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MyD88 Interacts with Interferon Regulatory Factor (IRF) 3 and IRF7 in Atlantic Salmon (*Salmo salar*)

TRANSGENIC SsMyD88 MODULATES THE IRF-INDUCED TYPE I INTERFERON RESPONSE AND ACCUMULATES IN AGGRESOMES[*][S]

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Background: MyD88 directly interacts and affects IRF activation in mammals, but no information has been available about lower vertebrates.
Results: Transgenic MyD88 interacts with IRF3 and IRF7A/B and modulates the IRF-induced IFN response and accumulates in aggresomes in Atlantic salmon.
Conclusion: MyD88 is involved in the regulation of the IRF-induced IFN response in Atlantic salmon.
Significance: The results shed light on the evolution of the innate immune response in vertebrates.

MyD88 is an intracellular adaptor protein that transmits signals downstream of immune receptors such as the IL-1 receptor and the majority of the known mammalian toll-like receptors. Homologs of MyD88 have been identified in many vertebrate species; however, the adaptor has been studied mostly in mammals, and little is known about its function in lower vertebrates. The results presented in the current paper demonstrate, for the first time, that the teleost MyD88, through its Toll/Interleukin-1 receptor domain, interacts with SsIRF3 and two SsIRF7 paralogs: transcription factors that are critically involved in the virus-induced IFN responses. The data further highlight the potential of transgenic SsMyD88 to modulate the IRF-induced type I IFN response as the adaptor synergized with SsIRF3 to activate IRF-E/IFN-stimulated response element-containing reporter gene constructs and endogenous myxovirus resistance homolog expression. Microscopy analyses demonstrated that, similar to mammalian MyD88, both endogenous and transgenic SsMyD88 accumulated in intracellular aggregates. However, unlike the endogenous SsMyD88 clusters, which co-localized with endocytosed CpGs and probably represented myddosomes, overexpressed SsMyD88 accumulated in aggresomes. Although these structures accumulated ubiquitinated proteins, they did not associate with the autophagosome markers p62 and light chain 3-like protein, indicating that they are most likely classical aggresomes rather than aggresome-like induced structures, aggregates of ubiquitinated proteins induced by toll-like receptor/MyD88 signaling in antigen-presenting cells. The significance of the accumulation of transgenic MyD88 in aggresomes is currently unknown; nevertheless it is tempting to speculate that it might represent a defense mechanism against the potentially harmful effects of excessive MyD88 signaling.

MyD88 (myeloid differentiation primary response protein 88) is a phylogenetically conserved intracellular adaptor and a myeloid differentiation marker with important functions within the innate immune system (1, 2). It belongs to a family of Toll/IL-1 receptor (TIR)[2] domain-containing adaptor proteins that, most notably, are involved in the signal transduction downstream of toll-like receptors (TLRs) (3). The TLRs are pattern recognition receptors that recognize conserved microbial and viral molecules and that can quickly initiate an innate immune response upon encounter with potential pathogens (4–6). Among the currently characterized TLR adaptors, MyD88 has attracted a great deal of attention because it interacts and commences signaling downstream of all known mammalian TLRs with the exception of TLR3 (7). Instead of MyD88, TLR3 (a receptor for double-stranded RNA) interacts with the TIR domain-containing adaptor inducing IFN-β (TRIF) in a process that leads to activation of IFN regulatory factor 3 (IRF3)-dependent expression of type I IFN (8). In contrast with TRIF, which mediates only ephemeral Nuclear Factor κB (NF-κB) activation and, as a result, relatively weak expression of proinflammatory genes, the MyD88 signaling pathway induces robust production of both type I IFNs and proinflammatory cytokines such as IL-1 and TNF-α. This potential is conferred by the modular structure of MyD88, which along with the TLR-interacting TIR domain also includes a "death domain" (DD) that recruits IL-1 receptor-associated kinase (IRAK)-1, IRAK-2, and IRAK-4 (6). The IRAK activation precipitates a signaling cascade resulting in the activation of NF-κB, a transcription factor essential for the up-regulation of numerous immune genes. In addition, it has been demonstrated that

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[2] The abbreviations used are: TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; MyD88, Myeloid Differentiation primary response protein 88; IRF, Interferon Regulatory Factor; ISRE, IFN-stimulated response element; TRIF, TIR domain-containing adaptor inducing IFN-β; DD, death domain; IRAK, IL-1 receptor-associated kinase; ICC, immunocytochemistry; RGA, reporter gene assay; ALIS, aggresome-like induced structures; WB, Western blot; MTOC, microtubule organizing center; LC3, microtubule-associated protein light chain 3; IRF, interferon regulatory factor; Ab, antibody; EGFP, enhanced GFP; Mx, myxovirus resistance homolog; NF-κB, Nuclear Factor-κB.

MyD88, through its DD, can directly interact with IRF7, and this interaction results in activation of type I IFN promoters (9).

Studying the function of MyD88 has been greatly facilitated by the fact that it can become spontaneously activated through ectopic overexpression in different cell lines. In this regard, it has recently been found that MyD88 activation either through interaction with activated TLRs or via its overexpression brings about the formation of a helical oligomer, named myddosome, that consists of six MyD88, four IRAK4, and four IRAK2 DDs (10–12).

MyD88 has been extensively studied in invertebrates (*Drosophila*) and in different vertebrate organisms. However, the vast majority of the studies in vertebrates have been devoted to characterizing the function of the mammalian MyD88 homologs. The few studies on piscine MyD88 that have been published so far indicate that, compared with other TLR adaptors, the structure of MyD88 is well conserved (13, 14). It has also been revealed that the MyD88 homologs in fish species may function similarly to the mammalian counterparts. More specifically, a MyD88 knockdown in zebrafish embryos impaired the clearance of a bacterial infection (15), whereas its overexpression in HEK293T and carp leukocyte cells activated both an NF-κB and an IFN-β promoters (16). In salmonids, including Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*), MyD88 was shown to be involved in the positive regulation of an NF-κB and a serum amyloid A promoters, respectively (17, 18), whereas in zebrafish a knockdown study indicated that the adaptor was involved in peptidoglycan and lipoteichoic acid-induced IL-1β up-regulation (16).

The aim of the current study was to further characterize the function of a salmon MyD88 homolog (SsMyD88) in regards to its capacity to interact with and modulate the function of different IRFs. The presented results demonstrate that SsMyD88 had the capacity to interact with different IRF members and to modulate the IRF-induced IFN response. The study also revealed that compared with the endogenous protein, ectopically expressed MyD88 had a distinct distribution because it accumulated in detergent-insoluble aggresomes. It is possible that the sequestration of SsMyD88 in detergent-insoluble aggresomes might be another mechanism for control of excessive and/or prolonged MyD88 activation.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The ORFs of SsIRF3 (GenBank™ accession number ACN11005.1), SsIRF7A (GenBank™ accession number ACI33478.1), SsIRF7B (GenBank™ accession number ACI33478.1), SsTRIF (GenBank™ accession number DW540088.1, DW551436.1, DY693081.1), SsLC3 (GenBank™ accession number ACH70617.1), and SsMyD88 (GenBank™ accession number EF672332.1) were directionally cloned in pENTR/D-TOPO vector (Invitrogen). Using the Gateway technology (Invitrogen), the inserts were transferred to pDEST12.2 (Invitrogen), which expresses untagged proteins and pDEST-EGFP C1 (19). In addition, the full ORF of SsMyD88 was cloned in pDEST-HA, and its DD and TIR domains were cloned in pDEST-FLAG (19). All of the tags were positioned upstream of the inserts resulting in expression of N-terminally tagged proteins.

Antibodies—The affinity purified SsMyD88 antibody was produced in rabbits injected with the full-length SsMyD88, and it was used at 1 μg/ml in all of the applications. The GFP antiserum was purchased from Abcam (catalog number ab290) and used at 1:10,000 dilution in WB. The salmon myxovirus resistance (Mx) polyclonal antibody was provided by Dr. Hilde Hansen (University of Tromsø) and used at 1:3000 and 1:1000 dilutions for WB and ICC, respectively. The actin antibody was purchased from Sigma (catalog number 2066) and used at 1:200 dilution in WB. The monoclonal FLAG antibody was obtained from Stratagene (catalog number 200472) and was used at 1:1000 dilution in WB. The HA monoclonal (12CA5) antibody was obtained from Roche Applied Science (catalog number 200472) and used at 0.5 μg/ml for ICC. Monoclonal ubiquitin antibody (P4D1) was purchased from Covance and used at 1:100 dilutions for ICC. Monoclonal tubulin-γ antibody was obtained from Sigma (T6557) and used at 1:1000 dilutions for ICC. The secondary anti-rabbit Ig Alexa 546-conjugated antibody was purchased from Invitrogen (catalog number A11071).

Cell Cultures—HEK293T cells were cultured in minimum essential medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2. TO cells (20) were obtained from Dr. Heidrun Wergeland (University of Bergen, Bergen, Norway). Chinook salmon embryo CHSE-214 cells originate from Chinook salmon (*Oncorhynchus tschawytscha*) embryo cells (21). TO and CHSE cells were cultivated at 19 °C in Eagle's minimum essential medium supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine, 1% nonessential amino acids, and 5 and 8% FBS for TO and CHSE cells, respectively. Primary leukocytes from Atlantic salmon head kidney were isolated as previously described (22). The density of the leukocyte suspensions was adjusted to 7×10^6 cells/ml, and the cells were further incubated at 14 °C in 24-well plates in L-15, 5% FBS. The adherent leukocytes used for immunostaining were allowed to adhere in 8-well coverslip chambers (Lab-Tech) in L-15, 0.1% FBS overnight, washed, and further cultured in 5% FBS at 14 °C.

Co-immunoprecipitations—HEK293T cells incubated in 6-well plates were transfected with 3 μg of each plasmid using Lipofectamine 2000 (Invitrogen). Approximately 48 h after transfection, the cells were washed two times with ice-cold phosphate-buffered saline and harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100) with a protease inhibitor mixture (Complete EDTA-free; Roche Applied Science). The cell lysates were cleared by centrifugation for 15 min at 15,000 $\times$ *g*. The lysates were then incubated with anti-GFP rabbit antiserum (5 μg/ml) (Abcam) or with the same amount of normal rabbit Ig (Santa Cruz) for 1 h. The antibody was precipitated using preblocked protein A-agarose beads (Santa Cruz) for 1 h. The beads were then washed four times with lysis buffer and eluted in 40 μl of 1$\times$ LDS sample buffer (Invitrogen).

Reporter Gene Assays—The reporter that contains the promoter region I of the Atlantic salmon IFNa1 (23) was provided by Prof. Børre Robertsen (University of Tromsø, Norway). The rainbow trout Mx1 promoter construct was described earlier (24). The IFN-stimulated response elements (ISRE) reporter

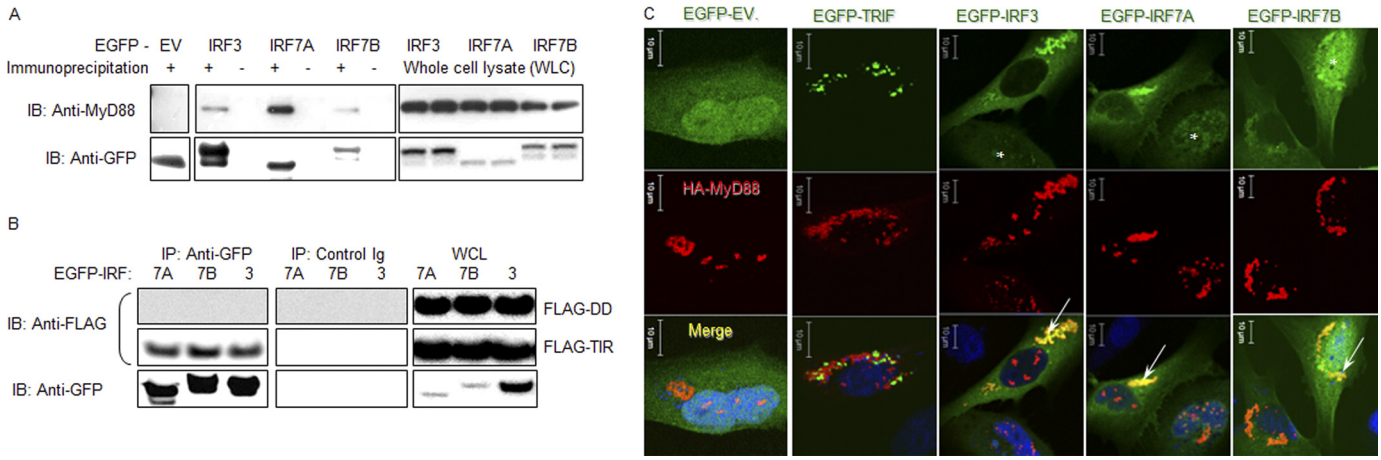

FIGURE 1. **SsMyD88 interacts with IRFs through its TIR domain.** *A*, protein extracts from HEK293 cells co-transfected with plasmids expressing SsMyD88 and EGFP-tagged SsIRF3 and IRF7A/B were immunoprecipitated with an EGFP antibody (+) or a control, nonspecific Ig (−) as indicated. The samples were analyzed with WB using antibodies against SsMyD88 and EGFP. The results indicate that SsMyD88 co-immunoprecipitated with all of the tested IRFs. The IRFs are visualized as two bands, the lower of which is most likely a truncated form. *B*, HEK293 cells overexpressing the FLAG-tagged TIR and DD of SsMyD88 and different GFP-tagged SsIRFs were analyzed as in *A*. *C*, co-localization of SsMyD88 with IRFs. TO cells were co-transfected with plasmids expressing HA-SsMyD88 (*red*) and the indicated EGFP-fusion proteins (*green*). The HA tag was stained with a monoclonal primary Ab and an Alexa 546-labeled secondary Ab (*red*). The nuclei were stained with TO-PRO3 (*blue*). Co-localization (*yellow*) is indicated with *arrows*. *Asterisks* designate nuclei with significant translocation of IRFs. *IP*, immunoprecipitation; *IB*, immunoblot; *WCL*, whole cell lysate.

was purchased from SABiosciences (catalog number CCS-008L). CHSE and TO cells grown in 96-well plates were co-transfected with the reporters and pDEST12.2 constructs, which express the untagged protein of interest (25 ng of each plasmid/well) using FuGENE HD (Roche Applied Science) and analyzed 72 h later using the dual luciferase reporter assay system (Promega) as described earlier (25). The firefly luciferase values were normalized using the *Renilla* luciferase levels, and the values were presented as relative luciferase units. IFNa1 used to stimulate TO and CHSE cells was produced as described earlier (26). Poly(I·C) was purchased from Invivogen.

TaqMan Real Time PCR—Total RNA was extracted from CHSE cells using NucleoSpin RNA/protein isolation kit (Macherey-Nagel). The RNA (100 ng in a 20-μl reaction) was reverse transcribed using TaqMan® reverse transcription reagents (Applied Biosystems). Each of the cDNA samples was analyzed in duplicate PCRs, which included 2.5 ng of reverse-transcribed RNA and were prepared using FAST PCR Mastermix (Applied Biosystems). The Mx probe and primers were designed, as described earlier (27), to amplify all of the known salmon Mx isoforms. The reactions were run using ABI Prism 7500 FAST Cycler (Applied Biosystems), and the Mx expression was normalized against the levels of elongation factor 1A and analyzed as described earlier (25).

Western Blot—The eluates from the co-immunoprecipitations were directly subjected to WB analysis. Protein samples from CHSE cells incubated in 24-well plates (Nunc) and transfected with 125 ng of each plasmid with FuGENE HD (Roche Applied Science) were obtained using NucleoSpin RNA/protein isolation kit (Macherey-Nagel) and prepared in 30 μl of 1× LDS sample buffer supplemented with 20 mM DTT. Primary salmon leukocytes were stimulated in 24-well plates and were lysed directly in 100 μl of 1× LDS, 20 mM DTT. The samples were run on NuPAGE Novex Bis-Tris 4–12% gels (Invitrogen). The proteins were transferred to PVDF membranes, blocked with 5% dry milk or BSA, and incubated overnight with different primary Abs followed by 1 h of incubation with the secondary HRP-conjugated antibodies. The WB was developed using SuperSignal West Pico Chemiluminesccent Substrate (Pierce).

Immunocytochemistry—The TO cells were transfected with 125 ng of each plasmid using FuGENE HD, stained 48 h after transfection, and analyzed as previously described (22) using Axiovert 200 microscope equipped with an LSM510-META confocal module. The CHSE cells were incubated for 72 h after transfection and stained using the same protocol. The cells were visualized with an Axiovert 40 inverted microscope equipped with a fluorescent illumination system, and the images were taken with Axiocam MRm (Zeiss).

Data Analyses—Statistical analysis was carried out using the unpaired Student *t* test. The *p* values ≤0.05 were considered to indicate a statistically significant difference (*, $p < 0.05$; **, $p < 0.005$).

RESULTS

SsMyD88-IRF Interactions—Preliminary results (not shown) from a yeast two-hybrid screening of a salmon leukocyte cDNA library with an SsMyD88 bait indicated that the adaptor might interact with SsIRF7B. This initial finding inspired the work presented in the current paper in which co-immunoprecipitations were used to investigate the potential of SsMyD88 to interact with different Atlantic salmon IRFs. HEK293T were used for this assay because of their significantly higher transfectability as compared with any of the currently available salmon cell lines. The results in Fig. 1*A* clearly show that SsMyD88 co-immunoprecipitated with all of the tested, EGFP-tagged SsIRFs, but not with EGFP alone. Different parts of MyD88 interact with different IRFs; for example, in mouse the DD of MyD88 interacts with IRF7 (28), whereas the intermediate and a part of the TIR domain are involved in direct interactions with IRF1 (29). The co-immunoprecipitations presented here show that salmon MyD88 homolog interacts with all of the

tested SsIRFs through its TIR domain, and no interactions are observed between the IRFs and the DD of SsMyD88 (Fig. 1*B*).

Transgenic SsMyD88 Accumulates in Cytoplasmic and Nuclear Granules and Co-localizes with IRF3 and IRF7A/B—Immunocytochemistry and confocal microscopy techniques were used to confirm the interactions between SsMyD88 and the IRFs in salmonid cell lines. The results shown in Fig. 1*C* were obtained using TO cells, a cell line derived from head kidney of Atlantic salmon, and the same results were also acquired with CHSE cells (not shown). The cells were co-transfected with the indicated plasmids and incubated for 3 days prior to the analysis. When overexpressed alone, the EGFP tag alone and the EGFP-fused IRFs were distributed mostly homogeneously throughout the cytoplasm, and relatively few cells exhibited IRF accumulation in granules and vesicles (not shown). Co-transfection with HA-SsMyD88-expressing plasmid induced accumulation of the IRFs in cytoplasmic granules and larger polymorphic structures. Staining of cells with an HA antibody revealed that SsMyD88 was mostly found in granules in both the cell nuclei and the cytoplasm, which in the latter tended to form perinuclear aggregates. As clearly visible in Fig. 1*C*, all of the EGFP-IRFs co-localized with SsMyD88 in these aggregates. As mentioned above, like MyD88, TRIF is an important TLR adaptor molecule, and it also shows aggregated cytoplasmic distribution. To our knowledge, the possibility of interaction/co-localization between the two adaptors has not been studied so far. In the current study, although both SsMyD88 and SsTRIF showed similar, granular distribution, no significant co-localization was detected between the two adaptors. No EGFP aggregation was observed when SsMyD88 was co-transfected with the empty EGFP vector, which was used as a control. Altogether, the results demonstrate that SsMyD88 interacts with both SsIRF3 and SsIRF7A/B. Regarding the functional studies, it is also relevant to mention here that the IRFs could also be detected in cell nuclei, which is indicated with *asterisks* in Fig. 1*C*. Co-transfection with SsMyD88-expressing plasmids did not appear to either enhance or inhibit the nuclear translocation of any of the IRFs, and co-localization with SsMyD88 was observed both in the cytoplasm and in the nuclei.

SsMyD88 and SsIRF3 Synergistically Activate ISRE-dependent Gene Expression in CHSE Cells—Reporter gene assays (RGAs) are commonly used to study the regulation of the activity of transcription factors. The firefly luciferase reporter gene constructs used in this study include a minimal IFNa1 promoter from Atlantic salmon (containing two IRF-E binding sites and one NF-κB binding site) and an Mx1 promoter of rainbow trout (containing only one ISRE), both of which have previously been used to study activity of IRFs (30, 31). A commercial luciferase reporter from SABiosciences that contains tandem ISRE repeats was also included in this study. The results presented in Fig. 2*A* show that when overexpressed alone, SsIRF3 induced the highest IFNa1 promoter activity as compared with IRF7A/B, which confirms previously published data (30). Co-transfection of CHSE cells with SsIRF3 and SsMyD88 resulted in a synergistic up-regulation of all of the tested reporters, whereas no significant cooperation was detected between SsMyD88 and SsIRF7A/B. SsMyD88 alone up-regulated the IFNa1 promoter weakly (~2-fold), as compared with the control transfected with the empty vector, and it did not seem to have affected the activity of the Mx1 and the ISRE reporters.

The cooperation between SsMyD88 and the IRFs was also confirmed by the up-regulation of endogenous Mx mRNA and protein in CHSE cells. The results shown in Fig. 2*B* indicate that the synergy between SsMyD88 and SsIRF3 was even more pronounced as compared with the results from the RGAs. Furthermore, unlike the results from the RGAs, SsMyD88 enhanced the IRF7A/B-induced endogenous Mx expression.

To determine whether the expression of endogenous Mx had been significantly affected by the positive feedback action of type I IFN, initially up-regulated by the IRFs in transfected cells, the Mx protein levels were analyzed using an immunocytochemistry approach. Endogenous Mx was stained in CHSE cells transfected either with EGFP-IRF3 alone or in combination with SsMyD88 (Fig. 2*C*). The results from the immunocytochemistry analysis demonstrated that endogenous Mx was induced not only in the cells co-transfected with EGFP-SsIRF3 and SsMyD88 but also in neighboring cells, which did not appear to be transfected.

In TO Cells, SsMyD88 Synergizes with SsIRF3 in the Induction of Mx1 and ISRE Reporters, but It Suppresses the IRF7A/B-induced Promoter Activation—The ability of SsMyD88 to modulate the IRF-induced IFN response was also analyzed in TO cells using the Mx1 and the ISRE reporters (Fig. 3). Unlike in CHSE cells, in TO cells SsIRF7A/B induced much higher promoter activity as compared with SsIRF3. Nevertheless, like in CHSE cells, synergistic induction of the Mx1 promoter by SsMyD88 and SsIRF3 was also detected in TO cells. However, overexpression of SsMyD88 suppressed the IRF7A/B-induced Mx1 and ISRE promoter activity. Unlike the Mx1 and the ISRE reporters, the IRFs did not significantly influence the activity of an NF-κB promoter construct. The latter was up-regulated upon SsMyD88 overexpression, and in the cells co-transfected with SsIRF7A/B, the NF-κB activation was not significantly affected.

Disparate Effects of SsMyD88 on IRF- and IFNa1-induced ISRE Reporter Activation—Stimulation of CHSE cells with poly(I·C) resulted in substantially enhanced ISRE reporter activation in IRF-transfected cells (Fig. 4*A*). In the poly(I·C)-stimulated cells co-transfected with SsIRF7A/B and SsMyD88, the mean ISRE promoter activity was lower compared with that in cells transfected with SsIRF7A/B alone. In the case of SsIRF3, SsMyD88 had a positive effect on the ISRE activity both in non-stimulated and in poly(I·C)-activated cells. To investigate whether SsMyD88 may modulate the response to type I IFN, TO (Fig. 4*B*) and CHSE cells (Fig. 4*C*) were stimulated with 100 ng of salmon IFNa1. The results clearly demonstrate that SsMyD88 has an inhibitory effect on the IFNa1 activity in both cell lines. In SsMyD88-transfected CHSE cells, the IFNa1 activity was down-regulated by ~50% but nevertheless it remained well above the ISRE activity in SsIRF3/SsMyD88-stimulated cells.

Endogenous SsMyD88 Protein Is Up-regulated by TLR Ligands in Primary Leukocytes and, Unlike Transgenic SsMyD88, It Co-localizes with Endocytosed CpG—The mRNA expression of SsMyD88 has been previously studied in salmon leukocytes stimulated with TLR ligands (18). In the current study, the up-regulation of the SsMyD88 protein was analyzed

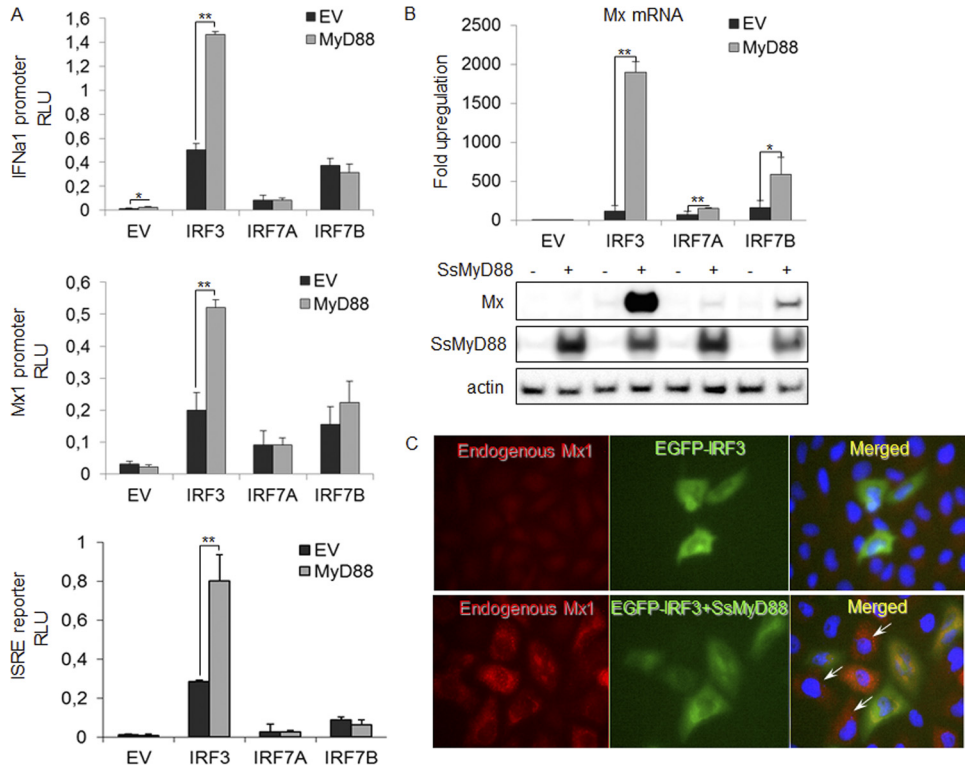

FIGURE 2. **Transiently overexpressed SsMyD88 synergizes with SsIRF3 to activate IRF-E/ISRE-containing reporter gene constructs and endogenous Mx expression in CHSE cells.** *A*, SsMyD88 and SsIRF3 synergistically induce IFNa1, Mx1, and a commercial ISRE firefly luciferase reporter construct. The cells were co-transfected as described under "Experimental Procedures" for 72 h prior to analysis of firefly luciferase levels. *EV* stands for empty vector. The data are presented as relative luciferase units calculated as the ratio between the firefly *versus* the *Renilla* luciferase activities ($n = 5$). *B*, SsMyD88 synergizes with SsIRF3 and to a lesser extent with IRF7A/B to induce the expression of endogenous Mx. CHSE cells were co-transfected as in *A*, and the RNA and protein samples were collected 72 h later. The histogram shows the results from a TaqMan® assay with an Mx probe. The data are presented as "fold up-regulation" as compared with the empty vector control ($n = 3$). Protein samples from the same cells were analyzed with Western blot using the indicated antibodies. Representative results from one of three identical experiments are shown. *C*, Mx is up-regulated not only in the cells that overexpress SsIRF3 and SsMyD88. CHSE cells overexpressing EGFP-SsIRF3 (*green*) alone or together with SsMyD88 were fixed, and endogenous Mx was stained with an anti-Mx1 primary and a secondary Alexa 546-conjugated antibody (*red*). The results demonstrate that Mx is up-regulated in cells co-transfected with SsIRF3 and SsMyD88, as well as neighboring cells that do not appear to be transfected (*arrows*). This is most likely due to the paracrine action of type I IFNs secreted by the transfected cells. The cell nuclei were counterstained with DAPI (*blue*).

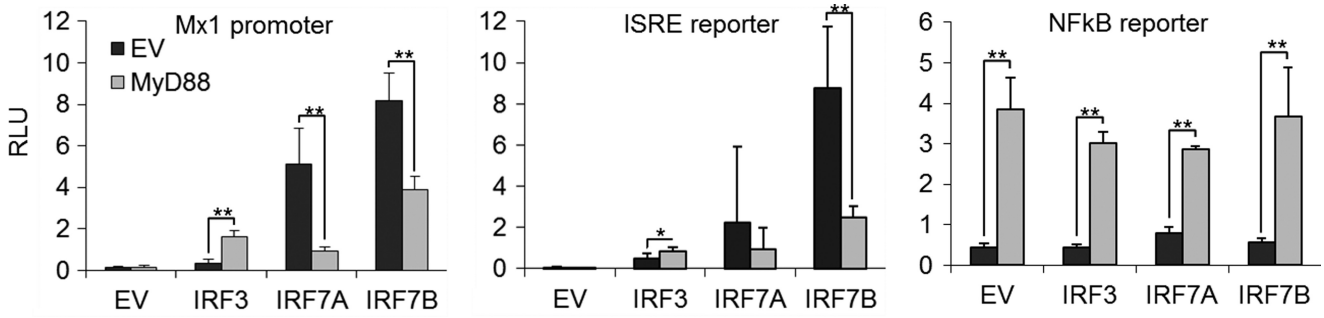

FIGURE 3. **SsMyD88 synergizes with SsIRF3 in the activation of the Mx1 reporter, but it suppresses the IRF7A/B-induced Mx1 and ISRE reporter activity in TO cells.** The cells were transfected, and the samples were analyzed as described in Fig. 2*A* ($n = 5$). *EV*, empty vector.

in head kidney-derived leukocytes stimulated with poly(I·C), CpG-B, and LPS. The WB results shown in Fig. 5*A* demonstrated that SsMyD88 was induced following stimulation with different TLR ligands, which was most pronounced after 48 h of stimulation with CpG-B, a TLR9 ligand. To examine the distribution of endogenous SsMyD88 and its potential to associate with an endocytosed TLR ligand, adherent salmon leukocytes were incubated with Cy5-labeled CpG-B for 24 h, and, following fixation and permeabilization, endogenous SsMyD88 was stained with a primary SsMyD88 antibody and a secondary Alexa 546-conjugated antibody. The specificity of the immunostaining with the SsMyD88 was confirmed in HEK293T cells overexpressing SsMyD88 (supplemental Fig. S1). In Fig. 5*B*, endogenous SsMyD88 appears in small granules and speckles distributed homogeneously throughout cell cytoplasm. Stimulation with CpG-B resulted in formation of larger SsMyD88 aggregates, which co-localized with the endocytosed CpGs. To determine whether the granules and the aggregates of transgenic SsMyD88 could also associate with endocytosed CpGs, adherent primary salmon leukocytes were transfected with a plasmid expressing EGFP-SsMyD88, and 48 h later the cells were stimulated with Cy5-labeled CpG-B for 24 h. To label the

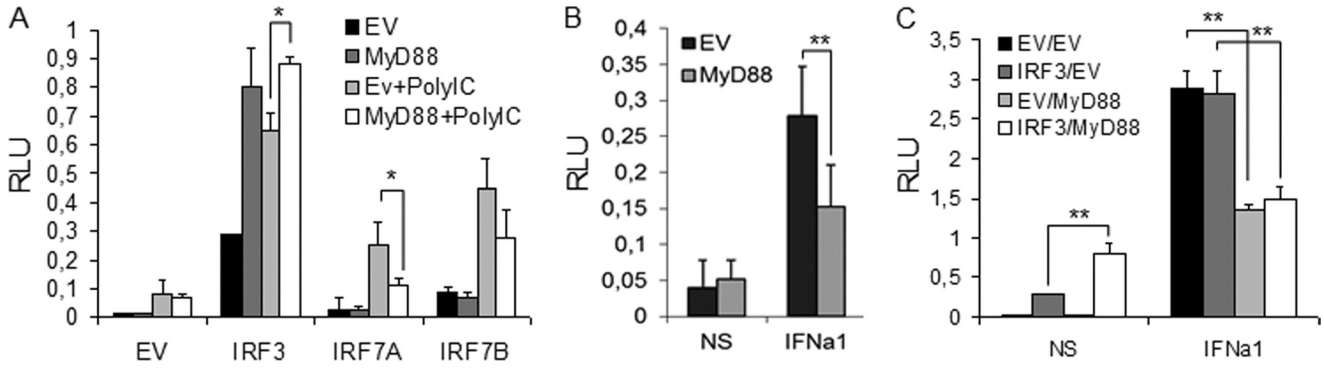

FIGURE 4. **Disparate effects of SsMyD88 on IRF- and IFNa1-induced ISRE reporter activation.** *A*, SsMyD88 affects negatively the poly(I·C)-induced ISRE activation in IRF7A/B-transfected but not in IRF3-transfected CHSE cells. The cells were stimulated for 24 h with 20 μg/ml of poly(I·C). *B* and *C*, both in TO cells (*B*) and in CHSE cells (*C*), SsMyD88 attenuates the ISRE activation by salmon IFNa1. In CHSE cells, co-transfection with IRF3 was included as a control to directly compare the magnitude of the synergistic ISRE activation and the suppression of the IFNa1 activity. Despite the fact that the IFNa1-induced ISRE activation was significantly suppressed in SsMyD88-transfected cells, its magnitude remained well above the SsIRF3/SsMyD88-induced ISRE activation. The cells were stimulated with 100 ng/ml of salmon IFNa1 ($n = 3$). *EV*, empty vector.

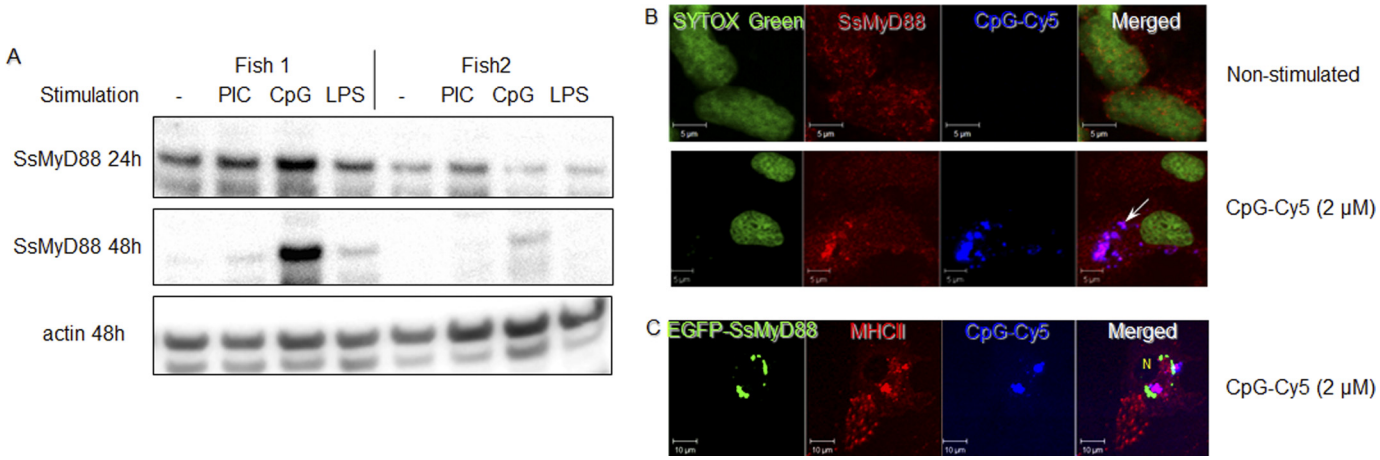

FIGURE 5. **Expression and intracellular localization of endogenous SsMyD88 protein.** Unlike the endogenous adaptor, transgenic SsMyD88 does not accumulate in CpG-containing endosomes. *A*, SsMyD88 is up-regulated in primary leukocytes by CpG stimulation. Western blot analysis of proteins from head kidney leukocytes stimulated *in vitro* with 20 μg/ml of poly(I·C), 2 μM CpG-B, and 50 μg/ml of LPS for either 24 or 48 h. *B*, endogenous SsMyD88 is distributed relatively uniformly in nonstimulated salmon leukocytes, and upon stimulation it concentrates in CpG-containing endosomes. Adherent leukocytes were incubated with Cy5-labeled CpGs (*blue*) for 24 h before fixation and immunostaining of endogenous SsMyD88 with a primary anti-SsMyD88 and a secondary Alexa 546-conjugated antibody. The nuclei were counterstained with SYTOX Green. Co-localization between SsyD88 and CpGs is indicated with an *arrow*. *C*, transgenic EGFP-SsMyD88 aggregates are not found in endosomal MHCII compartments. Adherent mononuclear phagocytes were transiently transfected with a construct expressing an EGFP-SsMyD88 fusion protein (*green*). The cells were incubated with fluorescent CpGs (*blue*) for 24 h, and MHCIIβ was stained with a polyclonal primary antibody and an Alexa 546-conjugated secondary antibody (*red*) to label the MHCII endosomal compartments. The images were taken with a laser scanning microscope (Zeiss 510 Meta) using "multi-track" acquisition to avoid cross-excitation. *EV*, empty vector.

endocytic MHCII compartments, the cells were stained with anti-MHCIIβ primary and Alexa 546-conjugated secondary antibodies. The representative image in Fig. 5*C* demonstrates that, unlike endogenous SsMyD88, the aggregates of the EGFP-labeled adaptor did not co-localize with the endocytosed CpGs and/or any of the intracellular MHCII compartments.

Transgenic SsMyD88 Accumulates "Classical" Aggresomes and Not in ALIS—Surprisingly low levels of transiently overexpressed SsMyD88 in cell lysates were detected in preliminary experiments, in which cells were transfected with plasmids expressing SsMyD88 and lysed with buffers containing non-ionic detergents. In the experiment presented in Fig. 6*A*, untagged and HA-tagged SsMyD88 was transiently overexpressed in TO cells, and the Triton-soluble and -insoluble fractions were separated and analyzed on a WB using the SsMyD88 antibody to detect simultaneously both the endogenous and the overexpressed adaptor. As seen in the figure, unlike endogenous SsMyD88, the overexpressed protein accumulated mostly in the insoluble fraction, whereas minor amounts were found in the supernatant. The untagged SsMyD88 was also included to demonstrate that the aggregation was not caused by the tag. It has been previously shown that MyD88 is implicated in ALIS formation and the regulation of autophagy (32). The results from the immunostaining presented in Fig. 6*B* indicated that transiently overexpressed SsMyD88 aggregated around the microtubule organizing centers (MTOCs), which were seen as small perinuclear dots, labeled with tubulin-γ antibody and a secondary Alexa 546-conjugated antibody (*red*). Disruption of microtubule polymerization with 1 μM nocodazole inhibited the aggregation of the EGFP-SsMyD88 granules around the MTOCs, indicating that the process required an intact microtubule cytoskeleton. Fig. 6*C* shows that although EGFP-SsMyD88 associated with aggregates of ubiquitinated proteins in TO cells, it did not co-localize with endogenous p62, which is an autophagy substrate. To determine whether the SsMyD88 granules might associate with autophagosomes, TO cells were

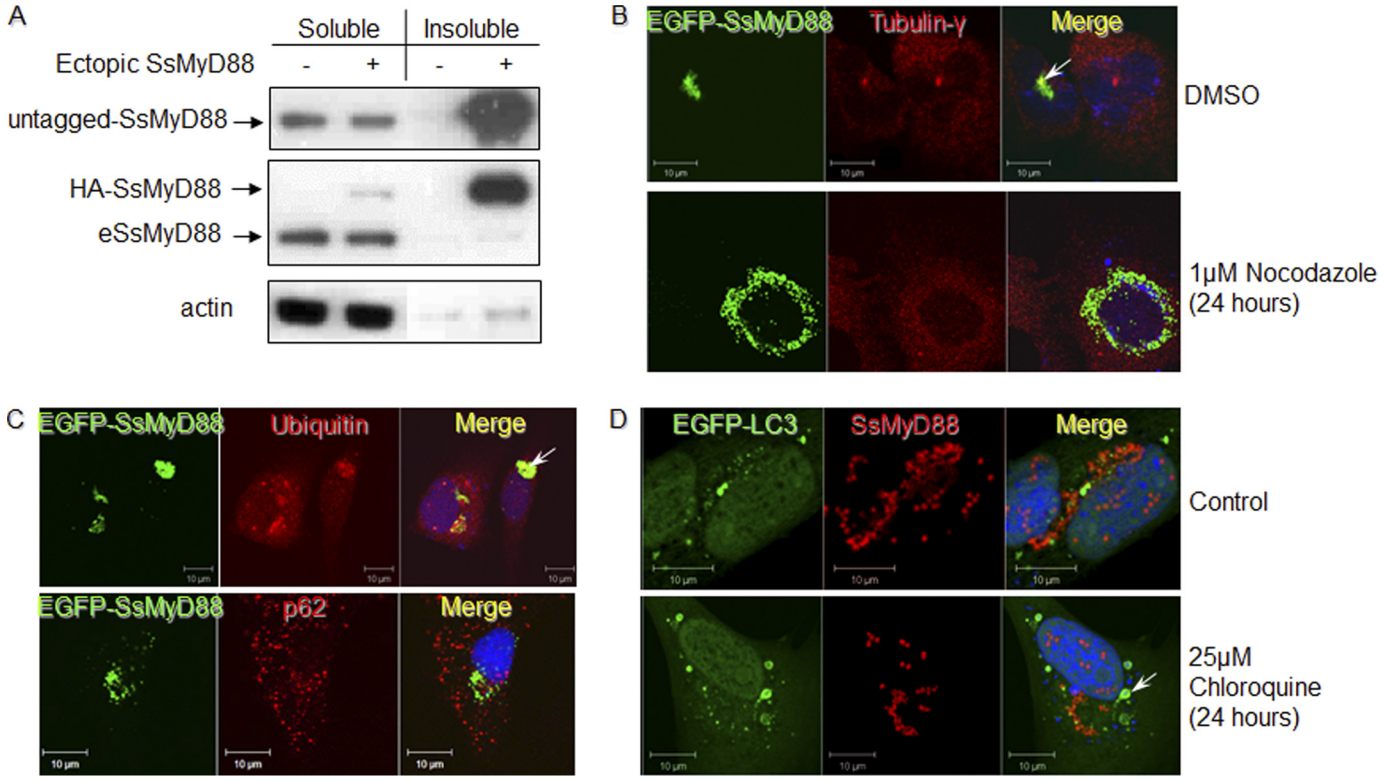

FIGURE 6. **The Triton-insoluble granules in which overexpressed SsMyD88 accumulates appear to be "classical" aggresomes and not ALIS.** *A*, Western blot analysis of samples from TO cells transfected with an untagged or a HA-tagged SsMyD88 construct as indicated. The cells were lysed with buffer containing 1% Triton, the samples were centrifuged, and the supernatant (soluble fraction) and the pellet (insoluble fraction) were prepared with LDS buffer and subjected to SDS-PAGE. The endogenous (eSsMyD88) and the transgenic HA-SsMyD88 were detected with an anti-SsMyD88 Ab. *B*, SsMyD88 granules accumulate in aggresomes as revealed by their accumulation around the MTOC (*arrow*) through a microtubule-dependent mechanism. TO cells were transfected with a construct expressing EGFP-SsMyD88, and the cells were treated either with vehicle (dimethyl sulfoxide, *DMSO*) or 1 μM nocodazole for 24 h prior to fixation and staining with a primary tubulin-γ antibody and a secondary Alexa 546-conjugated antibody to label the MTOCs, seen as perinuclear dots (*red*). *C*, TO cells transfected with an EGFP-MyD88-expressing plasmid were stained with Abs that recognize ubiquitinated proteins and p62 (*red*). Co-localization between MyD88 and aggregates containing ubiquitin is indicated with an *arrow*. No co-localization was observed between EGFP-SsMyD88 and endogenous p62. *D*, transgenic SsMyD88 is not found in autophagosomes. TO cells were co-transfected with plasmids expressing SsMyD88 and EGFP-LC3 (a marker for autophagosomes). The cells were either left untreated or incubated with chloroquine to block acidification of autophagosomes and to induce their accumulation before staining SsMyD88 (*red*). No granules or larger SsMyD88 aggregates were detected in any of the observed autophagosomes, seen as EGFP-positive vesicles in the chloroquine-treated, but not found in the control cells.

co-transfected with plasmids expressing SsMyD88 and EGFP-microtubule-associated protein light chain 3 (LC3), another autophagy-related protein and were treated with chloroquine for 24 h. Chloroquine blocks endosomal acidification and is commonly used to induce accumulation of autophagosomes, which in Fig. 6*D* are seen as EGFP-LC3 positive vesicles (*arrow*). The representative image indicates that SsMyD88 granules (red) do not associate with any of the observed autophagosomes.

DISCUSSION

A major finding in the current study is the interaction of the TIR domain of SsMyD88 with both SsIRF3 and SsIRF7A/B. It has been found that in mammals MyD88 interacts directly with several IRFs including IRF1, IRF4, IRF5, and IRF7 (28, 29, 33, 34). An initial study has suggested that human MyD88 does not interact with IRF3 (9); however, later research demonstrated that murine MyD88 does interact with IRF3 (35). Unlike MyD88, whose amino acid sequence is relatively well conserved, the sequences of the IRFs have diverged to a much greater extent throughout the vertebrate evolution (36). Among the IRFs, IRF3 and IRF7 share the greatest amino acid sequence identity; however, their homology is limited mainly to the N-terminal DNA-binding domain, whereas the IRF association domain is much less conserved (30). This domain is important for the homo- or heterodimerization of the IRFs, and it is also involved in their interactions with other transcriptional and signaling mediators, including MyD88 (9). Therefore, it appears that the interactions of the IRFs with upstream signaling mediators might differ significantly between different vertebrate species.

Of the currently known nine members of the IRF family in mammals, IRF3 and IRF7 seem to be most critically involved in the virus-induced IFN responses (37). It has been demonstrated that IRF3 is phosphorylated by TANK-binding kinase-1 and IκB kinase-ϵ in a TRIF-mediated signaling cascade downstream of TLR3/4 (38). On the other hand, upon TLR9 stimulation in plasmacytoid dendritic cells, IRF7 is activated both through phosphorylation and TNF receptor-associated kinase 6-mediated ubiquitination (9). This process depends on the formation of a "transductional-transcriptional" complex in which MyD88 interacts directly with IRF7 (9, 28). Once activated, the IRFs form homo- or heterodimers, translocate to the nucleus, and activate type I IFN and IFN-stimulated genes by binding to the

IRF-E/ISRE sites in their promoters (39). A major difference between IRF3 and IRF7 is that, unlike IRF3, which is ubiquitously expressed and noninducible by IFN stimulation or viral infections, IRF7 has more restricted expression and is critical for the robust IFN production by dendritic cells (40). However, this is not the case for Atlantic salmon, where recent results have revealed that transcription of both salmon IRF3 and IFR7 in TO cells is induced by IFNa1 (30). In addition to IFNs, both IRF3 and IRF7 are known to activate the expression of other genes that contain ISRE elements (41, 42), and it has been shown that IRF7 is more "liberal" in respect with its ISRE binding specificity (43), a property that can explain its ability to trigger the expression of larger number of genes as compared with IRF3.

It has been shown that transient co-transfection of mouse MyD88 with IRF7 results in synergistic activation of a reporter gene containing a type I IFN promoter (28). However, in the case of IRF3, the data published so far is somewhat controversial. Recently it has been found that MyD88 has an inhibitory effect on the TLR3-mediated signaling, which is due to suppression of the IκB kinase-ϵ-dependent IRF3 activity (35). On the other hand, in B-cells the CpG-induced IFN production is dependent on a pathway that includes both MyD88 and IRF3 but not TANK-binding kinase-1 (44).

The type I IFN inducing activity of different salmon IRF isoforms has already been studied (30) through reporter gene assays in CHSE cells. In the current paper, using the same reporter construct and a similar experimental setup, SsMyD88 synergized with IRF3 in the activation of the salmon IFNa1 promoter (Fig. 2*A*). This "minimal IFN promoter" construct contains two ISRE elements and an NF-κB binding site, both of which are critical for the up-regulation of IFNa1 by poly(I·C) (23). Upon ectopic overexpression, SsMyD88 alone strongly activates NF-κB (Fig. 3); however, the synergy between SsMyD88 and SsIRF3 was also observed with a commercial ISRE reporter and the trout Mx1 promoter construct, which has only one predicted ISRE element and no NF-κB-binding sites (24), indicating that the cooperation is independent of the activation of NF-κB. Although there was no obvious cooperation between SsMyD88 and SsIRF7A/B in the induction of the reporter gene activity, significant (albeit considerably weaker as compared with SsIRF3) cooperation was detected when endogenous Mx was analyzed both at mRNA and protein levels (Fig. 2*B*). This indicates that the expression of endogenous Mx might have been influenced post-transcriptionally by SsMyD88. In this regard, the up-regulation of many immune genes is dependent on the stabilization of their mRNAs, and MyD88 signaling is known to result in stabilization of mRNAs, which contain adenine-uridine-rich element in their 3′-untranslated regions (45). Therefore, the SsMyD88-dependent up-regulation of endogenous Mx could also be due to its mRNA stabilization, because the core AUUUA elements of adenine-uridine-rich elements are present in all of the known salmon Mx isoforms.

The reporter gene assay is a relatively sensitive method for measuring promoter activity; however, this technique detects activity only in the transfected cells, whereas the paracrine action of secreted IFNs and other factors on nontransfected cells remains undetected. In the SsIRF3/SsMyD88 co-transfected samples, endogenous Mx up-regulation was detected both in transfected and in neighboring, non-transfected cells as revealed by the immunostaining (Fig. 2*C*). Therefore, it seems that the paracrine action of type I IFNs or other factors secreted by the IRF3-transfected cells has contributed to the more pronounced induction of endogenous Mx as compared with the analysis of the promoter activity alone as detected by the RGAs.

CHSE is a relatively heterogeneous Chinook salmon embryo cell line that has been preferred for RGAs because of its relatively high (compared with other fish cell lines) transfectability (up to 30% using FuGENE HD). In the present study, RGAs were also performed with TO cells, a cell line derived from salmon head kidney that possesses some characteristics of dendritic cells (46). Surprisingly, although the synergy between SsMyD88 and SsIRF3 was confirmed, SsMyD88 overexpression suppressed the superior IRF7A/B-induced activation of the Mx1 and the ISRE reporters in TO cells (Fig. 3). The disparate influence of SsMyD88 on the IRF3 and IRF7A/B activity was also observed in CHSE cells (Fig. 4*A*). The poly(I·C)-induced ISRE promoter activity was substantially enhanced in cells transfected with SsIRF3 and SsIRF7A/B, suggesting involvement of these IRFs in the poly(I·C)-induced signaling. Interestingly, upon stimulation with poly(I·C), SsMyD88 attenuated the ISRE activity only in IRF7A/B-transfected and not in IRF3-transfected cells, indicating that the negative effect of SsMyD88 on the IRF7A/B activity is not restricted to TO cells, and it may depend on regulation of upstream signaling events that are specifically involved in the IRF7A/B activation. In this regard, in mouse, MyD88 has been shown to suppress the response to poly(I·C), by inhibiting a specific mechanism which involves the IκB kinase-ϵ-dependent but not the TANK-binding kinase-1-dependent IRF3 activation (35).

It should also be considered that the MyD88 signaling leads to up-regulation of molecules such as the suppressor of cytokine signaling 1, which functions as an inhibitor of the Jak/STAT signaling (47), and in the present study SsMyD88 significantly attenuated the IFNa1-induced ISRE reporter activation in both TO (Fig. 4*B*) and CHSE cells (Fig. 4*C*). Overall, these data highlight, for the first time, the potential of a piscine MyD88 homolog to modulate both positively and negatively the IFN response, functions that are, undoubtedly, critical for the proper regulation of the antiviral immune response.

The TLRs recognize their ligands and initiate signaling events either at the plasma membrane or at the endosomal compartments, and it has been shown that induction of IFN response by TLRs requires signaling initiated from endosomes (48). Therefore, it was intriguing to identify the intracellular compartments where SsMyD88 interacts with the SsIRFs. When overexpressed alone, EGFP-tagged IRF3 and IRF7A/B are distributed mostly homogeneously throughout cytoplasm; however, co-transfection of the IRFs with SsMyD88 induces their accumulation in polymorphic structures in which the co-localization with MyD88 is clearly visible (Fig. 1*C*). In zebrafish, transgenic TRIF has been shown to accumulate in aggregates within the Golgi apparatus (49); however, in the present study SsMyD88 aggregated in distinct structures, because no co-localization was observed between the two adaptors (Fig. 1*C*). In

mammals, it is well known that transgenic SsMyD88 accumulates in granules and larger, condensed structures distributed both throughout the nucleus and the cytoplasm (50). Earlier studies have failed to identify whether the aggregates are associated with any particular organelles, and it has more recently been proposed that these might represent myddosomes (51). In the current study, the up-regulation of the endogenous SsMyD88 protein varied considerably between individuals (Fig. 5*A*). Although it was not always detectable following stimulation with poly(I·C) and LPS, SsMyD88 was consistently up-regulated after 48 h of stimulation with CpG, which reflects the superior potential of this TLR ligand to activate expression of both proinflammatory cytokines and type I IFN in piscine leukocytes (52–55). Furthermore, the CpG stimulation induced accumulation of endogenous SsMyD88 in larger aggregates that might reflect its activation state. The fact that that these structures co-localized with endocytosed fluorescent CpGs indicates that they might represent myddosomes or other protein assemblies involved in the CpG-induced signaling (Fig. 5*B*). It has been shown that, in antigen presenting cells, the MHCII-restricted antigen presentation is controlled through a phagosome-autonomous mechanism that involves MyD88-dependent TLR signaling (56, 57). In the current study, unlike endogenous MyD88, aggregates of EGFP-tagged SsMD88 co-localized neither with endocytosed CpGs nor with any of the endosomal MHCII compartments, which questions the notion that these structures represent functional myddosomes.

MyD88 is a relatively small molecule (~33 kDa) and, apparently, it lacks any large hydrophobic regions. Therefore, it was quite surprising to find out that overexpressed SsMyD88 accumulated mostly within the Triton-insoluble cell fractions (Fig. 6*A*). The SsMyD88 aggregates formed around the MTOC through a microtubule-dependent mechanism indicating that these structures were aggresomes. The aggresomes are proteinaceous inclusion bodies involved in the sequestration of polyubiquitinated proteins, a defense mechanism that protects the cells from potentially cytotoxic aggregates (58). It has recently been shown that the aggregation of MyD88 depends on its interaction with p62 and HDAC6, which are involved in accumulation of polyubiquitinated proteins and autophagy (59). More specifically, p62 was found to deliver polyubiquitinated proteins to autophagosomes because it can directly bind and link polyubiquitinated proteins to the autophagosome marker LC3 (60). The autophagy is a mechanism for elimination of old or damaged organelles and insoluble proteins aggregates (61). In addition, autophagy has important immune functions because it is implicated in elimination of intracellular pathogens, the delivery of TLR ligands in endosomal compartments (62), and antigen presentation (63). TLR signaling is involved in the autophagy regulation both through MyD88-dependent and -independent mechanism(s) (64–67), and it has been found that activation of macrophages and dendritic cells with TLR ligands results in formation of ALIS in which polyubiquitinated aggregates are directed by p62 to autophagosomes (32). In the current study, transgenic SsMyD88 co-localized with aggregates of ubiquitinated proteins, but there was no detectable co-localization with endogenous p62, and no SsMyD88 aggregates were detected in LC3-labeled autophagosomes (Fig. 6*D*), indicating that these are, most likely, not ALIS but "classical" aggresomes.

Overall, the data in the current paper are the first to demonstrate that, like in mammals, teleost MyD88 associates with IRFs and can modulate the IRF-dependent IFN response. The sequestration of overexpressed SsMyD88 into aggresomes may represent a defense mechanism for control of the potentially harmful effects of excessive and/or prolonged MyD88 activation.

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