dsRNA sensing in fish cells: Using CHSE-214 as a model for the study of class A scavenger receptors

Andrea Monjo
Wilfrid Laurier University, monj6630@mylaurier.ca

Follow this and additional works at: https://scholars.wlu.ca/etd

Part of the Cell Biology Commons

Recommended Citation
https://scholars.wlu.ca/etd/1902

This Thesis is brought to you for free and open access by Scholars Commons @ Laurier. It has been accepted for inclusion in Theses and Dissertations (Comprehensive) by an authorized administrator of Scholars Commons @ Laurier. For more information, please contact scholarscommons@wlu.ca.
DSRNA SENSING IN FISH CELLS: USING CHSE-214 AS A MODEL FOR THE STUDY OF CLASS A SCAVENGER RECEPTORS

By

Andrea Lilly-Anne Monjo

Honours Bachelor of Science Biology, Wilfrid Laurier University, 2013

THESIS

Submitted to the Department of Biology

Faculty of Science

In partial fulfillment of the requirements for the

Master of Science in Integrative Biology

Wilfrid Laurier University

2016

Andrea Monjo 2016 ©
ABSTRACT

When infecting cells, most viruses produce dsRNA molecules at some point in their replicative cycle. When an infection results in cell lysis, these molecules are released into the extracellular space. Class A scavenger receptors (SR-As) on the surface of animal cells bind extracellular dsRNA and bring it into the cell where dsRNA triggers an innate immune response, enabling the cell to protect itself from an impending virus infection. Very few cells are unable to bind and respond to extracellular dsRNA, thus cell-based assays for studying extracellular dsRNA sensing via SR-As have been limited. CHSE-214 cells are a promising model for SR-A study as preliminary reports suggest they are unable to initiate an antiviral response when treated with extracellular dsRNA, but are able to respond to intracellular dsRNA (via transfection); possibly due to a lack of functional SR-As. Using qRT-PCR to measure the expression of innate immune gene transcripts and a cytopathic effect assay to investigate whether immune gene expression leads to an antiviral state, the present study found that CHSE-214 cells express immune genes and establish an antiviral state only when dsRNA is transfected directly into the cell, not in response to extracellular dsRNA. Immunocytochemistry showed that CHSE-214 cells cannot bind or internalize dsRNA, furthering the hypothesis that they may lack functional SR-As. To further investigate the feasibility of CHSE-214 functioning as a cell model for SR-A binding and signaling, novel rainbow trout SR-A sequences were cloned into expression vectors and sequenced. Bioinformatics analysis shows that these sequences have similar protein architecture to those in other species and phylogenetic analysis shows a strong relationship to other fish MARCO and SCARA5 sequences. Preliminary data have shown that overexpression of exogenous SR-As in CHSE-214 enables it to bind extracellular dsRNA. Developing a cell line for use as a model for the study of class A
scavenger receptors will facilitate the understanding of these receptors’ functions in innate immunity.

ACKNOWLEDGEMENTS

I would like to thank all members of the DeWitte-Orr lab for providing a supportive and enthusiastic research environment where many friendships were formed. Specifically, I would like to thank Sarah Poynter for her constant help, support and guidance, which contributed to all aspects of this project. Most importantly, thank you to Dr. Stephanie DeWitte-Orr for the wonderful experience and opportunity of working with you. Your enthusiasm for science is contagious and your guidance and support throughout my time in the lab has been very much appreciated.
# TABLE OF CONTENTS

**Chapter 1: General introduction and literature review**

1.1 Innate immunity.................................................................2

1.2 Pathogen-associated molecular patterns (PAMPs).........................2

1.3 Pathogen recognition receptors (PRRs)..................................3

   1.3.1 Intracellular PRRs......................................................3

      1.3.1.1 Toll-like receptors (TLRs).......................................3

      1.3.1.2 Rig-I-like receptors (RLRs).....................................4

      1.3.1.3 Cytosolic DNA sensors (CDSs).................................5

   1.3.2 Surface PRRs.............................................................6

      1.3.2.1 Class A scavenger receptors (SR-As).........................6

1.4 Type I interferons (IFNs)..................................................8

1.5 Interferon-stimulated genes (ISGs) and the antiviral state...............10

1.6 Fish innate immunity........................................................11

   1.6.1 Pathogen recognition receptors (PRRs)..........................11

   1.6.2 Interferon (IFN) and interferon stimulated genes (ISGs)........12

1.7 CHSE-214.................................................................14

1.8 Research objectives and hypotheses.....................................14

1.9 Co-authorship...............................................................15

**Chapter 2: CHSE-214 is defective in its dsRNA uptake mechanism: Implications for dsRNA sensing studies**

2.1 Introduction.............................................................................20

2.2 Materials and methods.......................................................22

   2.2.1 Cell culture.................................................................22
2.2.2 CSV propagation and infection .........................................................22
2.2.3 Cell treatments ............................................................................22
2.2.4 qRT-PCR: IFN and ISG transcript expression analysis ......................23
   2.2.4.1 RNA extraction and cDNA synthesis ........................................23
   2.2.4.2 qRT-PCR reactions ................................................................23
2.2.5 Cytopathic effect assay .................................................................24
2.2.6 Immunocytochemistry .................................................................25
2.2.7 Overexpression of hSR-AI and AcLDL binding assay .........................25
2.2.8 Statistical analysis ........................................................................26

2.3 Results and discussion ....................................................................26
   2.3.1 IFN and ISG transcripts are only upregulated in response to intracellular
       dsRNA ............................................................................................26
   2.3.2 Intracellular dsRNA pretreatment reduces CSV’s cytopathic effects.......27
   2.3.3 dsRNA is not bound or internalized by cells treated with dsRNA .......27
   2.3.4 CHSE-214 cells transfected with a human SR-AI-containing expression
       plasmid bind AcLDL .......................................................................28

2.4 Conclusions ....................................................................................28

Chapter 3: Novel MARCO and SCARA5 sequences in Oncorhynchus mykiss

3.1 Introduction ....................................................................................40
3.2 Materials and methods .................................................................42
   3.2.1 Cell culture ..............................................................................42
   3.2.2 Full-length sequence identification .............................................42
      3.2.2.1 Primer design ....................................................................42
      3.2.2.2 RT-PCR ...........................................................................43
      3.2.2.3 Cloning and sequencing .....................................................44
3.2.3 Cell and tissue expression screening ............................................... 44
3.2.4 Bioinformatics analysis .................................................................... 45
3.3 Results and discussion .......................................................................... 46
3.4 Conclusions .......................................................................................... 47

Chapter 4: General discussion

4.1 Major research findings and contributions to the field ............................. 58
4.2 Summary .................................................................................................. 61

Literature cited ............................................................................................. 66
LIST OF TABLES

Chapter 1

Table 1.1 Class A scavenger receptors, examples of their ligands and expression profiles.................................................................16

Chapter 2

Table 2.1 qRT-PCR primers used for transcript expression analysis of immune genes…30

Chapter 3

Table 3.1 RT-PCR primers used for amplification of full-length and fragment MARCO and SCARA5.................................................................48

Table 3.2 Amino acid sequence identity scores between Oncorhynchus mykiss MARCO and SCARA5 sequences and other species represented in Figure 3.1.................49
LIST OF FIGURES

Chapter 1

Figure 1.1 PRR signaling pathways leading to type I IFN induction and signaling……17

Chapter 2

Figure 2.1 IFN and ISG transcripts are only upregulated in response to intracellular dsRNA in CHSE-214………………………………………………………………………………31

Figure 2.2 Intracellular but not extracellular dsRNA treatment protects CHSE-214 from CSV-induced syncytia………………………………………………………………………………33

Figure 2.3 dsRNA is not bound or internalized by CHSE-214 cells treated with dsRNA………………………………………………………………………………………………35

Figure 2.4 CHSE-214 cells transfected with a human SR-AI-containing expression plasmid are able to bind AcLDL…………………………………………………………………37

Chapter 3

Figure 3.1 The protein domain architecture of class A scavenger receptors MARCO and SCARA5 in different species, including those sequenced in Oncorhynchus mykiss………………………………………………………………………………51

Figure 3.2 Phylogenetic tree illustrating the relationship between the Oncorhynchus mykiss MARCO and SCARA5 protein sequences and those reported in representative fish, mammalian, reptile and avian species………………..53

Figure 3.3 RT-PCR based detection of MARCO and SCARA5 transcripts in rainbow rout cell lines and tissues……………………………………………………………………….55

Chapter 4

Figure 4.1 CHSE-214 cells transfected with a rainbow trout MARCO-containing GFP expression plasmid are able to bind polyI:C. …………………………………………..64
Chapter 1

General introduction and literature review
1.1 Innate immunity

In all vertebrates, the first line of defense against viral infections is the production of type I interferon (IFN) by the innate immune system, which hampers viral replication and recruits the adaptive immune system (a branch of immunity unique to jawed vertebrates). While the adaptive immune response is antigen specific and relies on the action of highly specialized immune cells, the innate immune response is fast and broadly specific. Innate immunity relies on the ability of germ-line encoded pathogen recognition receptors (PRRs) to do exactly what their name suggests, recognize pathogens. Viruses are made up of and/or produce pathogen-associated molecular patterns (PAMPs) during replication, which bind to their cognate PRR either on the surface, in the cytoplasm or in the endosome of a host cell. In the innate antiviral immune response, the binding of PAMPs to PRRs initiates a signaling cascade that ultimately leads to increased expression of IFN and interferon-stimulated genes (ISGs). Through their diverse functions, ISGs protect the host cell by circumventing the virus’ attack.

1.2 Pathogen-Associated Molecular Patterns (PAMPs)

Viral PAMPs are molecules produced by a virus, and are essential to that virus’ existence/replication. Viral PAMPs are usually in the form of nucleic acids, including both double-stranded (ds) and single stranded (ss) RNA, as well as DNA. dsRNA molecules are produced by most viruses at some point during their replication cycle in a host cell and are discriminated from endogenous molecules based on factors like sequence, length, molecular modifications and localization (Nellimarla and Mossman, 2014). dsRNA is produced differently depending on the virus’ genome; it can be the virus’ genome in the case of dsRNA viruses, it can come from replicative intermediates
of ssRNA viruses or from convergent transcription of DNA viruses (Jacobs and Langland, 1996). DNA from DNA viruses can also be transcribed into dsRNA by RNA polymerase III, which enables sensing by cytoplasmic dsRNA PRRs (Chiu et al., 2009). Viral PAMPS can also take the form of glycoproteins from viral envelopes (Haynes et al., 2001), though these are less common as viruses can easily mutate their proteins, whereas nucleic acid detection is based on structure, which viruses cannot change.

1.3 Pathogen Recognition Receptors (PRRs)

1.3.1 Intracellular PRRs

Intracellular PRRs can be found both in the cytoplasm and in endosomes. Toll-like receptors (TLRs) that sense viral nucleic acids are found within the endosome, while RIG-I-like receptors (RLRs) and cytosolic DNA sensors (CDSs) sense these molecules in the cytoplasm (Figure 1.1).

1.3.1.1 Toll-Like Receptors (TLRs)

TLRs are a well-studied evolutionarily conserved group of membrane bound receptors that recognize a wide variety of PAMPs. To date, 13 TLRs have been cloned in mammals (10 in humans and 12 in mice; Beutler, 2004). In mammals, TLR3 senses dsRNA (Alexopoulou et al., 2001), TLR9 senses unmethylated CpG DNA (Latz et al., 2004) and TLR7 and 8 sense ssRNA (Gorden et al., 2005). These nucleic acid-sensing TLRs are localized to the endoplasmic reticulum prior to PAMP stimulation, after which they move to the endosome for activation (Leifer et al., 2004). The remaining TLRs (TLR1, 2, 4, 5, 6 and 10) are surface-bound and will not be discussed as they bind bacterial, fungal and viral protein PAMPs (Medzhitov, 2001; Akira et al., 2006; Schumann et al., 1990; Hayashi et al., 2001).
TLRs contain an extracellular N-terminus with a leucine-rich repeat region (LRR), a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor domain (TIR) (Palti, 2011). The diversity of LRR domains allows each TLR member to recognize a specific pathogen-associated molecular pattern (Bell et al., 2003) and TIR domains are responsible for transmitting downstream signaling through a range of adaptor molecules (Funami et al., 2004).

TLR3, 7/8 and 9 signaling is known to activate type I IFN production (Perry et al., 2005). Upon PAMP recognition TLR3 recruits TRIF, which results in the production of IFN through the activation of interferon regulatory factor-3 (IRF3; Kawai and Akira, 2010). TLR7/8 and 9 recruit MyD88, which results in the production of IFN through the activation of interferon regulatory factor 7 (IRF7; Negishi et al., 2006).

1.3.1.2 RIG-I-Like Receptors (RLRs)

Retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2) are members of the RLR family of cytoplasmic viral RNA sensors. MDA5 and LGP2 recognize cytoplasmic long dsRNA while RIG-I recognizes ssRNA with a 5’ triphosphate and dsRNA less than 1000 bp in length (Baum and Garcia-Sastre, 2010). RIG-I and MDA5 have two N-terminal caspase recruiting domains (CARDs), a central DexD/H-box RNA helicase domain (HD) and a C-terminal regulatory domain (RD), whereas LGP2 contains a DExH/D-box RNA helicase domain and a C-terminal regulatory domain but lacks the CARD domain (Zhang and Gui, 2012), suggesting that it is not directly involved with downstream signaling. In mammals, LGP2 assists MDA5-RNA interactions (Bruns et al., 2014).
Following dsRNA binding, the N-terminal CARDs of RIG-I and MDA5 interact with the CARD domain of the mitochondrial antiviral signaling protein, interferon promoter stimulator-1 (IPS1). IPS1 then associates with TRAF3 and the adaptor STING, which links signaling to downstream cytosolic kinases (Ishikawa and Barber, 2008), leading to IRF3 phosphorylation and IFN production.

1.3.1.3 Cytosolic DNA Sensors (CDSs)

Cytosolic DNA is sensed by a CDSs, which include: DNA-dependent activator of IFN-regulatory factors (DAI; Takaoka et al., 2007), absent in melanoma 2 (AIM2; Hornung et al., 2009), RIG-I via RNA polymerase III (RNA; Ablasser et al., 2009), leucine-rich repeat (in Flightless I) interacting protein-1 (Lrrfip1; Yang et al., 2010), DExD/H-box helicases DDX41, DHX9, and DHX36 (Zhang et al., 2011), cyclic GMP-AMP synthetase (cGAS; Sun et al., 2013) and IFI16 (Unterholzner et al., 2010).

Most CDSs mediate type I IFN expression in response to cytosolic DNA, namely: DAI (Takaoka et al., 2007); DHX9, DHX36, DDX41 (Zhang et al., 2011); and cGAS (Sun et al., 2013). For these CDSs, signaling is mediated by a number of adaptor molecules, which activate signaling cascades to induce IFNs via IRF3. STING is regarded as the key adaptor protein for cytosolic DNA sensing (Burdette and Vance, 2013). Lrrfip1 is unique in that it recognizes both dsRNA and dsDNA and induces IFN expression without directly regulating the IFN transcription factors (Yang et al., 2010) and RNA pol III can transcribe viral DNA into a 5’ triphosphate dsRNA intermediate, which can induce type I IFNs via RIG-I (Ablasser et al., 2009).
1.3.2 Surface PRRs

1.3.2.1 Class A Scavenger Receptors (SR-As)

Scavenger receptors are cell surface receptors that were originally defined by their ability to bind and internalize modified low-density lipoproteins (mLDL) (Goldstein et al., 1979). For this reason, research has largely focused on their involvement in the development of atherosclerosis. Scavenger receptors are now known as a diverse group of PRRs that recognize a variety of polyanionic ligands, including PAMPs, as well as modified host molecules (damage-associated molecular patterns, DAMPs). Scavenger receptors are subdivided into eight classes (A to H) and although members of each class share structural features, there is little to no homology between classes (Canton et al., 2013). Class A scavenger receptors (SR-As) are of particular interest as, in mammals, they have been shown to be involved in cell-cell recognition, macrophage adhesion, endocytosis, phagocytosis, and the detection of pathogens as part of the innate immune system (Platt and Gordon, 2001). SR-As are a family of type II membrane glycoproteins, which consists of SR-AI/II/III, MARCO, SCARA3, SCARA4 and SCARA5. All members contain cytoplasmic, transmembrane, α-helical, and collagenous domains, though they differ in the lengths of their α-helical and collagenous domains and in the composition of their C-terminal domains (Whelan et al., 2012). SR-AI, MARCO and SCARA5 possess a terminal Scavenger Receptor Cysteine Rich (SRCR) domain while SCARA4 possesses a C-type lectin domain and SCARA3 terminates at the collagenous domain (Whelan et al., 2012). All 5 SR-A members form homotrimers, which are stabilized by α-helical coiled-coil motifs and by their collagenous regions (Pearson, 1996). The collagenous region mediates ligand binding and therefore pathogen
recognition in all the SR-As (Bowdish and Gordon, 2009; Doi et al., 1993) except for MARCO, in which the SRCR domain mediates this function (Novakowski et al. 2016; Bowdish et al., 2009). Human SR-AI, -AII and –AIII are coded by a single gene that generates the three isoforms through alternative RNA splicing (Murphy et al., 2005). SR-AII lacks SR-AI’s C-terminal SRCR domain but is still functional, while SR-AIII is a truncated form of SR-AI that is retained in the endoplasmic reticulum (Martinez et al., 2011). Along with the leucine-rich repeat of the TLRs (Roach et al., 2005), the SRCR domain is one of the most ancient pattern recognition domains associated with innate immunity. Although the SRCR domain is found in many proteins and highly conserved across various deuterosome species (Martinez et al., 2011), no full SR-A sequence has been identified in non-vertebrate genomes, indicating that the modern SR-A structure arose after the divergence of vertebrates from other species (Whelan et al., 2012).

SR-As vary in their cellular and tissue level expression profiles and in their ligand binding abilities (see Table 1.1 – a list of the SR-As, examples of their ligands and expression profiles). SR-AI/II is expressed on macrophages but is also found on endothelial, smooth muscle and dendritic cells (Murphy et al., 2005; Pitas, 1990; Harshyne et al., 2003). MARCO is mainly expressed on macrophages of the spleen marginal zone and lymph nodes (Elomaa et al., 1995) as well as on splenic dendritic cells (Granucci et al., 2003). SCARA3 is expressed on fibroblasts and protects cells by scavenging reactive oxygen species during oxidative stress (Han et al., 1998). SCARA4 is expressed on endothelial cells, specifically those of the placenta, lungs and tonsils (Selman et al., 2008). SCARA5 is expressed on epithelial cells associated with mucosa, (Jiang et al., 2006). In addition to binding mLDLs, SR-As bind a wide range of
polyanionic ligands. Their role in innate immunity is highlighted by their ability to internalize the synthetic dsRNA polyI:C and mediate a subsequent antiviral response (DeWitte-Orr et al., 2010). SR-AI, MARCO, SCARA4 and SCARA5 have all been shown to bind Gram-positive and -negative bacteria and so are hypothesized to be involved in host defense (Peiser et al., 2000; Selman et al., 2008; Jiang et al., 2006).

SR-As have been shown to cooperate with pattern recognition receptors, such as TLRs, to initiate cytokine secretion after nucleic acid recognition. MARCO cooperates with TLRs as it has been shown to deliver CpG DNA to endosomal TLR9 (Jozefowski et al., 2006). SR-As bind and internalize dsRNA through clathrin-mediated endocytosis in mammals (DeWitte-Orr et al., 2010). Once within the endosome, dsRNA is then detected by TLR3 and subsequently by the cytoplasmic dsRNA-sensing PRRs, though it is still unclear by what mechanism dsRNA escapes the endosome to the cytoplasm. Though previous research suggested that SR-As may be capable of initiating an antiviral response as signaling receptors, recent research has shown that SR-A–mediated dsRNA internalization is independent from downstream signaling and that in the absence of TLR3 and RLR signaling, SR-As do not contribute to antiviral responses (Nellimarla et al., 2015).

1.4 Type I Interferons (IFNs)

In mammals, IFNs are divided into three groups based on biological features as well as cognate receptor structure: type I (α, β, ω, ε, and κ), type II (γ), and type III (λ). Type I and III act in the innate antiviral response and so are often called the “virus-induced IFNs”, whereas type II regulates the innate and adaptive immune systems (Langevin et al., 2013). Mammalian type I IFN genes are classified into two groups: early
phase IFN genes (including IFNβ) that are induced rapidly and late-phase genes (including most IFNαs) that display delayed induction (Marie, et al., 1998). In mammals, all cell types are able to produce IFNβ upon sensing a virus and some specialized cells can produce IFNα. Mammalian type II IFN (IFNγ) is produced by T cells, natural killer (NK) cells and dendritic cells (Tominaga, et al., 2000; Fricke et al., 2006). Upon PAMP sensing, PRRs induce signaling cascades that ultimately lead to IRF3 phosphorylation by the serine-threonine kinases TANK-binding kinase-1 (TBK1) or the inducible IκB kinase (IKK-i) (Fitzgerald, et al., 2003). IRF3 then dimerizes, translocates into the nucleus, and cooperatively binds to the IFNβ promoter with transcription factors c-Jun/ATF-2 and NF-KB (Malmgaard, 2004). Following production, IFNβ initiates a positive feedback loop by binding to IFNAR (interferon alpha/beta receptor) in an autocrine and paracrine manner (Taniguchi and Takaoka, 2002). IFNAR activates JAK protein tyrosine kinases (JAK1 and Tyk2), which phosphorylate STAT1 and STAT2; STAT1 and STAT2 together with IRF9 form a transcription factor complex termed IFN-stimulated gene factor 3 (ISGF3; Malmgaard, 2004). ISGF3 translocates into the nucleus and binds IFN-stimulated response elements (ISREs), to induce expression of a large group of IFN stimulated genes (ISGs), including IRF7 and many others with anti-viral functions (Taniguchi and Takaoka, 2002). IRF7 also becomes activated by phosphorylation by TBK1 and/or IKK-i, and acts to further type I IFN expression by activating transcription of IFNα (Sharma et al., 2003). The ISGs produced following IFN signaling accumulate in the cell, causing an ‘antiviral state’ (see Figure 1.1 for signaling pathways that lead to IFN and ISG induction).
1.5 Interferon-Stimulated Genes (ISGs) and the antiviral state

Type I IFNs do not possess direct antiviral activity but interfere with viral infection through the induction of a repertoire of ISGs that work together to limit every step of virus replication. The autocrine and paracrine signaling of type I IFN through their IFNAR receptor allows them to initiate an antiviral state in the infected cell from which they originate but also in uninfected neighboring cells, giving uninfected cells time to accumulate ISGs in preparation for a potential infection. While hundreds of ISGs have been identified, precise antiviral mechanisms have only been elucidated for a small subset (Poynter and DeWitte-Orr, 2016). Healthy cells maintain these proteins at low levels to limit inappropriate activation while keeping the cell prepared to respond to legitimate viral infections (Schneider et al., 2014). The Mx family of proteins is one of the best studied ISGs. Mx proteins are dynamin-like members of the GTPase family. In mammals, Mx1 induces a broad antiviral state by forming oligomers around viral nucleocapsids, ultimately targeting them for degradation (Schneider et al., 2014). ISG15 (also known as vig-3) is an ubiquitin-like protein that covalently binds to its target protein in a process known as ISGylation (Schneider et al., 2014). ISG15 is also secreted and has cytokine activity (Bogunovic et al., 2013). ISGs use varied mechanisms to impede virus replication; for example they can control viral replication at the level of transcription by degrading ssRNA molecules (Silverman, 2007), limit viral replication by inhibiting protein synthesis (Saunders and Barber, 2003) and induce apoptosis of host cells in order to control viral spread to other cells (Kumar et al., 1997).
1.6 Fish innate immunity

1.6.1 PRRs

Though fish genomes have been molded by various whole genome duplication and contraction events, they have still conserved the many processes that make up the IFN system, which highlights the importance of innate antiviral immunity in vertebrates (Langevin et al., 2013). Of the 20 TLRs that have been identified in fish, 8 are conserved from the 10 human TLRs. Fish are missing TLR6 and 10 found in humans but uniquely express TLR13, 14, 18, 19, 20, 21, 22, 23, 24, 25 and 26 (Zhang et al., 2014); though the ligand specificities for many TLRs in fish still need to be elucidated. Specifically, the nucleic acid binding human TLRs 3, 7/8 and 9 are conserved in fish. TLR3 and TLR9 have been shown to be endosomal like their human counterparts (Jorgensen et al., 2001), whereas there is contradictory evidence surrounding the localization of TLR7/8 (Zhou and Sun, 2015; Palti et al., 2010). The signaling pathways downstream of TLRs, including the involvement of MyD88 and TRIF have been confirmed in several fish species (Poynter et al., 2015). MDA5 and LGP2 seem to be conserved in all fish species while RIG-I has only been identified in some groups including salmonids and cyprinids (Hansen et al., 2011). The signaling pathways downstream of RLRs, including the involvement of STING, are well conserved in fish (Biacchesi et al., 2009). DDX41 is the only CDS that has been identified in teleost fish (Quynh et al., 2015) and STING, the adaptor molecule in its signaling pathway has been identified in several fish species and was found to function analogously to mammalian STING in the induction of IFN and ISGs (Biacchesi et al., 2012). Research to date does indicate that fish SR-A sequence, structure and function share many similarities with mammalian SR-As (Poynter et al.,
Several SR-A homologs have been identified in fish but more functional studies are needed to characterize their expression and function in different fish cells and tissues. *In silico* analysis has shown that SCARA5 is conserved in all fish species, SCARA3 is conserved specifically in Ostariophysian and Salmonidae fish species and SCARA4 is conserved in these genomes as well as in the Acanthopterygii fishes (Whelan et al., 2012). MARCO, SCARA3 and SCARA5 have been identified in large yellow croaker (*Pseudosciaena crocea*) and common carp (*Cryprinus carpio*) and their expression was upregulated in both species *in vivo* following bacterial infection (He et al., 2014; Feng et al., 2016). A SCARA4 homolog has been cloned in zebrafish (*Danio rerio*), in which it binds both mLDLs and bacteria and is involved in vasculogenesis (Fukuda et al., 2011); fragments of the SCARA4 sequence have also been identified in rainbow trout (*Oncorhynchus mykiss*; Poynter et al., 2015; Dumont et al., 2008). A SCARA5 homolog has been cloned in puffer fish (*Tatraodon nigroviridis*), and is able to bind LPS (Meng et al., 2012). Functionally, the same competitive ligands used to define SR-A function in mammals have been used to define function in fish, such as fucoidan, DxSO₄, polyI and polyC (Poynter et al., 2015); formaldehyde-treated albumin (FSA) (Seternes et al., 2007); and LTA and formaldehyde treated bovine serum albumin (fBSA; Froystad et al., 2002).

### 1.6.2 IFN and ISGs

Fish virus-induced IFNs are divided into two groups based on the number of cysteine residues predicted to be engaged in disulfide bridges, with two cysteines in group I and four in group II; group I IFNs are further subdivided into subgroup-a and subgroup-d, and group II IFNs into subgroup-c and subgroup-b (Sun et al., 2009). As in mammals, fish IFNs appear to be expressed by discrete cell populations. Some IFNs are expressed in
an “IFNβ” pattern by fibroblasts and other cells whose tissues may be direct targets of infection, while others are expressed in an “IFNα” fashion by more specialized immune cells (Langevin et al., 2013). Group I IFNs have been identified in all investigated fish species, whereas group II is only found in more primitive teleost fish such as salmonids and cyprinids (Zou et al., 2007). Fish genomes contain all key components of the Jak-Stat signaling pathway (Stein et al., 2007), indicating that fish IFN also induces the expression of ISGs to establish a host antiviral state through Jak-Stat signaling, as seen in mammals.

Many well-characterized mammalian ISGs have been identified in fish; however, their antiviral mechanisms still require elucidation. As in mammals, fish Mx is IFN inducible and exhibits broad antiviral activity (Alvarez-Torres et al., 2013). Some fish species can express as many as 7 Mx isoforms whereas humans only express 2 (Lin et al., 2006). Interestingly, fish Mx isoforms have shown synergistic or antagonistic interactions, leading to different antiviral ranges, which have not been noted in mammals (Fernandez-Trujillo et al., 2015). ISG15 and its antiviral mechanism of ISGylation in mammals has been confirmed in many fish species (Langevin et al., 2013). Several other mammalian ISG homologs have been identified in fish and have been shown to have similar functions (i.e. inhibit transcription and translation, induce apoptosis) to those in mammals. Fish-specific ISGs have also been identified, such as Vig-B319 (Yeh et al., 2014), Gig-1 and -2 (Sun et al., 2013), which have been shown to be induced by synthetic dsRNA and to have antiviral activities but whose exact antiviral mechanisms remain to be uncovered.
1.7 CHSE-214

CHSE-214 is an epithelial cell line that was initiated from Chinook salmon embryonic tissue in 1964 (McCain, 1970). Since then it has been mostly used to study virus replication and has also been used to make a stably transfected cell line to study the induction and regulation of IFN signaling in teleost fish (Jorgensen et al., 2007). In 1979, MacDonald and Kennedy found that unlike Atlantic salmon cells, CHSE-214 cells did not initiate an antiviral response when infected with pancreatic necrosis virus (IPNV) or when treated with exogenous polyI:C and hypothesized that the cells had a defect in the IFN system. Recognizing that cells with such defects may serve as important tools in studies of host/virus interactions, Jensen et al. further investigated CHSE-214’s IFN system in 2002. They found that transfection of CHSE-214 cells with polyI:C induced expression of Mx protein and an antiviral state against IPNV infection, in which the antiviral activity was secreted from the cells and had characteristics of type I IFN-like activity. These findings suggested the presence of a potential defect in CHSE-214’s ability to internalize dsRNA and not necessarily in their ability to mount an IFN response once the dsRNA was intracellular. At the time the receptors and mechanisms responsible for dsRNA entry were not clearly established. Since then, no other inquiries have been made regarding CHSE-214’s defective dsRNA uptake.

1.8 Research objectives and hypotheses

To date, the study of class A scavenger receptors’ ability to bind dsRNA has been difficult due to ubiquitous expression patterns producing high background in most cell lines. According to previous findings suggesting a defect in CHSE-214’s dsRNA uptake mechanism, this project aimed to investigate whether CHSE-214 cells could be used as a
model for the study of SR-A binding and signaling. The project’s objectives included characterizing CHSE-214’s response to extracellular and intracellular dsRNA by treating (extracellular) or transfecting (intracellular) with dsRNA to confirm that the defect was really present at the extracellular dsRNA sensing step. The final objective aimed to use CHSE-214 as a model for scavenger receptors sensing extracellular dsRNA by transiently transfecting this cell line with novel full rainbow trout SR-A sequences and seeing whether their dsRNA uptake defect could be rescued. The hypotheses were that CHSE-214 cells would be able to mount an antiviral response when transfected with dsRNA but not when treated with it and that transfection of a full-length SR-A sequence would restore the cells’ ligand binding abilities.

1.9 Co-authorship

Gabi Micheli appears as a secondary author in manuscript 2 of this thesis. She contributed to this project by amplifying the full-length MARCO sequence from RTS11 cells (including primer design, RNA extraction, cDNA synthesis and RT-PCR), cloning it into a vector for sequencing and later into an expression vector for overexpression studies. She also performed all RNA extractions, cDNA syntheses and RT-PCR from rainbow trout cell lines and tissues for expression analysis of MARCO and SCARA5 (Figure 3.3).
### 1.10 Tables, figures and figure legends

**Table 1.1 Class A scavenger receptors, examples of their ligands and expression profiles**

<table>
<thead>
<tr>
<th>SR-As</th>
<th>Ligands</th>
<th>Tissue/cell expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-AI/II</td>
<td>oxLDL, acLDL (Suzuki et al., 1997) • dsRNA (DeWitte-Orr et al., 2010) • CpG DNA (Mukhopadhyay and Gordon, 2004) • <em>E. coli</em> (Peiser et al., 2000); <em>N. meningitides</em> (Pluddemann et al., 2009); <em>S. aureus, S. pyogenes, S. agalactidae, E. hirae, L. monocytogenes</em> (Dunne et al., 1994)</td>
<td>Macrophages, endothelial and smooth muscle cells (Murphy et al., 2005; Pitas, 1990); dendritic cells (Harshyne et al., 2003)</td>
</tr>
<tr>
<td>MARCO</td>
<td>acLDL (Kraal et al., 2000) • dsRNA (DeWitte-Orr et al., 2010) • CpG DNA (Jozefowski et al., 2006) • <em>N. meningitides</em> (Pluddemann et al., 2009); <em>E. coli, S. aureus</em> (Elomaa et al., 1995); <em>M. tuberculosis</em> (Bowdish et al., 2009)</td>
<td>Macrophage of the spleen marginal zone and lymph nodes (Elomaa et al., 1995); splenic dendritic cells (Granucci et al., 2003)</td>
</tr>
<tr>
<td>SCARA3</td>
<td>dsRNA (DeWitte-Orr et al., 2010) • Reactive oxygen species (Han et al., 1998) • Cell-penetrating peptides nanocomplexes with oligonucleotides (Ezzat et al., 2012)</td>
<td>Fibroblasts (Han et al., 1998)</td>
</tr>
<tr>
<td>SCARA4</td>
<td>oxLDL (Selman et al., 2008) • dsRNA (DeWitte-Orr et al., 2010) • <em>E. coli, S. aureus, S. cerevisiae</em> (Nakamura et al., 2001; Ohtani et al., 2001)</td>
<td>Placenta, lungs and tonsils (Selman et al., 2008)</td>
</tr>
<tr>
<td>SCARA5</td>
<td>dsRNA (DeWitte-Orr et al., 2010) • <em>E. coli, S. aureus</em> (Jiang et al., 2006) • Cell-penetrating peptides nanocomplexes with oligonucleotides (Ezzat et al., 2012)</td>
<td>Epithelial cells associated with mucosa (Jiang et al., 2006)</td>
</tr>
</tbody>
</table>
Figure 1.1 PRR signaling pathways leading to type I IFN induction and signaling in mammals. PRRs that sense viral nucleic acids can be found on the cell surface, in the endosome or in the cytoplasm of host cells. Cytoplasmic viral nucleic acids arise from virus infection and replication and can bind to cytoplasmic PRRs whereas viral nucleic acids present in the extracellular space are bound and internalized by SR-As. SR-As deliver extracellular nucleic acids to endosomal PRRs via clathrin-mediated endocytosis, and through an unknown mechanism these nucleic acids can also escape the endosome to be sensed by cytoplasmic PRRs. The activation of host PRRs by viral PAMPs induces the production of type I IFN through several different pathways. Following their activation, host PRRs activate different adaptor molecules, which interact with intracellular kinases to mediate the activation of key transcription factors, IRF3 and IRF7. Once activated, these transcription factors translocate to the nucleus and associate with other transcription factors to cooperatively bind to the IFN promoter, inducing IFN production. Following induction, IFNs are secreted and bind to their cognate receptor, IFNAR, in an autocrine and paracrine fashion to induce the expression of ISGs, via ISGF3 binding to each ISG’s respective interferon-stimulated response element (ISRE).
Chapter 2

CHSE-214 is defective in its dsRNA uptake mechanism:
Implications for dsRNA sensing studies

Short Communication: will be submitted to Fish and Shellfish Immunology
2.1 Introduction

Double stranded RNA (dsRNA) is a potent inducer of the innate immune response. This molecule is produced by most viruses during replication and arises via different mechanisms depending on the virus’ genome type. It can come directly from the genome of dsRNA viruses, from replicative intermediates of ssRNA viruses binding to the complementary genome sequence or from convergent transcription of DNA viruses (Jacobs and Langland, 1996). dsRNA can also be made from viral DNA via RNA polymerase III (Ablasser et al., 2009). dsRNA molecules produced by viruses are discriminated from endogenous molecules based on factors such as sequence, length, molecular modifications and localization (Nellimarla and Mossman, 2014). When they are recognized by pathogen recognition receptors (PRRs) inside an infected cell, dsRNA molecules initiate a signaling cascade that ultimately leads to the expression of type I interferon (IFN) and interferon stimulated genes (ISGs). ISGs work together to limit virus replication and lead to the establishment of an antiviral state. If a virus infection becomes lytic, an infected cell’s contents are released into the extracellular space and dsRNA molecules may bind to neighboring cells via class A scavenger receptors (SR-As). These receptors facilitate dsRNA entry into the cell, delivering it to intracellular signaling PRRs, which initiate the signaling cascade that ultimately leads to an antiviral state, thereby preparing the cell for a potential virus infection.

CHSE-214 is an epithelial cell line that was initiated from Chinook salmon embryonic tissue in 1964 (McCain, 1970). Since then, two research groups have made interesting observations about this cell line’s ability to mount an antiviral response to extracellular dsRNA. In 1979, MacDonald and Kennedy found that unlike Atlantic
salmon cells, CHSE-214 cells did not initiate an antiviral response when infected with pancreatic necrosis virus (IPNV) or when treated with exogenous polyI:C (a synthetic dsRNA molecule) and hypothesized that the cells had a defect in the IFN system. Recognizing that cells with such defects may serve as important tools in studies of host/virus interactions, Jensen et al. further investigated CHSE-214’s IFN system in 2002. They found that transfection of CHSE-214 cells with polyI:C, essentially forcing dsRNA into the cell, induced expression of Mx protein (an ISG) and an antiviral state mediated by IFN-like activity against IPNV infection. Jensen et al.’s findings suggest the presence of a potential defect in CHSE-214’s dsRNA uptake mechanism and not necessarily in their ability to mount an IFN response. At the time the receptors and mechanisms responsible for dsRNA entry were not clearly established and since then, no other inquiries have been made regarding CHSE-214’s defective dsRNA uptake.

We aimed to elucidate whether CHSE-214’s inability to respond to extracellular dsRNA is due to a defect in dsRNA uptake or downstream, at IFN production. The present study used novel methods to study the dsRNA-induced antiviral pathway of CHSE-214 cells, directly comparing extracellular dsRNA-induced responses to intracellular dsRNA-induced responses. These methods include qRT-PCR to measure IFN and ISG transcripts and a cytopathic effect assay to measure antiviral activity. Lastly, CHSE-214’s ability to bind and internalize extracellular dsRNA was investigated using immunocytochemistry and the anti-dsRNA J2 antibody.
2.2 Materials and methods

2.2.1 Cell culture

CHSE-214 cell cultures were maintained at 20°C in Leibovitz’s L-15 (Thermo Fisher Scientific, Waltham MA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% (v/v) penicillin G/streptomycin sulfate (P/S; Thermo Fisher Scientific). Cells were grown in 75 cm² plastic tissue culture flasks (Falcon, Tewksbury MA, USA).

2.2.2 CSV propagation

Chum salmon reovirus (CSV) was propagated in semi-confluent monolayers of CHSE-214 cells in L-15 with 5% (v/v) FBS and 1% (v/v) P/S at 17°C for 5 d, or until complete cell death was observed. CSV-containing medium was collected and then filtered through a 0.2µm filter, and stored at -20°C. The virus titre was determined by infecting monolayers of CHSE-214 cells seeded into 96-well plates with serially diluted viral suspensions (10⁻¹ to 10⁻¹¹) and incubating the cells for 7 d at 17°C. Following this infection period, cells were scored for the presence of cytopathic effect (CPE) and a virus titre, expressed as a 50% Tissue Culture Infective Dose (TCID₅₀/mL), was estimated using the Karber method (Karber, 1931). TCID₅₀/mL values were then converted to multiplicity of infection (MOI) values, which refer to the number of virus particles per cell in a well. MOI = [(volume/well) x (PFU/mL)] / (# of cells), where PFU/mL = 0.7 x TCID₅₀/mL.

2.2.3 Cell treatments

Unless stated otherwise, CHSE-214 cells were plated at 1 x 10⁶ cells/well in a 6-well plate, 2 x 10⁴ cells/well in a 96 well plate and 3 x 10⁵ cells/well in a 12-well plate.
and incubated overnight in 10% (v/v) FBS media. The media was aspirated and cells were treated with 2 µg/mL polyI:C (Sigma-Aldrich) or transfected with 2 µg/mL polyI:C using Fugene 6 (Promega, Madison WI, USA) at a 1 to 1.5 ratio of polyI:C to Fugene 6 per well in L-15, as per manufacturer’s instructions. Cells were incubated with polyI:C treatment or transfection media for 24 h, treatment following this step is described below for each assay.

2.2.4 qRT-PCR: IFN and ISG transcript expression analysis

2.2.4.1 RNA extraction and cDNA synthesis

Cells were plated in a 6-well plate, following 24 h treatment (described above in section 2.3), L-15 was removed and RNA was extracted using TRIzol (Life Technologies, Carlsbad CA, USA) as per manufacturer’s instructions. RNA was quantified using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and stored at -80°C. cDNA was synthesized using 2 µg of total RNA, 0.5 µg Oligo(dT) primers (Sigma-Aldrich, St. Louis MO, USA), 1X GoScript reaction buffer (Promega), 1.5 mM MgCl₂ (Promega), 0.5 mM each deoxynucleotide triphosphate (dNTP, Sigma-Aldrich), and 160 U GoScript Reverse Transcriptase (Promega). Reactions were performed according to manufacturer’s instructions. 10 µL cDNA was diluted in 90 µL nuclease-free water (Fisher Scientific) and stored at 4°C until use.

2.2.4.2 qRT-PCR reactions

All PCR reactions contained: 2 µL of diluted cDNA, 2x SsoFast EvaGreen Supermix (Bio-Rad, Hercules CA, USA), 0.2 µM forward and reverse primer (Sigma
Aldrich) and nuclease-free water to a total volume of 10 µL (Fisher Scientific). The primer sets used are listed in Table 2.1. qPCR reactions were performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad). The program used for all qPCR reactions was: 98°C 2 min, 40 cycles of 98°C 5 s, 55°C 10 s, followed by 95°C for 10 s. A melting curve was completed from 65°C to 95°C with a read every 5 s. Product specificity was determined through single PCR melting peaks. Data were analyzed using the ΔΔCt method. Specifically, gene expression was normalized to the housekeeping gene (β-actin) and expressed as fold change over the untreated control group.

2.2.5 Cytopathic effect (CPE) assay

Cells were plated in a 96-well plate, following 24 h cell treatments (described above in section 2.3), L-15 was removed and replaced with 2% (v/v) FBS L-15 and cells were incubated for 48 h. Cells were then challenged with CSV (at an MOI of 0.1) by adding virus directly to the media. Cells were incubated at 17°C and were observed daily until areas of syncytia had formed (~5 d post infection). Media was removed and cells were rinsed with PBS. Cells were then fixed with 10% neutral buffered formalin (Fisher Scientific) for 15 min, rinsed with PBS, stained with 1% crystal violet (Thermo Fisher) for 5 min and rinsed twice each with PBS and water. Cells were imaged at 40X magnification with the Nikon Eclipse TiE with Qi1 camera and syncytia area boundaries were identified using Nikon NIS-Elements software. The cell monolayer area covered with syncytia was divided by the total monolayer area of the well and this value was converted to a % syncytial area. % Syncytial Area = [(Σ of syncytial areas in well) / (total area of cell monolayer)] x 100%.
2.2.6 Immunocytochemistry

Cells were plated on glass coverslips in a 12-well plate, cell treatments described above in section 2.3 were followed except that cells were treated with 100 µg/mL instead of 2 µg/mL polyI:C. L-15 was removed and cells were fixed with 10% buffered formalin (Fisher Scientific) for 10 min, permeabilized with a 0.1% Triton X-100 (Thermo Fisher) PBS solution for 15 min and blocked in blocking buffer (3% goat serum (Sigma-Aldrich), 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.02% Tween 20 (Thermo Fisher) in PBS) for 1 h. Coverslips were then incubated with the anti-dsRNA Ab, J2 (Scicons, Szirák, Hungary), at a 1/200 dilution for 1 h, followed by a TRITC-labeled anti-mouse secondary Ab (Scicons, Szirák, Hungary), also at diluted 1/200 for 1 h in a humidified chamber. All Ab dilutions were made in blocking buffer. Nuclei were stained with 0.01 mg/mL DAPI and coverslips were mounted using SlowFade Gold Antifade (Thermo Fisher) mountant. Mounting media was then cured by incubating slides overnight at room temperature in the dark. Cells were imaged at 200x magnification with an inverted fluorescence microscope (Nikon Eclipse TiE with Qi1 camera). TRITC mean fluorescence intensity was quantified using NIS-Elements analysis software.

2.2.7 Overexpression of hSR-AI and AcLDL binding assay

Cells were plated on glass coverslips in a 12-well plate and allowed to attach overnight. The following day cells were transfected with a human SR-AI (hSR-AI)-containing pcDNA3.1+ expression plasmid (obtained from Dr. Dawn Bowdish) or with an empty expression plasmid as a control using a 1.5 to 1 ratio of Fugene 6 to plasmid, as per manufacturer’s instructions. 72 h post transfection, media was removed and cells were treated with 5 µg/mL Alexa Fluor® 488 AcLDL in L-15 for 6 h. AcLDL was
then removed and cells were treated with 0.025% (v/v) Trypan Blue in PBS for 5 min to quench extracellular fluorescence. After 5 min, Trypan Blue was removed and cells were fixed with 10% buffered formalin (Fisher Scientific) for 10 min. Cells were then rinsed with PBS 1X and were stained with 0.01 mg/mL DAPI in PBS for 5 min. Cells were rinsed with PBS 2X and MilliQ 2X and were mounted using SlowFade Gold Antifade (Thermo Fisher) mountant. Mounting media was then cured by incubating slides overnight at room temperature in the dark. Cells were imaged at 200x magnification with an inverted fluorescence microscope (Nikon Eclipse TiE with Qi1 camera).

2.2.8 Statistical analysis

Data are expressed as means ± standard error of the mean. Intergroup differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test, using Kaleidagraph Software (version 4.1.0, Synergy Software, Reading PA). A p value of < 0.05 was considered statistically significant.

2.3. Results and discussion

2.3.1 IFN and ISG transcripts are only upregulated in response to intracellular dsRNA

As type I IFNs are key mediators of the innate antiviral state, expression of IFN1 as well as that of two of its downstream ISGs, Mx1 and Vig3, were measured in response to extracellular (treatment) or intracellular (transfection using Fugene 6) polyI:C for 24 h. Type I IFN, Mx1 and Vig3 were upregulated 281 ± 46.7, 41 983 ± 3811.7 and 741 ± 295.4 fold, respectively, in CHSE-214 cells transfected with polyI:C, compared to 4 ± 1.7, 7 ± 2.8 and 2 ± 0.5 fold in cells treated with polyI:C (Figure 2.1), demonstrating that
CHSE-214 cells can initiate IFN production, signaling and induction of ISGs in response to intracellular but not extracellular dsRNA.

2.3.2 Intracellular dsRNA pretreatment reduces CSV’s cytopathic effects

CSV’s cytopathic effect in CHSE-214 is the formation of syncytia (DeWitte-Orr and Bols, 2007); it forms large multinucleated cells. Therefore, in order to measure the antiviral state initiated by both intracellular and extracellular dsRNA, a cytopathic effect assay was developed to measure the extent of CSV-induced syncytia within a monolayer of CHSE-214 cells. With treatments similar to the qRT-PCR experiments, cells were transfected or treated with polyI:C for 24 h and incubated for another 48 h to allow an IFN-mediated antiviral state to be established in the cells prior to CSV challenge. Once syncytia formation was observed in infected, untreated control cells (~5 d post infection), cells were fixed, stained and imaged. Cells transfected and treated with polyI:C displayed 1.3% ± 1.3 and 88% ± 3.18 syncytia, respectively, while the infected control cells displayed 86% ± 4.16 syncytia (Figure 2.2). This assay clearly demonstrates that intracellular dsRNA is able to induce an almost complete antiviral state in CHSE-214, while extracellular dsRNA cannot.

2.3.3 dsRNA is not bound or internalized by cells treated with dsRNA

As the signaling pathways leading to IFN and ISG expression and eventually to an antiviral state were shown to be intact in CHSE-214 by the previous assays, the question remained whether CHSE-214 cells could bind or internalize dsRNA. To investigate this, cells were transfected or treated with polyI:C for 24 h and immunocytochemistry with the anti-dsRNA J2 antibody was used to detect polyI:C. Immunocytochemistry to detect
dsRNA (polyI:C) binding and entry has been previously shown to be successful in the rainbow trout gill cell, RTgill-W1 (Doherty et al., 2016). DsRNA was not detectable in CHSE-214 cells treated with polyI:C, whereas it was in transfected cells (Figure 2.3). Permeabilization of the cells before dsRNA detection allowed the J2 antibody to bind to dsRNA on the surface, as well as inside the cell. Since no signal was detected at all, this indicates that these cells have a defect in either their ability to bind or internalize extracellular dsRNA.

2.3.4 CHSE-214 cells transfected with a human SR-AI-containing expression plasmid bind AcLDL.

As AcLDL is a known SR-AI ligand, a fluorescently labeled version was used to test whether overexpression of the human SR-AI would lead to increased or restored binding of AcLDL in CHSE-214 cells. As Trypan Blue was used to quench extracellular fluorescence, only internalized fluorescently labeled AcLDL was observable. Green fluorescence corresponding to the fluorescently labeled AcLDL was only observed in cells transfected with the hSR-AI-containing expression plasmid (panel D of Figure 2.4), further suggesting that these cells may indeed lack functional SR-As and that they may serve as an appropriate model for the study of these receptors.

2.4 Conclusions

These data suggest that CHSE-214’s intracellular signaling is intact, as cells were able to express type I IFN and ISGs and establish an antiviral state in response to intracellular dsRNA. The defect in the dsRNA response appears to be at the binding and/or internalization step, which class A scavenger receptors have been shown to facilitate in both mammals and fish (DeWitte-Orr et al., 2010; Poynter et al., 2015). We
hypothesize that CHSE-214 may lack functional SR-As, which would explain their inability to mount an antiviral state in response to extracellular dsRNA but maintain their ability to do so in response to intracellular dsRNA. Identifying a cell line negative for these receptors would be very useful as the study of SR-As’ dsRNA binding abilities has been difficult due to ubiquitous expression patterns producing high background in most cell lines. Due to their function as modified low-density lipoprotein scavengers, much is known about SR-As in their role in the development of atherosclerosis but much more remains to be elucidated pertaining to their role in innate immunity and dsRNA sensing in fish, as well as in other species.
### 2.5 Tables, figures and figure legends

**Table 2.1 qRT-PCR primers used for transcript expression analysis of immune genes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’ – 3’)</th>
<th>Product length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN1</td>
<td>F – AAAACTGTGTGGATGGGAATATGAAA R – CGTTTCAGTCTCTCTTCAGGTT</td>
<td>141</td>
<td>Chaves-Pozo et al., 2010</td>
</tr>
<tr>
<td>Mx1</td>
<td>F – CCGAGTTCGTCTCAACGTCT R – CCCTCCACGGGTACGTCTTC</td>
<td>140</td>
<td>Poynter et al., 2015</td>
</tr>
<tr>
<td>Vig3</td>
<td>F – ACCCAGTTCAAAGCCAAGGT R – CCCTCCTGAAATCAGCCCTCTG</td>
<td>70</td>
<td>Poynter et al., 2015</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F – GTCACCAACTGGGAGCGACAT R – GTACATGGCAGGGGTGTTGA</td>
<td>174</td>
<td>Poynter et al., 2015</td>
</tr>
</tbody>
</table>
Figure 2.1 IFN and ISG transcripts are only upregulated in response to intracellular dsRNA in CHSE-214. Cells were treated (extracellular) or transfected (using Fugene 6; intracellular) with polyI:C for 24 h. RNA was extracted, cDNA synthesized and transcript levels measured using qRT-PCR. IFN1 and two ISG transcripts, Mx1 and Vig3 were measured. These data represent three independent experiments ± standard error of the mean (SEM). * indicates significant difference at p < 0.05, ** indicates significant difference at p < 0.001.
Fugene 6 | Poly I:C
---|---
-+| -+
---|---
+ | +

**IFN1 expression**

**Mx1 expression**

**Vig3 expression**
Figure 2.2 Intracellular but not extracellular dsRNA treatment protects CHSE-214 from CSV-induced syncytia. Cells were treated (extracellular) or transfected (using Fugene 6; intracellular) with polyI:C for 24 h and challenged with CSV 48 h later. A cytopathic effect assay was performed once syncytia were observed (~5 d pi). These data represent three independent experiments ± SEM. “a” is significantly different from “b” at p < 0.0001.
<table>
<thead>
<tr>
<th></th>
<th>Fugene 6</th>
<th>Poly I:C</th>
<th>CSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fugene 6 - 0
Poly I: C - 0
CSV - 0

% syncytial area

![Graph with bars and annotations]

- a
- b

Note: The graph shows the percentage of syncytial area for different treatments, with bars indicating the range of values.
Figure 2.3 dsRNA is not bound or internalized by CHSE-214 cells treated with dsRNA. Cells were treated (extracellular; panel A) or transfected (using Fugene 6; intracellular; panel B) with polyI:C for 24 h and dsRNA was detected by immunocytochemistry using the anti-dsRNA J2 Ab. Cell nuclei are stained blue with DAPI and dsRNA molecules are bound by the red secondary Ab. These data represent three independent experiments ± SEM. * indicates significant difference at p < 0.001.
100 μg/mL PolyI:C

2 μg/mL PolyI:C + Fugene

Fluorescent units

A

B

CTRL PolyI:C PolyI:C + Fugene

*
Figure 2.4 CHSE-214 cells transfected with a human SR-AI-containing expression plasmid are able to bind AcLDL. Cells were either treated with fluorescently labeled AcLDL for 6 h (B), transfected with an empty expression vector using Fugene 6 for 72 h and treated with fluorescently labeled AcLDL for 6 h (C) or were transfected with a human SR-AI-containing expression vector using Fugene 6 for 72 h and treated with fluorescently labeled AcLDL for 6 h (D). Cells were then imaged using fluorescence microscopy. Nuclei are blue (DAPI) and AcLDL is green.
Chapter 3

Novel MARCO and SCARA5 sequences in *Oncorhynchus mykiss*

Short Sequence report: will be submitted to Fish and Shellfish Immunology
3.1 Introduction

Class A scavenger receptors (SR-As) are surface receptors whose functions in mammals include: cell-cell recognition, macrophage adhesion, endocytosis, phagocytosis, and detection of pathogens as part of the innate immune system (Platt and Gordon, 2001). SR-As are type II membrane glycoproteins whose family consists of SR-AI (Scavenger Receptor class A), MARCO (MAcrophage Receptor with COllagenous domain), SCARA3 (SCAvenger Receptor class A, member 3), SCARA4 (SCAvenger Receptor class A, member 4) and SCARA5 (SCAvenger Receptor class A, member 5). All members share cytoplasmic, transmembrane, α-helical, and collagenous domains, though they differ in the lengths of their α-helical and collagenous domains and in the composition of their C-terminal domains with SR-AI, MARCO and SCARA5 possessing a terminal Scavenger Receptor Cysteine Rich (SRCR) domain, SCARA4 possessing a C-type lectin domain and SCARA3 terminating at the collagenous domain (Whelan et al., 2012). All 5 SR-A members form homotrimers, which are stabilized by α-helical coiled-coil motifs and by their collagenous regions (Pearson, 1996). The collagenous region mediates ligand binding and therefore pathogen recognition in SR-AI, SCARA3, SCARA4 and SCARA5 (Bowdish and Gordon, 2009; Doi et al., 1993), whereas the SRCR domain mediates this function in MARCO (Novakowski et al., 2016; Bowdish et al., 2009). The SRCR domain is one of the most ancient pattern recognition domains associated with innate immunity. Although it is found in many proteins and highly conserved across various deuterosome species (Martinez et al., 2011), no full SR-As have been identified in non-vertebrate genomes, indicating that the modern SR-A structure arose after the divergence of vertebrates from other species (Whelan et al., 2012).
SR-A expression profiles vary at the cellular and tissue level and each member possesses different ligand binding abilities. In mammals, MARCO is mainly expressed on macrophages of the spleen marginal zone and lymph nodes (Elomaa et al., 1995) as well as on splenic dendritic cells (Granucci et al., 2003). SCARA5 is expressed on epithelial cells associated with mucosa, (Jiang et al., 2006). The role of SR-As in innate immunity is highlighted by their ability to internalize the synthetic dsRNA polyI:C and mediate a subsequent antiviral response (DeWitte-Orr et al., 2010), as well as their ability to bind lipopolysaccharide (LPS) and lipotechoic acid (LTA) associated with Gram-negative and –positive bacteria, which both MARCO and SCARA5 have been shown to bind (Peiser et al., 2000; Jiang et al., 2006).

Little is known about SR-As in fish, although research to date does indicate that fish SR-A sequence, structure and function share many similarities with mammalian SR-As (Poynter et al., 2015). Functionally, the same competitive ligands used to define SR-A function in mammals have been used to define function in fish, such as fucoidan, DxSO₄, polyI and polyC (Poynter et al., 2015); formaldehyde-treated albumin (FSA) (Seternes et al., 2007); and LTA and formaldehyde treated bovine serum albumin (fBSA; Froystad et al., 2002). Rainbow trout (Oncorhynchus mykiss) SR-As have been shown to bind AcLDL and nucleic acids like their mammalian counterparts (Poynter et al., 2015). At the nucleotide level, MARCO and SCARA5 sequences have been identified in large yellow croaker (Pseudosciaena crocea) and common carp (Cyprinus carpio) where their expression was upregulated in vivo in both species following bacterial infection (He et al., 2014; Feng et al., 2016). In zebrafish (Danio rerio), MARCO mediated phagocytosis of Mycobacterium marinum and the initial pro-inflammatory response to this infection is
MARCO-dependent (Benard et al., 2014). Finally, SCARA5 has also been cloned in puffer fish (*Tetraodon nigroviridis*), and is able to bind LPS (Meng et al., 2012).

SR-As are clearly important mediators of innate immunity in mammals. Our understanding of SR-As in fish is less clear; however, data to date suggest that SR-As play an important innate immune role in fish as well. The present study reports the first confirmed MARCO and SCARA5 mRNA sequences identified in a salmonid species, *Oncorhynchus mykiss*. This is the first step to a better understanding of SR-As in this economically important aquatic animal.

### 3.2 Materials and methods

#### 3.2.1 Cell culture

RTgill-W1, RTgut-GC and RTS11 cell cultures were maintained at 20°C in Leibovitz’s L-15 (Thermo Fisher Scientific, Waltham MA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% (v/v) penicillin G/streptomycin sulfate (Thermo Fisher Scientific). RTgill-W1 and RTgut-GC cells were grown in 75cm² plastic tissue culture flasks and RTS11 cells were grown in 25cm² plastic tissue flasks (Corning, Corning NY, USA).

#### 3.2.2 Full-length sequence identification

##### 3.2.2.1 Primer design

The predicted salmon SCARA5 protein sequence (accession number XP_013999931.1) was entered in BLASTp and matched an unnamed rainbow trout protein product (accession number CDQ63372.1). This protein sequence was reverse translated and aligned to zebrafish and yellow croaker SCARA5 mRNA sequences (accession numbers NM_001030190.1 and NM_001303324.1, respectively). Degenerate
SCARA5 primers were designed from this alignment. MARCO rainbow trout primers were designed from the predicted salmon MARCO mRNA sequence (accession number XM_014173984.1) using NCBI primer BLAST. Primer sets used to amplify either full-length sequences or shorter regions for screening purposes, are listed in Table 3.1.

3.2.2.2 RT-PCR

RTgill-W1 and RTS11 were plated to confluency in 25 cm$^2$ flasks and incubated overnight in 10% (v/v) FBS media. RNA was extracted using TRIzol (Life Technologies, Carlsbad CA, USA) as per manufacturer’s instructions. RNA was quantified using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and stored at -80°C. cDNA was synthesized using 2ug of total RNA, 0.5 ug Oligo(dT) primers (Sigma-Aldrich, St. Louis MO, USA), 1X GoScript reaction buffer (Promega, Madison WI, USA), 1.5mM MgCl$_2$ (Promega), 0.5 mM each deoxynucleotide triphosphate (dNTP, Sigma-Aldrich), and 160 U GoScript Reverse Transcriptase (Promega). Reactions were performed according to the manufacturer’s instructions. PCR reactions were performed using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific). PCR reactions contained: 2 uL of cDNA, 1x Phusion HF Buffer (Thermo Scientific), 0.2 mM dNTP mix (Sigma-Aldrich), 0.4 U Phusion Hot Start II DNA Polymerase, 0.5 uM forward and reverse primer and up to 20 uL nuclease-free water (Thermo Fisher Scientific). The PCR reactions were conducted in a T100 Thermal Cycler (Bio-Rad, Hercules CA, USA) at the following cycle conditions: 98°C for 30 s, 33 cycles of: 98°C for 7 s, 64°C for 20 s, 72°C for 1 min, 72°C for 10 min and the reaction was held indefinitely at 4°C. 10 uL of the final MARCO and SCARA5 products were run on a 1% w/v agarose (Thermo Fisher Scientific) gel with 5 uL of O'GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher
Scientific). The resulting gel was stained with 0.5 mg/mL ethidium bromide (Sigma-Aldrich) for visualization with a VersaDoc Imager (Bio-Rad) set to UV transillumination. Bands corresponding to MARCO and SCARA5 PCR products were purified and gel extracted, respectively, using a High Pure PCR Product Purification Kit (Roche, Laval QC, Canada) and a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). To confirm their identity, PCR products were sent to Laboratory Services (University of Guelph) for sequence analysis.

3.2.2.3 Cloning and sequencing

Purified MARCO and SCARA5 PCR products were cloned into pGEM-T Easy Vectors as per manufacturer’s instructions (Promega). Plasmids were purified using a GenElute Plasmid Miniprep Kit (Sigma). Purified plasmids were sequenced by Laboratory Services (University of Guelph) using the T7 and S6 promoters. MARCO and SCARA5 PCR products were sequenced 5 and 4 times, respectively, each trial run independently. Full *Oncorhynchus mykiss* MARCO and SCARA5 accession numbers, respectively, are as follows: KX452014 and KX452177.

3.2.3 Cell and tissue expression screening

Rainbow trout cells and tissues were screened for the expression of MARCO and SCARA5 by using fragment primers that were designed based on full sequences (see Table 1). RTgill-W1, RTgutGC and RTS11 were chosen because they are two epithelial cell lines (in which SCARA5 is thought to be expressed), derived from gill and gut, respectively, and a macrophage/monocyte cell line (in which MARCO is thought to be expressed). Rainbow trout tissues were acquired from Dr. Brian Dixon (University of Waterloo), under his Animal Utilization Protocol. Tissues were stored in RNAlater-ICE.
overnight and RNA from both cells and tissues was extracted using TRIzol (Life Technologies) as per manufacturer’s instructions. cDNA synthesis was made as described above. PCR reactions were performed using GoTaq Flexi DNA Polymerase (Promega). PCR reactions contained: 2 uL of cDNA, 1x Green GoTaq Flexi Buffer (Promega), 0.2 mM dNTP mix (Sigma-Aldrich), 1.25 U GoTaq Flexi DNA Polymerase (Promega), 1.5 mM MgCl2 (Promega), 0.5 uM forward and reverse primer and up to 25 uL nuclease-free water (Thermo Fisher Scientific). The PCR reactions were conducted in a T100 Thermal Cycler (Bio-Rad, Hercules CA, USA) at the following cycle conditions: 95°C for 3 min, 34 cycles of: 95°C for 1 min, 55°C (SCARA5) or 53°C (MARCO and β-Actin) for 1 min, 72°C for 3 min, 72°C for 10 min and then stored at 4°C.

3.2.4 Bioinformatics analysis

Protein sequences used for Figure 3.1 were collected from NCBI. MARCO and SCARA5 rainbow trout mRNA sequences were translated into protein sequences using the ExPASy translate tool (http://web.expasy.org/translate/). The cytoplasmic and transmembrane domains were determined using TMHMM software (http://www.cbs.dtu.dk/services/TMHMM). Alpha-helical domains were identified using the PSIPRED protein sequence analysis workbench (http://bioinf.cs.ucl.ac.uk/psipred/). The collagenous and SRCR domains were determined using NCBI’s CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; E value ≤ 0.01. The protein representations were made using IBS (Illustrator for Biological Sequences; http://ibs.biocuckoo.org/). Amino acid sequence similarities in Figure 3.2 were determined using BLASTp. The phylogenetic tree in Figure 3.2 was constructed with MEGA7 software using the neighbor-joining method based on the amino acid alignment
(ClustalW) of full-length MARCO and SCARA5 proteins from representative species. The numbers beside the internal branches indicate bootstrap values based on 1000 replications. The scale indicates the genetic distance. The tree was rooted with an outgroup, colmedin from *Operophtera brumata*, as it is also a transmembrane protein containing a collagenous domain.

**3.3 Results and discussion**

The protein domain architectures of the MARCO and SCARA5 sequences cloned from rainbow trout are similar to those of other representative species and both contain the collagenous and SRCR domains that are characteristic of these proteins in other species (Figure 3.1). This suggests that rainbow trout MARCO and SCARA5 contain ligand-binding domains as described in other vertebrates, specifically the SRCR and collagenous domains respectively. Both proteins in all species examined contain cytoplasmic, transmembrane, α-helical, collagenous and SRCR domains. Relatively speaking, MARCO in all species contains a shorter α-helical domain and a larger collagenous domain whereas SCARA5 contains a larger α-helical domain and a shorter collagenous domain. Amino acid sequence similarity scores in Table 3.2 suggest that rainbow trout MARCO and SCARA5 sequences are conserved among vertebrates, MARCO ranging from 35% to 46% identity and SCARA5 ranging from 50% to 68%.

A phylogenetic tree was constructed using the neighbor-joining method to investigate the relationship between the cloned rainbow trout MARCO and SCARA5 sequences and those in other species (Figure 3.2). The tree reveals that the rainbow trout MARCO and SCARA5 amino acid sequences cluster strongly with corresponding fish
sequences; showing a closer relationship to yellow croaker (*Larimichthys crocea*), to which rainbow trout are most closely related (Broughton et al., 2013).

MARCO and SCARA5 expression was identified in rainbow trout cell lines and tissues at the transcript level using RT-PCR (Figure 3.3). MARCO was identified in RTS11, a monocyte/macrophage cell line and SCARA5 was identified in RTS11, RTgill-W1, a gill epithelium cell line, and RTgutGC, a gut epithelium cell line. These results suggest that the macrophage-restricted expression profile observed in mammals for MARCO is likely conserved in rainbow trout. SCARA5 was detected in both macrophages and epithelial cell types, suggesting its expression profile may be broader in fish than mammals. MARCO and SCARA5 were identified in all rainbow trout tissues examined: gut, spleen, gill, head kidney and liver. This broad expression across tissue types is likely due to the mixed cell-type populations within tissues, such as the presence of tissue-resident macrophages, which the present study shows express both MARCO and SCARA5 (in RTS11).

### 3.4 Conclusions

MARCO and SCARA5 are members of the class A scavenger receptor family of surface receptors, which play an important role in modulating LDL homeostasis as well as innate immunity. This is the first report of MARCO and SCARA5 sequences identified from rainbow trout. Functional analyses of these two receptors are currently in progress, in order to better understand how these important receptors modulate innate immunity in fish.
### 3.5 Tables, figure and figure legends

**Table 3.1 RT-PCR primers used for amplification of full-length and fragment MARCO and SCARA5**

<table>
<thead>
<tr>
<th>Gene</th>
<th>RT-PCR primers (5’ – 3’)</th>
<th>Product length (bp)</th>
<th>Annealing temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length MARCO</td>
<td>F – ATGGAGACATCAGTAGACAG R – TCACACACACTGCACCCAG</td>
<td>1347</td>
<td>64</td>
</tr>
<tr>
<td>Full-length SCARA5</td>
<td>F – ATGGAGAATAGGGCGATGTA R – TTAGGTGTGCAGGTCAAGC</td>
<td>1497</td>
<td>64</td>
</tr>
<tr>
<td>Fragment MARCO</td>
<td>F – TCCTCAAAGCTTCCACCAGGC R – ACTGGTCCAGCTCTCTTC</td>
<td>550</td>
<td>53</td>
</tr>
<tr>
<td>Fragment SCARA5</td>
<td>F – CCGTGTATCCGTCCTGAACC R – TGTACCGTGCACTCATGGCTT</td>
<td>269</td>
<td>55</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F – GTCACCAACTGGGACGACAT R – GTACATGGGCAGGGTGTGTA</td>
<td>514</td>
<td>54</td>
</tr>
</tbody>
</table>
Table 3.2 Amino acid sequence similarity scores between *Oncorhynchus mykiss* MARCO and SCARA5 sequences and other species represented in Figure 3.1.

Amino acid query cover and identity values were determined using BLASTp. Accession numbers for the protein sequences are listed in Figure 3.1.
<table>
<thead>
<tr>
<th></th>
<th>Oncorhynchus mykiss</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MARCO</td>
<td>SCARA5</td>
</tr>
<tr>
<td></td>
<td>Query cover</td>
<td>Identity</td>
</tr>
<tr>
<td>Larimichthys crocea</td>
<td>100%</td>
<td>46%</td>
</tr>
<tr>
<td>Cyprinus carpio</td>
<td>94%</td>
<td>38%</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>100%</td>
<td>39%</td>
</tr>
<tr>
<td>Alligator mississippiensis</td>
<td>90%</td>
<td>40%</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>92%</td>
<td>36%</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>93%</td>
<td>35%</td>
</tr>
</tbody>
</table>
Figure 3.1 The protein domain architecture of class A scavenger receptors MARCO and SCARA5 in different species, including those sequenced in *Oncorhynchus mykiss*. Structures are scaled based on the length of each domain, the total amino acids in each sequence is indicated by the number at the end of each protein. The cytoplasmic and transmembrane domains were determined using TMHMM software. The collagenous and SRCR domains were determined using NCBI’s CDD. Alpha-helical domains were identified using the PSIPRED protein sequence analysis workbench. The white regions represent areas of the sequence not supported by bioinformatics analysis. Accession numbers for the protein sequences are as follows. MARCO: *Larimichthys crocea* AHY18726.1, *Cyprinus carpio* BAU33570.1, *Danio rerio* AI173713.1, *Alligator mississippiensis* KYO18128.1, *Mus musculus* AAA68638.1, *Homo sapiens* NP_006761.1. SCARA5: *Larimichthys crocea* AHY18728.1, *Cyprinus carpio* BAU33576.1, *Danio rerio* NP_001025361.1, *Alligator mississippiensis* KYO32290.1, *Mus musculus* NP_083179.2, *Homo sapiens* NP_776194.2.
Figure 3.2 Phylogenetic tree illustrating the relationship between the *Oncorhynchus mykiss* MARCO and SCARA5 protein sequences and those reported in representative fish, mammalian, reptile and avian species. The tree was constructed with the MEGA7 software using the neighbor-joining method based on amino acid alignment (ClustalW) of full-length proteins. Numbers beside the internal branches indicate bootstrap values based on 1000 replications. 20 scale indicates the genetic distance. The tree was rooted with an outgroup (colmedin protein from *Operophtera brumata*). Accession numbers for the protein sequences are as follows. MARCO: *Homo sapiens* NP_006761.1, *Mus musculus* AAA68638.1, *Rattus norvegicus* NP_001102481.1, *Tyto alba* KFV48160.1, *Alligator mississippiensis* KYO18128.1, *Larimichthys crocea* AHY18726.1, *Cyprinus carpio* BAU33570.1, *Danio rerio* AII73713.1. SCARA5: *Homo sapiens* NP_776194.2, *Callithrix jacchus* JAB05721.1, *Mus musculus* NP_083179.2, *Rattus norvegicus* NP_001129327.1, *Alligator mississippiensis* KYO32290.1, *Larimichthys crocea* AHY18728.1, *Cyprinus carpio* BAU33576.1, *Danio rerio* NP_001025361.1. Colmedin: *Operophtera brumata* KOB75227.
Figure 3.3 RT-PCR based detection of MARCO and SCARA5 transcripts in rainbow trout cell lines and tissues. MARCO and SCARA5 transcript presence was measured using RT-PCR and primers to amplify fragments of the genes of interest in either (A) rainbow trout cell lines, RTS11, Rtgill-W1 and RTgut GC, or (B) tissues from three healthy, untreated rainbow trout. β-actin was used as an internal control.
A

RTS-11  Rigill-W1  RTgutGC

MARCO  SCARA5  β-Actin

B

Gut A  Gut B  Gut C

Spleen A  Spleen B  Spleen C

Gill A  Gill B  Gill C

HK A  HK B  HK C

Liver A  Liver B  Liver C

MARCO  SCARA5  β-Actin
Chapter 4

General discussion
4.1 Major research findings and contributions to class A scavenger receptor research

Class A scavenger receptors have diverse functions; in mammals they have been shown to be involved in cell-cell recognition, macrophage adhesion, endocytosis, phagocytosis, and the detection of pathogens as part of the innate immune system (Platt and Gordon, 2001). Due to their function as modified low-density lipoprotein (mLDL) scavengers, SR-A research has largely focused on their role in the formation of atherosclerosis. More recent research has elucidated their function in bacterial recognition, especially SR-AI and MARCO, but little is known about SCARA3, SCARA4 and SCARA5, and the family as a whole’s role in dsRNA sensing as part of the innate immune response. The innate antiviral immune response is paramount to any organism’s survival and as such, the study of its diverse receptors and subsequent signaling pathways is crucial. To date, the study of class A scavenger receptors has been difficult due to ubiquitous expression patterns producing high background in most cell lines.

The first part of this present work has paved the way for the use of the CHSE-214 cell line as a model for SR-A study by demonstrating four novel findings about this cell line. The CHSE-214 study has shown: 1) CHSE-214 cells express IFN and ISG transcripts in response to intracellular but not extracellular dsRNA, as measured using qRT-PCR; 2) intracellular dsRNA mounts a sufficient antiviral state to protect CHSE-214 cells from CSV-mediated CPE, while extracellular dsRNA did not; 3) it is CHSE-214’s uptake and internalization of dsRNA that appears to be defective in this cell line, as determined using immunocytochemistry and the J2 anti-dsRNA antibody; and 4) overexpression of human SR-AI restored AcLDL binding capabilities in these cells, as
determined using fluorescence microscopy. To summarize, this project has shown that CHSE-214 cells’ intracellular signaling pathway is intact in response to intracellular dsRNA and suggests that the defect in these cells’ ability to respond to extracellular dsRNA lies in their inability to bind and internalize the molecule, pointing to a lack of functional SR-As.

The second part of this work has identified two novel full-length rainbow trout SR-A sequences, MARCO and SCARA5. Protein sequence analysis has revealed that these proteins share similar domain architecture to MARCO and SCARA5 sequences from other species, including fish, reptile and mammalian species. Phylogenetic analysis has also shown each SR-A studied grouped with members of that same protein, and the fish MARCO and SCARA5 sequences grouped together for both proteins respectively. RT-PCR was used to investigate constitutive expression of these receptors in rainbow trout cell lines and tissues and showed expression of MARCO in RTS11, a rainbow trout macrophage/monocyte cell line, and of SCARA5 in RTS11, as well as RTgill-W1 and RTgutGC, two rainbow trout cell lines derived from gill and gut epithelium, respectively. Both scavenger receptors were identified in all rainbow trout tissues examined: gut, spleen, gill, head kidney and liver.

This work has shown that CHSE-214 has a defect in dsRNA uptake, suggesting it may be deficient in functional SR-As. Also, rainbow trout MARCO and SCARA5 sequences have been cloned into expression vectors. CHSE-214 cells can thus be used to overexpress SR-A sequences and perform binding and blocking studies to study SR-A binding in a background-free system. Additionally, mutation studies will also help to elucidate the function of SR-As’ diverse domains. Preliminary results show that
overexpression of a GFP-tagged rainbow trout MARCO (in an expression plasmid) rescued CHSE-214’s ability to bind dsRNA as detected by immunocytochemistry (Figure 4.1). Cells were plated on glass coverslips in a 12-well plate and allowed to attach overnight. The following day cells were transfected with the rainbow trout MARCO-eGFP-C1 expression plasmid using a 1.5 to 1 ratio of Fugene 6 to plasmid, as per manufacturer’s instructions. 72 h post transfection, media was removed and cells were treated with 100 µg/mL polyI:C for 24 h. Following polyI:C treatment, the same immunocytochemistry protocol outlined in section 2.2.6 was followed.

The DeWitte-Orr lab studies many facets of the innate antiviral response. Research to date has included investigating the effects of dsRNA length and sequence on antiviral responses, using different cell types as models for understanding innate immunity to specific viruses, identifying novel PRRs in rainbow trout, and characterizing SR-A expression and function in rainbow trout cells. Though much is known about innate antiviral responses already, much more remains to be elucidated especially in lower vertebrates such as fish. By furthering our knowledge of all aspects of innate antiviral immunity in different species, we will be able to learn more about specific host-virus interactions, which will facilitate the development of more effective therapeutics. This project has been integrative in many ways. Firstly, by utilizing several different techniques and approaches: qRT-PCR to quantify immune gene expression, a cytopathic effect assay to investigate antiviral activity, immunocytochemistry to detect the presence of a ligand, RT-PCR and cloning to identify novel rainbow trout SR-A sequences, bioinformatics to determine protein structure and phylogeny. Secondly, the use of the CHSE-214 cell line as a model will facilitate the study of class A scavenger receptors in
fish but as well as in all species as these proteins have been shown to be fairly well conserved across species.

4.2 Summary

4.2.1 Subject

Class A scavenger receptors are multifunctional proteins but due to their function as mLDL receptors and their role in atherosclerosis formation, their role as pathogen sensors in innate immunity, especially as sensors of viral dsRNA, has been largely overlooked. SR-As’ dsRNA-binding capabilities have been difficult to determine due to ubiquitous expression patterns causing high background in most cells. This study aimed to test whether a fish cell line, CHSE-214, could be an effective model for scavenger receptor study, as preliminary studies have suggested a defect in the type I IFN response in these cells. This study also aimed to identify novel rainbow trout class A scavenger receptor sequences that could be studied using CHSE-214.

4.2.2 Findings

4.2.2.1 IFN and ISG transcripts are only upregulated in CHSE-214 in response to intracellular dsRNA: CHSE-214 cells upregulated IFN and ISG transcripts in response to intracellular dsRNA (polyI:C transfected using Fugene 6) but did not in response to extracellular dsRNA (polyI:C treatment).

4.2.2.2 Only intracellular dsRNA pretreatment reduces CSV’s cytopathic effects in CHSE-214: CSV’s cytopathic effect (syncytia) was diminished considerably in CHSE-214 cells pretreated with intracellular dsRNA (polyI:C transfected using Fugene 6), when
compared to cells that were pretreated with extracellular dsRNA (polyI:C treatment), demonstrating that only intracellular dsRNA establishes an effective antiviral state.

4.2.2.3 dsRNA is not bound or internalized by CHSE-214 cells treated with dsRNA: dsRNA was detected in CHSE-214 cells that were transfected with polyI:C (using Fugene 6) but was not detected in cells that were treated with it, demonstrating it is dsRNA’s binding/uptake that is defected in CHSE-214 cells.

4.2.2.4 CHSE-214 cells overexpressing human SR-AI bind AcLDL: CHSE-214 cells transfected with a human SR-AI containing expression plasmid were able to bind AcLDL, a known SR-AI ligand, demonstrating the suitability of this cell line as a model for class A scavenger receptor study.

4.2.2.5 Cloned rainbow trout MARCO and SCARA5 sequences share similar domain architecture with these proteins in other species: Rainbow trout MARCO and SCARA5 sequences both contained cytoplasmic, transmembrane, α-helical, collagenous and SRCR domains characteristic of class A scavenger receptors. As in other species examined, MARCO contains a relatively short α-helical domain and a large collagenous domain, whereas SCARA5 contains a large α-helical domain and a short collagenous domain.

4.2.2.6 Phylogenetic analysis shows that rainbow trout MARCO and SCARA5 sequences cluster with other fish proteins: A neighbor-joining tree was used to investigate the relationship between the cloned rainbow trout MARCO and SCARA5 sequences and those in other species, revealing that the rainbow trout MARCO and SCARA5 amino acid sequences cluster strongly with corresponding fish sequences.
4.2.2.7 *MARCO and SCARA5 transcripts were identified in rainbow trout cell lines and tissues:* MARCO was identified in RTS11, a monocyte/macrophage cell line and SCARA5 was identified in RTS11, RTgill-W1, a gill epithelium cell line, and RTgutGC, a gut epithelium cell line. MARCO and SCARA5 were identified in all rainbow trout tissues examined: gut, spleen, gill, head kidney and liver.
4.3 Figures and figure legends

Figure 4.1 CHSE-214 cells transfected with a rainbow trout MARCO-containing GFP expression plasmid were able to bind polyI:C. Cells were transfected with the rainbow trout MARCO-containing eGFP-C1 expression plasmid using Fugene 6 for 72 hs, after which immunocytochemistry with the anti-dsRNA J2 antibody was performed. Cells were then imaged using fluorescence microscopy. Nuclei are blue (DAPI) and dsRNA is red.
LITERATURE CITED


Feng, S., Jiang, Y., Zhang, S., Dong, C., Jiang, L., Peng, W., ... & Xu, P. (2016). Genome wide identification of scavenger receptors class A in common carp (Cyprinus carpio) and their expression following Aeromonas hydrophila infection. Fish & shellfish immunology, 54, 60-67.


Novakowski, K. E., Huynh, A., Han, S., Dorrington, M. G., Yin, C., Tu, Z., ... & Bowdish, D. M. (2016). A naturally occurring transcript variant of MARCO reveals the SRCR domain is critical for function. *Immunology and cell biology.*


Tominaga, K., Yoshimoto, T., Torigoe, K., Kurimoto, M., Matsui, K., Hada, T., ... & Nakanishi, K. (2000). IL-12 synergizes with IL-18 or IL-1β for IFN-γ production from human T cells. *International Immunology, 12*(2), 151-160.


