Characterization of SIGIRR/IL-1R8 Homolog from Zebrafish Provides New Insights into Its Inhibitory Role in Hepatic Inflammation

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Single Ig IL-1R–related molecule (SIGIRR, also called IL-1R8 or Toll/IL-1R [TIR]8), a negative regulator for Toll/IL-1R signaling, plays critical roles in innate immunity and various diseases in mammals. However, the occurrence of this molecule in ancient vertebrates and its function in liver homeostasis and disorders remain poorly understood. In this study, we identified a SIGIRR homology from zebrafish (Danio rerio [DsSIGIRR]) by using a number of conserved structural and functional hallmarks to its mammalian counterparts. DsSIGIRR was highly expressed in the liver. Ablation of DsSIGIRR by lentivirus-delivered small interfering RNA in the liver significantly enhanced hepatic inflammation in response to polyinosinic-polycytidylic acid [poly(I:C)] stimulation, as shown by the upregulation of inflammatory cytokines and increased histological disorders. In contrast, depletion of TIR domain–containing adaptor inducing IFN-β (TRIF) or administration of TRIF signaling inhibitor extremely abrogated the poly(I:C)-induced hepatic inflammation. Aided by the zebrafish embryo model, overexpression of DsSIGIRR in vivo significantly inhibited the poly(I:C)- and TRIF-induced NF-κB activations; however, knockdown of DsSIGIRR promoted such activations. Furthermore, pull-down and Duolink in situ proximity ligation assay assays showed that DsSIGIRR can interact with the TRIF protein. Results suggest that DsSIGIRR plays an inhibitory role in TRIF-mediated inflammatory reactions by competitive recruitment of the TRIF adaptor protein from its TLR3/TLR22 receptor. To our knowledge, this study is the first to report a functional SIGIRR homolog that existed in a lower vertebrate. This molecule is essential to establish liver homeostasis under inflammatory stimuli. Overall, the results will enrich the current knowledge about SIGIRR-mediated immunity and disorders in the liver. The Journal of Immunology, 2016, 197: 151–167.

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Abbreviations used in this article: DIGIRR, double Ig IL-1R–related molecule; Drex-SIGIRR, extracellular region of DsSIGIRR; DsSIGIRR, SIGIRR homology from zebrafish (Danio rerio); EGFP, enhanced GFP; HEK293T, human embryonic kidney 293T; hpf, hour postfertilization; IFN, interferon regulatory factor; IVF, intravascular infection; MO, morpholino oligonucleotide; PLA, proximity ligation assay; poly(I:C), polyinosinic-polycytidylic acid; shRNA, short hairpin RNA; SIGIRR, single Ig IL-1R–related molecule; siRNA, small interfering RNA; TIR, Toll/IL-1R; TRIF, Toll/IL-1R domain–containing adaptor inducing IFN-β; UTR, untranslated region.

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signaling pathways may result in almost all the liver diseases examined, such as acute hepatitis, alcoholic liver injury, autoimmune hepatitis, and hepatocellular carcinoma (19, 22, 23). For example, Con A–induced autoimmune-associated hepatitis can be prevented in TLR3-deficient mice, and the mortality caused by acetaminophen-induced hepatotoxicity can be reduced by TLR9 antagonists (24, 25). Thus, TLR activation is a double-edged sword, which must be tightly regulated to keep a check on deregulated activation of TLR-dependent immune responses, thereby preventing inflammatory disorders. However, the negative regulatory mechanisms underlying the TLR-dependent inflammatory responses in the liver remain incompletely understood and are waiting to be determined.

In the present study, we describe the molecular and functional identifications of a SIGIRR homology from zebrafish (*Danio rerio*, DrSIGIRR), an emerging attractive model organism for the study of human diseases and comparative immunology. DrSIGIRR exhibits a number of conserved structural and functional hallmarks to its mammalian counterparts, including similar genomic synteny and organization, compositions of Ig-like and TIR domains,
substitutions of the two functional amino acid sites required for signal transduction in the TIR domain, subcellular localization on the cellular surface membrane, and an inhibitory role in the MyD88-dependent NF-κB signaling pathway. Remarkably, DrSIGIRR was found to be highly expressed in the liver. Ablation of DrSIGIRR by small interfering RNA (siRNA) significantly increased the liver inflammatory responses to polyinosinic-polycytidylic acid (poly[I:C]) stimulation. In contrast, depletion of TIR domain–containing adaptor inducing IFN-β (TRIF) or administration of a TRIF signaling inhibitory peptide extremely abrogated the poly(I:C)-induced hepatic inflammation. A zebrafish embryo model was used to demonstrate the overexpression of DrSIGIRR in embryos inhibited in both poly(I:C)- and TRIF-induced NF-κB activation; in contrast, knockdown of DrSIGIRR promoted such activations. Furthermore, pull-down and Duolink in situ proximity ligation assay (PLA) assays showed that DrSIGIRR can interact with TRIF. These observations suggest that DrSIGIRR may play a negative regulatory role in TRIF-mediated inflammatory reactions by competitive recruitment of TRIF adaptor protein from the TLR3/22 receptor, thus providing new insights into the inhibitory role of SIGIRR in hepatic inflammation. To our knowledge, the above findings provide the first evidence that a functional SIGIRR homolog exists in a lower vertebrate, which is essential to establish liver homeostasis. Overall, this study will enrich the current knowledge about SIGIRR-mediated immunity in the liver and provide significant implications for developing a novel zebrafish model to investigate SIGIRR-based liver diseases and clinical therapies.

Materials and Methods

Experimental fish and embryo

Wild-type AB zebrafish (D. rerio) were maintained in a circulating water bath at 28°C under standard laboratory conditions (26). Embryos were collected at different stages of embryonic development as determined using previous protocols (27).

Molecular cloning

DrSIGIRR cDNA was generated by RT-PCR using the homologous sequences predicted from the University of California Santa Cruz and National Center for Biotechnology Information genome databases (28). The encoding sequences of zebrafish MyD88 (D/MyD88), TLR3 (D/TLR3), and TLR22 (D/TLR22) (NM_212814, NM_001013269.3, and NM_001128675.1, respectively) were amplified according to their published data in the National Center for Biotechnology Information database. The primers used in cloning are listed in Supplemental Table I. PCR products were purified using a gel extraction kit (Qiagen), inserted into pGEM-T vector (Promega), and transformed into competent Escherichia coli TOP10 cells (Invitrogen). Plasmid DNAs were purified using a miniprep kit (Mega) and sequenced on an ABI 3730 sequencer (Invitrogen). Gene and protein structures, multiple amino acid sequence alignment, and phylogenetic analysis were executed using databases and software programs as described previously (29–31).

Real-time PCR for gene expression analysis

Total RNAs from developing embryos and various tissues (heart, spleen, liver, intestine, kidney, gill, brain, skin, and muscle) from healthy fish or liver tissues from poly(I:C)-stimulated fish were isolated using TRIzol bath at 28˚C under standard laboratory conditions (26). Embryos were collected at different stages of embryonic development as determined using previous protocols (27).

Plasmid constructions

The sequence encoding the extracellular region of DrSIGIRR (DresSIGIRR) was inserted into pET32b (Invitrogen) between the BamHI and XhoI sites to construct the prokaryotic expression vector pET32b-exSIGIRR. The full coding sequence of DrSIGIRR was subcloned into pEGFP-N1 (Clontech, Palo Alto, CA), pFLAG-CMV1 (Sigma-Aldrich), and pCDNA6/myc-HisB (Invitrogen) to construct the eukaryotic expression vectors pEGFP-SIGIRR, pFLAG-SIGIRR, and pCDNA6-SIGIRR to obtain the recombinant fusion proteins of DrSIGIRR with enhanced GFP (EGFP), Flag-tag, and Myc-tag, respectively. The encoding sequences of D/MyD88, D/TLR3, and D/TLR22 were subcloned into pCMV-Tag2B (Invitrogen) or pCDNA6/myc-HisB. The plasmids of pEGFP-TRIF and pCMV-TRIF were previously constructed in our laboratory (33). The NF-κB luciferase construct was purchased from Clontech, and the pRL-TK (Renilla luciferase reporter plasmid) vector was obtained from Promega. The primers used for construct generation are listed in Supplemental Table I. All constructs were sequenced to verify the correct sequences and orientations. Plasmids for transfection and microinjection were prepared free of endotoxin using an EZN plasmid mini kit (Omega Bio-Tek).
**FIGURE 3.** Inhibition of IL-1β- and CpG-ODN-induced NF-κB activation by overexpressing DsSIGIRR in zebrafish embryos. (A and B) Expression patterns of DsMyD88 and DsSIGIRR at the embryonic stages of 6, 12, 24, 36, 48, and 96 hpf against β-actin. The relative expression value was averaged from three replicates, each containing 30–50 embryos. (C and D) One-cell stage embryos were coinjected with the DrIL-1β (60 pg/embryo) (C) or CpG-ODN 1670 (1.5 ng/embryo) (D) alone or with the plasmid pcDNA6-SIGIRR (60 pg/embryo) plus the NF-κB luciferase reporter and the pRL-TK Renilla luciferase reporter. At 24 h after microinjection, luciferase activity was detected and expressed as the fold induction over the control with three replicates as described in *Materials and Methods*. Values represent the mean ± SD. ***p < 0.01.

Subcellular localization

Human embryonic kidney 293T (HEK293T) cells were seeded into multiwell plates (Corning) and cultured in DMEM (HyClone Laboratories) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂ to allow growth into 70–90% confluence. These cells were transfected with pEGFP-SIGIRR plasmid DNA (1–2 μg) using FuGENE HD transfection reagent (Promega) in accordance with the manufacturer’s instructions (31, 32). At 24 h posttransfection, the cells were fixed in 4% paraformaldehyde for 10 min and stained with 10 μM DiI (Beyotime) at 37°C for 5 min. Fluorescence images were obtained using a laser scanning confocal microscope (Zeiss LSM 710) (31).

Morpholino oligonucleotide and capped mRNA

The morpholino oligonucleotide (MO) against *DsSIGIRR* mRNA (DrMO) (5′-GTGACACGCACATTGCAGCCCTC-3′) and the standard control MO (5′-CTCTTACCTCAGTTACAATTTATA-3′) were designed and synthesized using Gene Tools. For DrMO validation, the *DsSIGIRR* cDNA fragment that encompassed partial 5′-untranslated region (UTR) and the ATG site were amplified and cloned into pEFGP-N1 to generate a *DsSIGIRR*-EGFP vector. This vector (50 pg/embryo) was then injected into one-cell stage embryos with control or DrMO (4 ng/embryo). GFP fluorescence was visualized at 24 h after microinjection via fluorescence microscopy (Zeiss Axiovert 40 CFL; Carl Zeiss, Jena, Germany). The capped mRNA of *DsSIGIRR* was transcribed in vitro using a Message Machine kit (Ambion), according to the user’s manual, and then solubilized in diethyl pyrocarbonate water for microinjection.

Evaluation of *DsSIGIRR* in NF-κB activation and IFN production

The effects of *DsSIGIRR* on MyD88- and TRIF-induced NF-κB activation or TRIF-induced IFN production were determined via MO-mediated knockdown and mRNA rescue assays. For NF-κB activation assay, the indicated expression plasmid (pCMV-MyD88 or pEGFP-TRIF) alone or with the *DsSIGIRR* mRNA (or pcDNA6-SIGIRR) plus NF-κB luciferase reporter plasmid (50 pg/embryo) and pRL-TK Renilla luciferase reporter plasmid (5 pg/embryo, as an internal control) was injected into one-cell stage embryos. The empty plasmid was added to ensure that all samples received equal amounts of DNA. At 24 h after microinjection, embryos were collected for luciferase activity detection as previously described (32, 33). For IFN production assay, plasmids in various combinations (pEFGP-TRIF alone, pEFGP-TRIF plus DrMO, and pEFGP-TRIF plus DrSIGIRR mRNA) were microinjected into one-cell stage embryos. Empty plasmid (pEFGP-N1) or *DsSIGIRR* mRNA alone was injected as negative control. At 24 h after microinjection, the embryos with strong and GFP expression were used for further analysis. Real-time PCR was performed to measure the mRNA levels of IFN regulatory factor (IRF)3 and IFN1 as described earlier (33, 34).

Preparation of recombinant protein and polyclonal Ab

The pET32b-exSIGIRR was transformed into *Escherichia coli* BL21 (DE3). The recombinant *DrexSIGIRR* protein was induced by 0.5 mM isopropyl β-d-thiogalactoside and purified through Nip-NTA agarose affinity chromatography (Qagen) in accordance with the manufacturer’s instructions. Subsequently, 6-wk-old male New Zealand White rabbits weighing ∼1.5 kg each were immunized with 100 μg purified *DrexSIGIRR* protein in CFA or IFA four times, followed by collection of anti-serum. The Ab titer was determined by microplate-based ELISA, and specificity to the *DsSIGIRR* protein was assessed via Western blot analysis (35, 36).

Western blot analysis

Tissue samples were treated with a lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 50 mM Tris-HCl [pH 7.4]) containing protease inhibitor mixture (Roche). The proteins were separated by 12% SDS-PAGE and transferred onto 0.45-μm polyvinylidenefluoride membranes (Bio-Rad Laboratories, Hercules, CA), then blocked.
with 5% (w/v) nonfat dry milk at 4°C for overnight. After washing with TBST three times for 30 min, the blots were incubated with rabbit anti-DrSIGIRR Ab or other mAbs (Abcam), detected with HRP-conjugated goat anti-rabbit/mouse IgG Ab (Abcam), and visualized with ECL reagents (GE Healthcare), all in accordance with the manufacturers’ protocols.

Coimmunoprecipitation and pull-down assay

Coimmunoprecipitation was performed in either the HEK293T cell line or the zebrafish embryo model. pcDNA6-MyD88 and pFLAG-SIGIRR were transfected into HEK293T cells using FuGENE HD, or pcDNA6-MyD88 and pcDNA6-TRIF were microinjected into one-cell stage zebrafish embryos. At 48 or 24 h posttransfection of microinjection, cells or embryos were lysed with cold lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 7.4]) containing protease inhibitor mixture (Roche) for 30 min at 4°C. Lysates were centrifuged for 15 min at room temperature, washed with PBS twice for 20 min, permeabilized with 0.2% Triton X-100, fixed in 4% paraformaldehyde for 15 min at room temperature, washed with PBS twice for 20 min, permeabilized with 0.2% Triton X-100, blocked with Duolink blocking buffer for 30 min at 37°C, and finally incubated with mouse anti-MyD88 mAb and rabbit anti-Flag mAb. PLA detection was subsequently performed in accordance with the manufacturer’s instructions, and the signals were detected using a laser scanning confocal microscope (Zeiss LSM 710) with a 63 oil immersion objective. The specificity of PLA was identified using negative controls, which consisted of samples incubated with each single species primary Ab but with both species of secondary Abs.

Preparation of lentiviruses

siRNAs against DrSIGIRR or DrTRIF were designed using related siRNA template design tools (31, 37). Short hairpin RNA (shRNA) oligonucleotides containing the selected siRNAs (four for DrSIGIRR and five for DrTRIF) (Supplemental Table I) were synthesized (Invitrogen), annealed, and ligated into the pSUPER.retro.puro vector (Oligoengine, Seattle, WA) digested with BglII and HindIII to generate the constructs of si1, si2, si3, si4, and TRIF1–5. The most efficient shRNAs and H1 promoter were subcloned into a PLB plasmid containing a CMV-EGFP expression cassette...
to produce EGFP-tagged infective viruses (38, 39). Subsequently, the lentiviruses were packaged and propagated in HEK293T cells via cotransfection of PLB, pcMV-VSVG, and pcMV-dR8.2 using FuGENE HD, followed by concentration via ultracentrifugation. The titers of the produced lentiviruses were determined by transduction and flow cytometric analysis of EGFP expression in HEK293T cells (31, 39). The silencing efficacy of the lentiviruses (LV4 for DrSIGIRR and LVTrif for DrTRIF) was evaluated in the liver by real-time PCR after the zebrafish were injected with LV4, LVTrif, or LVC (lentivirus harboring scrambled shRNA as negative control) (2 × 10^5 transduction units/fish) once a day for 3 d.

**Poly(I:C)-induced liver inflammation**

Poly(I:C)-induced liver inflammation was evaluated in zebrafish using previously reported protocols in mice with slight modifications (40). Each zebrafish was i.p. injected with poly(I:C) (Sigma-Aldrich) at a gradient dosage of 25, 50, and 75 μg/g body weight. A moderate concentration (50 μg/g body weight) and a high concentration (50 μg/g body weight + 0.1% DMSO) of poly(I:C) was then administrated into zebrafish once a day for 2 d, and the liver samples were collected at 3, 6, 12, 24, and 48 h after the final injection. Liver inflammatory responses were determined by the enhanced histopathological alterations and the upregulated inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, and IFN1) and IRF3 via real-time PCR. The control fish received PBS at the same doses and time points.

**Histological analysis**

Liver samples were harvested at the indicated time points after poly(I:C) stimulation, fixed in 10% paraformaldehyde, and then embedded in paraffin. Sections (~6 μm) were collected for H&E staining and TUNEL assay using an in situ cell death detection kit, POD (Roche). Histological changes were examined under a light microscope (Zeiss Axiostar Plus) at ×1000 magnification.

**Evaluation of DrSIGIRR and DrTRIF in liver inflammation**

The functions of DrSIGIRR and DrTRIF in poly(I:C)-induced liver inflammation were examined by lentivirus-based siRNA knockdown and antagonist peptide–based inhibition assays. For knockdown assay, zebrafish were injected i.p. with the lentivirus (LV4 or LVTrif or LV4 plus LVTrif) once a day for 3 d as described earlier. For antagonist inhibition assay, zebrafish were pretreated 1 h via i.p. with two TRIF antagonist peptides (with a penetration sequence at the N terminus) derived from a mice model (41, 42). The sequences of the peptides for DrTRIF (DrT1 and DrT2) and control are shown as follows: RQIKIWFQNRRMKWKK-CIEDAI (DrT1) and RQIKIWFQNRRMKWKK-CIEDAI (DrControl). These peptides were synthesized by Leon Chemical (Shanghai) with purity of >95% as determined by HPLC. At 3 d after the final administration of lentiviruses or 1 h after antagonist peptide pretreatment, poly(I:C) was injected i.p. into the zebrafish (50 μg/g body weight), and the control groups received mock PBS only. Liver samples were collected at 6 h after poly(I:C) challenge (10–15 fish in each group) to evaluate the level of liver inflammation based on the changes in cytokine expression and histology.

**Statistical analysis**

Statistical evaluations of the differences between the means of experimental groups were conducted by multiple Student t tests and the data are expressed as mean ± SD. A p value <0.05 was considered statistically significant. All experiments were replicated at least three times independently.

**Results**

**Molecular identification of DrSIGIRR**

The full-length DrSIGIRR cDNA (GenBank accession no. KJ574205) comprises a 1281-bp open reading frame that encodes 426 aa, an 84-bp 5′-UTR, and a 462-bp 3′-UTR (Supplemental Fig. 1). The DrSIGIRR gene consists of nine exons and eight introns, in agreement with many other species (Fig. 1A). Genes adjacent to the DrSIGIRR locus on chromosome 25 (such as Pdxs2, Pkph3, and Ano9) show an overall conservation with those of other species (Fig. 1B). The DrSIGIRR protein is predicted to exhibit a molecular mass of ~48 kDa, with a theoretical isoelectric point of ~5.96. This protein contains a single extracellular Ig-like domain (34–128 aa) distinct from double Ig IL-1R–related molecule (DIGIRR; with two) and IL-1R (with three) molecules, a transmembrane domain (135–157 aa), an intracellular TIR domain (181–327 aa), and a peculiar terminal intracellular tail with 95 aa, all of which are similar to those of the other SIGIRR proteins (Fig. 1C). Multiple sequence alignment showed that DrSIGIRR shares 58–59% amino acid similarities to its mammalian counterparts, with the highest similarities (77–78%) among the TIR domains (Supplemental Fig. 2). Similar to the TIR domains in other SIGIRR proteins, the DrSIGIRR TIR domain is predicted to display three TIR boxes with a conserved tertiary structure containing a central five-stranded parallel β-sheet (βA–βE) surrounded by a total of five α helices (αA–αE) (Fig. 1D) (33). The β-sheet and α helix structures are connected by a number of loops (such as AB, BB, CD, DD, and EE...
loops) based on the strands and helices that they connect. The BB loop, a prominent feature on the surface of the TIR domain, is conserved between zebrafish and mammals (Fig. 1D). The two amino acid sites (Ser 447 and Arg-Tyr536) in the BC loop and a helix E, which are functionally crucial for signal transduction of IL-1R/TLR family members, are substituted by Cys238 and Ala-Leu 322, respectively, and distinguish SIGIRR TIR domains from those of the other family members (Supplemental Fig. 3). Clearly, a high structural conservation of TIR domains exists between DrSIGIRR and other SIGIRRs. Phylogenetic analysis revealed that fish SIGIRRs initially cluster together with fish DIGIRRs, the ortholog of SIGIRR recently identified in fish, then merge with the avian and mammalian SIGIRRs into a large group with high bootstrap probability (Fig. 2). Finally, DrSIGIRR is found to be predominantly distributed on the cell surface membrane, with structural localization similar to that of mammalian SIGIRRs (Fig. 1E) (7, 12).

Functional identification of DrSIGIRR

The classical function of SIGIRR proteins in mammals is its exertion as a negative regulator of MyD88-dependent NF-κB activation in IL-1R/TLR signaling pathways. Therefore, such characteristic of DrSIGIRR was examined using NF-κB Dual-Luciferase reporter system in a zebrafish embryo model for in vivo functional identification purpose. The expression patterns of zebrafish MyD88 (DrMyD88) and DrSIGIRR during embryogenesis were initially analyzed to optimize the time points for their signaling study. The results showed that DrMyD88 was constitutively expressed at different developmental stages (Fig. 3A), whereas DrSIGIRR presented a lower expression before 12 h postfertilization (hpf) and a higher expression at 24 hpf (Fig. 3B). These findings indicated that both the DrMyD88-dependent signaling pathway and the DrSIGIRR-mediated regulatory mechanism were established in the embryo before 24 hpf, suggesting that the embryo is a promising model for in vivo study of DrMyD88 and DrSIGIRR. Nevertheless, the supposed biological significance of the expressed DrMyD88 and DrSIGIRR in the early defense against pathogen invasion or in the regulation of embryonic development remains to be further clarified. As expected, the administration of zebrafish embryos with IL-1R/TLR stimuli (IL-1β and CpG-oligodeoxynucleotides [ODNs]) significantly (p, 0.01) induced NF-κB activation at 24 h after microinjection. In contrast, overexpression of DrSIGIRR in the embryos substantially (p, 0.01) inhibited such activations (Fig. 3C, 3D). To evaluate whether DrSIGIRR plays an inhibitory role in a MyD88-dependent manner, pCMV-MyD88 or pCMV-MyD88 combined with DrSIGIRR mRNA was injected into the embryos. The results showed that overexpression of DrMyD88 significantly (p, 0.01) induced NF-κB activation at 24 h after microinjection, whereas coexpression of DrSIGIRR significantly (p, 0.01) suppressed such activation (Fig. 4B). For further clarification, MO-mediated DrSIGIRR knockdown and mRNA-based rescue experiments were performed at 24 h after microinjection. Accordingly, a SIGIRR-EGFP expression

FIGURE 6. Poly(I:C)-induced liver inflammation. (A) Dosage dependence of poly(I:C)-induced liver inflammation. Adult zebrafish were injected with PBS or 25, 50, and 75 μg/g body weight of poly(I:C) for 3 h. Real-time PCR measured the mRNA levels of different inflammatory cytokines. (B) Liver sections were prepared for H&E staining or TUNEL assay to detect apoptosis. H&E staining of poly(I:C)-treated livers showed evidently distorted hepatic plates, less compact and homogeneous hepatocytes, edema, and spotty necrosis (indicated by the arrow). TUNEL assay demonstrated that more cells underwent apoptosis as compared with the controls. Scale bars, 5 μm. Values in (A) represent the mean ± SD of 10–15 fish for each group from three independent experiments. *p < 0.05, **p < 0.01.
vector containing the DrMO target sequence was first generated to validate the efficacy of DrMO against DrSIGIRR. The SIGIRR-EGFP vector was coinjected into one-cell stage embryos with control MO or DrMO. The data revealed that DrMO can efficiently suppress the expression of DrSIGIRR in zebrafish embryos (Fig. 4A). Correspondingly, DrMyD88-induced NF-κB activation in DrSIGIRR morphants was significantly ($p < 0.01$) upregulated compared with that in wild-type embryos, and such upregulation can be remarkably ($p < 0.01$) inhibited by the administration of DrSIGIRR mRNAs (Fig. 4B). Moreover, coimmunoprecipitation assay showed that DrMyD88 can interact with DrSIGIRR in HEK293T cells (Fig. 4C) and bind to the endogenous DrSIGIRR in zebrafish embryo cells (Fig. 4D). The above observations demonstrate that DrSIGIRR plays a conserved negative regulatory role in MyD88-dependent NF-κB signaling pathways. Thus, SIGIRR is structurally and functionally conserved from fish to mammals throughout the vertebrate evolution, making zebrafish an attractive model for the study of SIGIRR biology.

Inhibitory function of DrSIGIRR in hepatic inflammation

Tissue expression analysis showed that DrSIGIRR yielded the highest expression in the liver at both mRNA and protein levels, which provides initial insights into the involvement of DrSIGIRR in hepatic biology (Fig. 5A, 5B). To investigate the role of DrSIGIRR in liver inflammation, we performed a poly(I:C)-induced hepatic inflammatory assay by administering the adult zebrafish with various concentrations of poly(I:C) at different times. The results showed that poly(I:C) induced a moderate inflammation in the liver at the dosage of $\sim 50 \mu g/g$ body weight during a 3–12 h time period, as determined by the upregulated inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IFN1) and IRF3 (Figs. 6A, 7), as well as the appearance of histological disorders, including distorted hepatic plates, less compact and homogeneous hepatocytes, edema, spotty necrosis via H&E staining, and increased apoptosis, as detected by TUNEL staining (Fig. 6B). Notably, the expression of DrSIGIRR in the liver was found to be significantly declined ($p < 0.01$ or $p < 0.05$) at mRNA and protein levels during the poly(I:C) stimulation, implying that DrSIGIRR plays a profound role in poly(I:C)-induced hepatic inflammation (Fig. 5C, 5D). To provide evidence for this notion, we performed an in vivo DrSIGIRR knockdown assay followed by a lentivirus-based siRNA delivery protocol. Among the four generated constructs harboring four candidate siRNAs targeted to different regions of DrSIGIRR, pSUPER-DrSIGIRR-4 (si4) was identified to be the most effective ($\geq 70\%$) in inducing DrSIGIRR.

**FIGURE 7.** Time dependence of poly(I:C)-induced liver inflammation. Adult zebrafish were injected with poly(I:C) at 50 µg/g body weight once daily for 2 d. Liver samples were collected at 3, 6, 12, 24, and 48 h after the final injection. The mRNA levels of cytokines were measured by real-time PCR. Values represent the mean ± SD of 10–15 fish at each time point from three independent experiments. *$p < 0.05$, **$p < 0.01$. [Liver Imaging Diagram]
mRNA degradation (Fig. 8A). This siRNA encoding sequence was then used to produce the recombinant lentivirus. The generated lentivirus (LV4) after concentration showed a highly infectious efficacy, as determined by most of the HEK293T cells with strongly expressed GFP (Fig. 8D), and reached a titer above $1 \times 10^6$ transduction units/ml, as shown by flow cytometry (Fig. 8E). The LV4 also exhibited a high efficiency (>60%) for DrSIGIRR interference in zebrafish liver via real-time PCR and Western blot analysis (Fig. 8B, 8C). The LV4 was then administered into the fish once a day for 3 d before stimulation with poly(I:C). As expected, knockdown of DrSIGIRR considerably increased the inflammatory responses of liver to poly(I:C) challenge, as shown by the significant upregulation (p < 0.01 or p < 0.05) of TNF-α, IFN-γ, IL-1β, IL-6, IFN1, and IRF3 (Fig. 9A), as well as enhanced histological defects, including massive liver degeneration, necrosis, and apoptosis, compared with those in the control group that received the lentivirus harboring scrambled siRNA (LVc) (Fig. 9B) at 6 h.

**TRIF signaling participates in poly(I:C)-induced hepatic inflammation**

TLR3 is known as the receptor for poly(I:C) that activates downstream signaling through the adaptor protein TRIF in mammals (43, 44). TLR22 is also found to be another functional homolog to TLR3 in teleost fish (45–47). These observations suggest that the TLR3/22-mediated TRIF signaling pathway may participate in poly(I:C)-elicited hepatic inflammation in zebrafish. Therefore, the expression levels of TLR3 and TLR22 were detected to be substantially elevated in the liver after poly(I:C) stimulation (Fig. 10A, 10B). To provide further clarification, two TRIF signaling inhibitory peptides derived from the sequences of the BB loop and the helix B in DrTRIF TIR domain (which were homologous to those in mouse TRIF) were used in the experiment. The results showed that pretreatment of zebrafish with one of these inhibitory peptides (DrT1, not DrT2; data not shown) at 2.5 nmol/g body weight for 1 h considerably inhibited the poly(I:C)-driven hepatic inflammation compared with that of the control group, as determined by the significant decline (p < 0.01 or p < 0.05) of the exaggerated expression of inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, and IFN1) and IRF3 (Fig. 10C). Furthermore, an in vivo TRIF knockdown assay was performed using the lentivirus-based siRNA delivery protocol as described above. A recombinant lentivirus (LVtrif) harboring an encoding sequence for siRNA with the most efficient silencing activity for DrTRIF (>70%) was constructed for this purpose (Fig. 11). This LVtrif was administered into fish once a
day for 3 d before poly(I:C) stimulation. As expected, knockdown of DrTRIF significantly abated the poly(I:C)-induced hepatic inflammation, as shown by the remarkable ($p < 0.01$ or $p < 0.05$) decreases in the excessive production of TNF-$\alpha$, IFN-$\gamma$, IL-1$\beta$, IL-6, IFN1, and IRF3 (Fig. 12), as well as the improvement of the histological damages, including reduced degeneration and apoptosis of the hepatocytes (Fig. 13). Clearly, the above observations suggest that TLR3/22-mediated TRIF signaling plays a major role in poly(I:C)-induced liver inflammation.

Association of DrSIGIRR with TRIF-mediated inflammatory reactions

The above-mentioned DrSIGIRR and DrTRIF knockdown/inhibitory assays, which were conducted independently in the liver under poly(I:C) stimulation, suggested that both DrSIGIRR and DrTRIF are involved in the TRIF-mediated inflammatory reactions. Both molecules play opposite roles in these processes. For further evaluation, the involvement of DrSIGIRR in the TRIF signaling pathway was examined in poly(I:C)-induced hepatic inflammation by a lentivirus-based double-knockdown assay. As expected, the simultaneous knockdown of DrSIGIRR and DrTRIF in the liver significantly impaired the enhanced inflammatory responses to poly(I:C) occurring in the DrSIGIRR single-deficient livers ($p < 0.01$ or $p < 0.05$), as determined by changes in the cytokines (TNF-$\alpha$, IFN-$\gamma$, IL-1$\beta$, IL-6, IFN1, and IRF3) and the histological damages between the two silenced livers (Figs. 12, 13). These results provide preliminary information that DrSIGIRR plays an inhibitory role in the TRIF signaling pathway. To clarify this observation, the zebrafish embryo model was used again to determine the effect of DrSIGIRR on the poly(I:C)- and TRIF-induced

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**FIGURE 9.** Knockdown of DrSIGIRR exhibits stronger liver inflammation after poly(I:C) stimulation. (A) Expression levels of cytokine mRNAs (normalized to $\beta$-actin) were detected by real-time PCR. Values represent mean $\pm$ SD of 10-15 fish in each group from three independent experiments. $^*$ $p < 0.05$, $^{**}$ $p < 0.01$. (B) Histological images of liver tissues from DrSIGIRR normal (LVc) and DrSIGIRR knockdown (LV4) adult zebrafish after injection with PBS or poly(I:C). H&E staining of liver sections showed severe liver necrosis and TUNEL assay revealed that more apoptotic hepatocytes were present in the LV4 group compared with the LVc group. The arrow indicates the site of spotty necrosis. Scale bars, 5 $\mu$m.
NF-κB activations. As expected, poly(I:C) administration of the embryos significantly induced the NF-κB activation at 24 h after microinjection (p < 0.01), whereas overexpression of DrSIGIRR in the embryos significantly inhibited such activation (p < 0.01; Fig. 14A). Similarly, the overexpression of DrTRIF in the embryos at 24 h after microinjection induced significant NF-κB activation in a dose-dependent manner (p < 0.01; Fig. 14B). In contrast, the overexpression of DrTRIF with DrSIGIRR during that stage substantially suppressed the NF-κB activation (p < 0.01; Fig. 14C). Then, MO-mediated DrSIGIRR knockdown assay was performed in the embryos at 24 h after poly(I:C) injection. The results showed that overexpression of DrTRIF induced significantly higher NF-κB activation (p < 0.01; Fig. 14C). The above data provide functional suggestions that DrSIGIRR plays a negative regulatory role in the DrTRIF-mediated inflammatory reactions, probably via the competitive recruitment of the DrTRIF adaptor protein from the TLR3/22-TRIF signaling pathway.

**Discussion**

SIGIRR is an important member of the IL-1R subgroup of the TIR superfamily, which encompasses a group of structurally homologous proteins essential for the modulation of host defenses against infection, inflammation, injury, and stress in mammals (48–50). The IL-1R subgroup members are distinguished by the presence of extracellular Ig-like domains and a conserved intracellular TIR domain activating the NF-κB, MAPK, and JNK pathways (50, 51). The IL-1R family members, including IL-1RI, IL-1RI accessory protein, IL-18 receptors (IL-18Ra and IL-18Rb), and IL-1R-related protein 2, usually contain three Ig-like domains in their extracellular regions and serve as positive regulatory receptors in the IL-1R signaling pathways (1, 52). However, SIGIRR contains only one Ig-like domain and functions as a negative regulator to suppress the Toll/IL-1R (such as TLR4 and IL-1R) signaling pathways (5, 6, 50). Evolutionally, SIGIRR is supposed to originate from a common IL-1R-like ancestor with the loss of distinct functional elements, including two extracellular Ig-like domains.
essential for IL-agonist binding, and the substitution of two conserved amino acid sites (Ser\textsuperscript{447} and Arg-Tyr\textsuperscript{536}) in the TIR domain, which are required for signal transduction (31). Thus, SIGIRR has long been considered as an orphan receptor with unknown ligand molecules. Recently, human SIGIRR was found to act as a coreceptor with IL-18R\textsubscript{a} for the recognition of IL-37, uncovering the existence of a previously unknown mechanism underlying the SIGIRR-mediated inflammatory regulation (3, 53). Although well identified in mammals and predicted to be conserved from chicken to humans on the basis of sequence and expression patterns (54), the occurrence and existence of the SIGIRR homolog in lower vertebrates, including teleost fish, are poorly understood. In the present study, we identified a SIGIRR homolog (\textit{DrSIGIRR}) from zebrafish. This \textit{DrSIGIRR} shares a number of conserved structural and functional features with its mammalian counterparts, as shown by the similar genome synteny, gene organization, tertiary structure of the TIR domain, existence of one Ig-like domain, two substitutions of the key functional amino acid sites, and the negative regulatory function of the MyD88-dependent NF-κB activation. To our knowledge, this work is the first to identify a SIGIRR homolog from teleost fish. SIGIRR originated at least as early as in the fish species, with conserved structures and functions throughout vertebrate evolution. Therefore, our study may contribute to the definition of the evolutionary history of the SIGIRR family and its associated signaling pathways, as well as the development of a zebrafish model for research on SIGIRR biology.

Functionally, SIGIRR has become increasingly attractive because of its critical negative regulation of various Toll/IL-1R–mediated mechanisms of immune homeostasis and tolerance. SIGIRR dysfunction results in various inflammatory diseases, such as lupus nephritis, rheumatoid arthritis, brain or psoriatic inflammation, and disorders of colonic epithelial homeostasis, the latter of which may lead to colonic inflammation and tumorigenesis (11–14, 55, 56). However, limited evidence shows the functions of SIGIRR in the liver. Previous tissue expression analyses showed that SIGIRR widely distributes in various tissues examined (2, 50). However, the expression levels in tissues differ.
Among various species. For instance, mice showed a higher expression of SIGIRR in the kidney, gastrointestinal tract, and lung, swine demonstrated a stronger expression in the kidney and lymph nodes, and chicken exhibited a higher expression in the kidney, gastrointestinal tract, and liver (54, 57). This phenomenon implies the functional diversity of SIGIRR among cellular activities, which was supposed to be the adaptation partly ascribed to the evolutionary pressure (54). In the present study, we showed that DrSIGIRR was highly expressed in the liver and kidney but moderately expressed in the spleen, gill, intestine, and skin, with expression patterns similar to those of several other species, especially humans (58). These observations indicate the important functional involvement of SIGIRR in the liver. To evaluate this notion, we initially performed a dynamic expression analysis of DrSIGIRR in response to poly(I:C) stimulation in the liver. The results showed that DrSIGIRR underwent a significant decline at 6 h after poly(I:C) treatment, which was accompanied by the

**FIGURE 12.** Functional evaluation of DrTRIF and DrSIGIRR in liver inflammation by knockdown assays. The mRNA expression levels of the inflammatory cytokines and IRF3 (shown in each panel) in response to poly(I:C) stimulation were measured by real-time PCR. Values are shown in means ± SD from three independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 13.** Histological changes and apoptotic alterations in DrTRIF and DrSIGIRR knockdown livers under poly(I:C) stimulation. The histological images of liver tissues were obtained from the normal (NM), negative control (LVc), DrTRIF knockdown (LVtrif), DrSIGIRR knockdown (LV4), and the DrTRIF and DrSIGIRR double knockdown (LV4+LVtrif) adult zebrafish after injection with PBS (for the NM group) or poly(I:C) (for the LVc, LVtrif, LV4, and LV4+LVtrif groups). H&E staining and TUNEL assay of liver sections showed the following: healthy tissue structures with little damages and apoptosis (in NM); the appearance of histological disorders with less compact, edema, spotty necrosis, as indicated by the arrow, and apoptotic hepatocytes (in LVc); the improvement of the histological damages with less degeneration and apoptosis of the hepatocytes (in LVtrif); enhanced histological defects with massive liver necrosis and more apoptotic hepatocytes (in LV4); and decreased histological damages with less degeneration and apoptosis of the hepatocytes (in LV4+LVtrif) as compared with the LV4 group. Scale bars, 5 μm.
upregulation of proinflammatory cytokines. As a negative regulator in inflammatory responses, the decreased expression of DrSIGIRR might be the prerequisite for the induction of inflammatory reactions. In support, a similar result was found in the expression pattern of SIGIRR after LPS or infection stimulation in several mouse models (59–62). Consequently, we developed an siRNA-mediated DrSIGIRR-deficient liver model in zebrafish and found that the ablation of DrSIGIRR significantly enhanced the hepatic inflammatory responses to poly(I:C) stimulation. Therefore, DrSIGIRR plays an inhibitory role in liver inflammation. Poly(I:C) is a synthetic analog of viral RNA widely used to mimic virus infection to activate the TLR3 signaling pathway (43). TLR3 is a well-recognized receptor for poly(I:C) that activates downstream signaling via the TRIF adaptor protein, which is conserved between teleost fish and mammals (45, 47, 63). TLR22 is a functional homolog of TLR3 in teleost fish (46). Therefore, the TLR3/22-mediated TRIF signaling pathway may participate in poly(I:C)-induced hepatic inflammation in zebrafish, and DrSIGIRR may serve as a negative regulator during the inflammatory responses. Our study showed that the siRNA depletion of DrTRIF or the administration of a TLR3/22-TRIF signaling inhibitory peptide in the liver significantly inhibited inflammation, whereas the double deficiency of DrTRIF and DrSIGIRR impaired the enhanced inflammation in the livers with single deficiency of DrSIGIRR. With the zebrafish embryo model, the inhibitory effect of DrSIGIRR on the TLR3/22-TRIF signaling pathway was further determined by the downstream NF-κB activation. As expected, the results displayed that overexpression of DrTRIF in DrSIGIRR morphants significantly induced NF-κB activation, whereas such activation can be significantly hampered by DrSIGIRR administration.

Detailed mechanisms of SIGIRR in the negative regulation of Toll/IL-1R signaling were well addressed by the MyD88-dependent signaling pathways. For example, SIGIRR inhibits IL-1R signaling by disturbing the heterodimerization of IL-1RI and IL-1R accessory protein through binding to these molecules with its extracellular Ig domain. The mutated cytoplasmic TIR domain inhibited signaling by attenuating the recruitment of receptor-proximal proteins, such as MyD88, TRAF6, and IRAK to the receptor (6). In contrast, only the TIR domain of SIGIRR was necessary to inhibit TLR4 signaling, probably through its interaction with the receptor via the BB loop region in the TIR domain to prevent the dimerization of the TLR4 and MyD88 complexes for signal transduction (14). However, the mechanism by which SIGIRR participates in the TRIF-mediated signaling pathways remains to be elucidated. In the present study, we explored the possible inhibitory mechanism of DrSIGIRR in the TLR3/22-TRIF signaling pathway. Pull-down binding assay results revealed that the recombinant DrSIGIRR protein can interact with the DrTRIF protein but not obviously with the TLR3 or TLR22 protein (data not shown). Moreover, Duolink in situ PLA assay results showed that the DrSIGIRR protein can bind with DrTRIF in zebrafish embryos. Many previous studies have shown that teleosts TLR3, TLR22, and TRIF share conserved functional domains with their mammalian homologs; zebrafish TRIF can associate with TLR3, RIP1, and TBK1 to initiate NF-κB activation and IFN production (45, 46, 64). Moreover, the TIR domain of TLR3 is responsible for its interaction with TRIF (45). These observations imply that DrSIGIRR may play its negative regulatory role in the TLR3/22-TRIF signaling pathway by competitive recruitment of DrTRIF from the TLR3/22 receptor based on the TIR–TIR interaction. However, further investigations are still needed to clarify the precise molecular mechanisms underlying DrSIGIRR activity in the TLR3/22-TRIF signaling pathway. Additionally, the cellular mechanism of poly(I:C)-induced liver inflammation in mice models is characterized to be NK-mediated, which is dependent on Kupffer cell activation (40). The Kupffer cells activated by poly(I:C) via TLR3 are supposed to express surface Rae-1 and produce soluble IL-12, IL-18, and TNF-α. Rae-1 expression on Kupffer cells directly activated NK cells by its interaction with NKG2D. IL-12, IL-18, and TNF-α indirectly stimulated NK cells to produce IFN-γ. Finally, the NK cell–derived IFN-γ and the Kupffer cell–derived TNF-α synergistically induced
hepatocyte damage (65, 66). However, the cellular mechanisms underlying the influence of SIGIRR on poly(I:C)-triggered hepatic inflammation remain to be clarified. These mechanisms may largely depend on completely understanding the precise cellular distribution of SIGIRR in Kupffer cells, NK cells, or other cell types in the liver.

Animal models are powerful tools for exploring major issues in immunology and the disease mechanisms that are difficult to address.

**FIGURE 15.** Detection of the negative regulation of DrSIGIRR in TRIF-mediated IFN production and interaction between DrSIGIRR and DrTRIF. (A) DrSIGIRR negatively regulates TRIF-mediated IFN production. pEGFP-TRIF (60 pg/embryo) alone, with DrMO (4 ng/embryo), or with capped DrSIGIRR mRNA (100 pg/embryo) and DrMO was injected into zebrafish embryos in the one-cell stage. Empty plasmid (pEGFP-N1) or DrSIGIRR mRNA alone was injected into respective one-cell stage embryos as negative controls. Real-time PCR was performed to detect the expression levels of IRF3 and IFN1 at 24 h after microinjection with three replicates (each containing 30–50 embryos). Values represent the mean ± SD. *p < 0.05, **p < 0.01. (B) Association of DrSIGIRR with DrTRIF detected by pull-down assay. Myc-tagged DrTRIF and Flag-tagged DrSIGIRR were transiently transfected into respective HEK293T cells. At 24 h posttransfection, cells were lysed and supernatants of Flag-SIGIRR and Myc-TRIF were incubated at 4˚C overnight. The proteins were immunoprecipitated with rabbit anti-DrSIGIRR Ab or rabbit IgG as a negative control for 6 h at 4˚C, followed by Western blot analysis with mouse anti-Myc mAb (Myc) and rabbit anti-Flag mAb (Flag). The experimental groups (c and d) were incubated with mouse anti-Myc mAb and rabbit anti-Flag mAb (Myc+Flag) simultaneously. Red fluorescence was shown in groups (c) and (d), which indicated the protein interaction signal. The nuclei were stained with DAPI in blue. Scale bar, 5 μm.
in humans. Aside from the widely used mouse models, the zebrafish model has attracted considerable interest from researchers because of its conserved innate immunity and numerous advantages for investigations, such as its high fecundity, rapid development, easy visualization, and genetic manipulation. The zebrafish has been extensively used in studies of developmental and comparative immunology and in modeling human diseases, such as T cell acute lymphoblastic leukemia (67), diabetes mellitus (68), Alagille syndrome, alcoholic liver disease (69), and hepatocellular carcinoma (70). In the present study, the zebrafish model showed its advantages for uncovering the involvement of SIGIRR in hepatic inflammation, which indicates a new function of SIGIRR in liver homeostasis. We think that the zebrafish model, as a complement to mammalian models, will greatly benefit the cross-species understanding of liver immunity and diseases.

Disclosures

The authors have no financial conflicts of interest.

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