**Cynoglossus semilaevis** ISG15: A Secreted Cytokine-Like Protein That Stimulates Antiviral Immune Response in a LRGG Motif-Dependent Manner

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**Abstract**

ISG15 is an ubiquitin-like protein that is induced rapidly by interferon stimulation. Like ubiquitin, ISG15 forms covalent conjugates with its target proteins in a process called ISGylation, which in mammals is known to play a role in antiviral immunity. In contrast to mammalian ISG15, the function of teleost ISG15 is unclear. In this study, we identified and analyzed the function of an ISG15 homologue, CnISG15, from tongue sole (*Cynoglossus semilaevis*). CnISG15 is composed of 162 residues and possesses two tandem ubiquitin-like domains and the highly conserved LRGG motif found in all known ISG15. Expression of CnISG15 occurred in a wide range of tissues and was upregulated in kidney and spleen by viral and bacterial infection. In vitro study with primary head kidney (HK) lymphocytes showed that megalocytivirus infection caused induction of CnISG15 expression and extracellular release of CnISG15 protein. Purified recombinant CnISG15 (rCnISG15) activated HK macrophages and enhanced the expression of immune genes in HK lymphocytes, both these effects, however, were significantly reduced when the conserveld LRGG sequence was mutated to LAAG. Further study showed that the presence of rCnISG15 during megalocytivirus infection of HK lymphocytes reduced intracellular viral load, whereas antibody blocking of CnISG15 enhanced viral infection. Likewise, interference with CnISG15 expression by RNAI promoted viral infection. Taken together, these results indicate that CnISG15, a teleost ISG15, promotes antiviral immune response and that, unlike mammalian ISG15, CnISG15 exerts its immunoregulatory effect in the form of an unconjugated extracellular cytokine. In addition, these results also suggest a role for the LRGG motif other than that in protein conjugation.

**Introduction**

Interferons (IFNs) play an important role in the innate immunity against viral infection. IFNs bind to their cognate receptors on the target cells and activate the signal transduction pathways involving Jak kinases and the transcription factors of the STAT family [1, 2], which in turn activate the transcription of hundreds of IFN-stimulated genes (ISGs) [3, 4]. Among the identified ISGs are a group of proteins called ISG15, which are small ubiquitin-like proteins induced rapidly by IFN stimulation [5, 6].

ISG15 was first identified in humans as a ~15 kDa protein derived from a 165-residue precursor [7]. Subsequently, ISG15 homologues were discovered in diverse vertebrate species including fish. All ISG15 proteins possess two ubiquitin-like (UBL) domains and a highly conserved C-terminal LRGG sequence, the latter being known as the ubiquitin conjugation motif [8]. In mammals, both intracellular and extracellular ISG15 have been detected. Intracellular ISG15 are conjugated, via the LRGG motif, to target proteins through a process called ISGylation, which resembles largely ubiquitination, the process of formation of ubiquitin conjugates. Ubiquitination involves three enzymes, i.e., ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). E1 catalyzes adenylylation of the C-terminal di-glycine sequence in the LRGG motif, which is exposed after proteolytic cleavage, while E2 and E3 catalyze transferring of the ubiquitin moiety to the substrate protein [9]. In most cases, the first ubiquitin molecule is attached to the substrate protein through a linkage formed between the C-terminal glycine residue of ubiquitin and a lysine residue of the substrate protein [10]. Poly-ubiquitination is achieved by successive attachment of new ubiquitin molecules to the conjugated ubiquitins. Similar to ubiquitination, ISGylation begins by adenylylation of the C-terminal di-glycine sequence of the LRGG motif, which is followed by successive transfer of ISG15 from E1, E2 and E3 enzymes to the target protein [11]. Unlike ubiquitination, which is known to function in protein and immune regulation [12, 13], the function and biological significance of ISGylation remain elusive. However, recent evidences suggest an involvement of ISG15, in the form of conjugated protein modifiers, in regulation of IFN signaling and in antiviral immunity [9, 14]. Both ubiquitin and ISG15 have been found to exist extracellularly in unconjugated forms. Extracellular ubiquitins are known to inhibit secretion of tumor necrosis factor
(TNF-α and TNF-β mRNA expression from peripheral blood mononuclear cells in response to endotoxin [15]. Likewise, unconjugated extracellular ISG15, which are released from several types of human and murine cells, are known to possess cytokine-like activity [16–20].

ISG15-like sequences from a number of teleost species have been reported, most of which were found to be positively regulated in expression by microbial challenge [21–26]. Recent studies showed that the ISG15 homologues of Atlantic cod (Gadus morhua L.), Atlantic salmon (Salmo salar), and goldfish (Carassius auratus) are capable of forming conjugates with cellular proteins [27–29], and the ISG15 of red drum (Sciaenops ocellatus) exhibits immunoregulatory property [30]. However, except these few cases, the activity and biological function of teleost ISG15 are essentially unknown. In this study, we identified an ISG15 homologue, CsISG15, from tongue sole (Cynoglossus semilaevis) and analyzed its potential function. Our results showed that CsISG15 is a secreted cytokine-like protein that, unlike mammalian ISG15, possesses antiviral capacity in the unconjugated form.

**Results**

**Characterization of the sequence of CsISG15**

The cDNA of CsISG15 is 1159 bp, which contains a 5'-UTR of 57 bp, an open reading frame of 489 bp, and a 3'-UTR of 613 bp (Fig. 1). The cDNA is terminated with a poly-A tail formed by 25 adenines. A putative polyadenylation signal, AATAAA, is located 16 bp upstream the poly-A tail. The deduced amino acid sequence of CsISG15 is 162-residue in length and has a calculated molecular mass of 18.3 kDa and a theoretical pI of 9.7. CsISG15 contains two tandem UBL domains (residues 1 to 76 and 81 to 151, respectively) and the conserved C-terminal LRGG motif, the latter being followed by seven residues that terminate the CsISG15 sequence. No putative signal peptide or glycosylation site was found in CsISG15. Blast analysis showed that CsISG15 shares 41.5–61.6% overall sequence identities with the ISG15 of the teleosts Oplegnathus fasciatus, Epinephelus coioides, Paralichthys olivaceus, Sebastes schlegeli, Anoplopoma fimbria, Channa argus, Oncorhynchus mykiss, Salmo salar, and Danio rerio (Figure S3). The overall sequence identities between CsISG15 and human and mouse ISG15 are 30.5% and 27.1% respectively.

Figure 1. The nucleotide and deduced amino acid sequences of CsISG15. The nucleotides and amino acids are numbered along the left margin. In the nucleotide sequence, the translation start and stop codons are in bold, and the polyadenylation signal is underlined. In the amino acid sequence, the two ubiquitin-like (UBL) domains are shaded, and the ubiquitin conjugation motif (LRGG) is boxed.

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Tissue specific expression of *CsISG15* under normal physiological conditions and during pathogen infection

qRT-PCR analysis showed that under normal physiological conditions, *CsISG15* expression was detected, in increasing order, in liver, gill, muscle, kidney, spleen, brain, heart, and gut (Fig. 2). The expression level in gill, muscle, kidney, spleen, brain, heart, and gut were, respectively, 1.1-, 4-, 5.9-, 6-, 11.9-, 20-, and 26.4-fold higher than that in liver. To examine whether *CsISG15* expression was affected by pathogen infection, tongue sole were infected experimentally with the viral pathogen megalocytivirus or the bacterial pathogen *V. anguillarum*, and *CsISG15* expression in kidney and spleen was subsequently determined by qRT-PCR at 1 h, 4 h, 12 h, 24 h, and 48 h post-infection. The results showed that both megalocytivirus and *V. anguillarum* induced *CsISG15* expression in time dependent manners (Fig. 3). In kidney, megalocytivirus infection caused significant inductions (3.2-, 38.2-, and 9.3-fold respectively) of *CsISG15* expression at 4 h, 12 h, and 24 h post-infection, while *V. anguillarum* infection caused significant induction (33.4 fold) at 48 h post-infection (Fig. 3A). In spleen, both megalocytivirus and *V. anguillarum* induced significant *CsISG15* expression at 1 h, 4 h, and 12 h post-infection, with peak inductions (20- and 32.6-fold respectively) occurring at 12 h and 4 h post-infection respectively (Fig. 3B). Consistent with the qRT-PCR results, Western blot analysis showed that *CsISG15* protein increased with megalocytivirus infection and reached peak levels at 12 h in kidney and spleen (Fig. 4).

CsISG15 expression and subcellular localization in HK lymphocytes

To examine the effect of pathogen infection on *CsISG15* expression at cellular level, tongue sole HK lymphocytes were infected with or without megalocytivirus, and *CsISG15* expression was determined by qRT-PCR at 1 h, 2 h, 4 h, 8 h, and 16 h post-infection. The results showed that significant inductions of *CsISG15* expression were observed at all the examined time points, with maximum induction (75 fold) occurring at 4 h post-infection (Fig. 5). To examine whether *CsISG15* was secreted into the...
extracellular milieu as it was observed with human ISG15, HK lymphocytes were infected with or without (control) megalocytivirus, and CsISG15 expression was determined by quantitative real time RT-PCR at various time points. At each point, the expression level of the control cells was set as 1. Values are shown as means±SE (N=3). N, the number of repeated experiments. *P<0.01, **P<0.05. (B, C, and D) Tongue sole HK lymphocytes were infected with (lane 3) or without (lane 2) megalocytivirus for 4 h, and extracellular proteins were subjected to immunoblot with antibodies against CsISG15 (B) or β-actin (C). To demonstrate that the β-actin antibody was able to detect β-actin when it was present, cytoplasmic proteins were prepared and blotted with β-actin antibody (D). Lane 1, protein marker.

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Biological effect of rCsISG15 and its dependence on the conserved LRGG motif

(i) Effect on macrophage activation. Since, as observed above, CsISG15 was a secreted protein, we examined its potential biological function by determining whether it had any effect on macrophage and lymphocyte immune response. In addition, to examine the functional importance of the LRGG motif, we also prepared and analyzed the effect of a mutant CsISG15, CsISG15M, which bears alanine substitutions at the RG residues of the LRGG sequence (i.e., LRGG was mutated to LAAG). HK macrophages were treated with or without rCsISG15, rCsISG15M, or LPS for various hours and then analyzed for respiratory burst and NO production. The results showed that significantly increased respiratory burst activity was observed in cells treated with rCsISG15 and LPS but not in cells similarly treated with rCsISG15M (Fig. 6A). NO production analysis showed that macrophages treated with rCsISG15M produced significantly higher amounts of NO after 0.5 h, 1 h, 2 h, 4 h, and 12 h treatment, while macrophages treated with rCsISG15M exhibited significant NO induction after 1 h, 2 h, 4 h, and 12 h treatment, however, the induction levels induced by rCsISG15M were significantly lower than those induced by rCsISG15 (Fig. 6B).
(ii) Effect on lymphocyte immune response. To examine the potential effect of rCsISG15 on lymphocyte immune response, HK lymphocytes were treated with or without rCsISG15 or rCsISG15M and then subjected to qRT-PCR analysis of the expression of IL-1β, CsIL8 (a tongue sole homologue of IL-8), TLR9, MHC IIβ, CsCCK1 (a CC chemokine), and CsCXCe1 (a CXC chemokine), which are the major immune relevant genes known in tongue sole. The results showed that treatment with rCsISG15 induced significant inductions of all the examined genes in a time-dependent manner, with drastic inductions (maximum levels of 21–73 fold) occurring in CsIL8, CsCCK1, and CsCXCe1 expression, and moderate inductions (maximum levels of 5–7 fold) occurring in IL-1β, TLR9, and MHC IIβ expression (Fig. 7A). In cells treated with rCsISG15M, the induction patterns of CsIL8, CsCCK1, and CsCXCe1 were similar to those in rCsISG15-treated cells but the induction folds were in general significantly lower than those in rCsISG15-treated cells. No significant difference was observed between the inductions of TLR9 induced by rCsISG15M and rCsISG15. For MHC IIβ and IL-1β, the induction patterns induced by rCsISG15M differed from those induced by rCsISG15. The peak induction of MHC IIβ induced by rCsISG15M was ~2-fold lower than that induced by rCsISG15, while the peak induction of IL-1β induced by rCsISG15M was comparable to that induced by rCsISG15.

(iii) Effect on lymphocyte resistance against viral infection. The above results led us to wonder whether the immunoregulatory effect observed with rCsISG15 on lymphocytes would have any effect on cellular resistance against viral infection. To examine this question, HK lymphocytes were treated with or without rCsISG15, rCsISG15M, or E. coli preparation before being infected with megalocytivirus. Viral copy numbers in the infected cells were then determined at 2 h and 4 h post-infection. The results showed that at both time points, the viral copies in rCsISG15- and rCsISG15M-treated cells were significantly lower than those in the control cells (Fig. 8A). However, compared to rCsISG15-treated cells,
To examine whether interference with CsISG15 expression would have any effect on viral infection, HK lymphocytes were subjected to RNAi assay by being transfected with pRiCsISG15 and pRNAT-CMV3.1 (control) and pRNAT-CMV3.1 (control) were infected with megalocytivirus. Viral numbers in the cells were determined at 4 h post-infection. Data are shown as means ± SE (N = 5). N, the number of repeated experiments. **P < 0.01. doi:10.1371/journal.pone.0044884.g009

Figure 9. Effect of CsISG15 RNAi on viral infection. Tongue sole head kidney lymphocytes transfected with pRiCsISG15, pRN1, and pRNAT-CMV3.1 (control) were infected with megalocytivirus. Viral numbers in the cells were determined at 4 h post-infection. Data are shown as means ± SE (N = 5). N, the number of repeated experiments. Significance between RNAi and the control is indicated with asterisks.

rCsISG15M-treated cells exhibited significantly higher numbers of viral numbers at 4 h post-infection. In contrast, E. coli preparation had no effect on viral infection.

Antibody neutralization of CsISG15 and its effect on viral infection

With the above results, i.e., CsISG15 was released into the culture supernatant of lymphocytes and able to stimulate lymphocyte immune response against viral infection, we wondered whether antibody blocking of the released CsISG15 would have any effect on viral infection. To examine this question, HK lymphocytes were infected with megalocytivirus in the presence or absence of anti-rCsISG15 serum or preimmune serum for 2 h and 4 h post-infection. Values are shown as means ± SE (N = 5). N, the number of repeated experiments. *P < 0.05; **P < 0.01. doi:10.1371/journal.pone.0044884.g008

Interference with CsISG15 expression and its effect on viral infection

To examine whether interference with CsISG15 expression would have any effect on viral infection, HK lymphocytes were subjected to RNAi assay by being transfected with pRiCsISG15 and pRN1, which expressed CsISG15-specific siRNA and a nonspecific siRNA respectively. As a control, the cells were also transfected with the backbone vector pRNAT-CMV3.1. To
Considering the E. coli exhibits cytokine-like activity and is able to induce natural killer cell proliferation and IFN-γ production by T lymphocytes [15–18]. In our study, we found that rCsISG15 stimulated the respiratory burst and NO production of HK macrophages and upregulated the expression of immune relevant genes in HK lymphocytes. These results suggest that rCsISG15 possesses immunoregulatory property. Since CsISG15, like some fish ISG15 such as those of Paralichthys olivaceous, Sciaenops ocellatus, and Omosychnus mykiss, has a C-terminal extension, which is a seven-residue sequence in CsISG15, beyond the LRGG motif, and this extension was not removed from rCsISG15, it is likely that cleavage of the C-terminal extension, as is observed with mammalian ISG15 during ISGylation, is not necessary for the biological activity of CsISG15 as an extracellular cytokine. Mutational study showed that, in contrast to rCsISG15, the mutant rCsISG15M had no apparent effect on the respiratory burst activity of HK macrophages and, compared to rCsISG15, much reduced though significant effect on NO production. These results suggest that the LRGG motif is required for the optimum activity of CsISG15 as an extracellular cytokine. It is possible that the LRGG motif, besides being participating in conjugate formation with intracellular substrate proteins, has additional functions such as being involved in interactions with cell surface proteins that serve as receptors for extracellular CsISG15.

qRT-PCR analysis showed that rCsISG15 drastically upregulated the expression of CsIL8, CsCCK1, and CsCXCl1, suggesting that rCsISG15 is a strong inducer of both CC and CXC chemokines. In addition to chemokines, rCsISG15 also upregulated the expression of TLR9 and the proinflammatory cytokine IL-1β. These results indicate that rCsISG15 is a stimulator of innate immunity, which may at least in part account for the anti-microbial property of rCsISG15. Compared to rCsISG15, rCsISG15M exhibited, in most cases, significantly lower stimulatory effects on the expression of immune genes, which is in line with the observation that rCsISG15M was impaired in the ability to activate HK macrophages and to block viral infection.

Previous studies have indicated that mammalian ISG15 possess a broad-spectrum antiviral activity against a wide range of DNA and RNA viruses, and in most cases the antiviral activity is dependent on ISGylation of either host or viral proteins [9,14]. However, a recent study of Chikungunya virus infection in a murine model showed that the antiviral effect of mouse ISG15 is independent on its ability to form conjugates with substrate proteins, which suggests a non-classical role for ISG15 during viral infection [33]. In our study, we found that treatment of HK lymphocytes with rCsISG15 before megalocytivirus infection reduced intracellular viral load, whereas interference with CsISG15 expression drastically increased intracellular viral load, suggesting that rCsISG15 is required for optimum resistance against megalocytivirus infection. Since rCsISG15 induced significant inductions of CsIL8, CsCCK1, CsCXCl1, IL-1β, and TLR9, which are involved in innate immunity against pathogen infection, it is likely that the anti-megalocytivirus effect of rCsISG15 is the result of augmented cellular immune response rather than a direct interaction between rCsISG15 and the viral pathogen. In line with these observations, the presence of rCsISG15 antisera in the culture medium of HK lymphocytes during megalocytivirus infection increased the intracellular viral load to significant extents, suggesting that interaction between anti-rCsISG15 antibodies and the released CsISG15 most likely blocked the activity of CsISG15, thus resulting in enhanced viral infection.

In conclusion, we showed in this study that CsISG15 is upregulated in expression by microbial pathogens and that, upon viral infection, CsISG15 is released into the extracellular milieu, where the protein probably acts as a cytokine and activates the response of various immune cells, which leads to elevated host defense against viral infection. Hence, it appears that, unlike mammalian ISG15, whose antiviral effects are mediated mostly through intracellular protein modification, CsISG15 exerts its immunoregulatory activity extracellularly as an unconjugated protein. Our results also indicate for the first time that the conserved LRGG motif of a teleost ISG15 is not essential to but required for the optimum activity of ISG15 as an extracellular cytokine, which suggests a role for the LRGG motif other than that in ISGylation. Taken together, these results support the conclusion that CsISG15 functions as an unconjugated extracellular cytokine that promotes antiviral immunity in a LRGG motif-dependent manner.

Materials and Methods

Ethics statement

Experiments involving live fish and rats were conducted in accordance with the “Regulations for the Administration of Affairs Concerning Experimental Animals” promulgated by the State Science and Technology Commission of Shandong Province.

Fish

Tongue sole (Cynoglossus semilaevis) were purchased from a commercial fish farm in Shandong Province, China and maintained at 22°C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before each experiment, fish were randomly sampled for the examination of bacterial recovery from internal organs (blood, liver, kidney, and spleen), and no bacteria were detected from any of the examined fish. Fish were euthanized with tricaine methanesulphonate (Sigma, St. Louis, MO, USA) before tissue collection.

Cloning of CsISG15

A cDNA library of tongue sole head kidney and spleen was constructed as described previously [34]. One thousand and five hundred clones of the library were randomly selected and subjected to DNA sequence analysis; one clone was found to contain the cDNA of CsISG15 with 5′ - and 3′ - untranslated regions (UTRs). The nucleotide sequence of CsISG15 has been deposited in GenBank database under the accession number JN967757.

Sequence analysis

The cDNA and amino acid sequences of CsISG15 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple
modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Multiple sequence alignment was performed with the ClustalX program. Signal peptide search was performed with SignalP 3.0.

Plasmid construction and mutation of CsiSG15

To construct pETCsISG15, which expresses a His-tagged CsiSG15, the coding sequence of CsiSG15 was amplified by PCR with primers CsiSGF1 (5'-GATATCTGGAATAACCATCACAA3'; underlined sequence, EcoRV site) and CsiSGR1 (5'-GATATGCACAATGAGCGTTG3'; underlined sequence, EcoRV site); the PCR products were ligated with the T-A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsiSG15-containing fragment, which was inserted into pET259 [35] at the EcoRV site. To construct pETCsISG15M, which expresses a mutant CsiSG15, CsISGM15M, bearing alanine substitutions at R153 and G154 (so that the conserved LRGG motif was mutated to LAAG), a two-step PCR was carried out as follows: the first PCR was performed with primers CsISGF1 and MR3 (5'-GCCAATGACGGTGTGTCCTGCCTGCGCAGACGCACTG3'); the PCR products were diluted 100×, and the dilution was used as a temperate for the second PCR with primers CsISGF1 and CsISGR1. The PCR products were inserted into pET259 at the EcoRV site as described above, resulting in pETCsISG15M.

Purification of recombinant protein and preparation of antiserum

Escherichia coli Rosetta (DE3) (Biotek, Beijing, China) was transformed with pETCsISG15, pETCsISG15M, and the empty vector pET259, resulting in transformants DE3/pETCsISG15, DE3/pETCsISG15M, and DE3/pET259 respectively. The transformants were cultured in Luria-Bertani broth (LB) medium at 37°C to mid-log phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM. After growth at 18°C for an additional 10 h, recombinant protein was purified using nickel-nitrilotriacetic acid columns (GE Healthcare, USA) as recommended by the manufacturer. Purified proteins and the preparation from DE3/pET259 (named E. coli preparation) were dialyzed for 24 h against phosphate-buffered saline (PBS) and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250 (Figure S1). The concentrations of the purified proteins were determined using the Bradford method with bovine serum albumin as a standard.

To prepare anti-CsISG15 antibodies, adult rats (purchased from the Institute for Drug Control, Qingdao, China) were immunized via subcutaneous injection with rCsISG15 mixed in complete Freund’s adjuvant. The rats were boosted at 20, 32, and 45 days after the initial immunization. The rats were bled 14 days after the last boost, and sera were obtained from the blood. The titer and specificity of the serum antibodies were determined by enzyme-linked immunosorbent assay and Western immunoblot analysis as described previously [36]. Western immunoblot analysis showed that anti-rCsISG15 serum could bind to rCsISG15 and rCsISG15M but not to purified rCsFerM, a recombinant ferritin of tongue sole [34] (Figure S2 and data not shown).

CsiSG15 expression in fish tissues

(i) CsiSG15 expression under normal physiological conditions. Brain, muscle, heart, gut, kidney, spleen, gill, and liver were taken aseptically from five fish (~12.7 g) and used for total RNA extraction with the RNeasy Kit (Qiagen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real time reverse transcriptase-PCR (qRT-PCR) was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [37]. PCR efficiency (99.9%) was determined as described previously [38]. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of CsiSG15 was analyzed using comparative threshold cycle method (2−ΔΔCT) with β-actin as a control. The primers used to PCR β-actin gene were reported previously [39]. The primers used to PCR CsiSG15 were CsISG15RTF1 (5'-CTACGGTCTTCGTTTCTGAC-3') and CsISG15RTR1 (5'-CACCCCTGCGCTTAAAGTTGG-3'). All PCR were performed in triplicate, and the data are given in terms of mRNA levels relative to that of β-actin and expressed as means plus or minus standard errors of the means (SE).

(ii) CsiSG15 expression in response to pathogen infection. The fish bacterial pathogen Vibrio anguillarum C312 [37] was cultured in LB medium and resuspended in PBS to 1×106 colony forming units (CFU)/ml. Tongue sole (~12.7 g) were divided randomly into three groups (N = 30) and injected intraperitoneally with 100 μl PBS containing 106 CFU V. anguillarum or 104 copies of the megalocytivirus RBIV-C1 [40] or with 100 μl PBS alone (control). Fish (five for each time point) were sacrificed at 1 h, 4 h, 12 h, 24 h, and 48 h post-infection, and tissues were taken under aseptic conditions. For mRNA expression analysis, total RNA was extracted and used for qRT-PCR as described above. For Western blot analysis, proteins were extracted from 33 mg of tissues with the Sample Grinding Kit (GE Healthcare, New Jersey, USA), and equal volumes of protein preparations were loaded into SDS-PAGE gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham, Cambridge, UK). Immunoblot was performed as described previously [41] using anti-CsISG15 antibodies or anti-β-actin antibody (Bioss, Beijing, China).

CsiSG15 expression and localization in head kidney (HK) lymphocytes

HK lymphocytes were prepared as described previously [42] and cultured in 96-well tissue culture plates containing L-15 medium (Thermo Scientific HyClone, Beijing, China) supplemented with 10% calf serum. The culture was maintained at 25°C. To examine the effect of viral infection on CsiSG15 expression, megalocytivirus RBIV-C1 was suspended in L-15 to 104 copies/ml. HK lymphocytes were washed and added with fresh L-15 containing or not containing megalocytivirus (104 copies/well). The cells were incubated at 25°C for 1 h, 2 h, 4 h, 8 h, and 16 h respectively and washed 3× with PBS. The cells were then examined for CsiSG15 mRNA expression and extracellular production of CsiSG15 protein. For mRNA expression analysis, total RNA was prepared from the cells with Total RNA Kit I of Omega Bio-tek (Norcross, GA, USA) and used for qRT-PCR analysis of CsiSG15 expression as described above. For extracellular protein production analysis, the culture supernatant of 5×105 cells was collected by centrifugation and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica,
Effect of rCsISG15 on macrophage activation

(i) Preparation of HK macrophages. HK macrophages were prepared based on the method of Chung and Secombes [43]. Briefly, HK was removed from two healthy tongue sole (Sangon, Shanghai, China) in L-15 medium containing 10% calf serum, 1% penicillin and streptomycin (Sangon, Shanghai, China), and 20 U/ml heparin (Sigma, St. Louis, MO, USA). The cells were added with 96-well tissue culture plates (~2×10^5 cells/well) and incubated at 25°C for 2 h. After incubation, the cells were adhered to a final concentration of 100 ng/ml. After incubation at 25°C for 2 h, respiratory burst activity was determined as reported previously [43]. In brief, 100 μl of 1 mg/ml nitroblue tetrazolium (Sangon, Shanghai, China) in L-15 was added to macrophages. After incubation at 25°C for 2 h, the reaction was stopped by adding 100% methanol. The plate was washed with 70% methanol, and the reduced formazan was solubilized in 100 μl of 2 M KOH and 120 μl of DMSO. The plate was read at 560 nm with a microplate reader.

(ii) Respiratory burst assay. rCsISG15 and rCsISG15M were diluted in L-15 medium to 1000 ng/ml. Macrophages were added with or without rCsISG15, rCsISG15M, or E. coli lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) in L-15 to a final concentration of 100 ng/ml. After incubation at 25°C for 2 h, respiratory burst activity was determined as described previously [43]. In brief, 100 μl of 1 mg/ml nitroblue tetrazolium (Sangon, Shanghai, China) in L-15 was added to macrophages. After incubation at 25°C for 2 h, the reaction was stopped by adding 100% methanol. The plate was washed with 70% methanol, and the reduced formazan was solubilized in 100 μl of 2 M KOH and 120 μl of DMSO. The plate was read at 560 nm with a microplate reader.

(iii) Nitrite assay. HK macrophages as described above were added with or without rCsISG15 or rCsISG15M in L-15 to a final concentration of 100 ng/ml. After incubation at 25°C for 2 h, nitric oxide (NO) production was determined as described by Tafalla and Novoa [44]. In brief, the supernatants were removed to a separate 96-well plate (50 μl/well) and 100 μl of 0.1% N-naphthylethylene-diamine (Sigma) in 2.5% phosphoric acid was added to each well, followed by adding 100 μl of 0.1% N-naphthylethylene-diamine (Sigma) in 2.5% phosphoric acid. The plate was read at 540 nm, and the molar concentration of nitrite was determined from standard curves generated using known concentrations of sodium nitrate.

Effect of rCsISG15 and rCsISG15M on immune gene expression

HK lymphocytes prepared above were added with or without rCsISG15 or rCsISG15M in L-15 to a final concentration of 100 ng/ml. The plates were incubated at 25°C for 1 h, 2 h, 4 h, and 8 h, and total RNA was prepared from the cells with the Total RNA Kit I of Omega Bio-tek (Beijing, China) and used for qRT-PCR analysis of the expression of the genes encoding toll-like receptor 9 (TLR9), interleukin (IL) 1β (IL-1β), CsIL8 (a homologue of IL-8) [39], CsCCK1 (a CC chemokine) [45], CsCXCl1 (a CXC chemokine) [46], and major histocompatibility complex (MHC) class IIβ as described above. These genes were selected because they are the major immune relevant genes that are available in sequence. The primers used to PCR the immune genes have been reported previously [46].

Effect of rCsISG15 and rCsISG15M on cellular resistance against viral infection

HK lymphocytes prepared above were added with or without rCsISG15 or rCsISG15M in L-15 to a final concentration of 100 ng/ml. As a control, the preparation from DE3/pET259, i.e., E. coli preparation, prepared above under the same conditions as rCsISG15 was also added to lymphocytes at the same volume as rCsISG15. The plates were incubated at 25°C for 2 h. After the incubation, the cells were added with L-15 containing or not containing megalocytivirus (10^5 copies/well). The plates were incubated at 25°C for 2 h and 4 h respectively, followed by washing 3× with PBS. Viral copy number in the infected cells was determined by absolute quantitative real time PCR as reported previously [40].

Effect of anti-r-CsISG15 serum on viral infection

Anti-r-CsISG15 serum and preimmune serum were diluted 2000× in L-15. HK lymphocytes prepared above were added with or without 100 μl of the diluted anti-r-CsISG15 serum or preimmune serum, followed by adding 100 μl L-15 containing or not containing megalocytivirus (10^5 copies/well). The plates were incubated at 25°C for 2 h and 4 h respectively. After incubation, the plates were washed 3× with PBS, and intracellular viral copy number was determined by absolute quantitative real time PCR as reported previously [40].

RNAi and its effect on viral infection

RNAi was performed with the DNA vector-based siRNA technology. For this purpose, the siRNA expression vector pRNAT-CMV3.1 (GenScript, Piscataway, NJ, USA) was used, in which the CMV promoter drives the expression of siRNA that can be inserted into the vector between BamH I and All II sites. In our study, the CsISG15-specific siRNA (5'-GCTCTGACACCGATT-GACG-3') and a nonspecific siRNA (5'-GCTTGTAGTCTCAGGACGAG-3') were inserted into pRNAT-CMV3.1, resulting in plasmids pRiCsISG15 and pRiNC1 respectively. RNAi was carried out by transient transfection of HK lymphocytes with pRiCsISG15, pRiNC1, and pRiNT-CMV3.1 (backbone vector) respectively using Lipofect (Tiangen, Beijing, China) according to manufacturer’s instructions. The transfectants were infected with megalocytivirus for 4 h and then examined for CsISG15 expression by qRT-PCR and immunoblot analysis as described above. To examine the effect of RNAi on viral infection, pRiCsISG15-, pRiNC1-, and pRiNT-CMV3.1-transfected cells in 96-well culture plates were infected with megalocytivirus for 4 h and determined for intracellular viral load as described above. The assay was performed independently for five times.

Statistical analysis

All statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as P<0.05.

Supporting Information

Figure S1 SDS-PAGE analysis of rCsISG15 and rCsISG15M. Purified rCsISG15 (lane 2) and rCsISG15M (lane 3) were analyzed by SDS-PAGE and viewed after staining with Coomassie blue. Lane 1, protein marker. (TIF)
Figure S2 Western blot analysis of the specificity of anti-rCsISG15 antibodies. rCsISG15 (lane 2) and rCSFerM (lane 3) were subjected to SDS-PAGE (upper panel) or immunoblot analysis with antiserum against rCsISG15 (lower panel). Lane 1, protein marker. (TIF)

Figure S3 Alignment of the amino acid sequences of ISG15 homologues. Dots denote gaps introduced for maximum matching. Numbers in brackets indicate overall sequence identities between CsISG15 and the compared sequences. The residues that are conserved among all the aligned sequences are in black; the residues that are ≥75% identical among the aligned sequences are in grey. The GenBank accession numbers of the aligned sequences are as follows: Ogopogopus fasciatus (BAJ3635), Epinephelus cooides (AAEG78371), Paralichthys olivaceus (BA98419), Sebastes schlegeli (BA G72218), Anoplopoma fimbria (ACQ57871), Chanana auga (ABK63480), Scianops ocellatus (ADJ57326), Osteochromis mukis (NP_01118081), Salmo salar (NP_001171172), Gadus morhua (ABD60150), Danio rerio (NP_001191098), Homo sapiens (AAH09507), Mus musculus (AAH31424). (TIF)

Figure S4 CsISG15 production in lymphocytes subjected to RNAi. Tongue sole head kidney lymphocytes transfected with pRNA-T-CMV3.1 (control), pRIN1, and pRiCsISG15 (lanes 2, 3, and 4 respectively) were incubated with megalovirus for 4 h. Extracellular (A and B) and cytoplasmatic (C and D) proteins were prepared and subjected to immunoblot with antibodies against rCsISG15 (A and C) or β-actin (B and D). Lane 1, protein marker. (TIF)

Author Contributions
Conceived and designed the experiments: LS. Performed the experiments: WW. Analyzed the reagents/materials/analysis tools: MZ ZXX. Wrote the paper: LS.

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