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Essential Role of IL-4 and IL-4Rα Interaction in Adaptive Immunity of Zebrafish: Insight into the Origin of Th2-like Regulatory Mechanism in Ancient Vertebrates

Ly-yun Zhu, Ping-ping Pan, Wei Fang, Jian-zhong Shao, and Li-xin Xiang

The roles of IL-4 and IL-4Rs in Th2-mediated immunity have been well characterized in humans and other mammals. In contrast, few reports have been documented in ancient vertebrates. Several putative IL-4- and IL-4Rα-like molecules were identified recently from a few fish species, providing preliminary insight into the occurrence of Th2-type immunity in teleosts. However, functional determination still is required to address this hypothesis. To this end, these two molecules were characterized functionally in zebrafish (Danio rerio). Besides the identification of a full-length IL-4Rα molecule and an isoform lacking most of the cytoplasmic region as predicted previously, two novel alternatively spliced soluble variants with the extracellular domain only also were identified. Zebrafish IL-4Rα (DrIL-4Rα) shared overall conserved structural features of the IL-4Rα family. Immunofluorescence staining showed that DrIL-4Rα distributed on B cells. In vitro binding assays demonstrated that zebrafish IL-4 (DrIL-4) can bind specifically to DrIL-4Rα. In vivo administration of DrIL-4 significantly upregulated B cell proliferation and Ab production. These DrIL-4–elicited immune responses were downregulated by the administration of zebrafish soluble IL-4Rα or by DrIL-4Rα blockade using anti–DrIL-4Rα Abs. In addition, Th2-related cytokines or transcription factors were upregulated by DrIL-4. The DrIL-4–DrIL-4Rα interaction promoted CD40 expression on B cells and enhanced the CD154–CD40 costimulatory response, both of which are critical for the initiation of Th2-type immunity. To our knowledge, this is the first report showing that a possible Th2-mediated regulatory mechanism may have appeared before the divergence of teleosts and mammals. These results add greater insight into the evolutionary history of adaptive immunity. The Journal of Immunology, 2012, 188: 000–000.

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The sequences presented in this article have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers EF523375.1, EF523376.1, EF523377.1, and EF523378.1.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BS3, bis(sulfosuccinimidyl) suberate; γc, common γ-chain; DC, dendritic cell; DrIL-4, zebrafish IL-4; DrIL-4Rα, zebrafish IL-4Rα; DrIL-4Rα-iso, zebrafish IL-4Rα isoform; EST, expressed sequence tag; FN III, fibronectin type III; KLH, keyhole limpet hemocyanin; ORF, open reading frame; sCD154, soluble CD154; sDrIL-4Rα, zebrafish soluble IL-4Rα; sDrIL-4Rα-iso, zebrafish soluble IL-4Rα isoform; UTR, untranslated region.

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ROLE OF IL-4–IL-4R Interaction in Th2-Like Immunity in Fish

TATA box and GATA3 binding motif in the proximal promoter regions of the gene. However, IL-4/13B lacks such a box and motif in its proximal promoter regions. This result suggests that IL-4/13B lacks the complete transcription elements. In addition, a search of the expressed sequence tags (ESTs) in the National Center for Biotechnology Information database revealed several ESTs for zebrafish IL-4/13A, whereas none was found for zebrafish IL-4/13B. As a consequence, IL-4/13A might be the real zebrafish IL-4, but more functional evidence still is needed. Two putative IL-4Rs were identified recently from rainbow trout (Oncorhynchus mykiss) and predicted in zebrafish and Atlantic salmon (Salmo salar) (38, 39). These observations provide preliminary insights into Th2-type immunity before the divergence of teleost fish and mammals ~400 million years ago. However, functional determination still is required to address this hypothesis.

In the current study, both structural and functional features of IL-4Rα were characterized further in zebrafish. Besides the identification of two membrane-bound IL-4R–like molecules as predicted previously, two novel alternatively spliced soluble variants with only the extracellular domain were identified initially in fish species. As expected, IL-4 and IL-4Rα are shown to be reciprocally regulated in zebrafish. The interaction of these two molecules is essential for B cell proliferation and Ab production, which are the two hallmark events in Th2-type immune responses. However, the zebrafish soluble IL-4Rα (sDrIL-4Rα) variant plays a negative regulatory role in this process. The reciprocal regulation of IL-4 and IL-4Rα promotes CD40 expression on B cells and increases B cell responses to stimulation by CD154 signals. To our knowledge, this result was the first observation that the IL-4–IL-4Rα interaction promotes Th2-type immune responses by upregulating the CD154–CD40-mediated costimulatory signaling pathway in B cells. Results give functional insights into the existence of a conserved Th2-like regulatory mechanism in fish, enrich our knowledge of fish immunology, and contribute to a better cross-species understanding of the evolutionary history of the IL-4 and IL-4R family, as well as the Th subset and T regulatory mechanisms.

Materials and Methods

Experimental fish

One-year old zebrafish (D. rerio) from both sexes, weighing ~0.5–1.0 g, with a body length of 1–2 cm, were kept in a circulating water bath at 26°C and fed with commercial pellets at a daily ration of 0.7% of their body weight. All of the fish were held in the laboratory for at least 2 wk before the experiments for acclimatization and evaluation of overall fish health. All of the fish were held in the laboratory for at least 2 wk before the experiments for acclimatization and evaluation of overall fish health. Only healthy fish, as determined by general appearance and level of activity, were used for the studies.

Database and bioinformatics analysis

Genome assemblies, including Homo sapiens, Mus musculus, Gallus gallus, and D. rerio, were searched at the University of California at Santa Cruz genome bioinformatics Web site (http://genome.ucsc.edu). Genome locations were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/mapview) and University of California at Santa Cruz Web sites as well. Gene organizations were elucidated by comparing IL-4Rα DNA with genome sequences. Figures were drawn using GeneMaper 2.5. Homologous ESTs were searched by WU-BLAST at the Computational Biology and Functional Genomics Laboratory. Gene predictions were carried out by the FGENEshH program. Sequences were compared with the database by the BLASTP algorithm. Multiple alignments were generated by the Clustal X program (version 1.8) (40). Putative signal peptides and transmembrane helices were predicted by the SigP program (version 3.0) and TMHMM 2.0 (41). Secondary structure was predicted using the PHD program (42). Structural features of the peptides were predicted in PROSITE (43, 44).

Sequence cloning

Total RNA was isolated from spleen and kidney tissues collected from 20 fish using a TRIzol reagent (Life Technologies BRL) treated with RNase-free DNase I (Qiagen). Zebrafish IL-4Rα (sDrIL-4Rα) and soluble IL-4Rα (sDrIL-4Rα) cDNAs were cloned using the primers shown in Table I. The 3′ and 5′ full RACE core sets were used following the manufacturer’s instructions (Takara). Seminested or nested PCR was adopted in RACE. PCR products were purified using a gel extraction kit (Qiagen), ligated into a pUCm-T vector (Takara), and transformed into Escherichia coli Top10 competent cells ( Invitrogen). Plasmid DNA was extracted by the plasmid Miniprep method and then sequenced on a MegaBACE 1000 sequencer (GE Healthcare) using a Dye Terminator cycle sequencing kit (Pharmacia). Full-length cDNAs were amplified by catabolite gene activator protein (CAP 3.0) (45).

Phylogenetic analysis

Protein sequences of the IL-4Rα family members, including IL-4Rα, IL2Rβ, and IL-21R, from 14 representative vertebrates, including human (H. sapiens), chimpanzee (Pan troglodytes), rhesus monkey (Macaca mulatta), cattle (Bos taurus), horse (Equus caballus), mouse (M. musculus), rat (Rattus norvegicus), dog (Canis lupus), wild boar (Sus scrofa), chicken (Gallus domesticus), bird (Taeniopygia guttata), rainbow trout (O. mykiss), Atlantic salmon (S. salar), and zebrafish (D. rerio), were identified. The neighbor-joining trees were constructed from pairwise Poisson correction distances with 2000 bootstrap replications by the MEGA 3.0 software (46).

Tissue distribution analysis

To determine the distribution of DrIL-4Rα and DrIL-4, the liver, gut, kidney, spleen, brain, muscle, skin, gill, ovary, and peripheral lymphocytes were collected carefully and flash-frozen in liquid nitrogen. Total RNA was extracted as described above and reverse-transcribed using the RNA PCR kit (AMV), version 3.0 (Takara). The PCR program consisted of treatment at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 36 cycles to amplify β-actin (as a standard), DrIL-4, and DrIL-4Rα using the corresponding pair of primers (Table I) in parallel tubes, respectively. Then, 5 μl of PCR products was loaded on a 1.5% (w/v) agarose gel and visualized by staining the gel in 0.1 μg/ml ethidium bromide. To distinguish two variant forms of DrIL-4Rα, restriction enzymes EcoRV and EcoRI (Takara) were used for specifically cutting at one unique site of DrIL-4Rα to generate another IL-4R–like isoform. PCR products were purified before digestion using Micro-PCR (Millipore). Finally, 5 μl of PCR products or enzyme digests was loaded on a 1.5% (w/v) agarose gel and visualized by gel staining with 0.1 μg/ml ethidium bromide.

Preparation of recombinant proteins

Sequences encoding DrIL-4 and sDrIL-4Rα were amplified by RT-PCR with primers (Table I) containing a BamHI or EcoRI site added on the 5′ end and a XhoI site added on the 3′ end. The resulting amplifications of DrIL-4 and sDrIL-4Rα were digested and ligated into pET41a and pET32b (Invitrogen Life Technologies), respectively. Plasmid DNAs were transformed into BL21 star cells (DE3; Invitrogen Life Technologies). A single colony of BL21 harboring the expression plasmids was inoculated into 100 ml of Luria-Bertani medium containing chloramphenicol (100 mg/ml), kanamycin (62.5 mg/ml), or ampicillin (25 mg/ml), and the culture was shaken and incubated at 37°C until OD600 value reached 0.6. Isopropyl-β-D-thiogalactoside then was added to a final concentration of 1 mM, and the culture was shaken continually at 250 rpm and incubated at 37°C for 6 h. After ultrasonication, the soluble supernatant liquids were collected. Recombinant Trx-DrIL-4Rα and GST-DrIL-4Rα proteins in these supernatant liquids were purified by nickel-nitrilotriacetic acid agarose affinity chromatography according to the QIAexpressionist manual (Qiagen) and assessed by 12% SDS-PAGE.

Preparation of Ab

Six-week-old male New Zealand white rabbits and BALB/c mice were immunized eight times at biweekly intervals with 100 and 20 μg purified recombinant Trx-DrIL-4Rα protein in CFA, respectively. A week after the final immunization, the rabbits and mice were bled when Ab titers were above 1:10,000, as determined by ELISA using recombinant protein adsorbed to the solid phase. Anti–DrIL-4Rα Abs were affinity-isolated into the IgG isotype using a protein A-agarose column (Qiagen) and purified further using an immunosorbent-based protocol, as described previously with slight modifications (47). In brief, recombinant DrIL-4Rα was electrotransferred (350 mA for 1 h) from a 12% SDS-polyacrylamide gel onto a nitrocellulose membrane. After being washed with PBS, small sections of the membrane with the target protein were cut and blocked with 2% BSA for 30 min. The membrane was incubated with protein A affinity-isolated Ab (500- to 1000-fold dilution with 2% BSA) for 16 h at 4°C and washed with 0.15 M NaCl and PBS, respectively. Afterward, elution buffer...
Flow cytometric analysis for B cell proliferation

Flow cytometric analysis was used to determine the proliferation of mlgM+ B cells. For this, whole-blood cell suspensions were collected with heparinized EDTA and lymphocytes were isolated from the peripheral blood of each group by Ficol-Hypaque (1.077 g/ml) centrifugation at 2500 rpm for 25 min at room temperature and were washed with PBS. After being fixed with 4% paraformaldehyde for 20 min at room temperature and blocked with 5% normal goat serum for 30 min at 37°C, they were incubated with primary Ab (rabbit anti-mlgM, 1:2000) (43, 44) or rabbit IgG as a non-related primary Ab for 60 min at 37°C. The samples then were incubated with a secondary FITC-labeled anti-rabbit Ab (IgG, 1:100; Chemicon) for 45 min at 37°C. After the samples were washed, cell fluorescence signals were determined immediately using a FACSscan flow cytometer (BD Biosciences) equipped with an argon laser with an emission at 488 nm. At least 10,000 events were acquired from the lymphocyte gate (49). The analysis was performed using CellQuest software (BD Biosciences).

Immunofluorescence staining

Lymphocytes were separated from the whole-blood cell suspensions as described above and fixed with cold methanol. They then were incubated with primary Abs, including rabbit anti-DrlgM (produced in our laboratory) and mouse anti-dI–dIL–4Ra, at 37°C for 1 h. In parallel, the nonrelated Abs, rabbit IgG and mouse IgG, were used as negative controls. The secondary Abs, including tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), mouse anti-mouse IgG FITC-conjugated (Cruz Biotechnology), were used according to the manufacturer’s instructions. Samples were photomicrographed under a confocal laser-scanning microscope (CLSM 510; Carl Zeiss).

Binding assays between DrlIL-4 and DrlIL-4Ra

GST pull-down and direct binding ELISA assays were performed to measure the interaction between DrlIL-4 and DrlIL-4Ra. For the pull-down assays, purified GST-DrlIL-4Ra protein (or GST protein alone as the negative control) was mixed with glutathione beads (Qiagen) and incubated for 2 h at 4°C. The beads then were washed with GST binding buffer [200 mM NaCl, 20 mM Tris-HCl, and 1 mM EDTA (pH 7.4)] five times and washed again with PBS (pH 7.5) five times. Afterward, soluble equivalent soluble Trx-DrlIL-4 was incubated with GST-DrlIL-4 or GST-coupled glutathione beads for 1 h at 4°C. The mixture then was cross-linked using bis(sulfo-succinimidyl) suberate (BS3) at a final concentration of 1 mM for 30 min. The mixture then was washed with GST binding buffer five times and washed with GST binding buffer. After being blocked with 5% normal goat serum for 30 min at 37°C, they were incubated with anti-Trx-tag mAb (Sigma-Aldrich) at 37°C for 1 h. After being washed, the membranes were incubated with a HRP-conjugated secondary Ab and visualized with an ECL detection kit (Amersham Bioscience). The Ab solution was adjusted to pH 7.4, and the titers were examined by flow cytometric analysis and real-time PCR, respectively. For direct binding ELISA, recombinant GST-DrlIL-4Ra was used as a competitive control and GST-DrlIL-4Ra was used as a positive control. Afterward, the membranes were blocked with 5% normal goat serum the same as above. For real-time PCR, total RNA was isolated from the lymphocytes of both experimental and control fish injected with GST-DrlIL-4Ra (0.1 μg per fish i.p.) and GST tag, respectively. B cell proliferation was evaluated by the expression of selected B cell markers, including mlgM, MHC class II, and CD80, using the primers shown in Table 1. The total amount of mRNA was normalized to endogenous β-actin mRNA. The related gene expression level was calculated using the 2−ΔΔCt method. Expression of the control groups was set arbitrarily to 1. Each sample was from the whole peripheral blood of 10 fish and run in three parallel reactions using a Mastercycler ep reallplex and detection software (Eppendorf). At least three independent experiments were performed for each panel. For Ab production analysis, fish were administered different doses (0.01, 0.1, and 1 μg per fish i.p.) of GST-DrlIL-4Ra three times with a 12-h time interval. This was followed by immunization with keyhole limpet hemocyanin (KLH) together with CFA at a dose of 10 μg per fish. In negative control groups, fish were administered KLH in combination with PBS or GST tag protein (1 μg per fish i.p.). After 28 d of immunization, the serum samples were collected, and IgM in response to KLH in these samples was measured by ELISA as described previously (43). In brief, 96-well plates were coated with 5 μg/ml KLH overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with 2% BSA for 1 h at 37°C. After the plates were washed, serially diluted serum samples and a background control serum sample with PBS administration were incubated for 1 h at 37°C. After the plates were washed again, rabbit anti-IgM was added and incubated for 1 h at 37°C. The plates were washed, and peroxidase-conjugated streptavidin rabbit IgG was added. The color was developed using the tetramethyl benzidine substrate solution. The plates were analyzed on an ELISA reader at 450 nm.

Functional evaluation for the interaction between DrlIL-4 and DrlIL-4Ra

In vivo blockade and administration of GST-DrlIL-4Ra assays were performed for the functional evaluation of the interaction between GST-DrlIL-4 and Trx-DrlIL-4Ra. For the administration assay, fish were injected with different concentrations of recombinant Trx-DrlIL-4Ra, GST-DrlIL-4, and Ag (KLH), including preinjections with Trx-DrlIL-4Ra (0.1 μg per fish i.p.), GST-DrlIL-4 (0.1 μg per fish i.p.), or GST-DrlIL-4Ra (0.1 μg per fish i.p.) with Trx-DrlIL-4Ra (0.1 μg per fish i.p.) three times with a 12-h time interval, respectively. Meanwhile, preinjections with GST tag (1 μg per fish i.p.), Trx tag (1 μg/fish i.p.), and PBS alone were performed as negative controls. The fish then were immunized with KLH (10 μg per fish i.p.). The Ab (IgM) production in response to KLH in combination with GST-DrlIL-4Ra, Trx-DrlIL-4Ra, and controls was determined by ELISA at 28 d after immunization, followed by the methods described above. B cell proliferation in response to the administration of GST-DrlIL-4Ra and Trx-DrlIL-4Ra also was examined by flow cytometric analysis at 72 h postimmunization with PBS, Trx (1 μg per fish i.p.), Trx-DrlIL-4Ra (0.1 μg per fish i.p.), GST-DrlIL-4 (0.1 μg per fish i.p.), GST-DrlIL-4Ra (0.1 μg per fish i.p.) with Trx-DrlIL-4Ra (0.1 μg per fish i.p.) and GST-DrlIL-4Ra (0.5 μg per fish i.p.), respectively. For the blockade assay, fish were injected with anti-DrlIL-4Ra at a dose of 1 μg per fish i.p. followed by administration of GST-DrlIL-4Ra (0.1 μg per fish i.p.) as described above. In parallel, the same dose of nonspecific rabbit IgG (1:100; Chemicon) in combination with GST-DrlIL-4Ra was administered as a negative control. After 72 h, lymphocytes were collected from the peripheral blood, and B cell proliferation was examined by flow cytometric analysis as described above.

Expression of Th1- and Th2-related cytokines and transcription factors

Total RNA was isolated from the spleen tissues of experimental fish that were injected with recombinant GST-DrlIL-4Ra (0.1 μg per fish i.p.) for 72 h or control fish that were injected with GST tag. The PCR primers are shown in Table 1. The expression levels of the selected Th1 and Th2 cytokines or transcription factors (T-bet, IRF1, STAT1, IFN-γ, IL-10, GATA3, c-maf, and STAT6) were quantified by real-time PCR using the Mastercycler ep realplex and detection software (Eppendorf) according to the protocol described above. Each sample was collected from the spleens of 10 fish and was run in three parallel reactions. At least three independent experiments were performed for each panel.

Effect of DrlIL-4 on B cell proliferation and IgM production

Total RNA was isolated from the spleen tissues of experimental fish that were injected with recombinant GST-DrlIL-4Ra (0.1 μg per fish i.p.) for 72 h or control fish that were injected with GST tag. The PCR primers are shown in Table 1. The expression levels of the selected Th1 and Th2 cytokines or transcription factors (T-bet, IRF1, STAT1, IFN-γ, IL-10, GATA3, c-maf, and STAT6) were quantified by real-time PCR using the Mastercycler ep realplex and detection software (Eppendorf) according to the protocol described above. Each sample was collected from the spleens of 10 fish and was run in three parallel reactions. At least three independent experiments were performed for each panel.

Effect of DrlIL-4 on the CD154–CD40-mediated costimulatory pathway

Fish were injected with either recombinant GST-DrlIL-4Ra (0.1 μg per fish i.p.), GST tag (negative control, 1 μg per fish i.p.), PBS (mock control), or

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different combinations of GST-DrIL-4 and various recombinant proteins including GST-DrIL-4 (0.1 μg per fish i.p.) plus Trx, GST-DrIL-4 (0.1 μg per fish i.p.) plus Trx-DrIL-4Rα (0.1 μg per fish i.p.), or GST-DrIL-4 (0.1 μg per fish i.p.) plus soluble CD154 (sCD154; 1 μg per fish i.p.) (43). After 3 d of stimulation, lymphocytes were separated from peripheral blood as described above and fixed in 4% paraformaldehyde at room temperature for 20 min. After being blocked in 5% normal goat serum for 30 min at 37°C, cells were incubated with primary Abs, mouse-anti-IgM (1:2000) and rabbit-anti-CD40 (1:2000) (43), for 60 min at 37°C. The nonrelated rabbit IgG and mouse IgG Abs used as negative controls were incubated with peripheral lymphocytes in the negative control groups at 37°C for 1 h. The samples then were incubated with a secondary FITC-labeled goat anti-mouse Ab (IgG, 1:100; Chemicon) and a secondary PE-labeled anti-rabbit Ab (IgG, 1:100; Chemicon) for 45 min at 37°C. After the samples were washed, FACSCalibur (BD Biosciences) was used for multicolor flow cytometry. At least 10,000 events were acquired from the lymphocyte gate, and the data were analyzed with CellQuest software (49).

Statistical analysis
Statistical evaluation of differences between means of experimental groups was done by ANOVAs and multiple Student tests. Both p values <0.05 and p < 0.01 were considered to be significant. The sample number for each group was >10 fish of equal mean body weight. Data points represented the means of three independent experiments.

Table I. Primers used for gene cloning and expression

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<tr>
<td>STAT6-R</td>
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<tr>
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Results
Characterization of DrIL-4Rα
Although the IL-4Rα–like gene has been predicted previously in silico in zebrafish, further molecular cloning, structural, and functional characterization of this gene still remains to be performed (39). In this study, we cloned the full-length DrIL-4Rα cDNA from spleen and kidney tissues using the primers shown in Table I (GenBank accession number, EF523375.1; http://www.ncbi.nlm.nih.gov/nuccore/EF523375). It consists of a 1716-bp open reading frame (ORF), a 100-bp 5’-untranslated region (UTR), and a 39-bp 3’-UTR (Supplemental Fig. 1A). This cloned sequence is mostly consistent with the predicted one. The genomic structure of the DrIL-4Rα gene consists of 11 exons and 10 introns, which is consistent with that of many other species, including mouse and chicken. However, human IL-4Rα, which possesses 12 exons and 11 introns, is slightly different from the others (Fig. 1A).

DrIL-4Rα is a membrane-bound protein that consists of a 206-aa extracellular region, a single 24-aa transmembrane domain, and a 321-aa cytoplasmic region. Although sequence alignment shows that DrIL-4Rα shares low identity with those of human and other vertebrates (20–25%), including rainbow trout (26%), residues important for the maintenance of structure and function, such as six...
cysteines in the extracellular region and three tyrosine residues in the intracellular domain, are conserved completely across all of the aligned species (Supplemental Fig. 2A). However, the “WSXWS” motif, which is critical for maintaining ligand binding and signal transduction in human and other mammalian IL-4Rα (50, 51), is not classic in zebrafish (YSXQS) or in rainbow trout and salmon (FSXWS) (38). This suggests that the “WSXWS” motif might have arisen after the divergence of tetrapods from fish.

With human IL-4Rα as the template, the tertiary structure of the DrIL-4Rα extracellular domain was modeled. The results show that the DrIL-4Rα extracellular domain has an overall tertiary structure similar to that of human IL-4Rα (23). The extracellular region of DrIL-4Rα exhibited an “L” shape organized with two FN III domains that show structural homology with the human FN III domain.

residues engaged in three disulfide bridges (Fig. 1B). However, the disulfide bridge derived from Cys49–Cys80 known to be essential and unique to IL-4Rα family members was found to be well conserved both in DrIL-4Rα and other IL-4Rα molecules. Structural feature analyses of DrIL-4Rα showed that the D2 domain had no disulfide bridges and contained no classic “WSXWS” motif but contained a “YSXQS” motif. The membrane-bound region of DrIL-4Rα was composed of 20 aa rich in hydrophobic amino acids. The cytoplasmic region of DrIL-4Rα contained five tyrosine residues, three of them (Tyr400, Tyr477, and Tyr566), known to be binding sites critical for IRS-1/2-, STAT6-, and ITIM/SHP2-mediated signaling pathways. Interestingly, two tyrosine residues (Tyr284 and Tyr369) predicted to be the potential STAT3 and STAT5 binding sites were seen only in teleost fish IL-4Rαs rather than in tetrapods IL-4Rαs, suggesting that functional diversity may exist between them (Supplemental Fig. 2A). In addition, a short proline-rich sequence in the membrane-proximal region of IL-4Rα, termed the “box1 motif,” which is found in a number of hematopoietin receptor family members, also existed in DrIL-4Rα (Fig. 1C). The structural characteristics of DrIL-4Rα and a comparative analysis between DrIL-4Rα and human IL-4Rα are summarized in Table II.
A phylogenetic analysis showed that all of the IL-4Rα molecules examined formed a single well-supported cluster, in which the teleost fish IL-4Rα formed an exclusive group, as opposed to human and other higher vertebrates, with high bootstrap probability. In addition, another two family members (IL-21R and IL-2Rβ) were analyzed together with IL-4Rα (Supplemental Fig. 2B). These results showed that close phylogenetic relationships existed among these molecules.

**Characterization of an IL-4Rα isoform**

We also cloned a unique IL-4Rα-like isoform (designated as DrIL-4Rα; GenBank accession number, EF523377.1; http://www.ncbi.nlm.nih.gov/nuccore/EF523377) from zebrafish. It consists of a sequence of 1872 bp comprising a 101-bp 5′-UTR, a 1107-bp ORF, and a 664-bp 3′-UTR, encoding a membrane-bound peptide with 368 aa (Supplemental Fig. 1B). The DrIL-4Rα iso gene is located ∼36 kb downstream of the IL-4R locus, and it also consists of 11 exons and 10 introns (Fig. 1A). The most significant difference between DrIL-4Rα and DrIL-4Rα-iso genes is the appearance of an early stop codon in exon 11 of the isoform, although several nucleotide variations exist in other exons (Supplemental Fig. 3). Exon 11 in the DrIL-4Rα or DrIL-4Rα-iso gene is the largest among the 11 exons, consists of 853 bp, and contributes to the encoding of the majority of the intracellular region. The stop codon (UAA) of DrIL-4Rα appears at the 812-bp locus, which is distributed near the 3′ end of exon 11. In DrIL-4Rα-iso exon 11, an early stop codon (UGA) appears at the 203-bp locus, resulting in the early termination of protein translation. Therefore, the exact coding region in DrIL-4Rα-iso exon 11 consists of only 205 bp, and the corresponding 648-bp segment at the 3′ end of exon 11 actually is unused. For this reason, the DrIL-4Rα-iso gene may encode a receptor protein that lacks a 203-aa segment of the intracellular tail. Interestingly, an EcoRI site (1034, GAAATTC) and an EcoRV site (538, CTATAG) were found to exist in the DrIL-4Rα gene but not in the DrIL-4Rα-iso gene, respectively. This makes it possible to distinguish the mRNA from these two genes by digestion with the two restriction enzymes at the reverse-transcribed cDNA level.

**Identification of soluble IL-4Rα variants**

Two soluble forms of IL-4Rα (sDrIL-4Rα; GenBank accession number, EF523376.1; http://www.ncbi.nlm.nih.gov/nuccore/EF523376) and DrIL-4Rα-iso (sDrIL-4Rα-iso; GenBank accession number, EF523378.1; http://www.ncbi.nlm.nih.gov/nuccore/EF523378), which were variants derived from alternative splicing of the DrIL-4Rα and DrIL-4Rα-iso genes, were cloned from zebrafish. These two variants both contain a 100-bp 5′-UTR, a 663-bp ORF, and a 384-bp 3′-UTR, encoding a peptide with 220 aa (Supplemental Fig. 4).

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>Zebrafish IL-4Rα</th>
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<td>Gene size</td>
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<td>Intracellular C terminus/external N terminus</td>
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<td></td>
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<td>Cys64-Cys86</td>
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<td>Asn3, Asn167</td>
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<td>Box/JAK-1, Tyr377/IRS-1/2, Tyr570, Tyr601, Tyr993/STAT6, Tyr1177/ITIM (SHP2)</td>
<td>Box/JAK-1, Tyr284/STAT3, Tyr269/STAT5, Tyr209/STAT6, Tyr209/ITIM (SHP2)</td>
</tr>
<tr>
<td>Cysteine location</td>
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<td>Tertiary structure</td>
<td>An “L” shape comprising two FN III domains</td>
<td>An “L” shape comprising two FN III domains</td>
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</table>

**Characteristics Human IL-4Rα Zebrafish IL-4Rα**

Table II. Characteristics of zebrafish IL-4Rα and comparison with human IL-4Rα

**Potential binding sites for signal transduction**

Potential binding sites for signal transduction associated with the DrIL-4Rα and DrIL-4Rα-iso genes include Box/JAK-1, Tyr377/IRS-1/2, Tyr570, Tyr601, Tyr993/STAT6, Tyr1177/ITIM (SHP2) for DrIL-4Rα and Box/JAK-1, Tyr284/STAT3, Tyr269/STAT5, Tyr209/STAT6, Tyr209/ITIM (SHP2) for DrIL-4Rα-iso.

**Tissue distribution analysis**

In consideration of the high homology between the two genes mentioned above, both DrIL-4Rα and DrIL-4Rα-iso could be amplified using any primers. Moreover, because the PCR products are of the same size, it would be difficult to distinguish them. After a careful search over all of the substitutions between the two genes, two specific restriction enzyme cutting sites were found in the DrIL-4Rα sequences that disappeared in the corresponding sites of DrIL-4Rα-iso (an EcoRV site in exon 6 and an EcoRI site in exon 11). Thereby, these restriction enzymes were used to distinguish the transcripts of DrIL-4Rα from PCR products as well as the alternatively spliced forms. For this purpose, two pairs of primers (DrIL-4Rα-Fb at exon 5 and DrIL-4Rα-R1 at exon 7 as well as DrIL-4Rα-F2 at exon 10 and DrIL-4Rα-R2 at exon 11, as shown in Table I) were devised to amplify a 312-bp segment, representing the membrane-bound DrIL-4Rα and DrIL-4Rα-iso transcripts, in which DrIL-4Rα-iso-derived transcripts contained the EcoRI site, as well as a 496-bp segment, representing the sDrIL-4Rα and sDrIL-4Rα-iso transcripts, in which sDrIL-4Rα-derived transcripts contained the EcoRV site, respectively (Fig. 2A). As shown in Fig. 2B, the mixed transcripts of membrane-bound DrIL-4Rα and DrIL-4Rα-iso could be detected constitutively in most tissues examined, including the liver, gut, kidney, spleen, brain, skin, gill, ovary, and peripheral lymphocytes, but not in the muscle. In contrast, a mixture of the sDrIL-4Rα and sDrIL-4Rα-iso transcripts could be detected constitutively only in limited tissues, such as the liver, brain, and muscle. These different distribution patterns suggest the presence of functional diversity between soluble and membrane-bound forms of IL-4Rα.
To identify whether IL-4 specifically binds to IL-4Rα, two digested segments from the membrane-bound DrIL-4Rα transcripts with predicted sizes of 187 and 125 bp could be detected in the liver, kidney, spleen, brain, skin, gill, and peripheral lymphocytes, indicating that the tissue distribution pattern of DrIL-4Rα was overall consistent with that of DrIL-4Rα-iso, with the exception of several tissues, such as the gut, muscle, and ovary. Accordingly, after being cut by EcoRI, two digested segments from the sDrIL-4Rα transcripts with predicted sizes of 361 and 135 bp could be detected in the liver, brain, and muscle (data not shown for the smaller 135 bp segment). In addition, the tissue distribution of the DrIL-4Rα transcript also was examined. The result shows that DrIL-4Rα was widely detectable in all of the tissues examined, including the liver, gut, kidney, spleen, brain, muscle, skin, gill, ovary, and peripheral lymphocytes, in which stronger expression was observed in the gut, kidney, spleen, skin, gill, and peripheral lymphocytes could be detected.

Preparation of recombinant proteins and Ab

Recombinant GST-DrIL-4 and Trx-sDrIL-4Rα were expressed in E. coli BL21 cells as soluble GST or Trx fusion proteins with their corresponding molecular weights, respectively (Fig. 3A). Abs for DrIL-4Rα (anti-DrIL-4Rα) were affinity-purified from immunized rabbit serum or mouse serum. ELISA analysis showed that the purified Ab had an average titer greater than 1:10,000. Western blot analysis showed that the purified anti-DrIL-4Rα was bound specifically to endogenous DrIL-4Rα in different tissues and that no cross-reactions between anti-DrIL-4Rα and other proteins from tissue extracts were observed (Fig. 3B).

Binding assay between DrIL-4 and DrIL-4Rα

To identify whether DrIL-4Rα did act as a receptor for DrIL-4, a GST pull-down binding assay and a direct quantitative ELISA were performed. In the GST pull-down assay, a larger band with an expected m.w. of 94 kDa, which represents the GST-DrIL-4 and Trx-DrIL-4Rα complex, could be detected clearly by Western blot analysis (Fig. 3C). In the ELISA analysis, increased interactions between recombinant GST-DrIL-4 and Trx-DrIL-4Rα could be detected with the increase in GST-DrIL-4 protein coating, indicating that the specific interaction between GST-DrIL-4 and Trx-DrIL-4Rα was dose-dependent (Fig. 3D). However, in the control groups, recombinant Trx-DrIL-4Rα failed to bind to mouse IL-4, trout IL-2, and trout IL-15, and three γc cytokine family members of other species. These results showed that DrIL-4 specifically binds to DrIL-4Rα.

DrIL-4 plays a role in B cell proliferation and Ab production

To evaluate whether DrIL-4 plays a role in Th2-mediated immune responses, an in vivo administration assay with recombinant GST-DrIL-4 was performed. In vivo B cell proliferation and Ag-stimulated Ab (IgM) production were determined for this purpose. Flow cytometric analysis shows that the percentage of mlgM+ B cells in PBS-injected control fish was 11.04 ± 1.8%, whereas it reached 15.89 ± 2.66%, 36.64 ± 5.18%, and 34.57 ± 6.11% in GST-DrIL-4–administered fish that received 0.01, 0.1, and 1 μg per fish GST-DrIL-4, respectively. The results demonstrate that B cell proliferation could be upregulated significantly (p < 0.01) with the increase in administered GST-DrIL-4 in comparison with that of the PBS control groups (Fig. 4A). In contrast, the percentage of B cells in GST tag-injected control groups was 13.57 ± 2.06%, which was not significantly (p > 0.05) different from that of the PBS control groups. Real-time PCR analysis showed that the expression of B cell markers (mlgM, MHC class II, and CD80) also was upregulated significantly (p < 0.01) in peripheral lymphocytes after in vivo administration of recombinant GST-DrIL-4 (Fig. 4B). This result supports the conclusion that DrIL-4 plays a role in B cell proliferation. Accordingly, Ab (IgM) production in GST-DrIL-4–stimulated groups also could be upregulated significantly (p < 0.01) in 0.1 and 1 μg per fish groups with the increase in administered GST-DrIL-4 in comparison with that of KLH-immunized control groups. In contrast, the production of IgM in GST tag-inoculated control fish was 11.04 ± 1.8%, whereas it reached 13.57 ± 2.06%, which was not significantly (p > 0.05) different from that of the PBS control groups. Real-time PCR analysis showed that the expression of B cell markers (mlgM, MHC class II, and CD80) also was upregulated significantly (p < 0.01) in peripheral lymphocytes after in vivo administration of recombinant GST-DrIL-4. These results demonstrated that DrIL-4 plays an important role in B cell proliferation and Ab production, which are typical characteristics of Th2-mediated immune responses.

DrIL-4Rα distributes on B cell surfaces

To investigate whether DrIL-4Rα distributes on B cell surfaces, a double-immunofluorescence staining experiment was performed.
colocalized on these cellular membranes, respectively (Fig. 4D). By contrast, isotype controls utilizing rabbit IgG and mouse IgG as nonrelated primary Abs displayed no fluorescent signals (data not shown). This result suggested that the DrIL-4–upregulated B cell proliferation and activation described above probably were achieved by its interaction with DrIL-4Rα on B cell surfaces.

**Soluble DrIL-4Rα plays an inhibitory role in B cell proliferation and Ab production**

To identify the possible role that sDrIL-4Rα may play and to provide further functional observations that DrIL-4 and DrIL-4Rα are two reciprocal molecules, an in vivo recombinant Trx-sDrIL-4Rα administration assay was performed. As mentioned above, the percentage (36.64 ± 5.18%) of mlgM+ B cells in recombinant GST-DrIL-4–treated groups was upregulated significantly \( (p < 0.01) \) in comparison with that of PBS-injected control groups \((11.04 ± 1.8\%) \). In contrast, the amounts of B cells in the group treated with GST-DrIL-4Rα alone \((10.43 ± 1.33\%) \) and in the control groups administered Trx tag \((9.56 ± 2.01\%) \) and GST tag \((13.57 ± 2.06\%) \) did not increase significantly \( (p > 0.05) \). However, the percentage of B cells in Trx-sDrIL-4Rα and GST-DrIL-4 coadministered groups \((14.80 ± 0.95\%) \) was downregulated dramatically \( (p < 0.01) \) in comparison with that of GST-DrIL-4–treated groups (Fig. 4E). Accordingly, KLH-stimulated Ab (IgM) production in GST-DrIL-4–treated groups was increased more significantly \( (p < 0.01) \) than those of the control group and groups treated with GST or Trx tag proteins alone. In contrast, titers of IgM in Trx-sDrIL-4Rα and GST-DrIL-4 coadministered groups were decreased significantly \( (p < 0.05) \) compared with that of the GST-DrIL-4–treated group (Fig. 4F). All of these results demonstrated that administration of Trx-sDrIL-4Rα greatly abrogated GST-DrIL-4–elevated B cell proliferation and Ab production as well as Ab production under the “natural” immunization condition. sDrIL-4Rα may play a negative role in fish Th2-mediated immunity by its competitive binding to DrIL-4. Similar roles also were seen in mammal soluble IL-4Rα molecules. In addition, these results provided further functional evidence that IL-4 and IL-4Rα are two reciprocal molecules in zebrfish.

**Blockade of DrIL-4Rα downregulates B cell proliferation**

To investigate further whether DrIL-4 and DrIL-4Rα were a pair of reciprocal molecules whose signaling pathway is essential for Th2-mediated immune responses, an in vivo IL-4Rα blockade assay with DrIL-4Rα–specific Ab (anti–DrIL-4Rα) was performed. Flow cytometric analysis shows that the percentage \((27.33 ± 3.62\%) \) of B cells in recombinant GST-DrIL-4–treated groups \((11.04 ± 1.8\%) \) and GST-DrIL-4 coadministered groups \((13.57 ± 2.06\%) \) did not increase significantly \( (p > 0.05) \). However, the percentage of B cells in anti–DrIL-4Rα blockade groups \((14.80 ± 0.95\%) \) was downregulated dramatically \( (p < 0.01) \) in comparison with that of PBS-injected control groups \((11.04 ± 1.8\%) \). In contrast, the amount of B cells in GST tag protein administered groups \((13.57 ± 2.06\%) \) did not increase significantly \( (p > 0.05) \). However, the percentage of B cells in anti–DrIL-4Rα blockade groups \((13.11 ± 1.39\%) \) was downregulated dramatically \( (p < 0.01) \) in comparison with that of positive groups (Fig. 4G). These observations demonstrate that blockade of DrIL-4Rα significantly downregulates B cell proliferation, which suggests that DrIL-4Rα is essential for DrIL-4–upregulated immune responses and provides further evidence that the DrIL-4–DrIL-4Rα interaction is essential for Th2-type immunity in zebrfish.

**DrIL-4 upregulates the expression of Th2 cytokines and transcription factors**

To investigate whether DrIL-4 acts as a Th2 cytokine that can upregulate the expression of Th2 cytokines and transcription factors and downregulates Th1 cytokines and transcription factors,
FIGURE 4. Evaluation of DrIL-4 function and the DrIL-4–DrIL-4Ra interaction involved in Th2-mediated immune responses by in vivo administration assays. (A) Flow cytometric analysis of the percentage of mlgM+ B cells in isolated peripheral lymphocytes separated by Ficoll-Hypaque centrifugation from whole-blood cells after treatment for 72 h with recombinant GST-DrIL-4 (0.01, 0.1, or 1 μg per fish i.p.), GST (1 μg per fish i.p.), or PBS. The gray histograms show background fluorescence on isotype controls. (B) Effect of DrIL-4 on the expression of B cell markers. Transcripts from the peripheral lymphocytes of zebrafish after i.p. inoculation with recombinant GST-DrIL-4 or GST for 72 h were analyzed by real-time quantitative PCR. Relative gene expressions were calculated using the 2^(-ΔΔCT) method, with the initial normalization of genes against β-actin within each sample. The expression level of each gene in the GST-treated control groups was set arbitrarily to 1.0, and the levels of recombinant GST-DrIL-4-stimulated groups were adjusted accordingly. The relative expression value was averaged from 10 healthy fish and run in three parallel reactions. The data represent the results obtained from at least three independent experiments. (C) ELISA analysis for KLH-specific IgM Abs in the serum from KLH-immunized (Figure legend continues)
ROLE OF IL-4–IL-4Rα INTERACTION IN TH2-LIKE IMMUNITY IN FISH

representative Th2 (IL-10, GATA3, c-maf, and STAT6) and Th1 (T-bet, IRF1, STAT1, and IFN-γ) cytokines and transcription factors in response to the stimulation of DrIL-4 were analyzed (52–56). As expected, administration of recombinant GST-DrIL-4 significantly upregulates the expression of IL-10, GATA3, c-maf, and STAT6 but downregulates the expression of T-bet, IRF1, STAT1, and IFN-γ. This result provides the observation that DrIL-4 acts as a Th2 cytokine that might be involved in Th2 development in fish immunity (Fig. 5).

DrIL-4 promotes CD40 expression and the CD154–CD40 costimulatory interaction

To investigate whether DrIL-4 functions in B cell proliferation by upregulating CD40 expression on B cells and by promoting CD154–CD40-mediated costimulatory signaling in zebrafish, an in vivo DrIL-4–elicited CD40 expression and CD154–CD40 interaction assay was performed. Real-time PCR analysis showed that the CD40 transcripts were upregulated dramatically in lymphocytes from the recombinant GST-DrIL-4–treated groups compared with those of the PBS or GST tag-treated control groups (p < 0.01) (Fig. 6A). This stimulatory effect of GST-DrIL-4 was inhibited significantly by coadministration of Trx-sDrIL-4Rα (p < 0.01) but enhanced significantly by coadministration of scCD154 (p < 0.01). Flow cytometric analysis demonstrated that the expression of CD40 proteins on B cells (as determined by the geometric mean of fluorescence intensity of PE-labeled anti-CD40 on IgM*CD40+ B cell surfaces) in the GST-DrIL-4–treated groups (75.49 ± 3.56) was significantly higher (p < 0.01) than that of the PBS- (42.75 ± 2.53) or GST tag-treated (44.87 ± 2.47) control group. Significant proliferation of B cells was accompanied by upregulated expression of CD40 molecules (p < 0.01). These stimulatory effects of GST-DrIL-4 on CD40 expression and B cell proliferation also were inhibited significantly by coadministration with Trx-sDrIL-4Rα (58.77 ± 2.92) and enhanced by coadministration with scCD154 (111.27 ± 5.86) (both p < 0.01) (Fig. 6B, 6C). These observations indicate that DrIL-4 promotes CD40 expression on B cells by interacting with its receptor DrIL-4Rα. The upregulated CD40 molecules enhance the B cell response to CD154 stimulation, resulting in the enhanced CD154–CD40 costimulatory interaction critical for B cell proliferation and activation.

Discussion

In humans and other mammals, the adaptive immune system is composed of various T and B subsets and cytokines that consist of a precise regulatory network for the development of cellular or humoral immunity. The Th cell is one of the most important populations in such a network, and its two main subsets, Th1 and Th2, largely contribute to cellular (Th1-type) and humoral (Th2-type) immunity (56). The development of Th1- and Th2-type immunity is regulated by a number of subset-specific cytokines, in which the IL-4–IL-4Rα–mediated signaling pathway plays a critical role in Th2-type immune responses (57). However, little documentation exists about whether Th1- and Th2-type immune mechanisms originate from ancient vertebrates.

We reported recently on the molecular and functional characterization of CD154 and CD40 costimulatory molecules from zebrafish (43). It shows that the CD154–CD40–mediated T–B interactions are required for humoral immune responses to thymus-dependent Ags in zebrafish and provides a preliminary insight into the origin of the Th-like regulatory mechanism in early vertebrates. However, further study is needed to elucidate the Th1 and Th2 subsets involved in the underlying Th regulatory mechanism. Because a IL-4–IL-4Rα–mediated regulatory mechanism is believed to be a hallmark of Th2-type immunity, the functional identification of these two reciprocal molecules becomes a prerequisite for the evaluation of Th2-type immunity in fish. For this purpose, the biological role that IL-4 and IL-4Rα played in B cell proliferation, Ab production, and CD154–CD40–mediated costimulatory Th2-type immunity was characterized carefully in zebrafish.

In total, four IL-4Rα molecules were identified from zebrafish, which includes one typical DrIL-4Rα molecule, one DrIL-4Rα-like isoform, and two corresponding alternatively spliced soluble variants, the latter of which to our knowledge were first reported in fish. This result suggests that the composition and function of the fish IL-4 system is much more complicated than known previously. DrIL-4Rα shares a number of conserved structural features with its homologues in other species. These include similar chromosomal locations and gene organizations, overall conserved protein homology domains (such as the FN III domain in the extracellular region), and key residues (such as cysteines) that are important for the formation of disulfide bonds and the structural integrity of the molecules, although sequence alignment between zebrafish and mammal IL-4Rα showed only 20–25% similarity. DrIL-4Rα iso seems to be unique in fish species. In this study, a comparison of the organizations of the DrIL-4Rα and DrIL-4Rα iso genes, including alternatively spliced exons, is presented. Comparisons between every counterpart show extremely high identity, including the transcriptional starts, the boundary sites of each intron, and the polyadenylation signals, implying an obvious gene duplication event occurred between them. Nevertheless, a few remarkable differences also were identified in detail. For instance, the lengths of introns 5, 6a, 6b, and 8 varied between these two genes, and some nucleotides were distinct at several positions, including both synonymous and nonsynonymous substitutions. The most significant nonsynonymous one is the appearance of an early stop codon in zebrafish treated with recombinant GST-DrIL-4 (0.01, 0.1, or 1 μg per fish i.p.) and GST (1 μg per fish i.p.) and the control group immunized with KLH only. All of the groups were titered from 1:100 to 1:100,000, and the titer was ascertained based on multiple point analysis. The data of the background control group with PBS administration were subtracted from each experimental group. (D) Immunofluorescence staining of lymphocytes with rabbit anti-mlgM and mouse anti-DrIL-4Rα. Nonrelated Abs, including rabbit IgG and mouse IgG, were used as negative controls (data not shown). White arrows indicate the mIgM and IL-4Rα–injected groups, GST (1 μg per fish i.p.), and the control group immunized with KLH (1:5)–injected groups. (E) B cell proliferation is activated by GST-DrIL-4 that is blocked by Trx-sDrIL-4Rα. Flow cytometric analysis of the percentage of mIgM+ B cells with different treatments in the recombinant Trx-sDrIL-4Rα-injected groups (0.1 μg per fish i.p.), GST-DrIL-4–injected groups (0.1 μg per fish i.p.), GST-DrIL-4–Trx-sDrIL-4Rα (1:1)–injected groups, GST-DrIL-4/Trx-sDrIL-4Rα (1:5)–injected groups, GST (1 μg per fish i.p.)–injected groups, Trx-injected (1 μg per fish i.p.) groups, and control groups. (F) ELISA assays for KLH-specific IgM Abs in the serum from KLH-immunized zebrafish treated with recombinant Trx-sDrIL-4Rα (0.1 μg per fish i.p.), GST-DrIL-4 (0.1 μg per fish i.p.), GST-DrIL-4/Trx-sDrIL-4Rα (0.1/0.1 μg per fish i.p.), GST (1 μg per fish i.p.), and Trx (1 μg per fish i.p.) and the control groups immunized with KLH only. All of the groups were titered from 1:100 to 1:100,000, and the titer was ascertained based on multiple point analysis. The background control group with PBS administration were subtracted from each experimental group. (G) B cell proliferation is inhibited by the Ab of DrIL-4Rα blockade. Peripheral lymphocytes were isolated from fish after treatment with either recombinant GST-DrIL-4 and IgG or recombinant GST-DrIL-4 and rabbit anti–DrIL-4Rα. The gray histograms in (A) and (C) show the background fluorescence of isotype controls. All of the data represent results obtained from at least three independent experiments. *p < 0.05, **p < 0.01.
GST-molecules was investigated initially both in vitro and in vivo. The are two reciprocal molecules, the interaction between these two interaction would be needed to clarify this suggestion.

![Graph](https://www.jimmunol.org/content/110/5/2847/F5.large.jpg)

**FIGURE 5.** Effects of DrIL-4 on the expression of Th1- and Th2-type cytokines and transcription factors. The transcripts from the spleen of zebrafish after i.p. inoculation with recombinant GST-DrIL-4 or GST for 72 h were analyzed by real-time quantitative PCR. Relative gene expression was calculated using the $2^{-\Delta \Delta CT}$ method with initial normalization of genes against $\beta$-actin within each sample. The expression level of each gene in the GST-treated control group was set arbitrarily at 1.0, and the levels of the recombinant GST-DrIL-4-stimulated group were adjusted correspondingly. The relative expression value was averaged from 10 healthy fish and run in three parallel reactions, and the data represent the mean of these samples. The experiments were repeated three times with similar results. *$p < 0.05$, **$p < 0.01$.

exon 11 of DrIL-4Rα-iso, making the cytoplasmic region of DrIL-4Rα-iso much shorter than that of DrIL-4Rα. As a result, a number of potential phosphorylation sites that are produced in various signaling pathways, such as Tyr369/STAT-5, Tyr407/IRS-1/2, Tyr477/STAT6, and Tyr566/ITIM (SHP2), may be lost, resulting in functional diversity between these two receptors. The alternatively spliced soluble variants also are seen in humans and other mammals (58–60). In these species, exon 8 in the IL-4Rα genes is a “pseudoexon” that contains a stop codon (TAG) at its 5’ end. When exon 8 is spliced into the end of the upstream exon, the stop codon may appear much earlier in the alternatively spliced mRNA, resulting in the production of a soluble IL-4Rα variant that lacks the transmembrane and cytoplasmic regions. This soluble splicing is similar to that in DrIL-4Rα genes. Notably, DrIL-4Rα-iso and sDrIL-4Rα-iso presented here are two novel molecules of the IL-4Rα family, and their functions had never been reported previously. Their in-depth study may lead to the discovery of new features of the IL-4Rα family.

The distribution pattern of the sDrIL-4Rαs, expressed only in the liver, brain, and muscle, is quite different from that found for membrane-bound DrIL-4Rα. To our knowledge, the liver is generally an immune-relevant organ that produces various immune-related cytokines and their receptors in teleost fish (38, 61, 62). From this point of view, the liver produces sDrIL-4Rα for relevant immune responses. Considering the different distribution patterns between sDrIL-4Rα and membrane-bound DrIL-4Rα, we suggest that sDrIL-4Rα may play different roles aside from its antagonistic-related functions to membrane-bound DrIL-4Rα. Further investigation would be needed to clarify this suggestion.

To provide functional evidence that IL-4 and IL-4Rα in zebrafish are two reciprocal molecules, the interaction between these two molecules was investigated initially both in vitro and in vivo. The results from the in vitro binding assay showed that recombinant GST-DrIL-4 could bind specifically to recombinant Trx-DrIL-4Rα. The in vivo binding assay was evaluated functionally by administering the fish with recombinant Trx-sDrIL-4Rα to inhibit the interaction of natural or administered recombinant GST-DrIL-4 with Trx-DrIL-4Rα. This resulted in the inhibition of GST-DrIL-4-enhanced B cell proliferation and Ab production in response to Ag (KLH) stimulation. As expected, GST-DrIL-4-enhanced in vivo B cell proliferation and Ab (IgM) production could be downregulated significantly by administration of Trx-sDrIL-4Rα. This not only indicates that DrIL-4 and DrIL-4Rα are two reciprocal molecules but also shows that sDrIL-4Rα may be used as a negative regulator to downregulate the hyperimmune responses elicited by excessive DrIL-4. Such regulators have potential application in the control in immune-related diseases.

To optimize the concentration of IL-4 for in vivo administration assays, different dosages of the reagent were used in the experiments. The optimized concentration was determined by in vivo B cell proliferation by flow cytometric analysis. The results show that the most appropriate concentration of GST-DrIL-4 is 0.1 µg per fish. The optimized time point for in vivo B cell proliferation assays was chosen as 72 h poststimulation of GST-DrIL-4 based on our previous investigation. We found that the activation of APCs (as determined by the expression of CD-SIGN/CD209) could be upregulated dramatically, even after 5 d of in vivo inoculation with recombinant DrIL-4 (44). Likewise, the time point used around 72 h also had been reported in other animal models, including mammals. For example, in vivo treatment with recombinant IL-4 for 72 h directly increased dendritic cell (DC) maturation and T cell recruitment independent of innate immune signals in a mouse model (63). According to these observations, it could be considered that the function of IL-4 is a critical driver in the priming and initiation of Th2-type adaptive immunity and is crucial for the recruitment, maturation, and activation of DCs and T lymphocytes by upregulating a number of key regulatory factors, such as CD40, CD154, CD80/86, CD83, and DCSIGN/CD209. As a result, although the half-life of recombinant IL-4 in vivo is expected to be very short, a quick initial response could be induced considerably by this cytokine at the early stage of Th2-type immunity. This reaction may be powerful enough to trigger subsequent immune response cascades, including the maturation and activation of DCs and T and B lymphocytes, the latter of which may occur in 72 h, at least in fish species.

To provide further functional observations that DrIL-4 plays a role in adaptive humoral immunity as a Th2 cytokine and that the DrIL-4–DrIL-4Rα interaction is essential for this process, in vivo administration of recombinant GST-DrIL-4 and in vivo Trx-DrIL-4Rα blockade assays were performed. The B cell proliferation and Ab production in response to GST-DrIL-4 were examined for this purpose. The results show that B cell proliferation is upregulated dramatically after stimulating the fish with GST-DrIL-4, because the percentages of mIgM+ B lymphocytes in the stimulated fish and the expression of B cell markers in the stimulated lymphocytes were elevated significantly ($p < 0.01$). Accordingly, the production of Ab also was found to be enhanced significantly ($p < 0.01$) by GST-DrIL-4. However, blockade of DrIL-4Rα by specific anti–DrIL-4Rα Ab could decrease B cell proliferation significantly ($p < 0.05$). Immunofluorescence staining showed that DrIL-4Rα and mIgM were colocated on B cell surfaces. This, together with the observation that B cell proliferation and Ab production could be downregulated by in vivo administration of Trx-sDrIL-4Rα, demonstrates that DrIL-4 plays an important role in B cell proliferation and Ab production by interaction with DrIL-4Rα on B cells, which is a typical characteristic of Th2-mediated immune responses in mammals. In addition, the changes in expression levels of the key cytokines or transcription factors involved in Th1 and Th2 differentiation were investigated after stimulating the fish with recombinant GST-DrIL-4. As expected, GST-DrIL-4 was able to enhance the ex-
expression of Th2 cytokines and transcription factors (IL-10, GATA3, c-maf, and STAT6) but inhibit the expression of Th1 transcription factors (T-bet, IRF1, STAT1, and IFN-γ), as its counterparts did in the adaptive immunity of humans and other mammals (2–4). Therefore, it seems reasonable to suggest that DrIL-4 may play a role as a Th2 cytokine and that a Th2-mediated regulatory mechanism might be developed in adaptive immunity as early as fish during vertebrate evolution.

The essential roles of IL-4–IL-4Rα signaling and the CD154–CD40-mediated costimulatory pathway in Th2-type immunity have been well documented in mammals (28–32, 64–66). However, the regulatory relationship between these two signaling pathways was still controversial. CD154–CD40 costimulatory signaling can induce effector T cell-derived IL-4 (67), but whether IL-4–IL-4Rα signaling can promote a CD154–CD40-mediated costimulatory pathway in B cells still lacks evidence. There was a report showing that IL-4 has no effect on CD40 expression in human monocytes (68). At the same time, IL-4 can suppress IFN-γ–induced CD40 gene expression in both mice macrophages and microglia (69). In the current study, we demonstrated that the DrIL-4–DrIL-4Rα interaction promotes B cell proliferation and activation by upregulating CD40 expression on B cells. These B cells with upregulated CD40 were more responsive to CD154 stimulation. This result suggests the existence of a close regulatory relationship between the DrIL-4–DrIL-4Rα signaling pathway and the CD154–CD40-mediated costimulatory pathway in zebrafish, which may delineate a signal network that is essential for modulating Th2 immune responses. Further study of this model will lead to a better understanding of IL-4–regulated costimulatory signaling pathways in Th2-type immunity.

In conclusion, this study shows the role that zebrafish IL-4 and IL-4Rα play in fish adaptive humoral immunity and suggests that...
the IL-4–IL-4Rα-mediated Th2 regulatory machinery might originate from teleost fish during early vertebrate evolution. In mammals, IgM is considered the most primitive Ab among the five isotypes (IgM, IgA, IgE, IgD, and IgG). These are regulated by the Th2 immune response. In fish, IgM seems to be the main isotype involved in humoral immunity. Therefore, it seems reasonable to suggest that participation in IgM production may be the original role that IL-4 and IL-4R play in adaptive humoral immunity, and this function is conserved from fish to mammals during all of vertebrate evolutionary history.

Disclosures
The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE S1. A. Sequence of DrIL-4Rα cDNA and deduced amino acids. B, Sequence of DrIL-4Rα-iso cDNA and deduced amino acids. The asterisk indicates peptides ending. Putative polyadenylation signal (AGTAAA) is in bold italic. Signal peptide and transmembrane hydrophobic region are shaded and underlined, respectively. N-glycosylation sites are in bold.
SUPPLEMENTAL FIGURE S2. A, Multiple alignments of IL-4Rα molecules. Residues shaded in black are completely conserved across all species aligned, and residues shaded in gray are similar with respect to side chains. The dashes in the amino acid sequences indicate gaps introduced to maximize alignment. “▲” indicates the conserved cysteine, while lines between arrows indicate the potential disulfide bonds. Line under the alignment represents the transmembrane domain. “Y” shows the conserved tyrosine residues. Singal peptide and the “WSXWS” motif of each sequence are underlined and marked above. B, Phylogenetic analysis of IL-4Rα molecules. Phylogenetic tree shows the relationship of IL-4Rα between zebrafish and other species. The unrooted phylogenetic tree is constructed by the neighbor-joining method based on the amino acid alignment (Clustal W) of full-length protein sequences. The accession numbers of mammalian, chicken, frog, and fish IL-4Rα sequences included in the phylogenetic analyses are as follows: Homo sapiens IL-4Rα, NM_000418 (http://www.ncbi.nlm.nih.gov/nuccore/NM_000418); Pan troglodytes IL-4Rα (predicted), XM_510892 (http://www.ncbi.nlm.nih.gov/nuccore/XM_510892); Macaca mulatta IL-4Rα (predicted), XM_001093763.1 (http://www.ncbi.nlm.nih.gov/nuccore/XM_001093763.1); Equus caballus IL-4Rα (predicted), NM_001081774.1 (http://www.ncbi.nlm.nih.gov/nuccore/NM_001081774.1); Bos taurus IL-4Rα, NM_001075142.1 (http://www.ncbi.nlm.nih.gov/nuccore/NM_001075142.1); Sus scrofa IL-4Rα, NM_214340 (http://www.ncbi.nlm.nih.gov/nuccore/NM_214340); Canis familiaris IL-4Rα (predicted), XM_547077.2 (http://www.ncbi.nlm.nih.gov/nuccore/XM_547077.2); Mus musculus IL-4Rα, NM_001008700 (http://www.ncbi.nlm.nih.gov/nuccore/NM_001008700); Gallus gallus IL-4Rα (predicted), XP_414885.2
*Taeniopygia guttata* IL-4Rα (predicted), XM_002193419

*Oncorhynchus mykiss* IL-4Rα1, AJ634731

*Oncorhynchus mykiss* IL-4Rα2, FN824519

*Salmo salar* IL-4Rα, NP_001133565

*Danio rerio* IL-4Rα, EF523375.1

*Danio rerio* IL-4Rα-iso, EF523377.1

*Homo sapiens* IL-21R, NP_851565.1

*Mus musculus* IL-21R, NP_068687

*Monodelphis domestica* IL-21R (predicted), XP_001369990.1

*Bos taurus* IL-21R, NP_001180108.1

*Pan troglodytes* IL-21R (predicted), XP_510893.2

*Macaca mulatta* IL-21R (predicted), XP_001094102.2

*Danio rerio* IL-2Rβ, NM_001128267.1

*Homo sapiens* IL-2Rβ, NM_000878.2

*Mus musculus* IL-2Rβ, NM_008368.4
**SUPPLEMENTAL FIGURE S3.** Comparison of DrIL-4R and DrIL-4R-iso gene.

All different nucleotides and amino acids are shaded. Deduced amino acids of DrIL-4R-iso are below, while the difference residues in DrIL-4R at corresponding
sites are above. Stop codon are in bold, and the asterisks indicate peptides ending.
Exons and introns are in uppercase and lowercase, respectively. Some too big introns and corresponding sizes are in brackets. Splicing sites are boxed. Alternatively used exon-7 in both genes are in italic. Polyadenylation signals are underlined, while the ployA appending sites are marked.
SUPPLEMENTAL FIGURE S4. A. Sequence of sDrIL-4Rc cDNA and deduced amino
acids. B, Sequence of sDrIL-4Rα-iso cDNA and deduced amino acids. The asterisk indicates peptides ending. Putative polyadenylation signal (AATAAA) is in bold italic. Signal peptide is shaded. N-glycosylation sites are in bold.