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ORIGINAL ARTICLE

Immune stimulation of rainbow trout reveals divergent regulation of MH class II-associated invariant chain isoforms

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Abstract

Major histocompatibility complex (MHC) class II-associated invariant chain is a chaperone responsible for targeting the MHC class II dimer to the endocytic pathway, thus enabling the loading of exogenous antigens onto the MHC class II receptor. In the current study, in vivo and in vitro methods were used to investigate the regulation of the rainbow trout invariant chain proteins S25-7 and INVX, upon immune system activation. Whole rainbow trout and the macrophage/monocyte-like cell line RTS11 were treated with PMA at concentrations shown to induce IL-1β transcripts and homotypic aggregation of RTS11. S25-7 transcript levels remained unchanged in the gill, spleen, and liver and were found to be significantly decreased in head kidney beginning 24 h post-stimulation. Meanwhile, INVX transcript levels remained unchanged in all tissues studied. Both S25-7 and INVX proteins were produced in gill and spleen tissues but their expression was unaffected by immune system stimulation. Surprisingly, neither INVX nor S25-7 protein was detected in the secondary immune organ, the head kidney. Analysis of RTS11 cultures demonstrated that both INVX and S25-7 transcript levels significantly increased at 96 h and 120 h following PMA stimulation before returning to control levels at 168 h. Meanwhile, at the protein level in RTS11, S25-7 remained unchanged while INVX had a significant decrease at 168 h post-stimulation. These results indicate that neither INVX nor S25-7 is upregulated upon immune system activation; thus, teleosts have evolved a system of immune regulation that is different than that found in mammals.

Keywords Invariant chain · Phorbol myristate acetate · Antigen presentation · Rainbow trout · RTS11 · Adaptive immunity

Introduction

To effectively combat the unavoidable and continual interaction with potential pathogens, vertebrates have evolved two distinct antigen presentation pathways that, when paired with effective innate immune system activation, can stimulate long-term immunological memory. The major histocompatibility complex (MHC) molecules are critical in this important immune process yet the MHC gene equivalents in teleosts are not clustered on a single chromosome as they are in mammals, so they are not considered to be a "complex." Instead these genes can be found on more than one chromosome and as a result are simply referred to as major histocompatibility (MH) genes (reviewed by Dixon and Stet 2001). In the mammalian model, the endogenous antigen presentation pathway includes MHC class I molecules which are found within all nucleated cells. This pathway involves the processing of antigens from intracellular pathogens and their presentation via MHC class I to $CD8^+$ cytotoxic T lymphocytes (reviewed by Neefjes et al. 2011). In comparison, the exogenous antigen presentation pathway uses MHC class II dimers and, following phagocytosis, is responsible for processing peptides from extracellular pathogens and their subsequent presentation to $CD4^+$ T helper lymphocytes (reviewed by Neefjes et al. 2011). Because MHC class II is typically only found within specific cell types, such as professional antigen presenting cells (APCs) and a limited number of other types during inflammatory conditions (reviewed by Landsverk et al. 2009), it is possible for a cell to have both the class I and class II antigen presentation pathways. Furthermore, both MHCI and MHCII molecules are assembled in the endoplasmic reticulum (ER), but only

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MHC class I completes peptide loading within the ER. As a result, loading of appropriate antigens into the correct MHC molecules must be organized and separated to avoid improper immune system activation, an essential task coordinated by the multifunctional MHC class II-associated invariant chain (also referred to as Ii and/or CD74).

Invariant chain plays several vital roles to ensure successful presentation, assembly and loading of MHC class II receptors. This multifunctional molecule consists of an N-terminal cytosolic tail, a transmembrane domain, the CLIP (class-II-associated Ii chain peptide) region, and a C-terminal luminal domain (reviewed by Schroder 2016). Immediately following synthesis in the ER, invariant chain assembles into trimers, a process which is driven by the luminal and transmembrane domains of Ii (Marks et al. 1990; Bijlmakers et al. 1994; Gedde-Dahl et al. 1997). From there, the CLIP regions of the trimerized invariant chains occupy the peptide-binding grooves of newly synthesized MHCII α and β chains resulting in nonameric complexes $(\alpha\beta Ii)_3$ (Roche et al. 1991; Romagnoli and Germain 1994). Invariant chain thereby promotes the correct assembly of MHCII heterodimers and also prevents the binding of antigens while these molecules are within the ER. Through a pathway that is not fully understood, invariant chain enables the nonameric complexes to exit the ER (reviewed by Landsverk et al. 2009). Two dileucine-based sorting motifs within the cytoplasmic domain of Ii then direct the associated MHCII molecules to specialized endosomal compartments, known as the MHC class II-containing compartment (MIIC), where antigen processing and loading takes place (Bakke and Dobberstein 1990; Bremnes et al. 1994). Both during transport and within the MIIC, Ii molecules are progressively degraded by late endosomal proteases until only the CLIP region remains associated with the MHCII dimer (reviewed by Stumptner-Cuvelette and Benaroch 2002). Following fusion with phagosomes and appropriate processing of the resulting protein fragments within the MIIC, CLIP is then exchanged for antigenic peptides in an enzyme-like reaction catalyzed by the non-classical MHCII human leukocyte antigen (HLA)-DM (Vogt et al. 1996). HLA-DM (DM) stabilizes the MHCII dimer when it is devoid of ligands thus preventing deterioration while also supporting peptide exchange until an antigen capable of binding to MHCII with high-affinity is obtained (Sloan et al. 1995; Denzin et al. 1996). The exogenous antigen presentation pathway concludes with the release of mature MHCII molecules to the cell surface and the presentation of bound antigens to $CD4^+$ T cells.

Considering its essential role in exogenous antigen presentation, one would expect invariant chain to be highly conserved across taxa. However, although there is high sequence and functional similarity, there exists a variety of Ii protein isoforms depending on the species considered. In humans, invariant chain is a type II transmembrane protein encoded by a single gene that can produce four different protein isoforms: p33, p35, p41, and p43 (Strubin et al. 1986a; O'Sullivan et al. 1987). The isoforms p33 and p41 are differentiated by the alternative splicing of an additional exon encoding 64 amino acids (Strubin et al. 1986a). The inclusion of this segment results in the thyroglobulin domain, a region responsible for regulating cathepsin proteolysis within the MIIC so that antigens and CLIP are not degraded too quickly (Mihelic et al. 2008). In comparison, p35 and p43 arise by alternative translational initiation resulting in an additional 16 amino acids in the N-terminal domain (Strubin et al. 1986b; O'Sullivan et al. 1987) of each of the p33 and p41 isoforms respectively. When this is compared with another mammalian model, the mouse, only equivalents to the p33 and p41 isoforms are present and are referred to as p31 and p41 respectively (Yamamoto et al. 1985; Strubin et al. 1986a). Though there are different numbers of Ii isoforms depending on the animal studied, all act as chaperones to ensure proper folding, trafficking, and peptide loading of MHCII in their respective species (reviewed by Fortin et al. 2013). The biological significance of the different isoforms is not well understood but there are differences in proportion as p41/p43 isoforms only account for 10–40% of Ii in professional APCs (Yamamoto et al. 1985; Kampgen et al. 1991). Furthermore, the only negative consequences observed from knockouts and/or recoveries of individual Ii isoforms are subtle changes to the MHCII peptide repertoire (Bikoff et al. 1998; Geneve et al. 2012). When taken together it appears that the existence of invariant chain isoforms may be reflective of necessary redundancies to ensure efficient and continuous antigen presentation.

Despite the numerous advances in our understanding of mammalian invariant chain, a similar molecule in fish was not discovered until 1999 when Yoder and colleagues cloned Ii-like transcripts from zebrafish. Their study uncovered that, similarly to mammals, invariant chain in fish also existed in multiple forms (Yoder et al. 1999). Subsequent work in rainbow trout supported this concept when it revealed three invariant chain genes (Dijkstra et al. 2003; Fujiki et al. 2003). The first gene, referred to as 14-1, appears to be closely related to zebrafish Iclp2 without a similar sequence present in mammals (Fujiki et al. 2003). The other two, INVX and S25-7, will be the focus of the present study. These genes encode proteins which possess domain structures equivalent to the human p33 and p41 Ii isoforms respectively. Furthermore, S25-7 is very closely related to the zebrafish Iclp1 (Dijkstra et al. 2003, Fujiki et al. 2003). Interestingly, rainbow trout INVX and S25-7 are encoded by two paralogous genes (Fujiki et al. 2003) rather than through alternative splicing of a single gene as seen in mammals. In spite of this regulatory difference, domains in both INVX and S25-7 present strong sequence similarity to tetrapod invariant chains. Thus, the resulting proteins likely have functional similarity to analogous isoforms in mammals but there has been little research to support this

claim. To correct for this lack of understanding, the current study compares the transcript expression of INVX and S25-7 with their protein production profiles following immune stimulation with phorbol 12-myristate 13-acetate (PMA) in both in vitro and in vivo systems. This required the development and validation of polyclonal antibodies raised against rainbow trout INVX and the thyroglobulin (Tg) domain of rainbow trout S25-7. The results presented here will provide important information regarding the evolution of invariant chain function prior to the emergence of mammals. Furthermore, an improved understanding of adaptive immunity in fish may provide insights regarding immunological memory and vaccine design for the growing aquaculture industry.

Materials and methods

Fish

Rainbow trout (150–300 g) obtained from Lyndon Hatcheries (New Dundee, ON) were maintained in 700-L freshwater flow-through tanks at 13 °C at the University of Waterloo. All fish were kept and handled under a permit from the University of Waterloo Animal Care Committee according to CCAC guidelines. All procedures were performed following guidelines of the Animal Care Committee of the University of Waterloo.

Maintenance of RTS11

The rainbow trout spleen monocyte/macrophage cell line, RTS11 (Ganassin and Bols 1998) was maintained as described previously by Sever et al. 2014.

Immune challenge and sample collection

In vivo stimulation

Thirty rainbow trout were anesthetized with 0.01% tricaine methanesulfonate (MS-222) after which 20 fish received an intraperitoneal (i.p.) injection of 0.1 μg of PMA (Sigma) emulsified in Freund's incomplete adjuvant (FIA, Sigma) per gram of body weight. This concentration was chosen based on preliminary studies to determine the optimal concentration of PMA based on the ability of a variety of PMA doses to induce an immune response. The stimulated fish were kept in one 700-L freshwater tank. In a separate tank, five fish received i.p. injections of emulsified FIA containing the relevant volume of DMSO alone as a carrier control. Prior to stimulation (day 0), the remaining five fish were euthanized with an overdose of MS-222 and tissue samples were collected as described below.

At 8 h, 24 h, 48 h, 96 h (4 days), and 120 h (5 days) following stimulation, 4 fish were euthanized with an overdose of MS-222. Gill, spleen, head kidney (HK), and liver samples were collected from all fish and these samples were flash frozen in liquid nitrogen. For all five of the carrier control fish, samples were collected on day 5. All tissue samples were stored at − 80 °C until further use.

In vitro stimulation

The macrophage/monocyte-like cell line, RTS11, was also stimulated with PMA to examine changes in S25-7 and INVX levels in vitro. Upon dissociation, the cells were resuspended in L-15 media without fetal bovine serum (FBS) supplemented with 200 μg/mL of streptomycin and 200 μg/mL of penicillin. Cells were plated in six-well tissue culture plates at a concentration of 8.0×10^6 cells/well. The cells were treated with 2 μg/mL of PMA or an equivalent volume of DMSO as a carrier control and incubated at 18 °C. The concentration of PMA selected for this study has been previously shown to induce homotypic aggregation in RTS11, a characteristic of activated immune cells (Dewitte-Orr et al. 2007). Stimulated cells were then collected from triplicate wells at 8 h, 24 h, 48 h, 96 h (4 days), 120 h (5 days), and 168 h (7 days) post-stimulation. At each of these time points, cells were separated into two equal fractions and centrifuged at 4000 rpm so that one fraction could be used for protein isolation, while the other was used for RNA isolation and cDNA synthesis.

Detection of INVX and S25-7 transcripts in rainbow trout

Primer design and sequence validation for INVX and S25-7

Primers were designed to examine the transcript expression of rainbow trout INVX and S25-7 (see Table 1). For sequence validation of these primer sets, the following PCR parameters were used: denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, 30 s at 60 °C and extension at 72 °C for 30 s. A final extension at 72 °C was carried out for 8 min. The amplified PCR products were separated on 2% agarose gels containing 2% GelGreen (Biotium Inc.). The resulting products were cloned into the pGEM®-T Easy vector, transformed into *Escherichia coli* XL1-blue cells and the resulting plasmids from 8 clones were extracted as previously described by Semple et al. (2018a). Plasmids were sequenced using SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, ON). The resulting sequences were then analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm sequence identity.

Table 1 Primer sequences used in this study

Gene	Sequence (5′–3′)	Annealing temp.	Application	Accession no. or reference
INVX extracellular	F-CGCCGGATCCCTGGTCTTGAACCAGA GG R-CGCCCTCGAGGCTGTGGTTACTCATTC	51 °C	Cloning	Christie 2007
S25-7 Tg domain	F-CGCCGGATCCGCCGCTGGCCTG R-CTCGAGCGCCTTATCTGTCACAGGTGGC			Braunstein 2004
IL-1β	F-CCACAAAGTGCATTTGAAC R-GCAACCTCCTCTAGGTGC	60 °C	qRT-PCR	Semple et al. 2018c
INVX	F-CAACACGAGGGCCTCTAACA R-CTGTTTCTCAGCTGCTTGCG			NM_001124443.1
S25-7	F-CAGTAGCCCCTGTCCAGATG R-ATGGCAGTGGCCTCCAATTT			NM_001124442.1
EF1α	F-CGCACAGTAACACCGAAACTAATTAAGC R-GCCTCCGCACTTGTAGATCAGATG			Semple et al. 2018c

RNA extraction and cDNA synthesis

Depending on the sample used, either 50 mg of tissue or a pellet of approximately 1×10^7 cells was homogenized in 1 mL of Trizol reagent and RNA was extracted according to the manufacturer's instructions (Invitrogen). The RNA was dissolved in appropriate volumes of DEPC water and stored at − 80 °C. To remove any contaminating genomic DNA, RNA samples were treated with DNase I (Thermo Scientific) as described by the manufacturer. RNA samples were then quantified using the Take3 plate of a Synergy H1 plate reader (BioTek Instruments) and were stored at − 80 °C until further use. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA using the qScript cDNA Supermix (Quanta Biosciences) in accordance with the manufacturer's instructions. For a no template control, 500 ng of RNA was included in the cDNA synthesis reaction without reverse transcriptase.

Quantitative reverse transcriptase polymerase chain reaction

To measure transcript expression of IL-1β (for confirmation of immune system activation) as well as invariant chain genes INVX and S25-7, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was used. All qRT-PCR reactions were 10 μL and contained 2.5 μL of cDNA (25 ng/μL diluted 1:10 in RNase free water), 2× WISENT ADVANCED™ qPCR mastermix (Wisent), and forward and reverse primers (Sigma-Aldrich) to a final working concentration of 0.25 μM. The sequences for all qRT-PCR primer sets are outlined in Table 1. All qRT-PCR reactions were completed on the LightCycler® 480 II (Roche). Each experimental sample was run in triplicate. For each plate, triplicate wells of a no template control and RNA only control were also present. The program used for all qRT-PCR reactions was as follows: pre-incubation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 8 s. A melting curve was completed for every run from 65 to 97 °C with a read every 5 s. Product specificity was determined through single PCR melting peaks. Data were analyzed using the ΔΔCt method and are presented as the average of either 4-5 fish (in vivo) or triplicate experimental repeats (in vitro) with the standard deviation. Specifically, gene expression was normalized to the housekeeping gene (EF1α) and expressed as fold change over the day 0 control group.

Development of polyclonal rabbit anti-rtINVX and anti-rTg antibodies

Construction of rtINVX and rTg expression vectors

Primer sets were developed to amplify the extracellular portion of INVX as well as the thyroglobulin domain (Tg) of S25-7. For both primer sets (presented in Table 1), the forward primers contained *BamHI* sites while the reverse primers contained *XhoI* sites. Both fragments were amplified using the following PCR conditions: 5 min at 95 °C followed by 30 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min followed by a 5-min extension at 72 °C. Amplification was confirmed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Following amplification, the PCR fragments were digested with *BamHI* and *XhoI*, gel purified with a GenElute agarose spin column (Millipore Sigma) and ligated into the pRSET A expression vector that had been previously digested with *BamHI* and *XhoI*. The INVX and S25-7 ligations were transformed into Rosetta (DE3) pLysS and BL21 (DE3) *Escherichia coli* bacteria (Novagen) respectively. Plasmid sequencing was performed in both the forward and reverse direction. The resulting sequences were compared with the

known INVX and S25-7 sequences (Fujiki et al. 2003) and both were observed to be identical.

Production and purification of recombinant INVX and recombinant S25-7 (Tg)

For both recombinant INVX and S25-7, the proteins were produced and purified from the *E. coli* cultures as described previously by Sever et al. 2013.

Production of polyclonal antibodies to rainbow trout INVX and S25-7 (Tg)

Four New Zealand white rabbits (Charles River, ON, CA) were injected with an emulsion containing 500 μL of recombinant protein (two rabbits received rINVX while the remaining two received rTg), 500 μL of Freund's complete adjuvant (Sigma), and 20 μg of keyhole limpet hemocyanin (Calbiochem) as described previously by Sever et al. 2013. To reduce non-specific binding of anti-rtINVX and anti-rtTg, the resulting polyclonal antibodies were affinity purified using sulfolink resin (Pierce).

Protein levels of INVX and S25-7 in rainbow trout

Total protein isolation

Depending on the sample used, total cellular protein was extracted from either 100–200 mg of tissue or from approximately 1.0×10^7 cells. NP-40 lysis buffer was used to extract protein from samples as described previously by Sever et al. 2013. The protein concentration of the resulting lysate was measured by a Bradford assay (Bio-Rad).

SDS-PAGE and Western blot analysis

All procedures followed Semple et al. (2017). Briefly, total protein samples were denatured at 100 °C for 10 min in 6× Laemmli Sample Buffer and subjected to electrophoresis in a 4 to 12% acrylamide gradient slab gel. All protein samples were loaded at 20 μg per lane. Protein samples were separated for 1 h at 130 V, after which, samples were transferred to a nitrocellulose membrane overnight at 22 V using a Transblot cell (Bio-Rad). Following the transfer, the membrane was removed and stained with Ponceau S stain, for assessment of equal loading and successful protein transfer. The membrane was then washed with 1× TBS-T (tris-buffered saline and Tween-20) and blocked for 2 h at room temperature in blocking buffer (5% skim milk in TBS-T). Membranes were then probed with either anti-INVX (1:80 dilution in blocking buffer) or anti-Tg (1:150 dilution in blocking buffer) for 1 h at room temperature. After washing with TBS-T, the membrane was probed with anti-rabbit IgG conjugated to horse radish peroxidase (HRP, Promega) at a 1:2000 dilution in blocking buffer for 1 h at room temperature. After three TBS-T washes, 1 mL of ECL plus substrate (Amersham) was added to the membrane and incubated for 5 min in the dark. The blot was detected on the Fluorochem 8000 using the chemiluminescence filter.

Densitometry analysis of all Western blots was performed with the Total Lab 100 software (Fotodyne Inc.). As both S25-7 and INVX are detected as doublets, the intensity of both bands in each doublet was added together. As a control for equal sample loading, the intensity of a 50 kDa band from the ponceau stain was measured and the ratio of either INVX or S25-7 to this 50 kDa band was calculated to normalize protein production.

Deglycosylation of invariant chain

Gill lysates containing 500 μg of total protein were boiled for 10 min to denature secondary structures. All samples were then treated overnight at 37 °C with 2500 U of Endo H in a sodium citrate buffer pH 5.5, containing 0.5% SDS and 1% β-mercaptoethanol. This was followed by an additional overnight incubation at 37 °C using 20 U of N-glycosidase F in sodium phosphate buffer pH 7.5, containing 1% NP-40. Control samples contained equivalent volumes of purified water in place of enzymes. Reaction volumes equivalent to 50 μg of original protein were separated on 15% SDS-PAGE and blotted onto nitrocellulose membranes for subsequent probing using rabbit anti-invariant chain, anti-MHI (1:300), or anti-β_2m (1:200) antisera as described above.

Statistical analyses

Statistical analysis was performed using a one-way ANOVA followed by a Tukey's post hoc test to determine if there was a significant change of either INVX or S25-7 when compared with the unstimulated control. A probability of $P < 0.05$ was considered statistically significant. All statistical analyses were completed using the statistical software Statistica version 7 (StatSoft, Tulsa, OK).

Results

Detection of immune activation with PMA

To determine whether the concentration of PMA was successful in inducing immune system activation, transcript levels of IL-1β were examined in the gill, spleen, and head kidney tissues (Fig. 1). In gill tissue, IL-1β transcript expression

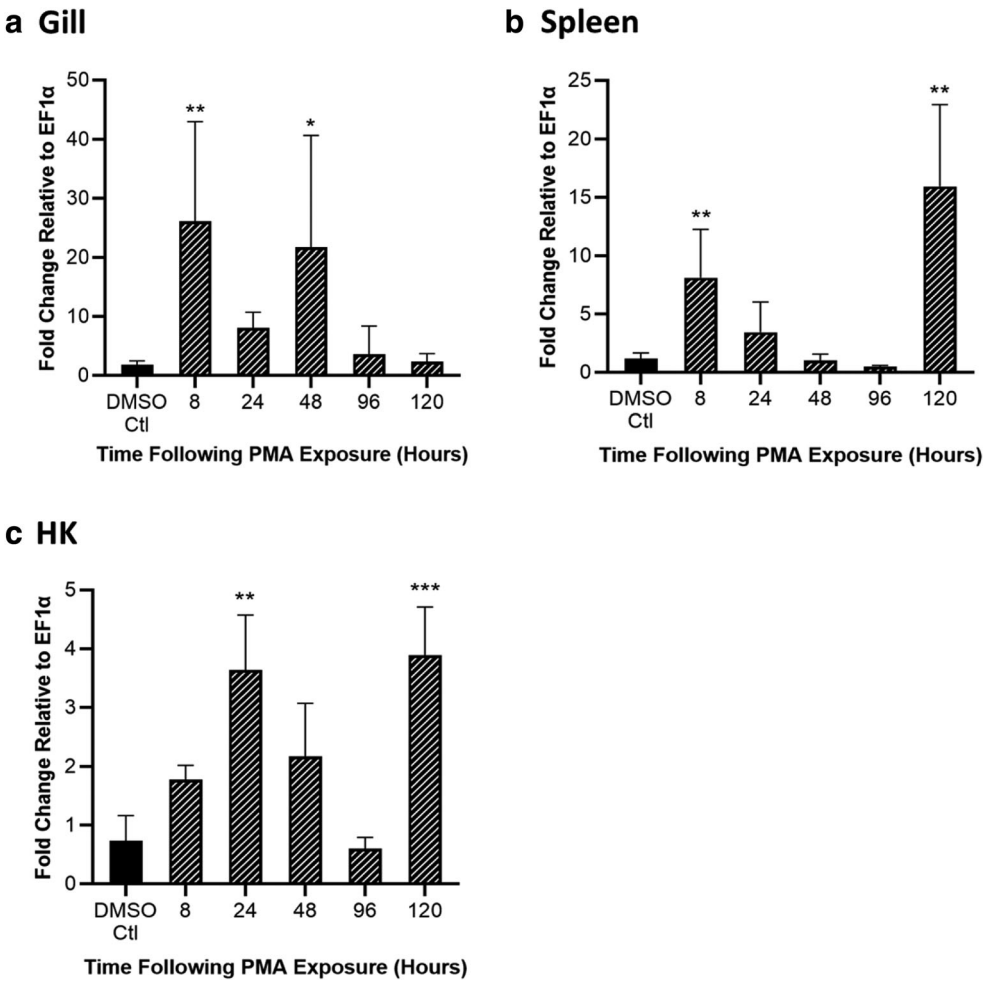

Fig. 1 Activation of the rainbow trout immune system by PMA. To assess immune activation, IL-β transcript levels were measured via qRT-PCR in the gill (panel **a**), spleen (panel **b**), and head kidney (HK, panel **c**) of rainbow trout throughout 5 days (120 h) of PMA stimulation. Each time point represents the transcript expression observed in three individual animals. Significant differences when compared with the control are represented by *($P < 0.05$), **($P < 0.01$), or ***($P < 0.001$). Vertical error bars represent the standard deviation

was very low in control fish but was observed to increase significantly 8 h and 48 h post-stimulation, while at all other time points, IL-1β transcript expression did not significantly differ from the DMSO injected control (Fig. 1a). In the spleen tissue, a significant increase in IL-1β transcript expression was only observed at 8 h and 120 h following injection with PMA (Fig. 1b). In head kidney, IL-1β expression was not observed to significantly increase until 24 h and this induction was not observed again until 120 h post-stimulation (Fig. 1c). As anticipated, IL-1β expression did not differ significantly at all time points analyzed in the liver tissue, the negative tissue control (data not shown).

Transcriptional profiles of INVX and S25-7 in PMA-stimulated tissue

Following injection with PMA, tissue samples from the gill, spleen, and HK were removed from stimulated fish so that transcript expression of INVX and S25-7 could be analyzed over 5 days (120 h, Fig. 2). Transcript levels of INVX did not appear to significantly change throughout 5 days of immune stimulation (Fig. 2 a–c) in the gill, spleen, and HK. Similarly, to INVX, S25-7 was also observed to have no significant change in transcript levels throughout the 5-day immune stimulation in the gill and spleen as presented in Fig. 2 d and e respectively. However, in head kidney samples, S25-7 had a significant decrease in expression following treatment, specifically at 120 h post-stimulation when compared with the untreated controls. Liver samples were also taken at all time points as a negative control and were observed to have little to no transcript expression of both INVX and S25-7 (data not shown).

INVX and S25-7 transcript levels in PMA-stimulated RTS11

When invariant chain transcripts were analyzed following immune stimulation in vitro with RTS11, genes for both invariant chain isoforms increased significantly at 96 h and 120 h post-stimulation when compared with both the DMSO and day 0 controls (Fig. 3). When INVX expression (Fig. 3a) was compared with S25-7 transcript levels (Fig. 3b), S25-7 had a slightly more pronounced fold change increase.

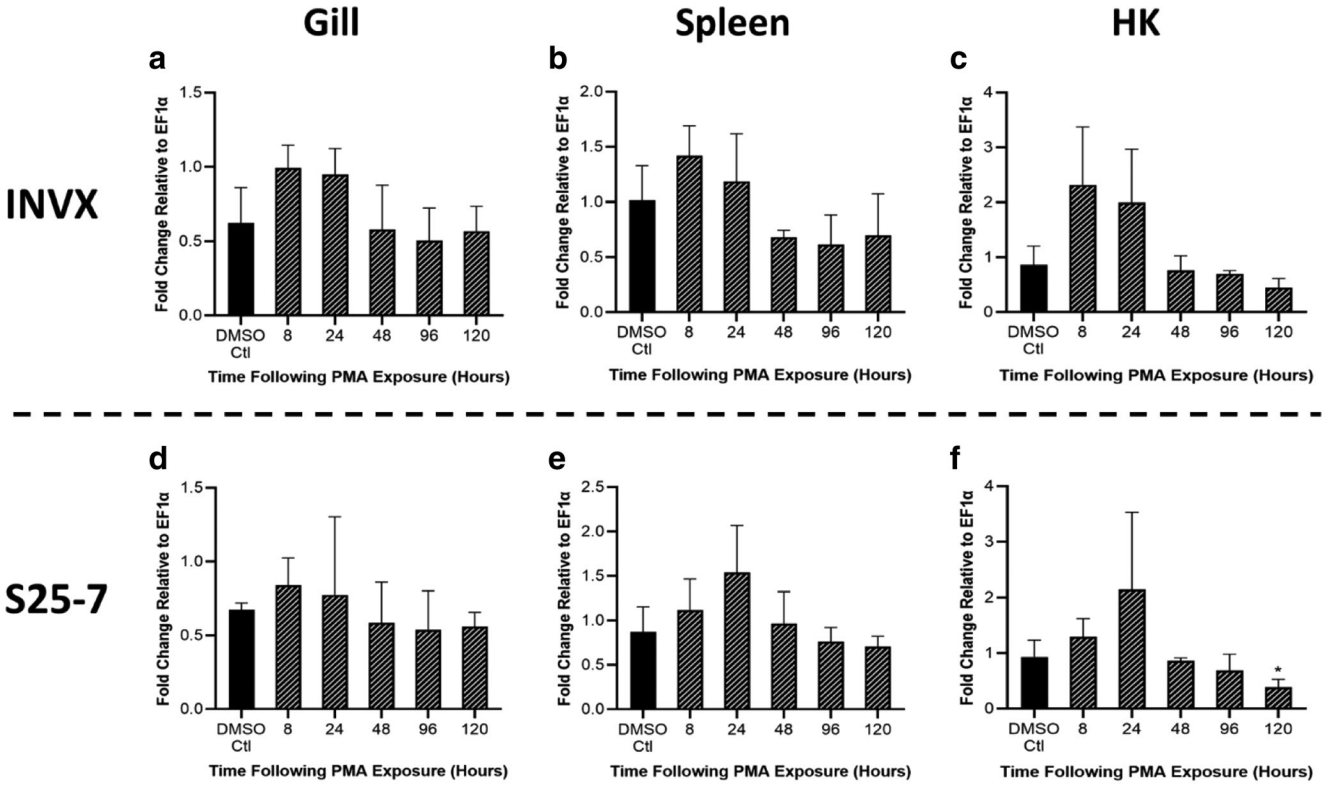

Fig. 2 Transcriptional profiles assessed by qRT-PCR of INVX (**a**–**c**) and S25-7 (**d**–**f**) throughout 5 days (120 h) of PMA stimulation in rainbow trout gill (**a** and **d**), spleen (**b** and **e**), and head kidney (**c** and **f**) tissues. The data presented for all time points was repeated using a minimum of three individuals. Significant differences when compared with the DMSO control are represented by *($P < 0.05$). Vertical error bars represent the standard deviation

Constitutive protein production of INVX and S25-7 in rainbow trout tissues

After the successful development of polyclonal antibodies to rainbow trout INVX and the thyroglobulin domain of S25-7, constitutive production of both proteins was tested in a variety of rainbow trout tissues (Fig. 4a). Protein from both INVX and S25-7 was constitutively produced in only the gill and spleen tissue. In all other tissues analyzed (head kidney, liver, intestine, muscle, and heart), neither INVX nor S25-7 proteins were detected (Fig. 4a). Additional data confirming the specificity of the INVX and S25-7 antibodies via blocking experiments can be found in online resource 1.

Both invariant chain proteins migrated at slightly larger sizes than what was predicted from the amino acid sequences with INVX migrating at approximately 37 kDa (expected size of 22.3 kDa) and S25-7 migrating at approximately 42 kDa (expected size of 31.8 kDa). In addition, both proteins migrated as doublets. These unanticipated protein variations could be explained by post-translational modifications such as glycosylation. To explore this possibility, a deglycosylation assay was performed wherein both INVX and S25-7 presented with

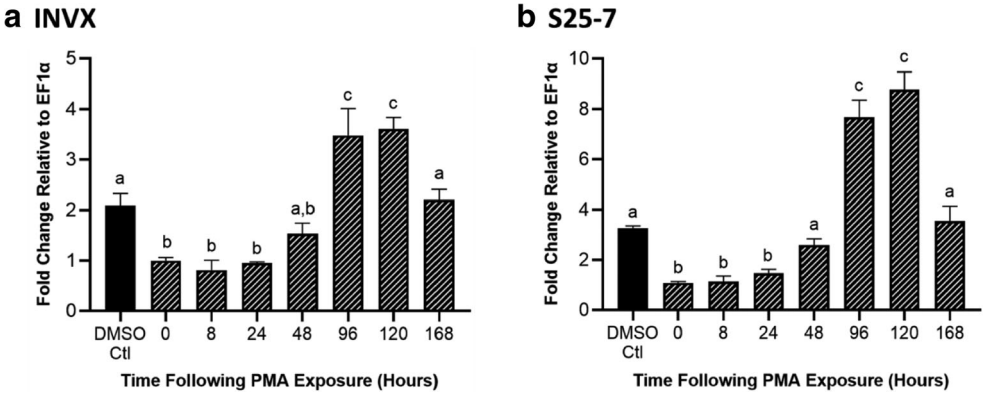

Fig. 3 Transcriptional profile of INVX and S25-7 throughout 1 week (168 h) of PMA stimulation in the monocyte/macrophage-like cell line RTS11. Transcript expression of INVX (**a**) and S25-7 (**b**) was determined using qRT-PCR analysis. Each time point was repeated a minimum of three times. Vertical error bars represent the standard deviation. Significant differences were only reported if $P < 0.05$

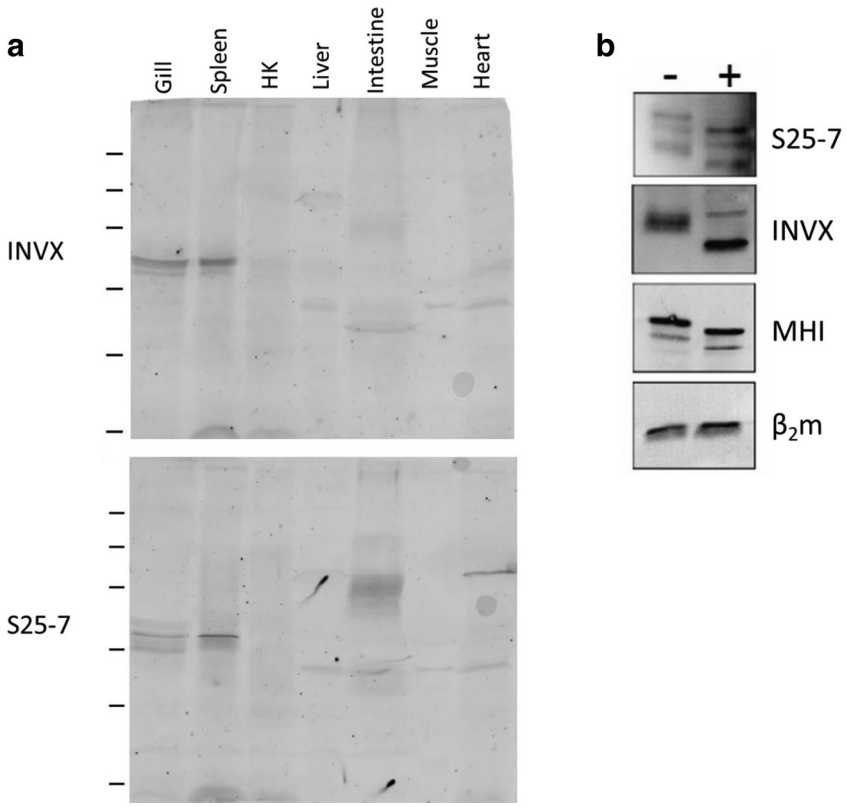

Fig. 4 Constitutive protein production of INVX and S25-7 in rainbow trout by Western blot analysis. Tissue distribution of INVX and S25-7 in unstimulated rainbow trout. Molecular weight markers from high to low were 98, 64, 50, 36, 30, and 16 kDa and are indicated by an en dash symbol on the left-hand side of the blots (**a**). N-type deglycosylation of INVX and S25-7 in rainbow trout gill lysate as well as appropriate positive and negative controls (**b**). As MHI is known to be glycosylated while β_2m is not in rainbow trout, these proteins were used as positive and negative controls respectively

N-linked glycosylation (Fig. 4b). However, even after deglycosylation, both invariant chains continued to migrate as doublets; thus, N-linked glycosylation is not responsible for these multiple bands. As a positive control, MH class I UBA was shown to be glycosylated while β_2m, the negative control, was not (Fig. 4b). The control results are comparable with what has been observed previously by our group when the rainbow trout MHI and β_2m antibodies were developed (Kales 2006).

Protein levels of INVX and S25-7 in fish tissues throughout immune stimulation

During PMA exposure, protein levels of the two invariant chains were assessed in the gill, spleen, and head kidney of stimulated fish (Fig. 5). In both gill and spleen, protein production of INVX did not differ significantly from control fish throughout the 7 days of PMA exposure (Fig. 5 a and b). This same trend in protein production was also observed for S25-7 in the gill and spleen of these fish (Fig. 5 d and e) wherein there were no significant differences during immune stimulation. Interestingly, at the protein level, both invariant chains were not observed at all in the head kidney of control or PMA-injected fish (Figs. 5c and 3f) despite differences noted previously at the transcript level in Fig. 2.

Protein levels of INVX and S25-7 in stimulated RTS11

After receiving a dose of PMA known to induce homotypic aggregation (Dewitte-Orr et al. 2007), protein production of neither INVX (Fig. 6a) nor S25-7 (Fig. 6b) was observed to significantly change when compared with the DMSO carrier control wells.

Discussion

Immune system activation with PMA

The purpose of this study was to explore both the transcriptional and protein production profiles of two rainbow trout invariant chain isoforms, INVX and S25-7, in both in vivo and in vitro systems. To do this, the phorbol ester PMA was used to stimulate either whole fish or the macrophage/monocyte-like cell line RTS11 through its activation of protein kinase C (PKC). PKC represents a family of protein kinase enzymes heavily involved in signal transduction pathways (reviewed by Newton 2009). This large family of enzymes functions by controlling activities of other proteins through phosphorylating the hydroxyl groups of specific serine or threonine residues in their structures (Stillwell 2016). An important activator of PKC is diacylglycerol (DAG), a neutral lipid functioning as a component of cellular

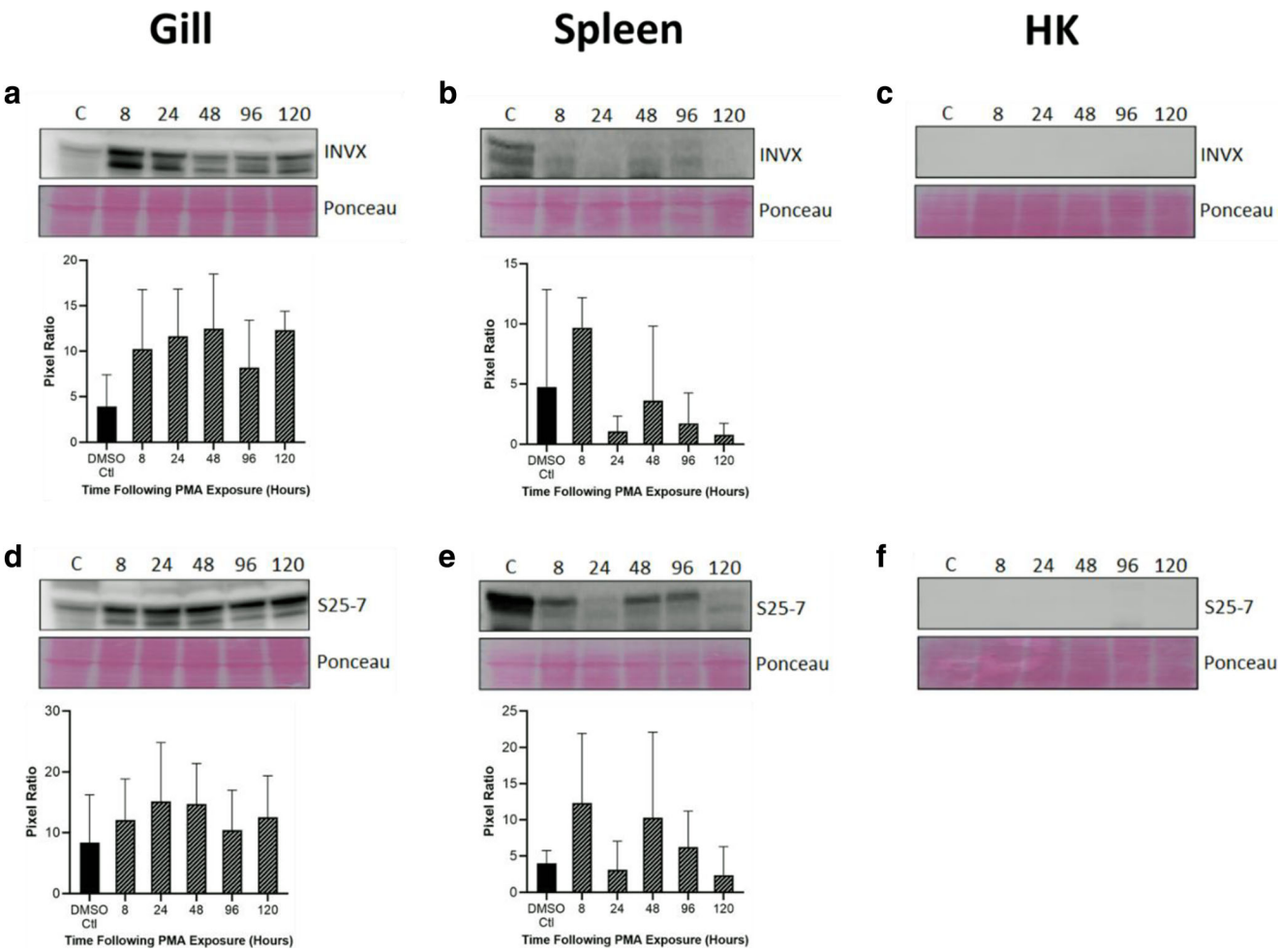

Fig. 5 Protein production profiles of INVX (**a**–**c**) and S25-7 (**d**–**f**) throughout PMA stimulation for 5 days (120 h) in rainbow trout gill (**a** and **d**), spleen (**b** and **e**), and head kidney (**c** and **f**) tissues. All bar graphs represent the densitometry analysis of Western blots in which the ratio of INVX or S25-7 to the ponceau 50 kDa band was calculated at each time point (repeated a minimum of three times). Error bars represent the standard deviation

membranes, building blocks for glycero (phospho) lipids, and/or as lipid second messengers (reviewed by Eichmann and Lass 2015). In immune responses, DAG primarily acts as a key secondary lipid messenger for transducing signals downstream of receptors expressed by hematopoietic cells such as G protein coupled receptors (GPCR) and immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors (reviewed by Singh and Kambayashi 2016). DAG is critical in driving the activation, proliferation, migration, and effector function of adaptive and innate immune cells including, but not limited to, the differentiation of monocytes to macrophages (Tsuchiya et al. 1982; Takashiba et al. 1999), T cell activation (Downward et al. 1990; Dower et al. 2000), homotypic aggregation of leukocytes (Yamauchi et al. 2002; Cho et al. 2003), and inducing antigen presentation by MHC class II (Majewski et al. 2007). Furthermore, through the activation of PKC, DAG is also involved in the regulation of the transcription factor, NF-κB, making this lipid essential for the production of cytokines like IL-1β and IFNγ (reviewed by Singh and Kambayashi 2016; Liu et al. 2017). The immune stimulus used in this study, PMA, is a plant-derived phorbol ester which can substitute for DAG in activating protein kinase C. However, unlike DAG, phorbol esters cannot be readily metabolized and thus can produce prolonged activation of PKC (Newton 1995). Given the wide immunostimulatory profile in mammalian models, and its similar effects in both fish species and fish cells (Hirono et al. 2000; Corripio-Miyar et al. 2007; Dewitte-Orr et al. 2007), PMA was an ideal candidate for understanding rainbow trout adaptive immunity both in vivo and in vitro.

Despite the extensive amount of research using PMA, there has been little to determine the appropriate dose required to induce immune stimulation in whole fish tissue. Though it has been observed in some studies (Corripio-Miyar et al. 2007; Kling et al. 2013), the majority of fish research has used PMA to stimulate either primary cultures of leukocytes or long-term cell cultures (Nagelkerke et al. 1990; Dewitte-Orr et al. 2007; Haghighi et al. 2017). The current study shows that an i.p.

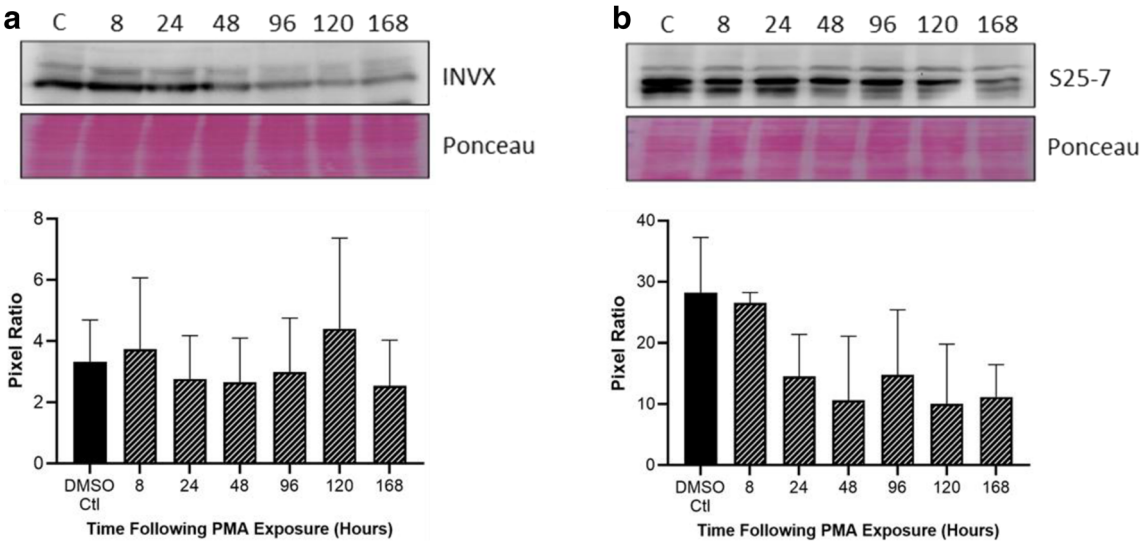

Fig. 6 Protein production of INVX (**a**) and S25-7 (**b**) in RTS11 cultures for 1 week (168 h) following 2 μg/mL stimulation with PMA determined through Western blot analysis. All bar graphs represent densitometry analysis in which the ratio of INVX or S25-7 to the ponceau 50 kDa band was calculated at each time point (repeated a minimum of three times). Significant differences respective to the control are represented by *($P < 0.05$). Vertical error bars represent the standard deviation

injected dose of PMA at 0.1 μg per gram of body weight could induce transcript expression of the pro-inflammatory cytokine, IL-1β, in the gill, HK, and spleen tissues of rainbow trout. This induction was not observed in the carrier control or in the non-immune tissue control, the liver. The concentration of PMA used here is consistent with previous experiments inducing immune system activation of fish species (Corripio-Miyar et al. 2007; Kling et al. 2013). However, the dose used in the present study is much higher than that used for mammalian models (Dzietko et al. 2015). In fact, when compared with their mammalian counterparts, fish sometimes require much higher doses of synthetic stimuli as has been observed with LPS in both in vivo and in vitro experiments (Iliev et al. 2005). This may also be the case with PMA but further research would be required to determine variability between individual fish species. For the purposes of the current study, the PMA concentration presented was successful in activating the immune system of rainbow trout and thus could be used to assess invariant chain gene/protein regulation.

INVX and S25-7 gene expression following PMA stimulation

Though PMA was shown to stimulate immune function in the gill, spleen, and HK tissues of rainbow trout, this stimulation did not appear to significantly influence INVX gene expression for any of the time points analyzed. Meanwhile, only HK samples of the stimulated fish experienced a significant reduction in S25-7 transcripts. As described above, stimulation with PMA would induce an inflammatory response (Gordon and Galli 1990; Dzietko et al. 2015) triggering many supporting cell types to produce MH class II that would not be expressed in an unstimulated individual (Benveniste et al. 1991; Thelemann et al. 2014). Thus, these supporting cells would also need to produce invariant chain, leading to an observable increase in its gene expression. When this is combined with the knowledge that inflammatory responses in mammalian immune cells/tissues result in an increase in invariant chain gene expression (Collins et al., 1984; Paulnock-King et al. 1985; Albanesi et al. 1998), upregulation would be expected in fish if they had comparable methods of Ii regulation. It is possible however that the supporting cells migrated away from the head kidney to the periphery before upregulating protein expression. Though rainbow trout present a very different Ii transcriptional pattern, a similar trend to mammals has been observed in the primitive fish, the Chinese sturgeon (*Acipenser sinensis*). This non-teleost species presents characteristics of both bony and cartilaginous fish; thus, it may provide insight regarding immune regulation prior to the divergence of salmonids or teleost fishes from the tetrapod lineage (Dixon 2012). In Chinese sturgeon, upregulation of Ii mRNA was observed in the intestine, spleen, and HK when stimulated with either poly I:C or a bacterial vaccine (Li et al. 2017). As has been predicted previously (Stet et al. 2003), it appears that the evolved bony fish species have their MH genes and regulation organized in a very different manner than that of mammals. This may extend to the gene function of MH class II-associated invariant chain as well, but more study would be required to provide a full comparison.

When observing invariant chain expression in a more homogenous cell mixture, such as that of RTS11, a conflicting trend was detected when compared with whole rainbow trout. Rather than presenting no expression, as observed in spleen tissue in vivo, PMA was able to induce stimulation of both

INVX and S25-7. This increase in expression was not observed until 96 h and 120 h after stimulation and then was seen to return to levels observed in the DMSO control. Because RTS11 consists of only monocyte-like and macrophage-like cells, rather than the complex blend of different cell types that may or may not be capable of exogenous antigen presentation, perhaps it is easier to observe a consistent trend than when analyzing whole tissue. As described above, mammalian invariant chain isoforms would be expected to increase following immune system activation. It is interesting that this was not observed at the tissue level in vivo but was seen in an immortalized rainbow trout immune cell line. Furthermore, there have been fish species, such as the Atlantic cod, where MH class II and invariant chain genes are completely absent (Star et al. 2011). This may indicate that MH class II, and thus invariant chain, is not always necessary for effective immune function in aquatic environments. When taken together, this would mean that regulation of the Ii isoform genes differs significantly from that of mammals similar to the alternative method of regulation of the endogenous antigen presentation pathway (Sever et al. 2018), but this would have to be supported at the protein level as well.

Antibody validation for anti-rtINVX and anti-rtTg (S25-7)

Functional analysis of the fish immune system is limited due to the lack of certain tools, primarily target-specific antibodies. The current study presents the development and characterization of the first antibodies raised against rainbow trout INVX and the thyroglobulin domain of S25-7 so that functional analyses of these molecules could be explored. Through northern blot analysis, Fujiki et al. (2003) previously revealed that S25-7 presented high gene expression in the spleen, gill, and PBLs of unstimulated rainbow trout while high expression was only observed in the gill and PBLs for INVX. Lower expression was detected in the heart, HK, and posterior kidney for S25-7 and in the HK, spleen, and heart for INVX (Fujiki et al. 2003). Based on these preliminary results, it was reasonable to believe that perhaps the two rainbow trout Ii isoforms had tissue-specific roles. However, following protein analysis in the present study, both INVX and S25-7 protein were constitutively produced in only the gill and spleen tissues of unstimulated rainbow trout. Though both of these tissues are important immunosurveillance organs in fish and would be expected to have cells capable of performing exogenous antigen presentation (Zapata et al. 1987; Ganassin and Bols 1998), these results did not follow the previously reported tissue transcriptional profile (Fujiki et al. 2003). This would not be the first example of transcript presence not correlating with functional protein in a fish species. Previous work by our group showed that even though embryonic Chinook salmon cells possessed transcripts for class A scavenger receptors (SR-As, Semple et al. 2018b), these cells could not bind to and internalize the SR-A ligand until they were transfected with a functional SR-A (Monjo et al. 2017). Therefore, transcript presence does not always guarantee the existence and proper function of the corresponding protein. Given that there are many required steps following transcription and translation to produce a fully functional protein, it is not surprising that gene expression does not always represent equivalent protein synthesis.

To ensure proper folding, activity, and stability, many cellular proteins undergo essential post-translational modifications (PTMs). These modifications are also seen in mammalian invariant chain isoforms where these proteins experience several PTMs including phosphorylation, N-linked glycosylation, O-linked glycosylation, and lipidation (Machamer and Cresswell 1982; Simonis and Cullen 1986; Koch 1988; Spiro and Quaranta 1989). Thus, when both INVX and S25-7 were larger than predicted and traveled unexpectedly as doublets, a deglycosylation assay was performed to provide an explanation for these unanticipated protein variations. Both INVX and S25-7 presented N-linked glycosylation, reducing the size of the protein, but this did not correct the doublet formation. Perhaps like the mammalian model, rainbow trout Ii isoforms undergo multiple PTMs which are capable of influencing their performance. Further study is required to have a full understanding of how PTMs influence invariant chain function in teleosts.

Protein levels of INVX and S25-7 throughout immune stimulation with PMA

Although transcriptional analysis provides some insight regarding the function of rainbow trout invariant chain, relating this to protein production will reveal a much more complete understanding of Ii regulation and function. When compared with unstimulated control fish, there were no significant differences in protein levels of both INVX and S25-7 in all of the stimulated tissues studied. Following immune stimulation, a lack of regulation in fish accessory molecules is common, even when the comparable mammalian molecules are regulated in similar circumstances. This has been seen with calreticulin (Kales et al. 2007; Sever et al. 2014) and ERp57 proteins (Sever et al. 2014) where these proteins were unaffected by immune stimulation. Unexpectedly, although HK was shown to have transcripts of both INVX and S25-7, neither of the isoforms were detected at the protein level in this tissue. The HK is believed to be responsible for blood cell differentiation making this tissue analogous to the bone marrow of mammals (Press and Evensen 1999). As this is the case, exogenous antigen presentation would be anticipated. However, the results presented here only consider two of the three invariant chain isoforms found in rainbow trout (Fujiki et al. 2003). The third Ii isoform, 14-1, may be present at the protein level in HK. Preliminary data from our research group

provides some evidence supporting that this may be the case (Reid 2005; manuscript in prep.), revealing a stark difference to mammalian Ii isoform regulation. In humans, although p33 is the most abundant Ii isoform and generates some homotrimers, it is characteristically part of heterotrimers resulting in all four isoforms existing in stimulated immune tissues (Lamb and Cresswell 1992). This does not appear to be the case in rainbow trout as the different Ii isoforms present tissue-specific functional profiles. Though 14-1 specific antibodies have been developed in our lab and preliminary results obtained, further research is required to fully understand invariant chain regulation in HK. When these results are combined with the fact that a DM-like molecule has not yet been discovered in a fish species and may be absent (Dijkstra et al. 2013), it is not surprising that teleosts may present different Ii regulation to correct for this. Overall, it appears that there is still a great deal to learn about exogenous antigen presentation in fish.

Similar to the in vivo results, the in vitro results did not present significant differences in protein production throughout PMA stimulation. Though INVX and S25-7 transcripts were both observed to increase during PMA challenge in vitro, this increase was not seen at the protein level. Because transcript expression in RTS11 cells was not seen to increase until later in the PMA challenge (96 h and 120 h) it is possible that observation of a significant increase in protein production may have required more than 168 h, although it is difficult to culture cells beyond that time point without significant cell death. A temporal delay before observing an increase in protein production would not be surprising, as time is required to translate mRNA into protein. This time difference between mRNA expression and functional protein production has also been shown in mammalian macrophages (Eichelbaum and Krijgsveld 2014). It is also possible that the transcriptional profile does not match the levels of functional protein due to alterations in translation rate and post-translational processing, but further experiments regarding the regulation of teleostean invariant chain would be necessary. Aside from the isoforms emphasized in this study, it would also be interesting to compare gene expression and protein production of 14-1 to see how this isoform reacts to immune challenge as well. Future studies will focus on comparing the protein production of all three isoforms between tissues/cells when undergoing immune system activation using various stimuli.

Conclusions

The results of the present study provide further evidence that rainbow trout invariant chain isoform gene are regulated in a manner that differs significantly from that of the mammalian model. All tissues and cells analyzed expressed transcripts of S25-7 and INVX. In vivo, head kidney tissue presented a surprising downregulation of S25-7 while all other tissues did not vary when compared with the control. At the protein level, there were no significant changes of either INVX or S25-7 in any of the tissues studied in vivo. Unlike mammals, there was no significant upregulation observed in vivo of either invariant chain isoform upon immune stimulation. These results were contrary to the in vitro results using the RTS11 cell line where transcript expression significantly increased at 96 h and 120 h following PMA stimulation. However, at the protein level, neither isoform of invariant chain differed significantly from the carrier control. Given some of the differences observed in this study, perhaps the Ii isoforms in rainbow trout have different functional roles depending on the tissue analyzed as well as the immune stimulus used. The development of the polyclonal antibodies specific to INVX and S25-7 presented here means that these questions can be explored in the future. Overall, it appears that the teleost immune system has evolved a unique method of immune system regulation upon stimulation and pathogen challenge.

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Compliance with ethical standards

All fish were kept and handled under a permit from the University of Waterloo Animal Care Committee according to CCAC guidelines. All procedures were performed following guidelines of the Animal Care Committee of the University of Waterloo.

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