Teleost B7 Expressed on Monocytes Regulates T Cell Responses[1,2]

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In mammals, professional APCs induce adaptive immunity via the activation of T cells. During this process, B7 family molecules present upon APCs are known to play crucial roles in optimal T cell stimulation. In contrast, the confirmation of APCs in a nonmammalian vertebrate has yet to be achieved. To obtain further insights into the evolutionary origin of APCs, we have identified three members of the B7 family in the teleost *Takifugu rubripes* (fugu): B7-H1/DC, B7-H3, and B7-H4. The three fugu B7s were expressed on the surface of blood monocytes. The $B7^+$ monocytes, which are composed of at least two distinct populations, expressed the MHC class II component gene. The fugu B7 molecules bound to activated T cells, indicating that putative B7 receptors were expressed upon T cells. Fugu B7-H1/DC inhibited T cell proliferation concomitant with increasing levels of both IL-10 and IFN-γ expression, whereas both B7-H3 and B7-H4 promoted T cell growth following IL-2 induction and the suppression of IL-10. These observations indicate that fugu B7s regulate T cell responses via receptors upon T cells. We suggest that fish $B7^+$ monocytes are APCs and that a costimulatory system has already developed in fish via the evolutionary process. *The Journal of Immunology,* **2009, 182: 6799–6806.**

In mammals, APCs induce adaptive immunity that in turn activates T cell responses (1). Dendritic cells (DCs),[4] monocytes/macrophages, and B cells have been identified as major professional APCs (1, 2). These APCs capture and process Ags and then present the processed antigenic peptides upon MHC molecules to the T cells. Subsequently, the APCs activate T cells with costimulatory molecules upon the APCs (1). Despite its importance, the evolutionary process of the T cell activation system via APCs remains poorly understood because of the lack of an effective model organism in lower vertebrates that could allow one to manipulate leukocytes and to use suitable markers for the identification of APCs.

In fish, a variety of specific immune responses such as acute allograft rejection indicate that T cells are involved in these immune activities in a similar manner to those of mammals (3). T cells have been recently identified by using key molecular markers such as CD4 and CD8 (4, 5).

In addition, orphan receptors similar to mammalian costimulatory receptors such as CD28 upon the T cell have been reported in fish species (6, 7). These observations suggest that fish species might have APCs and a costimulatory system. However, defined identification of APCs has yet to be achieved in lower vertebrates, including fish, due to a lack of basic knowledge of costimulatory ligands in such species. Thus, the evolutionary history of the T cell activation system via APCs remains unclear.

To characterize APCs in lower vertebrates, we focused upon costimulatory ligands, B7 family proteins, which play critical roles in the function of mammalian APCs (8). The B7 members expressed upon the APCs regulate T cell responses in both negative and positive manners (8). To date, B7 molecules have not been identified in lower vertebrates.

Previously, the identification of a number of fish orthologs to mammalian immune-related genes has been hampered because of the low similarity between such orthologous genes (9). However, recent studies have demonstrated that the genome databases of fish are powerful resources for identifying genes that evolved rapidly since the divergence of the fish and mammalian lineages (10). Tiger pufferfish (*Takifugu rubripes*, also known as fugu) is a model species of fish whose genome has been sequenced (10). A set of molecular markers for studying T cell function is now available for this species (9). Moreover, compared with other model fishes such as zebrafish and medaka, the body size of fugu is larger enough to allow simple collection of leukocytes (9). Considering the advantages of the fugu system, the present study proposes that the monocytes expressing B7s are professional APCs and that the T cell regulation depending upon B7s became established in fish during the evolutionary process.

Materials and Methods

Animals and cDNA preparation

Fugu, *T. rubripes* (~1,200 × *g*) were purchased from a local fish dealer and reared in a tank of running seawater maintained at 20°C. Fish were anesthetized with 2-phenoxyethanol and blood was collected immediately by heparinized syringes and needles from the caudal vasculature. A series of tissues were dissected from freshly killed fish and immediately fixed in RNAlater (Takara Bio) for subsequent RNA extraction. Samples were collected from the following tissues: skin, gill, thymus, head kidney, trunk kidney, spleen, liver, intestine, and muscle. PBLs were also isolated by density gradient centrifugation from the collected blood according to the

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[2] The sequences presented in this article have been submitted to GenBank under accession numbers AB453016 (fugu B7-H1/DC); AF317088 (mouse B7-H1; version AF317088.1); NM_021396 (mouse B7-DC); AB453017 (fugu B7-H3); NM_001024736 (human 4IgB7-H3); NM_133983 (mouse B7-H3, version NM-); AB453018 (fugu B7-H4); AY280973 (mouse B7-H4; version AY280973.1); X60958 (mouse B7-1); BC013807 (mouse B7-2); and NM_015790 (mouse B7-H2).

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[4] Abbreviations used in this paper: DC, dendritic cell; BLAST, basic local alignment search tool; EF, elongation factor.

method of Bei et al. (11) and stored at −80°C. The Animal Care and Use Committee of the University of Tokyo reviewed and approved this study.

Total RNA was extracted from fixed samples and PBLs using the RNA extraction reagent (Isogen; Nippon Gene) in accordance with the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA treated with DNase (TURBO DNA-*free*; Ambion) using the SMART RACE cDNA amplification kit (Clontech). A series of cDNA pools were used for expression analysis of fugu B7s and MHC class II transcripts by RT-PCR analysis.

cDNA cloning, genomic structure, and phylogeny of fugu B7 members

Basic local alignment search tool (BLAST) searches of the fugu genome database (genome.jgi-psf.org/Takru4/Takru4.home.html) were performed with the protein sequences of human B7 family molecules. Three types of full-length B7 cDNA were isolated from head kidney cDNAs by RACE with specific primers (supplemental Table I)[5] based on predicted B7 DNA sequences. Fugu B7 structures were analyzed with the following programs: SignalP (www.cbs.dtu.dk/services/SignalP) for signal peptide, SOSUI (sosui.proteome.bio.tuat.ac.jp/sosuimenu-0.html) for transmembrane region, and InterPro (www.ebi.ac.uk/interpro/) for Ig-like domains. The fugu genome database was queried with fugu B7 sequences for synteny. Genomic organization was analyzed by comparing the isolated cDNA sequences and genomic sequences in the fugu genome database. A multiple sequence alignment and phylogenetic tree were generated using ClustalW (www.ddbj.nig.ac.jp/Welcome-j.html) and Tree View version1.6.6 programs (evolution.genetics.washington.edu/phylip/software.html).

Preparation and activation of enriched T cells, B cells, and monocytes

PBLs were obtained from the blood of healthy fugu using the same methodology as described above. PBLs were suspended into RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% FBS, 1% penicillin/streptomycin/fungizone (Invitrogen), and 200 mM L-glutamine. To isolate monocytes and neutrophils by adherent properties onto plastic surfaces, the cell suspension was incubated for 1 h in a flask (TPP Techno Plastic Products) pretreated with heat-inactivated fugu serum. Following incubation, the lymphocyte-enriched supernatant in the flask was carefully collected to purify B cells and T cells as described below. Adhered cells were washed thoroughly several times with RPMI 1640 medium to remove neutrophils, which show less adhesive properties than monocytes. Following the washes, adherent monocytes were exposed with 1 mM EDTA in PBS and finally collected from the plastic surface.

The B cell population was isolated from the collected lymphocyte-enriched suspension using an anti-fugu IgM mAb (4H5; provided by Prof. T. Miyadai, Fukui Prefectural University, Obama, Japan) and MACS (Miltenyi Biotec). The lymphocyte-enriched supernatant was also filtered with nylon fiber columns (Wako Chemicals). The through fraction from the nylon column was pooled as a T cell population. The enriched monocytes, B cells, and T cells were used for cultures and RT-PCR analysis as described below. Morphology and gene expressions of these enriched leukocyte populations are shown in supplemental Fig. 1.

Production of Ig fusion proteins of B7s

Fugu B7-Ig fusion proteins (B7-Ig) were prepared by joining the complete extracellular region of each fugu B7 and Fc region of human IgG1 in pME-18S (provided by Prof. Arase, Osaka University, Osaka, Japan). The B7-Ig cDNAs were ligated into an insect cell expression vector, pIB/V5-His-TOPO (Invitrogen), and the constructs were transfected into insect High Five cells (Invitrogen) using FuGENE 6 transfection reagent (Roche). The transfectants were cultured in Grace's insect medium (Invitrogen) for 72 h, and the fusion protein secreted into the medium was collected and purified by HiTrap Protein G HP columns (GE Healthcare). The purity and predicted molecular mass of the fusion protein were confirmed by SDS-PAGE and Western blot analysis with anti-human IgG (Jackson ImmunoResearch Laboratories).

FACS analysis

Antisera against fugu B7-H1/DC, B7-H3, and B7-H4 were generated by immunizing Wistar rats (SLC) with 500 μg of each purified fugu B7-Ig emulsified with CFA (Sigma-Aldrich) and were collected 2 wk after immunization. To eliminate cross-reactivity to the other B7-Igs, each anti-fugu B7 serum was absorbed with the other two B7-Ig proteins. Specificities were determined by ELISA and FACS analyses before use in our experiments.

The monocyte, B cell, and T cell populations (2×10^7) were cultured in supplemented RPMI 1640 medium for 72 h at 20°C, either with each mitogen or alone. Both monocyte and B cell populations were treated with 50 μg/ml LPS (extracted from *Escherichia coli* O111: B4 strain by the phenol-water extraction; Wako Chemicals), and T cells were cultured with 20 μg/ml PHA (Sigma-Aldrich). All populations were also stimulated with a combination of 5 μg/ml PMA and 250 ng/ml ionomycin. The cultured monocytes, B cells, and T cells were reacted with the antiserum or normal serum as a control. Following washing with FACS buffer (PBS, 3% FBS, and 0.01% NaN_3), the cells were stained with FITC-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories). Thirty thousand events were analyzed by flow cytometry (Partec).

RT-PCR analysis

The expression profiles of each fugu B7 and MHC class II α-chain mRNA in different tissues of fugu were detected by RT-PCR with the specific primer pairs B7-H1/DC-F2/R2, B7-H3-F4/R4, B7-H4-F2/R2, and MHC-IIα-F/R, respectively (supplemental Table I). Fugu elongation factor (EF)-1α (EF-1F/R; supplemental Table I) was amplified as a control for the RT-PCR. PCR amplification was performed in a total volume of 10 μl with *Taq* DNA polymerase (Takara Bio) and subjected to 35–40 cycles (30 cycles for MHC class IIα, 35 cycles for B7-H3, B7-H4, and B7-H1/DC) consisting of 94°C for 10 s, 60°C for 5 s, and 72°C for 1 min. The PCR products were separated by electrophoresis on a 2.0% agarose gel and detected with ethidium bromide.

In addition, to examine the expression of fugu B7s and MHC class II α-chain in monocytes, we first prepared unstimulated and LPS-stimulated monocytes as mentioned above and then isolated B7$^+$ monocytes by MACS with anti-fugu B7 serum from the LPS-stimulated monocytes. Total RNA was isolated from each monocyte population, and RT-PCR was performed using the same methodology described above.

B7-Ig binding assays

The T cells were activated with PHA (20 μg/ml) for 24 h at 20°C in the supplemented RPMI 1640 medium. The activated T cells were collected and incubated on ice with 5 μg/ml B7-H1/DC-Ig, B7-H3-Ig, B7-H4-Ig, or human IgG (Wako Chemicals) in supplemented RPMI 1640 medium for 1 h on ice. The cells were washed and incubated for 30 min on ice in FACS buffer with FITC-conjugated goat anti-human IgG Ab. After washing with FACS buffer, three thousand events were analyzed by flow cytometry.

T cell proliferation assay

The proliferation of T cells was investigated in triplicate in flat-bottom 96-well plates (TPP Techno Plastic Products). Before this assay, we investigated optimal PHA concentrations and the associated time course for our study. We determined that a concentration of 5.0 μg/ml PHA did not influence T cell proliferation and that T cell growth was stimulated by 40 μg/ml PHA during culture for 96 h. The T cells (5.0×10^4) were cultured in RPMI 1640 medium supplemented with 1% FBS and 200 mM L-glutamine and 2% penicillin/streptomycin/fungizone with 5 μg/ml PHA for B7-H3 and B7-H4 or 40 μg/ml PHA for B7-H1/DC. Culture media containing B7-H1/DC-Ig, B7-H3-Ig, B7-H4-Ig, or human IgG at various concentrations (~20 μg/ml B7-H3-Ig and B7-H4-Ig, ~40 μg/ml B7-H1/DC-Ig, or a corresponding concentration of human IgG) were incubated at 20°C for 96 h. The B7-stimulated cells were pulsed with 10 μM BrdU per well for the last 4 h. BrdU incorporation into B7-Ig-stimulated T cells was quantified using cell proliferation ELISA and a BrdU (colorimetric) kit (Roche Diagnostics).

The stimulation index of the assay was defined as the ratio (R) of the absorbance signal in control and B7-Ig stimulated cells and was calculated as follows: $R = S - B/I - B$, where S represents the absorbance values of incorporated BrdU in costimulated or control cells and I and B indicate absorbance values for an initial stage (without Ig proteins) and background, respectively.

Cytokine assay

To investigate the relative mRNA level of cytokine expression in T cells, the cells cocultured with fugu B7-Igs (40 μg/ml B7-H1/DC-Ig, 15 μg/ml B7-H3-Ig, and 5 μg/ml B7-H4-Ig) or human IgG were harvested after 72 h of culture. Total RNA was extracted from the cells and treated with DNase by the same method as described above. Total RNA (500 ng) from each sample was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio). Using specific primer sets (supplemental Table II), the expressions of IL-2, IL-10, IL-4/13A, IL-4/13B, IFN-γ-1, and IFN-γ-2

[5] The online version of this article contains supplemental material.

were analyzed by real-time quantitative PCR with SYBR Premix EX *Taq* polymerase II (Takara Bio) and a 7300 real-time PCR system (ABI). The expression level of each cytokine was normalized to the expression of the EF-1α gene. Each PCR analysis was run in triplicate for each sample.

Statistical analysis

Statistical analysis of the T cell proliferation and cytokine assay were performed using the Student's *t* test for paired variables. Statistical differences were considered when the $p < 0.05$.

Results

cDNA cloning and identification of B7s

To identify the fugu B7 family genes, we surveyed the fugu genome database (version 4.0) by BLAST searches undertaken with mammalian B7 proteins and by analyzing sequences for synteny. We found three types of sequences homologous to mammalian B7s, but no others on this occasion. We then cloned the three full-length cDNAs of B7s from head kidney cDNA pools of fugu by RACE. Based on the structural and synteny analyses described below, we identified three B7 homologues to fugu B7-H1/DC (GenBank accession no. AB453016), B7-H3 (GenBank accession no. AB453017), and B7-H4 (GenBank accession no. AB453018), respectively (supplemental Fig. 2). Fugu B7-H1/DC exhibited the highest amino acid percentage identity with B7-DC (17.3%) followed by human B7-H1 (16.7%) (supplemental Table III). Fugu B7-H3 and B7-H4 exhibited the best identities to human B7-H3 (54.1%) and human B7-H4 (26.9%), respectively (supplemental Table III). A BLAST search also demonstrated that fugu B7-H1/DC exhibited the highest similarity with mammalian B7-H1s followed by B7-DCs and that fugu B7-H3 and B7-H4 exhibited the best homology with mammalian B7-H3s and B7-H4s, respectively. Phylogenetic analysis showed that the fugu B7-H1/DC is assembled in a cluster of mammalian B7-H1s and B7-DCs and that fugu B7-H3 and B7-H4 are in a distinct cluster of mammalian B7-H3s and B7-H4s, respectively (Fig. 1). In mammals, B7 family members are composed of a signal peptide, a single extracellular Ig V- and C-like (VC) domain, a transmembrane region, and a cytoplasmic tail (12–15). The overall structures of fugu B7s are conserved with the mammalian ones (supplemental Fig. 2). However, we found a few interesting differences between the B7 proteins of fugu and mammals. Fugu B7-H1/DC has a much longer cytoplasmic tail than those of mammalian B7-H1 and B7-DC. In this region, we found a putative nucleoside triphosphatase NTPase (NACHT-NTPase) domain that is absent in the cytoplasmic tail of mammalian B7-H1 and B7-DC (supplemental Fig. 2*A*). B7-H3 is known to have two types of VC domains, the 2-Ig and the 4-Ig type (16, 17). Fugu B7-H3 was 2-Ig type B7-H3 with a single VC domain like that of mouse B7-H3 (supplemental Fig. 2*B*).

Part of the amino acid sequence of fugu B7-H4 that corresponds to two conserved Cys residues in the Ig C-like domain of mammalian B7-H4 were replaced with the hydrophobic amino acid residues Ala and Leu, respectively (supplemental Fig. 2*C*). This is similar to mammalian CD2 and CD4, which can form an Ig-like domain without a disulfide bond (18). This finding suggests that fugu B7-H4 also forms an Ig C-like domain lacking a Cys pair.

We next investigated the genomic organization of fugu B7 members. The overall gene structure of fugu B7-H1/DC is basically similar to those of both human B7-H1 and B7-DC genes (data not shown). An exon encoding the Ig V-like domain in human B7-H1 and B7-DC was separated into two parts in fugu B7-H1/DC. The fugu B7-H1/DC gene also has an additional exon encoding the cytoplasmic region containing a putative NACHT-NTPase domain (Fig. 2*A*). The structures of the fugu B7-H3 and B7-H4 genes are also conserved with those of their human counterparts (Fig. 2*A*).

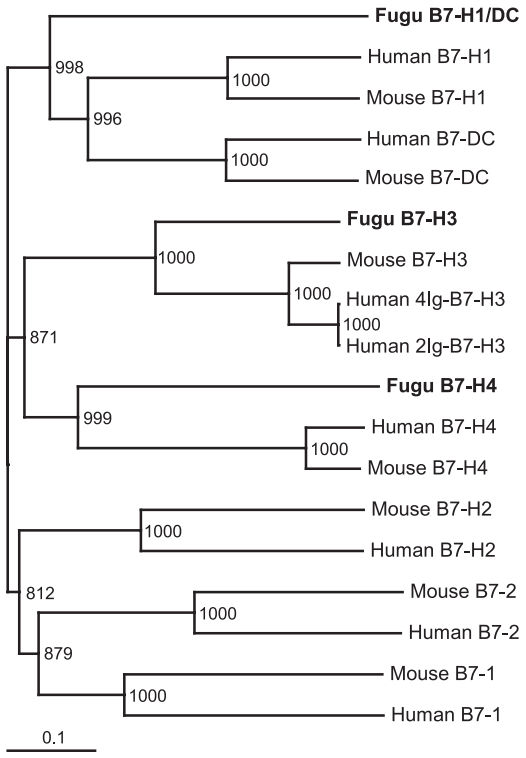

FIGURE 1. Phylogenetic analysis of B7 family. The phylogenetic tree was constructed by the neighbor-joining method based on the amino acid alignment (ClustalW). Node values represent bootstrap analysis of 1,000 replicants. Database accession numbers for the used sequences are given in footnote 2 at the bottom of the first page of this article. For ready access to GenBank for a particular sequence, use www.ncbi.nlm.nih.gov/nuccore/ plus the GenBank identifier or version number given in parentheses (for example, use www.ncbi.nlm.nih.gov/nuccore/225637169 for fugu B7-H1/DC): fugu B7-H1/DC (225637169); mouse B7-H1 (AF317088.1); mouse B7-DC (10946739); fugu B7-H3 (225637171); human 4IgB7-H3 (67188442); mouse B7-H3 (NM_133983.4); fugu B7-H4 (225637173); mouse B7-H4 (AY280973.1); mouse B7-1 (50111); mouse B7-2 (15489434); mouse B7-H2 (118131092). The other human B7 members are mentioned in supplemental Table III.

To further ascertain the level of orthology between the pair of fugu and human B7 loci, we compared gene clustering around the fugu B7s (Scaffold_243 on linkage group 6 for B7-H1/DC, Scaffold_418 on linkage group 13 for B7-H3, and Scaffold_433 on linkage group LG1 for B7-H4) with those around human B7s (chromosome 9 for B7-H1 and B7-DC, chromosome 15 for B7-H3, and chromosome 1 for B7-H4) (Fig. 2*B*). Interestingly, in the fugu locus corresponding to the human genomic region containing tandemly aligned B7-H1 and B7-DC (19), it was apparent that the fugu B7-H1/DC gene is the only gene belonging to the B7 gene family (Fig. 2*B*). Although a few changes in gene order were observed, syntenic relationships are well conserved between the regions around the fugu and human loci. Similarly, conserved syntenies are observed between the genomic regions around the fugu and human *B7-H3* loci and between the fugu and human *B7-H4* loci (Fig. 2*B*). Syntenic analysis also showed orthology of B7 genes in fish.

Expression of B7s and MHC class IIα genes in various tissues and monocytes

To investigate the tissue distribution of the three fugu B7s, we examined the expression of these genes by RT-PCR. All observed tissues showed expression of one or more of the three fugu B7s.

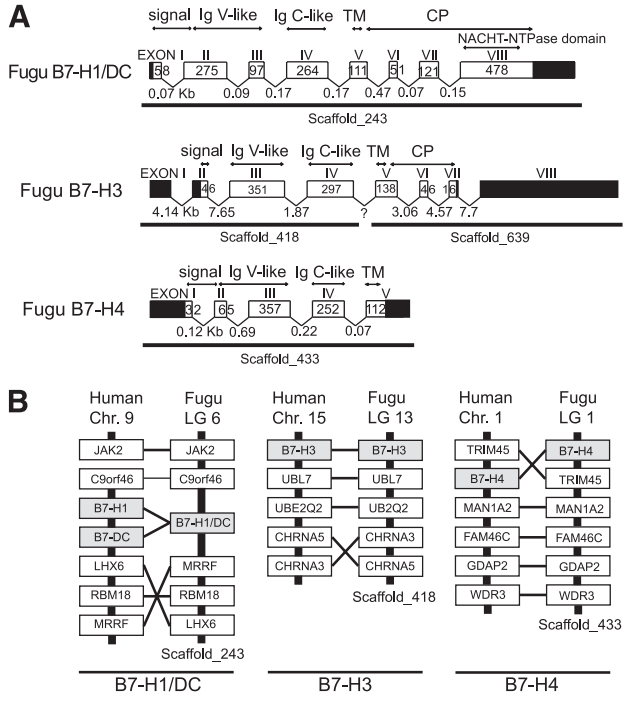

FIGURE 2. Genomic analysis of fugu B7 genes. *A*, Gene organizations of fugu B7s. The exons of each fugu B7 gene are represented as boxes, and the coding region (□) and 5′ and 3′ untranslated regions (■) are shown. Consensus motifs are also indicated in the corresponding regions on the exons. Numbers in the boxes represent the length of exons in base pairs, and numbers in kilobases (Kb) underneath the boxes mark the length of introns between exons. *B*, Syntenic relationships of the B7 family between fugu and human. Comparison is schematic and does not reflect gene distances. Gene locations in the fugu scaffolds and human chromosomes (Chr.) were obtained from the fugu genome database and human genome resource, respectively, of the National Center for Biotechnology Information. The B7 genes are highlighted as gray boxes. Assignment of the scaffolds on fugu linkage groups (LG 1, LG 6, and LG 13) hae been published previously by Kai et al. (20).

Fugu B7 genes were expressed in the skin and intestine in addition to the head kidney and trunk kidney (Fig. 3*A*). These tissues are regarded as mucosal and lymphoid organs in fish, respectively

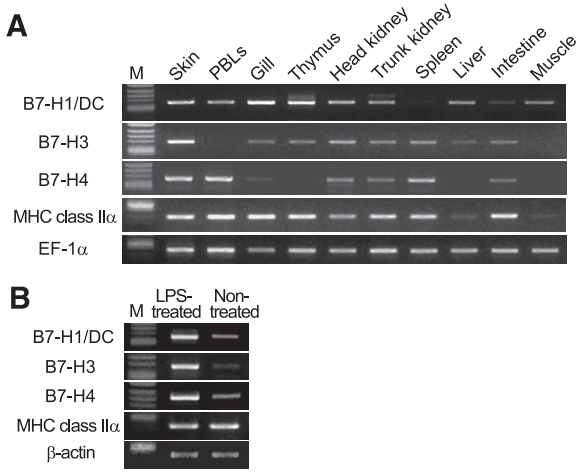

FIGURE 3. Distribution of fugu B7s and MHC class IIα mRNA. *A*, Expression of B7s and MHC class IIα genes in various tissues of fugu. *B*, mRNA expression of fugu B7s and MHC class IIα genes in nontreated and LPS-stimulated monocytes. Transcripts of EF-1α or β-actin were amplified as controls to ensure the quality of mRNA. Data are representative of results from three individuals. "M" indicates a ΦX174/*Hinc*II digest DNA size marker.

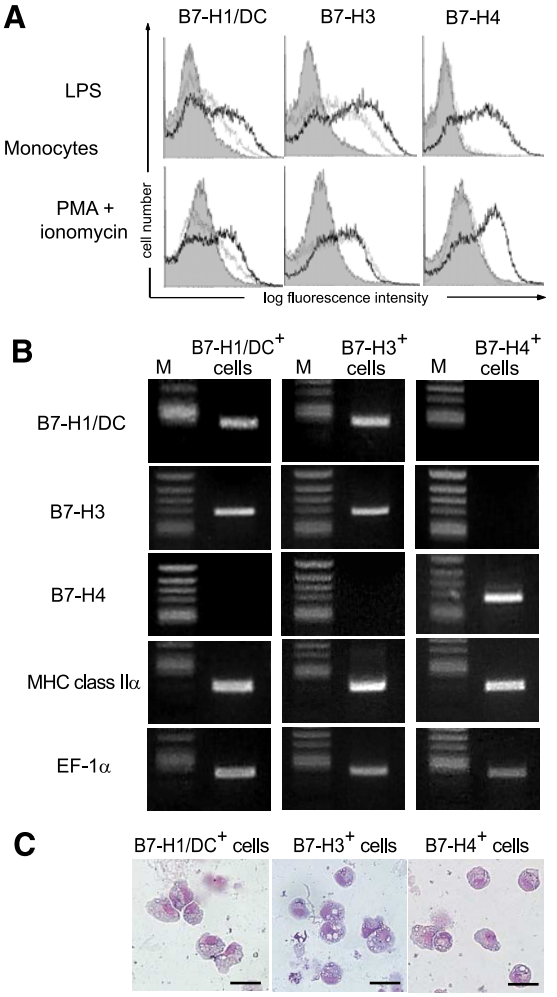

FIGURE 4. Expression analysis of fugu B7s in monocytes. *A*, Cell surface expression of fugu B7s on monocytes. Monocytes stimulated by LPS or PMA and ionomycin were stained with each anti-fugu B7 serum or control serum. Solid line open histograms and shaded histograms indicate stimulated cells stained with the anti-fugu B7 serum and control serum, respectively. Dashed line open histograms show nonstimulated cells stained with the anti-fugu B7 serum. The data are representative of three experiments. *B*, Expression profiles of fugu B7s and MHC class IIα genes in B7$^+$ cells isolated from LPS-stimulated monocytes. The data are representative of three experiments. "M" indicates ΦX174/*Hinc*II digest DNA size marker. *C*, Morphological observation of each B7$^+$ cell by Giemsa stain. Scale bar, 20 μm.

(21). In these immune-related organs we also demonstrated the coexpression of MHC class IIα (GenBank accession no. AB453019) (Fig. 3*A*), an essential component for Ag presentation.

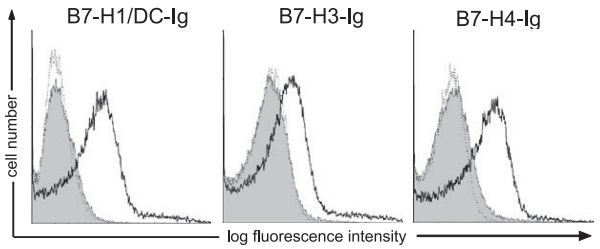

FIGURE 5. Binding of fugu B7-Ig proteins to an activated, enriched T cell population. Enriched T cells from fugu PBLs were cultured alone or with PHA for 24 h. PHA-activated T cells were stained with 5 μg of fugu B7-Igs (solid line open histograms) or control human IgG (shaded histograms). Nontreated T cells were also stained with fugu B7-Igs (dashed line open histograms). Data are representative of results from three individuals.

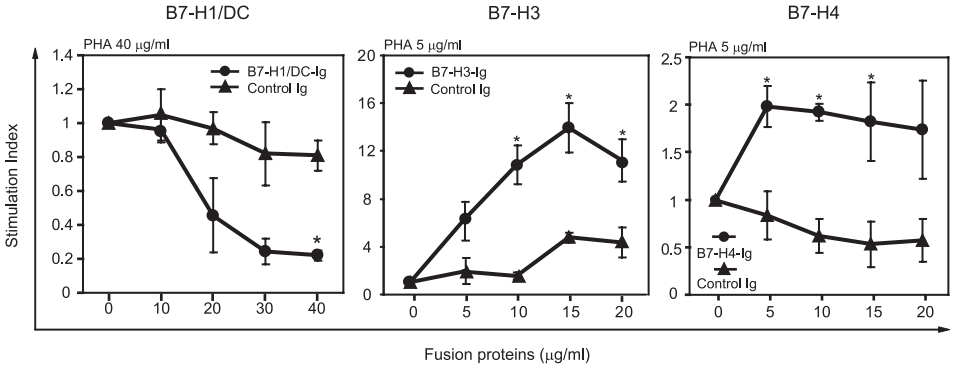

FIGURE 6. Regulatory functions of fugu B7-Igs for T cell proliferation. Enriched T cells were cocultured with the indicated concentrations of either B7-Ig or control Ig in the presence of PHA for 96 h. Proliferation was measured by BrdU incorporation and indicated as stimulation index. Data are expressed as mean ± SD of results from three individuals.

In nonlymphoid organs such as liver and muscle, we also observed the expression of fugu B7-H1/DC, and B7-H3 genes. This result is similar to the expression patterns of mammalian B7-H1, B7-DC, and B7-H3 genes (14, 22). However, the MHC class IIα gene was not expressed in these nonlymphoid organs, including liver tissue, in fugu (Fig. 3*A*).

In teleost species, monocytes/macrophages are considered to be candidates for APCs (23). Consequently, we isolated fugu monocytes by using their adherence property and examined their gene expression by RT-PCR under the presence or absence of mitogen (Fig. 3*B*). Fugu monocytes expressed three B7 and MHC class IIα genes, and B7 gene expression was stimulated by LPS stimulation (Fig. 3*B*). The coexpression of fugu B7s and the MHC class II component suggests that fish monocytes could have costimulatory and Ag-presenting properties.

Expression of B7s on the surface of fugu leukocytes

To determine whether fugu B7s are expressed on the surface of monocytes, we labeled monocytes with specific antisera against each B7. FACS analysis demonstrated that fugu B7-H1/DC and B7-H3 were expressed at low levels upon the surfaces of nontreated monocytes, whereas B7-H4 was not expressed at all (Fig. 4*A*). However, the expression of B7-H4 was induced by LPS stimulation. Moreover, such stimulation led to the up-regulation in the expression of fugu B7-H1/DC and B7-H3 (Fig. 4*A*). The expression of B7-H1/DC and B7-H4, but not B7-H3, was also enhanced on monocytes by exposure to PMA with ionomycin. In contrast, none of the three B7s were observed on T and B lymphocytes in fugu (supplemental Fig. 3). The up-regulation of B7 expression upon monocytes after mitogenic stimulation suggests that B7-related costimulation could be induced via the immune responses of fugu.

To examine the diversity evident within the monocytes expressing B7 molecules, we used MACS to isolate $B7^+$ cells from the LPS-activated monocytes with antiserum specific for each B7 (Fig. 4*B*). Subsequent RT-PCR revealed that fugu B7-H1/DC and B7-H3 genes were coexpressed in monocytes in which B7-H4 was not expressed. Moreover, we failed to detect expression of either gene in $B7\text{-}H4^+$ cells (Fig. 4*B*). The MHC class IIα gene was expressed in $B7^+$ cells isolated by each antiserum (Fig. 4*B*). Each

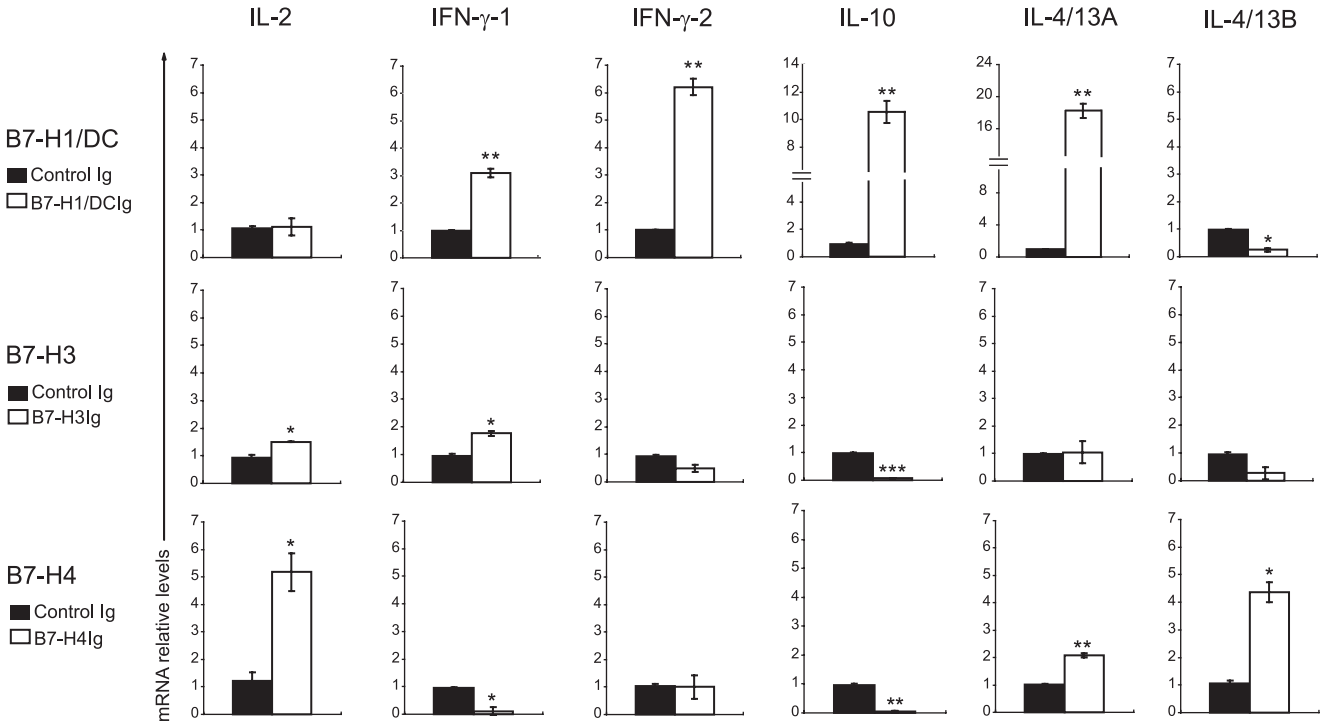

FIGURE 7. Cytokine expression of costimulated T cells by fugu B7-Igs. Enriched-T cells were cocultured with either B7-Ig or control Ig in the presence of PHA for 72 h. Cytokine mRNA levels in the cocultured cells were calculated relevant to EF-1α mRNA level. Data are expressed as mean ± SD of results from two individuals.

fugu B7$^+$ cell showed the morphological features of monocytes (Fig. 4*C*). Thus, fish B7$^+$ monocytes exhibit heterogeneity, suggesting the existence of phenotypic and functional diversities.

Binding of B7s to putative counter-receptors on activated T cells

In mammals, B7 family members interact principally with T cells via counter-receptors to induce T cell responses in a specific manner (8). To assess whether fugu T cells have counter-receptors to each B7, we investigated the binding of fugu B7-Ig fusion proteins (B7-Ig) to T cells by FACS. This analysis showed that each fugu B7-Ig bound onto PHA-activated T cells, but not onto resting T cells (Fig. 5). In contrast, human IgG did not bind to T cells, eliminating the possibility that fugu B7-Igs bind to Fc receptors on T cells (Fig. 5). Thus, we demonstrated that B7 counter-receptor(s) are induced on the T cells by activation with PHA.

Costimulatory roles of B7 molecules on T cell proliferation and cytokine production

To test costimulatory functions of fugu B7 members upon T cell responses, we costimulated T cells with B7-Igs in the presence of PHA and measured the proliferation of T cells. Compared with control Ig, fugu B7-H1/DC-Ig exhibited inhibitory effects upon T cell growth in the presence of PHA (Fig. 6). In contrast, fugu B7-H3-Igs and B7-H4-Igs enhanced T cell proliferation under PHA stimulation (Fig. 6). In the absence of PHA, all of the B7-Igs failed to exert any noticeable effect upon T cell proliferation (data not shown).

To examine whether fugu B7 molecules regulate cytokine production in T cells, we measured cytokine mRNA levels in T cells stimulated by fugu B7-Igs. Fugu B7-H1/DC-Ig significantly increased the mRNA levels of IL-4/13A, IL-10, IFN-γ-1, and IFN-γ-2, but reduced levels of IL-4/13B mRNA (Fig. 7). When T cells were costimulated with fugu B7-H3-Ig, the expression of IL-2 and IFN-γ-1 were moderately enhanced, but that of IL-10 was greatly reduced (Fig. 7). B7-H4-Ig enhanced IL-2 and both IL-4/13A and IL-4/13B expression concomitant with suppression of IL-10 and IFN-γ-1 expression (Fig. 7). Consequently, fugu B7s appear to regulate T cell responses by virtue of functional diversities.

Discussion

We identified three types of B7 in fugu: B7-H1/DC, B7-H3, and B7-H4. Our study revealed that activated fugu monocytes induced surface B7 molecules concomitant with the expression of the MHC class II gene. Fish B7s bound onto the activated T cells and regulated T cell proliferation concomitant with specific cytokine expression. We suggest that the B7$^+$ monocytes would be APCs and that a costimulatory system has developed during the evolutionary process.

Fugu B7-H1/DC showed common structural and genomic features with both mammalian B7-H1 and B7-DC (Figs. 1 and 2 and supplemental Fig. 2*A*). A similar case is observed in the CD3 gene family. CD3γ/δ molecules in nonmammalian vertebrates are similar to both mammalian CD3γs and CD3δs (24–27). In mammals these genes are tandemly aligned on the same genomic locus, suggesting that they are derived from the tandem duplication of an ancestral gene. We propose that fugu B7-H1/DC is an orthologue of both the mammalian B7-H1 and B7-DC. The mammalian B7-H1 and B7-DC genes might have emerged in a similar manner to that of mammalian CD3 genes (27).

In contrast to the mammalian B7-H1 and B7-DC, fugu B7-H1/DC possesses a long cytoplasmic tail containing a putative NACHT-NTPase domain (Fig. 2*A* and supplemental Fig. 2*A*). None of the B7 family members in mammals are known to have any domains or motifs in the cytoplasmic region (data not shown). This suggests that fugu B7-H1/DC functions in a novel manner as B7 molecules. The NACHT-NTPase domain is shared with a CIITA and a nucleotide-binding oligomerization domain (28). The CIITA activates NF-κB and the nucleotide-binding oligomerization domain stimulates NF-κB and caspase in APCs (29–31). It is possible that fugu B7-H1/DC regulates activation and apoptosis of APCs via this domain. Whether fish gained, or mammals lost, this particular domain during evolution remains an interesting question.

In this study, fugu B7-H1/DC inhibited T cell proliferation on day 4 of culture, and expression of IL-10, IFN-γ-1, and IFN-γ-2 was induced on day 3 of culture (Figs. 6 and 7). In mammals, both inhibitory and stimulatory roles have been reported for B7-H1 and B7-DC (8). For both B7s, this inhibitory function was mediated by a receptor, programmed death-1. However, programmed death-1 homologues have not as yet been identified in fish species (7). Furthermore, we were unable to detect this gene within the fugu genome (data not shown). Positive receptor(s) for B7-H1 and B7-DC have yet to be found in vertebrates. The identification of receptors for B7-H1/DC would provide new insights in our understanding of the costimulatory system and its evolution. Recently, an alternative idea for T cell proliferation via B7 was proposed (32). It has been recently shown that mouse B7-H1 stimulates Th1 cells to produce IFN-γ in the early phase of culture (day1–3) and that Tr1 (T regulatory 1) cells are developed by the IFN-γ secreted from Th1 and suppress Th1 proliferation with IL-10 induction during the later phase (day 4–6) (32). Thus, B7-H1 appears to be involved in the transition of T cell function from Th1 to Tr1 cells. Our results in fugu are consistent with this hypothesis. This implies that a similar regulatory system in T cell response might be mediated by B7-H1/DC in fugu.

Fugu B7-H3 has only a single VC form, similar to mouse B7-H3 (Fig. 2*A* and supplemental Fig. 2*B*), whereas the human has two types of B7-H3, B7-H3VC and B7-H3VCVC (16–17). The human *B7-H3* locus has two sets of VC domains, resulting in two splicing variants (17), whereas in the mouse *B7-H3* locus one of the two VC sets is known to be a pseudoexon (17). In contrast, the fugu *B7-H3* locus has only a single VC set. We failed to detect any other VC domains at the locus (data not shown). Ling and coworkers proposed that tandemly repeated VC domains in primate and rodent *B7-H3* loci arose from proto-B7-H3 with only a single VC set (17). Our results support this hypothesis and the idea that the fugu B7-H3 would thus conserve the features of ancestral B7-H3 during evolution.

In this study, fugu B7-H3 promoted T cell proliferation with modest increases of IL-2 and IFN-γ-1 expression concomitant with suppression of IL-10, similar to the stimulatory function of human B7-H3 with IFN-γ expression (14) (Figs. 6 and 7). In contrast, negative regulation of T cells by B7-H3 has been reported in humans and mice (8). These observations imply that fish B7-H3 could negatively regulate T cells. Suh and colleagues proposed that B7-H3 response depend upon two putative receptors with opposing functions such as CD28 and CTLA-4 in mammals (33). However, the counter-receptors of B7-H3 have not yet been identified in either teleosts or mammals. Identification of the receptors involved would greatly aid our understanding of the opposing functions of B7-H3.

Several lines of evidence indicate that fugu B7-H4 is an orthologue of mammalian B7-H4s (Figs. 1 and 2 and supplemental Fig. 2*C*). Fugu B7-H4 promoted T cell proliferation with the induction of IL-2 (Figs. 6 ans 7). However, most previous studies in mammals have reported the negative regulation of T cells by B7-H4 (8). Only one study in humans has shown that B7-H4, expressed on renal tubular epithelial cells, stimulates T cell growth with IL-2

production (34). These observations indicate that B7-H4 can be a positive regulator of T cell responses in vertebrates.

Our data also demonstrated the coexpression of B7-H1/DC and B7-H3 in a single monocyte population in fugu (Fig. 4). These fugu B7s have opposing functions on T cell proliferation (Fig. 6). Similar B7 redundancy in a single cell was also observed in mammals. B7-1 and B7-2, coexpressed on APCs, bind to two costimulatory receptors with opposing functions, CD28 and CTLA-4, coexpressed on activated T cells (35–37). In this case, T cell response would be directed by relative expression levels and affinity differences of the two costimulatory receptors (36). Similarly, receptors for B7-H1/DC and B7-H3 would play crucial roles in the regulation of T cell responses in teleosts.

In contrast, high redundancy of B7s and their receptor genes have been reported in mammals, although in a genomic survey of fish, B7s and their receptor genes have shown less redundancy than mammalian ones (7). This implies that fish might adopt the simple ligand-receptor combination in costimulation.

In mammals, professional APCs are mainly composed of monocytes/macrophages, B cells, and DCs (1), with other novel APCs being identified only recently (38, 39). These APCs possess abilities of costimulation and Ag-presentation via MHC class II molecules. In this study, we showed that three fugu B7 molecules have T cell costimulatory functions with cytokine inductions (Figs. 6 and 7) and that each $B7^+$ monocyte expressed the MHC class II component gene (Fig. 4*B*). In teleost species, it has been suggested that MHC class II present Ags (23). The MHC class II expression increases with pathogenic infection or vaccination in teleosts (40–42) and is induced on macrophages following mitogenic stimulation (43). Taken together, we therefore suggest that $B7^+$ monocytes would be APCs in fish.

We have demonstrated heterogeneity of fugu $B7^+$ monocytes, including at least two populations: B7-H1/DC^+ and/or B7-$H3^+$ cells and B7-$H4^+$ cells (Fig. 4*B*). Recently, functional heterogeneity was also reported in carp macrophages (44). These results indicate that fish monocytes are composed of many populations. In this article, we speculate that DCs, which are major APCs in higher vertebrates (1, 2), are present in these $B7^+$ populations, although DC subsets have not been identified definitively in fish. Our speculation is supported by the expressions of DC-specific marker genes such as CD8α and CD83 in macrophages of some fish species (45, 46). In addition, dendritic-like cells have been morphologically observed in a few teleost species (47, 48). In mammals, B7s were expressed on both macrophages and DCs (8), and some DC subsets were shown to differentiate from blood monocytes (49). Further studies involving motility, morphology, and maturation of these candidates are required to identify DCs in fish.

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Disclosures

The authors have no financial conflict of interest.

References

1. Weaver, C. T., and E. R. Unanue. 1990. The costimulatory function of antigen-presenting cells. *Immunol. Today* 11: 49–55.
2. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* 2: 151–161.
3. Nakanishi, T., U. Fischer, J. M. Dijkstra, S. Hasegawa, T. Somamoto, N. Okamoto, and M. Ototake. 2002. Cytotoxic T cell function in fish. *Dev. Comp. Immunol.* 26: 131–139.
4. Suetake, H., K. Araki, and Y. Suzuki. 2004. Cloning, expression, and characterization of fugu CD4, the first ectothermic animal CD4. *Immunogenetics* 56: 368–374.
5. Hansen, J. D., and P. Strassburger. 2000. Description of an ectothermic TCR coreceptor, CD8α, in rainbow trout. *J. Immunol.* 164: 3132–3139.
6. Bernard, D., B. Riteau, J. D. Hansen, R. B. Phillips, F. Michel, P. Boudinot, and A. Benmansour. 2006. Costimulatory receptors in a teleost fish: typical CD28, elusive CTLA4. *J. Immunol.* 176: 4191–4200.
7. Bernard, D., J. D. Hansen, L. D. Pasquier, M. P. Lefranc, A. Benmansour, and P. Boudinot. 2007. Costimulatory receptors in jawed vertebrates: conserved CD28, odd CTLA-4 and multiple BTLAs. *Dev. Comp. Immunol.* 31: 255–271.
8. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23: 515–548.
9. Suetake, H., N. R. Saha, K. Araki, K. Akatsu, K. Kikuchi, and Y. Suzuki. 2006. Lymphocyte surface marker genes in fugu. *Comp. Biochem. Physiol. Genomics Proteomics* 1: 102–108.
10. Aparicio, S., J. Chapman, E. Stupka, N. Putnam, J. M. Chia, P. Dehal, A. Christoffels, S. Rash, S. Hoon, A. Smit, et al. 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* 297: 1301–1310.
11. Bei, J. X., H. Suetake, K. Araki, K. Kikuchi, Y. Yoshiura, H. R. Lin, and Y. Suzuki. 2006. Two interleukin (IL)-15 homologues in fish from two distinct origins. *Mol. Immunol.* 43: 860–869.
12. Dong, H., G. Zhu, K. Tamada, and L. Chen. 1999. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat. Med.* 5: 1365–1369.
13. Tseng, B. S. Y., M. Otuji, K. Gorski, X. Huang, J. E. Slansky, S. I. Pai, A. Shalabi, T. Shin, D. M. Pardoll, and H. Tsuchiya. 2001. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J. Exp. Med.* 193: 839–845.
14. Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada, and L. Chen. 2001. B7-H3: a costimulatory molecule for T cell activation and IFN-γ production. *Nat. Immunol.* 2: 269–274.
15. Sica, G. L., I. H. Choi, G. Zhu, K. Tamada, S. D. Wang, H. Tamura, A. I. Chapoval, D. B. Flies, J. Bajorath, and L. Chen. 2003. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity* 18: 849–861.
16. Sun, M., S. Richards, D. V. R. Prasad, X. M. Mai, A. Rudensky, and C. Dong. 2002. Characterization of mouse and human B7-H3 genes. *J. Immunol.* 168: 6294–6297.
17. Ling, V., P. W. Wu, V. Spaulding, J. Kieleczawa, D. Luxenberg, B. M. Carreno, and M. Collins. 2003. Duplication of primate and rodent B7-H3 immunoglobulin V- and C-like domains: divergent history of functional redundancy and exon loss. *Genomics* 82: 365–377.
18. Jones, E. Y., S. J. Davis, A. F. Williams, K. Harlos, and D. I. Stuart. 1992. Crystal structure at 2.8 A resolution of a soluble form of the cell adhesion molecule CD2. *Nature* 360: 232–239.
19. Wang, S., J. Bajorath, D. B. Flies, H. Dong, T. Honjo, and L. Chen. 2003. Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. *J. Exp. Med.* 197: 1083–1091.
20. Kai, W., K. Kikuchi, M. Fujita, H. Suetake, A. Fujiwara, Y. Yoshiura, M. Ototake, B. Venkatesh, K. Miyaki, and Y. Suzuki. 2005. A genetic linkage map for the tiger pufferfish, *Takifugu rubripes*. *Genetics* 171: 227–238.
21. Zapata, A., B. Diez, T. Cejalvo, C. G. Frías, and A. Cortés. 2006. Ontogeny of the immune system of fish. *Fish Shellfish Immunol.* 20: 126–136.
22. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, et al. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2: 261–268.
23. Vallejo, A. N., N. W. Miller, and L. W. Clem. 1992. Antigen processing and presentation in teleost immune responses. *Annu. Rev. Fish Dis.* 2: 73–89.
24. Bernot, A., and C. Auffray. 1991. Primary structure and ontogeny of an avian CD3 transcript. *Proc. Natl. Acad. Sci. USA* 88: 2550–2554.
25. Dziano, R. C., and M. D. Cooper. 1997. An amphibian CD3 homologue of the mammalian CD3γ and δ genes. *Eur. J. Immunol.* 27: 1640–1647.
26. Araki, K., H. Suetake, K. Kikuchi, and Y. Suzuki. 2005. Characterization and expression analysis of CD3ϵ and CD3γ/δ in fugu, *Takifugu rubripes*. *Immunogenetics* 57: 158–163.
27. Alibaud, L., J. Arnaud, R. Llobera, and B. Rubin. 2001. On the role of CD3δ chains in TCR$\gamma\delta$/CD3 complexes during assembly and membrane expression. *Scand. J. Immunol.* 54: 155–162.
28. Koonin, E. V., and L. Aravind. 2000. The NACHT family—a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. *Trends Biochem. Sci.* 25: 223–224.
29. Inohara, N., and G. Nuñez. 2003. NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* 3: 371–382.
30. Harton, J. A., D. E. Cressman, K. C. Chin, C. J. Der, and J. P. Y. Ting. 1999. GTP binding by class II transactivator: role in nuclear import. *Science* 285: 1402–1405.
31. Wong, A. W., W. J. Bricky, D. J. Taxman, H. W. Deventer, W. Reed, J. X. Gao, P. Zheng, Y. Liu, P. Li, et al. 2003. CIITA-regulated plexin-A1 affects T-cell-dendritic cell interactions. *Nat. Immunol.* 4: 891–898.
32. Ding, Q., L. Lu, B. Wang, Y. Zhou, Y. Jiang, X. Zhou, L. Xin, Z. Jiao, and K. Y. Chou. 2006. B7H1-Ig fusion protein activates the $CD4^+$ IFN-γ $receptor^+$ type 1 T regulatory subset through IFN-γ-secreting Th1 cells. *J. Immunol.* 177: 3606–3614.
33. Suh, W. K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, et al. 2003. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat. Immunol.* 4: 899–906.
34. Chen, Y., C. Yang, Z. Xie, L. Zou, Z. Ruan, X. Zhang, Y. Tang, L. Fei, Z. Jia, and Y. Wu. 2006. Expression of the novel co-stimulatory molecule B7-H4 by renal tubular epithelial cells. *Kidney Int.* 70: 2092–2099.

35. Linsley, P. S., J. L. Greene, P. Tan, J. Bradshaw, J. A. Ledbetter, C. Anasetti, and N. K. Damle. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med.* 176: 1595–1604.
36. Bhatia, S., M. Edidin, A. C. Almo, and S. T. Nathenson. 2006. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. *Immunol. Lett.* 104: 70–75.
37. Chambers, C. A., M. S. Kuhns, J. G. Egen, and J. P. Allison. 2001. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu. Rev. Immunol.* 19: 565–594.
38. Laouar, A., V. Haridas, D. Vargas, X. Zhinan, D. Chaplin, R. A. W. Lier, and N. Manjunath. 2005. CD70$^+$ antigen-presenting cells control the proliferation and differentiation of T cells in the intestinal mucosa. *Nat. Immunol.* 6: 698–705.
39. Brandes, M., K. Willimann, and B. Moser. 2005. Professional antigen-presentation function by human γδ T cells. *Science* 309: 264–268.
40. Takano, T., A. Iwahori, I. Hirono, and T. Aoki. 2004. Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after vaccination. *Fish Shellfish Immunol.* 17: 367–374.
41. Sigh, J., T. Lindenstrøm, and K. Buchmann. 2004. The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout. *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* 27: 409–417.
42. Cuesta, A., M. A. Esteban, and J. Meseguer. 2006. Cloning, distribution and up-regulation of the teleost fish MHC class II α suggests a role for granulocytes as antigen-presenting cells. *Mol. Immunol.* 43: 1275–1285.
43. Knight, J., R. J. M. Stet, and C. J. Secombes. 1998. Modulation of MHC class II expression in rainbow trout *Oncorhynchus mykiss* macrophages by TNFα and LPS. *Fish Shellfish Immunol.* 8: 545–553.
44. Joerink, M., C. M. S. Riberiro, R. J. M. Stet, T. Hermsen, H. F. J. Savelkoul, and G. F. Wiegertjes. 2006. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *J. Immunol.* 177: 61–69.
45. Araki, K., K. Akatsu, H. Suetake, K. Kikuchi, and Y. Suzuki. 2008. Characterization of CD8$^+$ leukocytes in fugu (*Takifugu rubripes*) with antiserum against fugu CD8α. *Dev. Comp. Immunol.* 32: 850–858.
46. Haugarvoll, E., J. Thorsen, M. Laane, Q. Huang, and E. O. Koppang. 2006. Melanogenesis and evidence for melanosome transport to the plasma membrane in a CD83$^+$ teleost leukocyte cell line. *Pigm. Cell Res.* 19: 214–225.
47. Grayson, T. H., L. F. Cooper, A. B. Wrathmell, J. Roper, A. J. Evenden, and M. L. Gilpin. 2002. Host responses to *Renibacterium salmoninarum* and specific components of the pathogen reveal the mechanisms of immune suppression and activaton. *Immunology* 106: 273–283.
48. Lovy, J., G. M. Wright, and D. J. Speare. 2005. Morphological presentation of a dendritic-like cell within the gills of Chinook salmon infected with *Loma salmonae*. *Dev. Comp. Immunol.* 30: 259–263.
49. Ardavin, C., G. M. Hoyo, P. Martin, F. Anjuère, C. F. Arias, A. R. Marin, S. Ruiz, V. Parrillas, and H. Hernández. 2001. Origin and differentiation of dendritic cells. *Trends Immunol.* 22: 691–700.