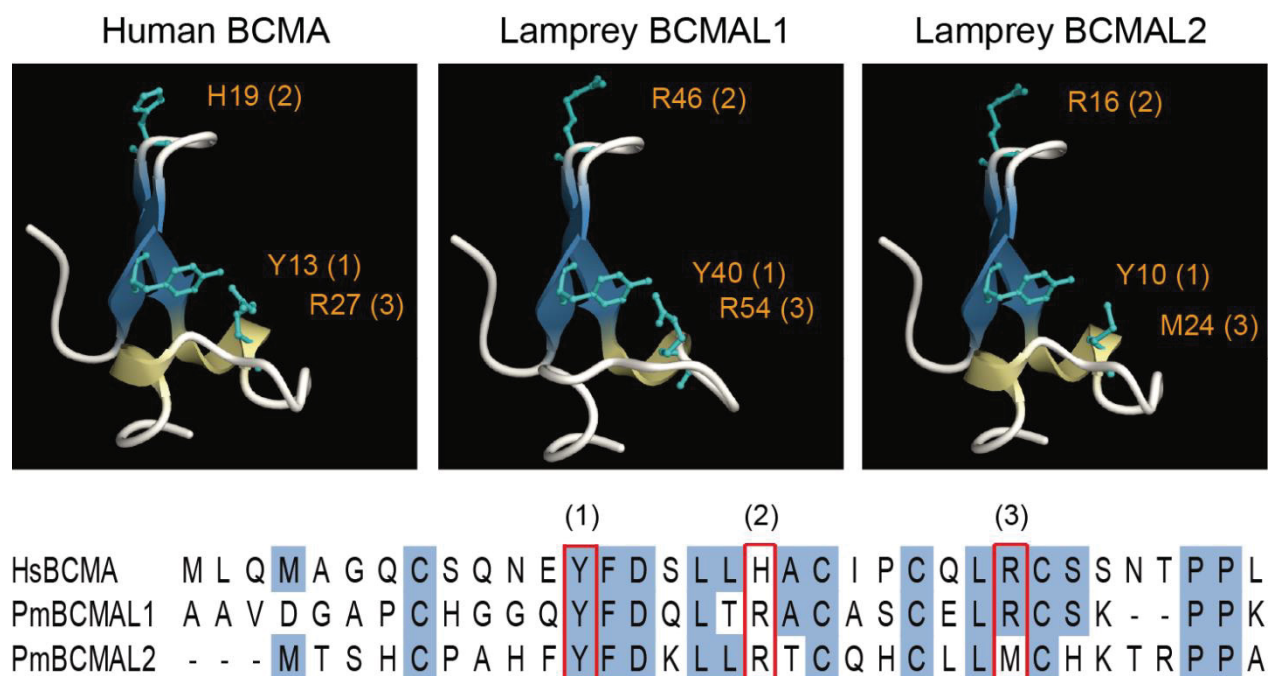
M.D.C. is a cofounder and shareholder of NOVAB, which produces lamprey Abs for biomedical purposes, and J.P.R. is a consultant for NOVAB. The other authors have no financial conflicts of interest.

References

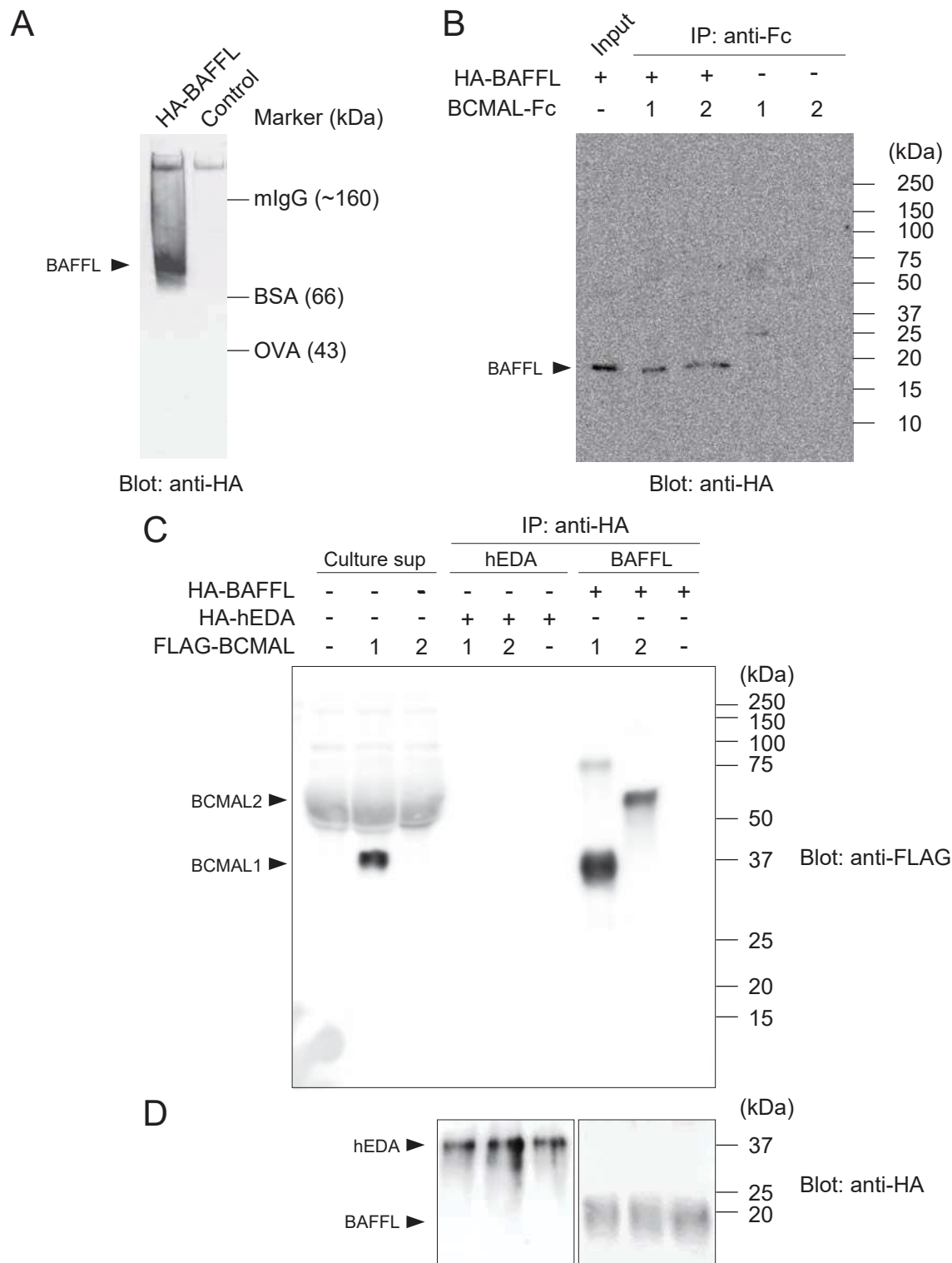
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Supplemental Fig. 1. Phylogenetic tree based on the maximum likelihood analysis of BAFF-interactive receptors. BAFFR, BCMA and TACI sequences in elephant shark and two BCMA-like sequences in lampreys are highlighted in bold letters. Bootstrap supports in interior branches are shown. The tree is rooted by EDAR sequences from vase tunicates (*Ciona intestinalis*) and elephant shark (*Callorhinchus milii*).



Supplemental Fig. 3. Conservation in the region demonstrated to mediate ligand binding in human BCMA. Models of human BCMA and lamprey BCMALs predicted by the SWISS-MODEL are shown in the top panels. Numbers in parentheses indicate the position of residues in the sequence alignment at the bottom. Conserved residues between human BCMA and lamprey BCMAL are highlighted in blue. The red boxes indicate residues that are essential for ligand binding. For example, an aromatic residue in the hairpin, present in human BCMA (Tyr13) is required for APRIL binding (red box located at left).



Supplemental Fig. 4. Analysis of the interaction between the lamprey BCMA-like receptors (BCMAL1 and BCMAL2) and the lamprey BAFF-like (BAFFL) ligand. (A) Culture medium of HA-tagged BAFFL- or vector-transfected 293T cells was mixed with glycerol and Ponceau S, run on a native PAGE gel, blotted and detected with an anti-HA antibody. Mouse IgG, BSA and ovalbumin were also run for size comparison. (B) The HA-BAFFL protein was incubated with the extracellular domains of BCMAL1 or BCMAL2 fused with human IgG Fc (BCMAL-Fc), pulled down by Protein G beads, separated by SDS-PAGE, blotted and detected using an anti-HA antibody. (C) The FLAG-tagged extracellular domains of BCMAL1 and BCMAL2 (FLAG-BCMAL1 and FLAG-BCMAL2) were incubated with HA-tagged BAFFL protein (or human ectodysplasin A (hEDA) protein as a control), pulled down by anti-HA beads, separated by SDS-PAGE and detected using an anti-FLAG antibody. BCMAL2 signal from the blotting of 12 μ l of loaded supernatant is difficult to detect above background (third lane from the left), but is enhanced after pull down from 250 μ l of supernatant (second lane from the right). (D) The same samples shown in (C) were separated by SDS-PAGE and blotted with an anti-HA antibody to detect the hEDA and BAFFL proteins. Signal levels in (D) were adjusted independently for each ligand. The experiments were repeated twice (B and D) and three times (A and C) with similar results.