CHARACTERIZATION OF ATLANTIC SALMON HEAD KIDNEY LEUKOCYTE CULTURE

By

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Abstract

The Atlantic salmon (*Salmo salar*) is an economically important farmed and wild fish in several countries including Canada. Macrophages are white blood cells of the immune system of fish and other vertebrates, that are essential in fighting infection and disease. Elucidating how macrophages differentiate and function is necessary to fully understand how the fish immune system functions and to enable the development of methods to maintain healthy fish. Therefore, the objective of my Ph.D. thesis was to characterize the Atlantic salmon adherent head kidney leukocyte (HKL) culture, a macrophage-like model commonly used in fish immunological studies, using various genomic and complementary techniques.

Using morphology (Giemsa stain) and functional (phagocytosis) assays, the results of this thesis showed that the Atlantic salmon adherent HKL population changes during culture time. At Day 1 of culture, the results suggest that adherent HKLs are a heterogeneous population of predominantly "monocyte-like", cells but by Day 5 of culture, the cells become more homogenous selectively enriched with macrophages. RNA-sequencing identified a change in the microRNA (miRNA) profile of Day 1 and Day 5 adherent HKLs, as well as the extracellular vesciles (EVs) released from them. Many of the identified miRNAs are involved in macrophage function and/or differentiation in other species. Furthermore, using a 44K microarray, changes in the mRNA transciprtome were profiled. Macrophage-related transcripts, lipid-related transcripts, immune-related transcripts and transcription factors were identified as differentially expressed between the two cell populations. In addition, GO term enrichment and network analysis identified immune-related and immune-cell differentiation related terms.

The results of this thesis provides evidence that the Atlantic salmon HKL culture changes to become predominantly "macrophage-like" by Day 5 of culture and this is something that

should be kept in mind when using HKLs for *in vitro* fish immunology studies. This research provides novel insight into the genes, miRNAs and molecular pathways involved in the differentiation of Atlantic salmon adherent HKLs from monocyte-like cells to macrophage-like cells.

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List of Abbreviations

AA	Amino acid
Abs	Antibodies
AGO	Argonaute
AID	Activation-induced cytidine deaminase
AIM	Absent in melanoma
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA-editing catalytic
APP	Acute phase protein
ARG1	Arginase-1
aRNA	Amplified RNA
BCR	B cell receptor
BLAST	Basic local alignment search tool
BMDMs	Bone marrow-derived macrophages
bp	Base pair
BP	Biological process
CC	Cellular component
CCR	CC chemokine receptor
cDNA	Complementary DNA
CEBP	CCAAT-enhancer-binding protein
cGRASP	Consortium for genomic research on all salmonids project
CLP	Common lymphoid progenitor
CLRs	C-type lectins
CMP	Common myeloid progenitor
CPG	Cytosine–phosphate–guanosine
CRP	C-reactive protein
CSF1	Colony stimulating factor 1
CSF1R	Colony stimulating factor 1 receptor
CSF3R	Granulocyte colony-stimulating factor receptor
CSR	Class switch recombination
C_t	Cycle Threshold
c-type	Chicken-type
CXCR	C-x-c chemokine receptor type
(D)	Diversity
D6FADC	Delta-6 fatty acyl desaturase
DAMP	Danger associated molecular pattern
DDX58	Dexd/h-box helicase 58
DE	Differentially expressed
DEGS	Differentially expressed genes

DEPS	Differentially expressed probes
DGCR8	Digeorge syndrome critical region 8
DHCR7	7-dehydrocholesterol reductase
DMSO	Dimethyl sulfoxide
dsRNA	Double stranded RNA
EF1A1	Elongation factor 1 alpha-1
EF1A2	Elongation factor 1-alpha-2
ELOVL	Elongation of very long chain fatty acids protein
ER	Endoplasmic reticulum
EST	Expressed sequence tag
EVS	Extracellular vesicles
Fab	Fragment, antigen-binding
FABP6	Fatty acid binding protein 6
FACR	Fatty acyl-coa reductase
FACS	Fluorescence-activated cell sorting
FADS	Fatty acid desaturation
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FC	Fold-change
Fc	Fragment, crystallizable
FDR	False discovery rate
GALT	Gut associated lymphoid tissues
GC	Germinal centers
gcGXM	Ginbuna curcian carp Granzyme
GI	Gastrointestinal
GIALT	Gill associated lymphoid tissues
GMP	Granulocyte macrophage progenitor
GO	Gene ontology
GRN	Granulin
g-type	Goose-type
HAMP	Hepcidin
HBSS	Hank’s balanced salt solution
HKL	Head kidney leukocyte
HSC	Hematopoietic stem cell
HSPS	Heat shock proteins
IFIT5	Interferon-induced protein with tetratricopeptide repeats 5
Ig	Immunoglobulin
IgNAR	Ig new/nurse shark antigen receptor
IL	Interleukin
IL1B	Interleukin 1 beta
IRF	Interferon regulatory factor

IFN	Interferon
ISAV	Infectious salmon anemia virus
ITAM	Immunoreceptor tyrosine-based activation motif
(J)	Joining
JBARB	Dr. Joe Brown Aquatic Research Building
JUN	Jun proto-oncogene
KLF	Krueppel-like factor
L-15	Leibovitz-15
LGMN	Legumain
LPS	Lipopolysaccharide
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
MEP	Megakaryocyte erythroid progenitor
mg	milligram
MHC I/II	Major histocompatibility complex I/II
MIQE	Minimum information for publication of qPCR experiments
miRISC	Mirna induced silencing complex
miRNAs	MicroRNA
ml	milliliter
mRNAs	Messenger RNA
MS	Mass spectrometry
MX2	Interferon-induced gtp-binding protein mx
MYA	Million years ago
NCBI	National Center for Biotechnology Information
ncRNAs	Non-coding RNAs
NEB	New England Biolabs
NETS	Neutrophil extracellular traps
NITRS	Novel immune-type receptors
NLRs	Nucleotide-binding domain, leucine-rich repeat containing proteins
NO	Nitric oxide
NSC	Norwegian High-Throughput Sequencing Centre
NTA	Nanoparticle tracking analysis
NTC	No template control
nts	Nucleotides
PABPC1	Polyadenylate-binding protein 1
PAHS	Polycyclic aromatic hydrocarbons
PAMP	Pathogen associated molecular pattern
pAPC	Professional antigen presenting cell
PBMCS	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline

PMA	Phorbol myristate acetate
PMT	Photomultiplier tube
POLR2	RNA polymerase 2
POLY(I:C)	Polyinosinic:polycytidylic acid
pre-MIRNA	Precursor-mirna
pri-MIRNA	Primary-mirna
PRR	Pattern recognition receptor
PU.1	Transcription factor PU.1
QC	Quality control
r-	Recombinant
RGNNV	Red spotted grouper nervous necrosis virus
RIG-I	Retinoic acid inducible gene I
RISC	RNA-induced silencing complex
RNA-SEQ	RNA-sequencing
ROS	Reactive oxygen species
RP	Red pulp
RPL32	60s ribosomal protein 32
RSAD2	Radical sam domain-containing 2
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SALT	Skin-associated lymphoid tissues
SAM	Significance analysis of microarrays
SAP	Serum amyloid proteins
SAV	Salmonid alphavirus
SCREBP	Sterol regulatory element binding proteins
SE	Standard error
SGIV	Singapore grouper iridovirus
SHM	Somatic hypermutation
SLO	Secondary lymphoid organ
SRA	Sequence read archive
SREBPS	Sterol regulatory element-binding proteins
STAT	Signal transducer and activator of transcription
sTLR	Soluble toll like receptor
TBST	Tris buffered saline plus tween
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TGFB1	Transforming growth factor beta 1
TLR	Toll-like receptor
TNFA	Tumor necrosis factor alpha
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1a
TNP-KLH	Trinitrophenylated-keyhole limpet hemocyannin

TREG	T-regulatory
UTR	Untranslated region
(V)	Variable
VHSV	Viral hemorrhagic septicemia virus
VIPERIN	Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible
VLRS	Variable lymphocyte receptors
WB	Western blot
WP	White pulp
µG	Microgram
µL	Microlitre
µM	Micromolar

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CHAPTER 1: Introduction

1.1. Introduction

Aquaculture, the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants, is a growing industry worldwide. While the capture fishery has remained static, the aquaculture industry has continued increasing production to maintain fish supply [1]. For example, in Canada, the value of aquaculture production has increased by 56% in the past decade (2008-2018) [2]. The Atlantic salmon (*Salmo salar*) is Canada's top aquaculture export, while all farmed salmon (Atlantic salmon, Chinook salmon (*Oncorhynchus tshawytscha*) and Coho salmon (*Oncorhynchus kisutch*)) represent over 70% of the overall aquaculture production volume in Canada [3]. With an increase in fish production, however, comes an increased risk for disease. Farmed salmon are susceptible to infectious diseases including viruses such as salmonid alphavirus (SAV) and infectious salmon anaemia virus (ISAV), bacteria such as *Aeromonas salmonicida* and *Piscirickettsia salmonis*, and parasites such as sea lice [4–6]. In addition, environmental conditions and overcrowding can negatively affect fish health.

Understanding the fish immune system, in particular fish immune cells and how they differentiate and function, is a key step in being able to develop tools to fight fish disease and infection and, therefore, being able to maintain healthy farmed fish. Aquaculture based genomic tools and methods, including microarrays and RNA-sequencing, allow for a broader understanding of the fish immune response at a molecular level. There are many genomic resources that support the study of genes involved in the immune response of Atlantic salmon including, but not limited to, the whole genome sequence, over 200 cDNA libraries constructed from various tissues, over 498,000 Expressed Sequence Tags (ESTs) and several microarray platforms, including the platform used in this thesis, the Genomic Research on All Salmonids

Project (cGRASP)-designed 44K salmonid oligonucleotide microarray [7–9]. This platform has been used in several studies, including those examining the immune response of Atlantic salmon to bacterial infection (i.e. *Renibacterium salmoninarum*), to parasite infection (i.e. sea-lice), to challenge with the synthetic analog of double stranded RNA, polyinosinic:polycytidylic acid (poly(I:C)), and to diet composition, among many other studies [10–13]. Through RNA-sequencing, Atlantic salmon microRNAs (miRNAs) were first characterized, creating miRNA reference sequences which have been used to investigate miRNAs involved in the immune response of Atlantic salmon [14–19]. Therefore, through the use of various genomic tools and complementary methods, including microarrays, RNA-sequencing and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), the aim of this thesis was to characterize Atlantic salmon macrophages, important cells of both the innate and adaptive immune systems.

1.1.1. The immune system

The immune system can be divided into two subsystems: the innate immune system and the adaptive immune system. The innate immune system is the first to respond to initial pathogens and does not retain memory of previous responses, while the adaptive immune system is highly specific to a particular antigen and can provide long-lasting immunity [20,21]. The innate immune system is found in all multicellular organisms and thus is evolutionarily older than the adaptive immune system [22]. The adaptive immune system is comparatively newer, and components of the adaptive immune system are assumed to have arisen approximately 450 million years ago (MYA) in the first jawed vertebrates – the Gnathostomata [23–26]. While jawless vertebrate have an adaptive immune system base on variable leukocyte receptors (VLRs), T-like and B-like cells, jawed vertebrate have an adaptive immune system based on immunoglobulins, T cell receptors and major histocompatibility complex I and II (MHC I and

II). The Gnathostomes are subdivided into the Chondrichthyes (cartilaginous fishes) and the Osteichthyes (bony fishes), of which the latter can be further subdivided into the Actinopterygii (ray-finned fish) and the Sarcopterygii (lobe-finned fish). The infraclass Teleostei, to which the Atlantic salmon belongs, accounts for 96% of extant fish species [27].

1.1.2. The innate immune system

Components of the innate immune system include physical barriers, cellular components and humoral responses [28]. The first line of defense against the entry of pathogens are physical barriers including the skin, scales, mucus and skin-associated lymphoid tissues (SALT), the gills and gill associated lymphoid tissues (GIALT) and the gastrointestinal (GI) tract and gut associated lymphoid tissues (GALT) [29,30]. While some physical barriers have been studied more extensively in certain fish species, such as teleosts, elements of these physical barriers can be found in all Gnathostomes.

If a pathogen invades the physical barriers, it will encounter the cellular and humoral responses. Cells of the innate immune system in fish include monocytes/macrophages (see section 1.2.0.-1.2.2. for discussion on macrophages), granulocytes such as neutrophils, dendritic cells, and natural killer cells. Initiation of an innate immune response begins when a pathogen associated molecular pattern (PAMP), found on a pathogen, or a danger associated molecular pattern (DAMP), found on biomolecules released from injured cells, binds to germline-encoded intracellular or extracellular pattern recognition receptors (PRRs), found on an immune cell [31]. PRRs can be classified into at least five major groups: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), C-type lectins (CLRs), the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), and absent in melanoma (AIM)-like receptors, of which TLRs are the most extensively studied in both fish and mammals [31–33].

While there are 13 TLRs in mammals, there are up to 28 TLRs between cartilaginous, ray-finned and lobe-finned fish [34,35].

Humoral responses are mediated by molecules released from cells following infection and include components of the complement system, lysozyme, antimicrobial peptides (AMPs) and acute phase proteins (APPs), among many others. The complement system is composed of approximately 30 proteins that make up three pathways, the classical pathway, the alternative pathway and the lectin pathway, which ultimately lead to the elimination of pathogens through opsonization and phagocytosis and the promotion of the inflammatory response [36]. Lysozyme is a lytic enzyme that acts on the peptidoglycan layer of bacterial cell walls by hydrolyzing 1–4 β-linked glycoside bonds resulting in lysis of the bacterium and is one of the most studied innate immune components in fish [37]. AMPs defend against pathogens through pore-forming action against bacterial membranes [38,39]. Over 90 fish AMPs have been identified and several have been cloned with subsequent functional studies demonstrating antiviral and antibacterial activities against a variety of pathogens, suggesting that AMPs from teleost fish exhibit many, if not all, of the characteristics of other vertebrate AMPs [38,39]. APPs are produced and released from hepatocytes following stimulation from cytokines released from immune cells in response to tissue injury, infection and/or inflammation [40,41]. APPs are well-conserved in arthropods, fish, amphibians and mammals [42]. If a pathogen persists, despite the innate defenses, the adaptive immune system will be activated.

1.1.3. The adaptive immune system

Similar to the innate immune system, the adaptive immune system includes both humoral (B cells) and cellular (T cells) components. Antigen specificity of B cells and T cells is determined by their B cell receptors (BCRs) or T cell receptors (TCRs) which are able to

recognize innumerable specific antigens and are unique to the adaptive immune system. In response to antigen, and in combination with helper T cell interactions, B cells will secrete antigen–specific antibodies (Abs). Three classes of Abs have been identified in both teleost fish and cartilaginous fish: IgM, IgD, and IgZ/T in teleost fish, and IgM, IgW, and IgNAR in cartilaginous fish, while in lobe-finned fish, IgM, IgW, and IgN are found in the lungfish and two forms of IgW has been discovered in coelacanths [43]. In addition to Ab production and immunological memory, a major function of B cells, as well as other antigen presenting cells, is to process and present antigen to activate T cells through MHC I or MHC II. T cells are divided into two subpopulations, CD4+ helper T cells and CD8+ cytotoxic T cells. Upon activation, CD4+ cells release cytokines that regulate responses elicited by the antigen while CD8+ cells secrete cytotoxins such as perforin and granzymes that initiate apoptosis in the target cells.

1.2. Macrophages

Macrophages are white blood cells (i.e. leukocytes) that play an important role in both the innate and adaptive immune systems. In the innate immune system, macrophages provide some of the first lines of defense by acting against foreign pathogens [44]. In the adaptive immune system, macrophages act as antigen presenting cells (APCs); presenting antigen via MHC I or II to activate T cells, bridging the innate and adaptive immune systems [44]. Macrophages respond to pathogen infection in several ways: by engulfing the pathogen via phagocytosis, by producing cytokines and other inflammatory (or anti-inflammatory) proteins, and by producing reactive nitrogen and oxygen species, among other responses [44–46]. Many of these responses are conserved between mammals and fish and have been demonstrated in many fish species [45,46].

In mammals, hematopoiesis, the process of blood cell formation, begins in the bone marrow, while in teleost fish, hematopoiesis begins in the anterior (or head kidney) [47]. A

heterogeneous population of adhered leukocytes, which include precursor cells, monocytes and macrophages, can be isolated from the head kidney, and are used as a monocyte/macrophage model in many fish immunology studies [48–53]. For example, in Atlantic cod, head kidney leukocytes (HKLs) were used to investigate the miRNA profile of macrophages following poly(I:C) stimulation at various time points (12, 24, 48 and 72 hours post stimulation), while HKLs from common carp (*Cyprinus carpio* L.) were used to examine M1/M2 polarization in macrophages (see section 1.2.2. for M1/M2 polarization) [48,51].

1.2.1. Macrophage differentiation

Hematopoiesis begins when a hematopoietic stem cell (HSC) differentiates into either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). The CLP will develop into a natural killer cell or a lymphocyte (B cell or T cell) and the CMP will develop into either a megakaryocyte erythroid progenitor (MEP) or a granulocyte macrophage progenitor (GMP). A MEP will give rise to either erythrocytes or platelets, while the GMP will give rise to either granulocytes or monocytes, of which the latter will differentiate into a macrophage [54,55]. This process is tightly regulated by growth factors, transcription factors and cytokines. In particular, monocyte-to-macrophage differentiation is regulated by transcription factors including PU.1, members of the CCAAT-enhancer-binding protein (CEBP) family and members of the interferon regulatory factor (IRF) family, and the growth factor colony stimulating factor 1 (CSF1) and its receptor (CSF1R)[56]. Through the use of antibodies for cell specific markers, this process has been very well characterized in mammals. For example, human monocytes express high levels of CD14 and low levels of CD16 ($CD14^{+}CD16^{-}$) while macrophages are $CD14^{-}CD16^{+}$. However, as few antibodies that recognize fish proteins are currently available, this area of research is limited in fish. Experiments in goldfish (*Carassius auratus* L.), zebrafish

(*Danio rerio*) and carp (*Cyprinus carpio*) have set the groundwork for studying monocyte-to-macrophage differentiation and through this work it was identified that several factors that regulate this process in mammals, also regulate this process in fish (reviewed in [46]). For instance, it is now well known that in both mammals and fish, the differentiation, proliferation and survival of monocytes and macrophages depends on CSF1 and CSF1R [45]. Similarly, IRF8 is also necessary for macrophage differentiation in both fish and mammals [57,58]. Work in goldfish kidney leukocytes defined 3 cell populations, progenitors, monocytes and macrophages, based on their different transcript expression [59]. Figure 1.1A depicts hematopoiesis and highlights some of the factors involved in macrophage differentiation. In addition to differentiation from monocytes, self-renewing macrophages that are seeded during embryonic hematopoiesis have been identified in mammals, however, this requires further investigation in fish [45]. While our knowledge of the factors involved in fish monocyte-to-macrophage differentiation is starting to expand, it remains to be described across all fish species.

1.2.2. M1 and M2 polarization

Macrophages demonstrate a large degree of plasticity and are divided into two subtypes depending on their activation and response: M1 (classically activated) and M2 (alternatively activated). M2 macrophages can be further subdivided into M2a, M2b and M2c [60]. M1 macrophages are considered pro-inflammatory. They are activated by bacterial products such as lipopolysaccharides (LPS) and pro-inflammatory cytokines including IFN-γ and TNF-α, and are characterized by increased production of pro-inflammatory cytokines (e.g. TNF-α, IL-1β and IL-12) and increased production of reactive nitrogen and oxygen intermediates [61]. In contrast, M2 macrophages are considered anti-inflammatory and immune suppressive. They are activated by

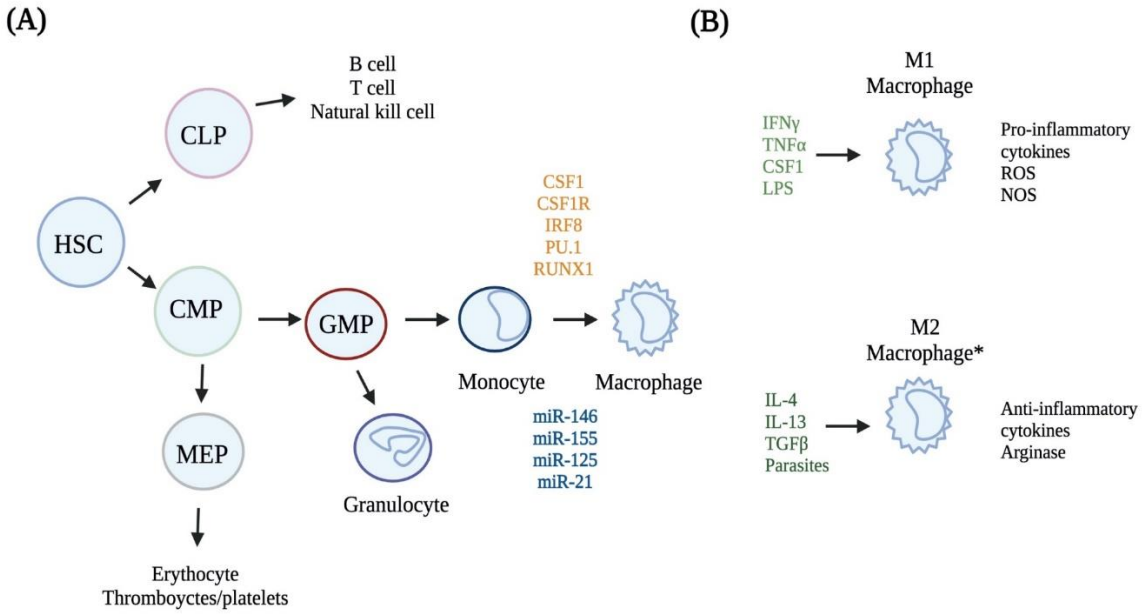

Figure 1.1. Macrophage differentiation. (A) Macrophage differentiation from a hematopoietic stem cell (HSC). Growth factors and transcription factors that are involved in macrophage differentiation are highlighted in orange, miRNAs involved are highlighted in blue. **(B)** M1 and M2 macrophages. Factors involved in the activation of a M1 or M2 macrophage are highlighted in green while the outcome is in black. * There are 3 subtypes of M2 macrophages (see page 7 for further explanation). CLP – common lymphoid progenitor; CMP – common myeloid progenitor; MEP – megakaryocyte; erythroid progenitor; GMP - granulocyte macrophage progenitor; miR - microRNA. Modified from [45,46,65]. Figure generated using BioRender.com.

IL-4, IL-13 or fungal infections (M2a), IL-1 and immune complexes (M2b) and IL-10, TGF-beta and glucocorticoids (M2c) and are characterized by the production of anti-inflammatory cytokines (such as IL-10), increased arginase activity and increased production of collagen and polyamines necessary for cell growth and healing [45,61–63]. M1 macrophages metabolize L-arginine to produce nitric oxide, while through increased arginase activity, M2 macrophages metabolize L-arginine to produce proline and polyamines for tissue healing and repair [64]. Figure 1.2B highlights some of the factors involved in M1 and M2 activation, as well as their outcome. The best characterized macrophage phenotype in fish is the M1 state, although characteristics of the M2 phenotype have also been defined [66]. Stimulation of goldfish and carp kidney cells with the M1 stimuli IFN-γ and TNF-α, induced the M1 phenotype, characterized by increased expression of TNF-α, IL-1β, IL-6 and IL-12 and increased expression of reactive nitrogen and oxygen intermediates [48,67,68]. IL-4-like and IL-13-like genes, associated with M2 macrophages, have been identified in several fish species (IL-4/13A, IL4-/13B) [63,67,69]. Addition of recombinant (r)-IL-4/13A and r-IL-4/13B to head kidney derived macrophages of several fish species stimulated the upregulation of the anti-inflammatory cytokines TGF-β and IL-10, decreased the expression of pro-inflammatory cytokines IFN-γ and IL-1β and increased arginase activity [45,48,67,68,70,71]. Furthermore, addition of cAMP, a M2 activator in mammals, to common carp head kidney leukocytes induced the upregulation of *arg2* gene expression and arginase activity [48,72]. Together, these studies suggest a conserved M1 and M2 phenotype in fish and mammals.

1.3. MicroRNA (miRNA)

MicroRNAs (miRNAs) are short (~22 nucleotide), non-coding RNAs that regulate gene expression post-transcriptionally [73–75]. miRNA biogenesis begins with transcription of the

primary-miRNA (pri-miRNA) which is cleaved by Drosha and its cofactor DiGeorge Syndrome Critical Region 8 (DGCR8) to form precursor-miRNA (pre-miRNA) (Figure 1.2). Pre-miRNA is then exported to the cytoplasm by Exportin5 and processed further by the RNase III endonuclease Dicer, which removes the terminal loop generating the mature miRNA duplex [74,75]. The miRNA duplex is subsequently loaded onto an Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC) [74,75]. The miRNA-RISC complex can then bind partially to its complementary sequence in the (usually) 3' untranslated region (UTR) of its target mRNA, resulting in the prevention of mRNA translation or inducing mRNA degradation. The name of the mature miRNA is determined by the directionality of the miRNA (i.e. the 5p strand originates from the 5' end of the pre-miRNA hairpin and the 3p strand originates from the 3' end) and miRNAs can be grouped into families that derive from a common ancestor, suggesting a common sequence or structure configuration [76]. miRNAs are involved in regulating many biological processes including cell development, growth, differentiation, apoptosis and immune function and response [77,78].

1.3.1. miRNAs and the immune response in teleost fish

In recent years, miRNAs have been characterized in a number of teleost species and have been reported to be involved in regulating cell development, growth, reproduction, and immune response, among other roles. For instance, miR-21 regulates the inflammatory response in miiuy croacker (Miichthys miiuy) and grass carp (Ctenopharyngodon idella) following Vibrio anguillarum and Aeromonas hydrophila infection, respectively, and miR-155 is associated with the immune response of several fish species following viral challenge [79–81]. High-throughput sequencing has been used to identify miRNAs that are differentially expressed (DE) in tissues following viral and bacterial challenge, with the idea that the responding miRNAs may regulate

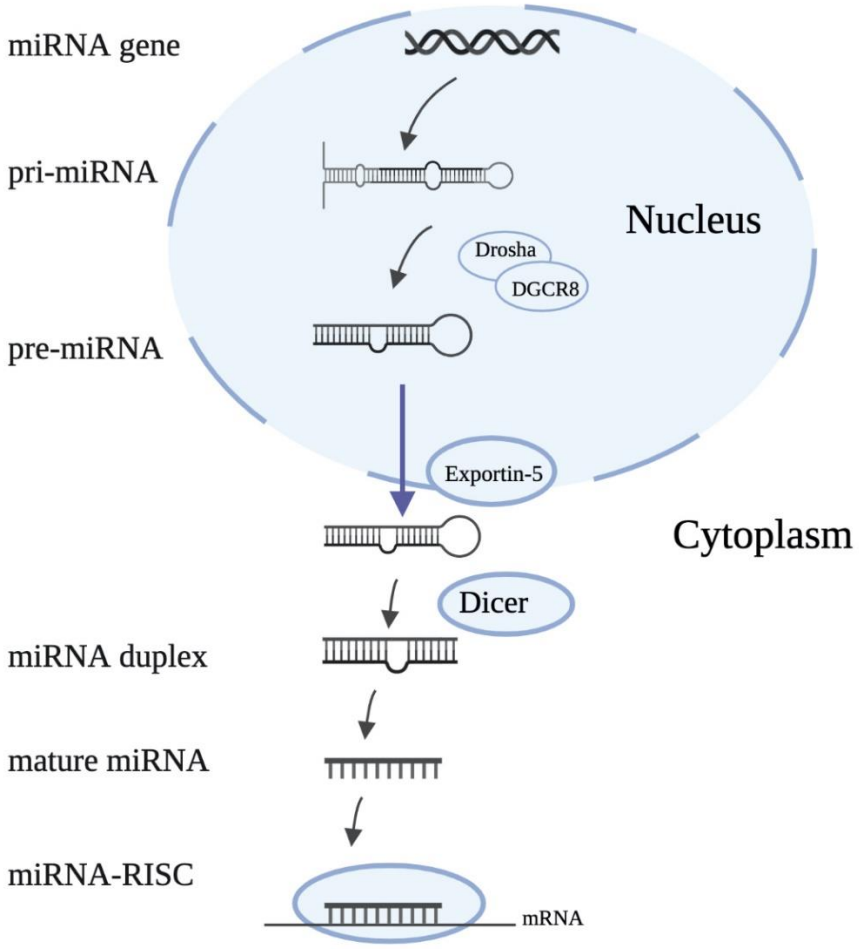

Figure 1.2. miRNA biogenesis. Modified from [73,75] and created with BioRender.com

immune response [14,15,51]. A review of the literature investigating miRNAs in response to viral and bacterial challenges in fish can be found in Andreassen et al. (2017) [79].

1.3.2. miRNAs in macrophage polarization, activation and differentiation

Through the use of microarrays and RT-qPCR, several studies have identified DE miRNAs in M1 and M2 macrophages. For example, Cobos Jimenez et al. [82] identified 303 DE miRNAs in M1 (induced by IFN-γ and TNF-α), M2a (induced by IL-4) and M2c (induced by IL-10) polarized human peripheral blood mononuclear cells (PBMCs). Zhang et al. [83] identified 109 DE miRNAs in M1 (induced by LPS and IFN-γ) and M2 (induced by IL-4) murine bone marrow-derived macrophages (BMDMs). Interestingly, there were some similarities and differences between the miRNA expression patterns between the two studies. For instance, while miR-221-5p was upregulated in M1 macrophages in both studies, it remained upregulated in human M2a macrophages but was downregulated in murine M2 macrophages. This may be due to species (human vs murine), cell type (PBMCs vs BMDMs) and/or M1 vs. M2 stimuli.

Functional (knockdown/overexpression) studies have further elucidated the roles of miRNAs in macrophage polarization and function. Through functional studies, it was demonstrated that some miRNAs (e.g. miR-155, miR-720, miR-125b, miR-127) regulate the M1 phenotype and promote the pro-inflammatory response, while other miRNAs (e.g.miR-146a, miR-125a, let-7c) regulate the M2 phenotype and the anti-inflammatory response [84]. For example, a large increase in miR-155 was observed in murine BMDMs when polarized to the M1 phenotype by LPS + IFN-γ, while polarization to the M2 phenotype by IL-4 had no effect on miR-155 expression [85]. Furthermore, miR-155 knock-out mice had reduced levels of pro-inflammatory genes (*Inos*, *Il-1b* and *Tnfa*) following M1 stimulation but had increased levels of the M2 marker *Arg1* following M2 stimulation [85]. Expression of miR-720 is downregulated in

primary human M2-polarized macrophages and overexpression of miR-720 in human THP-1 cells decreased the mRNA expression of M2 cytokines (*Il-10, Ccl17*) but had little effect on the production of M1 cytokines (*Tnfa, Il*-6) [86]. In addition, ectopic expression of GATA3, a potential target of miR-720 that promotes M2 polarization, resulted in the restoration of M2 macrophages in miR-720 overexpressed THP-1 cells [86]. miR-146a was the first miRNA associated with M2 macrophage polarization. Overexpression of miR-146a in the human THP-1 cell line resulted in decreased levels of M1 genes (*Il-6, Tnfa, Il-12*) and increased production of M2 markers (*Arg1, Ccl17, Ccl22*), while knockdown of miR-146a had the opposite effect [87]. Similarly, forced expression of let-7c in LPS-stimulated BMDMs reduced the expression of M1 markers (*Il-12, iNos*), while stimulating let-7c transfected BMDMs with the M2 stimuli IL-4 induced greater expression of the M2 markers *Arg1, FIZZ1 and YM-1* than in control transfected BMDMs [88].

In addition to regulating macrophage polarization, miRNAs have been implicated in hematopoiesis and macrophage differentiation. As previously stated, PU.1 is a main transcription factor involved in macrophage differentiation. PU.1 directly controls the expression of miR-342, miR-338, miR-146a and miR-155 [89]. Ectopic expression of miR-146a can direct the differentiation of murine hematopoietic stem cells (HSCs) into functional macrophages *in vivo* [89]. Furthermore, miR-155 upregulation is involved in the differentiation of RAW264.7 mouse monocytes to macrophages [90]. Finally, miR-15, miR-20a and miR-106a target RunX1, a transcription factor that controls expression of CSF1R, promoting the differentiation and maturation to the monocyte lineage [91]. While there is much known of miRNAs in mammalian macrophage biology, there is very little known of the miRNAs involved in fish macrophage differentiation, polarization and/or function.

1.3.3. miRNAs in teleost fish macrophages

Very few studies have examined the role of miRNAs in regulating fish macrophage activation and/or differentiation. DE miRNAs have been identified in Atlantic cod (*Gadus morhua*) head kidney macrophage-like cells following stimulation with the synthetic dsRNA poly(I:C) [51]. Infection of ayu (*Plecoglossus altivelis)* adhered head kidney cells with *V. anguillarum* increased the levels of miR-155 [92]. Overexpression of miR-155 in the *V. anguillarum*-infected macrophages enhanced the mRNA expression of pro-inflammatory cytokines (*il1b, tnfa*) and decreased the mRNA expression of anti-inflammatory cytokines (*il10, tgfb*) compared to the control group, while inhibition of miR155 had the opposite effect, suggesting that, similar to higher vertebrates, miR-155 may play a role in macrophage function and polarization in this fish species [92]. Infection of orange spotted grouper spleen cells with red spotted grouper nervous necrosis virus (RGNNV) increased the expression of miR-146a [93]. Overexpression of miR-146a facilitated the virus infection while knockdown of miR-146a decreased infection, suggesting that miR-146a plays a role in enabling viral infection [93]. A further understanding of the miRNA involved in macrophage function across all fish species, including the Atlantic salmon, will aid us in understanding the fish immune system.

1.4. Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer-enclosed particles that are derived from all cell types, including monocytes and macrophages, and have been isolated from diverse bodily fluids including urine, saliva, blood, breast milk and cerebrospinal fluid [94–96]. EVs participate in cell-to-cell communication via transfer of their cargo, which can include multiple miRNAs, mRNAs, DNAs and proteins, to recipient cells [94,95]. EVs can interact with their recipient cell by direct signalling through receptors on the recipient cells

surface or they are taken up by the recipient cells via membrane fusion, endocytosis or phagocytosis. Three categories of EVs have been described: exosomes, which are formed when multivesicular bodies fuse with the plasma membrane to release intraluminal vesicles; microvesicles, which are formed from direct budding of the plasma membrane; and apoptotic bodies, which are formed from the blebbing membrane of an apoptotic cell [94]. EVs share some common characteristics which enable their identification from cells and other vesicles and are commonly used as EV markers. These include membrane bound proteins such as heat shock proteins (HSP70, HSP90) and certain members of the tetraspanin superfamily of proteins (CD9, CD63, CD37, CD81, CD82) [97,98]. While EVs have been widely studied in mammals, there are few studies that examine EVs in teleost fish.

1.4.1. EVs and the immune response

EVs have been implicated in many physiological and pathological processes, including immune cell regulation and immune cell response [99,100]. Immune cells can release EVs to regulate the response of other cell types. For example, pathogen-challenged macrophages release EVs containing pathogen associated molecular patterns (PAMPs) that stimulate recipient cells to produce cytokines including TNFα, IL-10, IFNγ, and IL-1β [101–104]. Likewise, APCs, such as B cells, dendritic cells and macrophages, can stimulate T cell activation through release of EVs carrying membrane-bound MHC I and MHC II molecules and antigen [99,105,106]. EVs can also serve as biomarkers for health and disease; they are derived from cells under both normal and pathological conditions and therefore their molecular cargo is reflective of their cell of origin [107]. For instance, tumor cells have been shown release EVs containing tumor specific miRNAs [108].

1.4.2. EVs in teleost fish

While EVs have been widely studied in mammals, there are only a few studies that examine EVs in teleost fish. In rainbow trout, it was demonstrated that heat shock stimulated the release of HSP70 enriched exosomes *in vivo,* isolated from plasma, and *in vitro,* isolated from cultured hepatocytes [109]. Using Q-TOF mass spectrometry (MS), proteins including MHC IIB, HSP70 and HSP90 were identified in EVs derived from Atlantic salmon leukocytes that were stimulated with the PAMP analog cytosine–phosphate–guanosine (CpG) oligonucleotides, while proteins including granulin, MHC I, MHC II and proteasome subunits were identified in serum-derived EVs from Atlantic salmon infected with *Piscirickettsia salmonis* [110–112]. EVs contain a variety of miRNAs and the differential packaging of miRNAs in physiological states has been established in mammals [107,108]. Therefore, identifying signature miRNAs in fish EVs may be a relevant approach for evaluating fish health. For example, Atlantic cod reared in elevated water temperature had serum EVs with different protein and miRNA cargo than control Atlantic cod, suggesting that the packaged proteins or miRNAs may serve as a biomarker of environmental stress [113]. Additionally, signature miRNAs corresponding to sex differentiation were identified in serum EVs of Tongue sole (*Cynoglossus semilaevis)*, allowing early detection of sex differentiation, which may enhance the efficiency of reproduction and cultivation [114]. Therefore, studying the miRNA cargo of fish EVs is of sizable interest in understanding and identifying EV-based biomarkers in fish health and disease and characterizing immune-specific EVs is a key first step in determining immune-related EVs biomarkers.

1.5. Overall objectives of thesis

While our knowledge of fish macrophage biology is expanding, it remains to be fully characterized across all teleost species. The head kidney leukocyte (HKL) model is commonly

used in *in vitro* fish immune studies. However, HKLs change during the first 5 days of culture, and these changes have not been well characterized. Therefore, through transcriptomic profiling, the overall aim of this thesis was to better characterize Atlantic salmon HKLs during culture. My objectives were as follows:

1. Examine changes in morphology and function of Atlantic salmon adherent HKLs during culture duration.
2. Determine changes in the miRNA profile of Atlantic salmon adherent HKLs during culture.
3. Characterize EVs released from Atlantic salmon adherent HKLs during culture, including their size, concentration and miRNA profile.
4. Identify changes in the mRNA profile of Atlantic salmon adherent HKLs during culture and determine if any of the DE mRNAs are potential targets of the DE miRNAs.

1.6. Publications arising from this thesis

The results presented in this thesis have all been published as follows:

1. The research described in Chapter 2 has been published in *Frontiers in Immunology* as: Smith, N.C., Rise, M.L., Christian, S.L.C. A Comparison of the Innate and Adaptive Immune Systems in Cartilaginous Fish, Ray-Finned Fish, and Lobe-Finned Fish. *Front Immunol*. 2019, 10:2292.

Author Contributions: N.C.S. reviewed the literature, generated the figures, and wrote the manuscript. S.L.C. and M.L.R. revised and edited the manuscript. All authors contributed to the manuscript and approved the submitted version.

2. The research described in Chapter 3 has been published in the *International Journal of Molecular Science* as: Smith, N.C., Christian, S.L., Woldemariam, N.T., Clow, K.A., Rise, M.L., Andreassen, R., 2020. Characterization of miRNAs in Cultured Atlantic Salmon Head Kidney Monocyte-Like and Macrophage-Like Cells. *Int J Mol Sci.* 2020; 21(11):E3989.

Author Contributions: N.C.S, S.L.C, M.L.R. and R.A. conceptualized and designed the experiments. N.C.S. performed the HKL isolation, RNA isolation, RT-qPCR, morphology staining and analysis, and flow cytometry experiments. N.C.S and K.A.C. performed the Oroboros respirometer assay. N.T.W and R.A. performed the RNA-sequencing, analysis and *in silico* analysis. N.C.S. prepared the original draft of the manuscript. All authors contributed to the manuscript and approved the submitted version.

3. The research described in Chapter 4 has been published in *Frontiers in Immunology* as: Smith, N.C., Wajnber, G., Chacko, S., Woldemariam, N.T., Lacrois, J., Crapoulet, N., Ayre, D.C., Lewis, S.M., Rise, M.L., Andreassen, R., Christian, S.L. Characterization of miRNAs in extracellular vesicles released from Atlantic salmon monocyte-like and macrophage-like cells. *Front Immunol.* 2020; 11:2827

Author Contributions: N.C.S, D.C.A, S.L.C, M.L.R. conceptualized and designed the experiments. N.C.S performed HKL and EV isolation, RNA isolation, RT-qPCR, transmission

electron microscopy, Western blotting and nanotracking analysis. G.W., S.C., N.T.L., J.L., N.C., and S.M.L. performed the RNA-sequencing. R.A. and N.T.W., performed the RNA-sequencing analysis and *in silico* analysis. N.C.S. prepared the original draft of the manuscript. All authors contributed to the manuscript and approved the submitted version.

4. The research described in Chapter 5 has been published in *Frontiers in Immunology* as: Smith, N.C., Navaneethaiyer, U., Kumar, S., Woldemariam, N.T., Andreassen, R., Christian, S.L., Rise, M.L. Transcriptome profiling of Atlantic salmon adherent head kidney leukocytes reveals that macrophages are selectively enriched during culture. *Front Immunology.* 2021, 12:2916

Author contributions: N.C.S, S.L.C, M.L.R. conceptualized and designed the experiments. N.C.S. performed HKL isolation, RNA isolation, RT-qPCR. N.C.S and U.M. performed the microarray. N.C.S. performed the microarray analysis, GO-term enrichment and network analysis. S.K., N.T.W and R.A., performed the *in silico* analysis. N.C.S. prepared the original draft of the manuscript. All authors contributed to the manuscript and approved the submitted version.

1.7. References

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CHAPTER 2

A Comparison of the Innate and Adaptive Immune Systems in Cartilaginous Fish, Ray-Finned Fish, and Lobe-Finned Fish

2.1. Abstract

The immune system is composed of two subsystems – the innate immune system and the adaptive immune system. The innate immune system is the first to respond to pathogens and does not retain memory of previous responses. Innate immune responses are evolutionarily older than adaptive responses and elements of innate immunity can be found in all multicellular organisms. If a pathogen persists, the adaptive immune system will engage the pathogen with specificity and memory. Components of the adaptive system including immunoglobulins (Igs), T cell receptors (TCR) and major histocompatibility complex (MHC), are assumed to have arisen in the first jawed vertebrates – the Gnathostomata. This review will discuss and compare components of both the innate and adaptive immune systems in Gnathostomes, particularly in Chondrichthyes (cartilaginous fish) and in Osteichthyes (bony fish: the Actinopterygii (ray-finned fish) and the Sarcopterygii (lobe-finned fish)). While many elements of both the innate and adaptive immune systems are conserved within these species and with higher level vertebrates, some elements have marked differences. Components of the innate immune system covered here include physical barriers, such as the skin and gastrointestinal tract, cellular components, such as pattern recognition receptors and immune cells including macrophages and neutrophils, and humoral components, such as the complement system. Components of the adaptive system covered include the fundamental cells and molecules of adaptive immunity: B lymphocytes (B cells), T lymphocytes (T cells), Igs and MHC. Comparative studies in fish such

as those discussed here are essential for developing a comprehensive understanding of the evolution of the immune system.

2.2. Introduction

The vertebrate immune system is divided into 2 subsystems – the innate immune system and the adaptive immune system. The innate immune system is the first to respond to initial infection and disease and does not retain memory of previous responses. Components of the innate immune system include physical barriers such as the skin, cellular processes such as phagocytosis and humoral components such as soluble proteins [1]. If a pathogen persists, despite the innate immune defences, the adaptive immune system is recruited. The adaptive immune system is highly specific to a particular antigen and can provide long-lasting immunity [2]. While the innate immune system is assumed to have arisen >600 million years ago (MYA), specific components of the adaptive immune system, including immunoglobulins (Igs), T cell receptors (TCR), and major histocompatibility complex (MHC), are comparatively newer and are assumed to have arisen approximately 450 MYA in the first jawed vertebrates (i.e. Gnathostomata) [3–5]. In order to understand the evolution and functionality of the immune system in jawed vertebrates, a comparative analysis of the key branches of Gnathostomata (Chondrichthyes, Actinopterygii, and Sarcopterygii) is required.

2.2.1. Gnathostomata

Gnathostomes are subdivided into Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes). They diverged from a jawless common ancestor with the lineage leading to other bony vertebrates. While jawless fish have an adaptive immune system based on variable lymphocyte receptors (VLRs), B-like and T-like cells, Gnathostomes are the most distantly

related group to mammals that have an adaptive immune system based on Igs, TCR and MHC [3,6].

There are over 1000 species of cartilaginous fish, which are divided into two subclasses: Elasmobranchii (sharks, rays, skates and sawfish) and Holocephali (chimaeras) [7]. The Osteichthyes are a diverse group of fish that have skeletons composed of calcified bone rather than cartilage and consist of over 40,000 species of fish [8]. They are subdivided into two classes, the Actinopterygii (ray-finned fish) and the Sarcopterygii (lobe-finned fish) (Figure 2.1). The Actinopterygii have fins that are composed of webs of skin supported by bony spines, known as lepidotrichia. Ray-finned fish comprise 99% of the Osteichthyes, of which 96% are from the infraclass Teleostei [9,10]. Due to the large number of teleost species, as well their economic importance, there have been many genomic and functional immunological studies completed on teleost fish. The Sarcopterygii possess fleshy, lobed, paired fins, joined to the body by a single bone and are comprised of Actinistia (coelacanths) and Dipnoi (lungfish) [4]. The majority of immunological studies on the cartilaginous fish and lobe-finned fish are genomic analyses, with very few functional studies. However, due to their unique position in the evolution of adaptive immunity, more functional studies are now being applied to cartilaginous fish. While there are several reviews that examine the innate or adaptive immune systems of Chondrichthyes and Actinopterygii, and some studies on Sarcopterygii [3,11,12], a comprehensive comparison of both the innate and adaptive immune systems in all 3 classes of fish is lacking. Thus here, we will endeavor to provide a comprehensive comparison of the innate and adaptive immune systems in cartilaginous fish, lobe-finned fish (focusing on coelacanths and lungfish) and ray-finned fish, with a focus on Teleost fish.

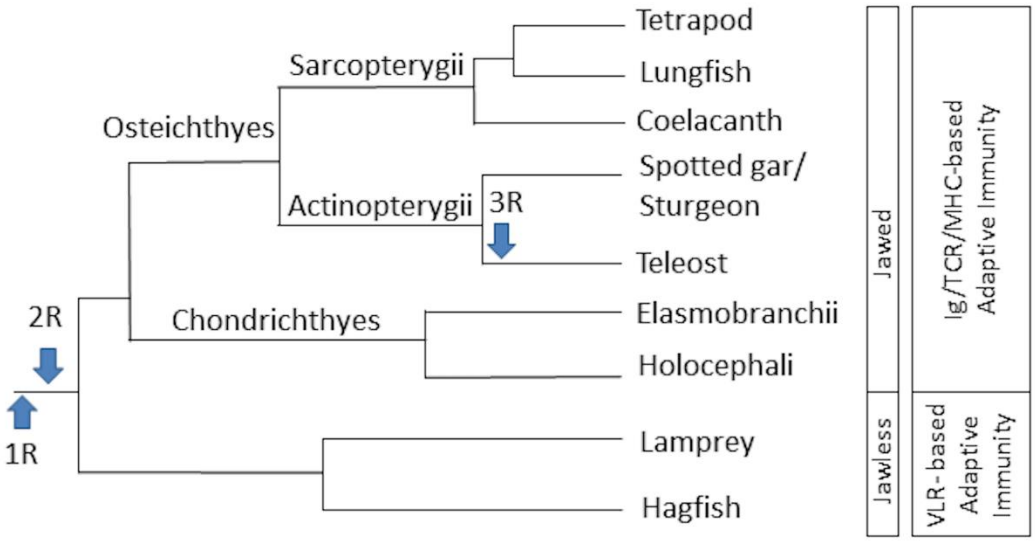

Figure 2.1. Schematic diagram of the evolution of jawed vertebrates and the immune system. Information sourced from multiple phylogenetic analyses [3,4,6,9,13,14]. R: genome duplication event.

2.3. The innate immune system

The innate immune system is the first to respond to an initial infection and/or disease. Elements of the innate immune response can be found in all multicellular organisms [12]. The innate immune system can be categorized into three defence mechanisms: (1) physical barriers, (2) cellular components, and (3) humoral responses [15]. As will be discussed, the functions of these defense mechanisms are highly conserved between fish and mammals.

2.3.1. Physical barriers

The first lines of defense in the fish innate immune system are physical barriers that prevent the entry of pathogens, which includes the skin (e.g. scales and mucus), gills, and epithelial layer of the gastrointestinal tract [15]. One of the first physical barriers encountered by a pathogen is the skin. Fish are constantly immersed in an aquatic environment and as a result are continuously exposed to potential pathogens or other harmful agents. Therefore, the skin is extremely important in early prevention of pathogen invasion. Teleost skin has been shown to contain skin-associated lymphoid tissue (SALT) that consists of multiple cell types including secretory cells (e.g. goblet cells), lymphocytes (B and T cells), granulocytes, macrophages and Langerhans-like cells [16,17]. In most teleost fish, the dermis layer of the skin consists of solid, bony scales known as leptoid scales. Interestingly, some teleost species, such as the catfish, have lost their scales during the course of evolution and instead some catfish species have regressed to having bony dermal plates covering their skin [18]. The skin of cartilaginous fish also contains many cell types, including melanocytes, lymphocytes, macrophages, and granular leucocytes [19]. The scales of cartilaginous fish are called placoid scales, also known as denticles [16]. The skin of lobe-finned fish contains keratinocytes, granulocytes and B cells [20]. Lobe-finned fish have cosmoid scales that includes a layer of dense, lamellar bone called isopedine. An equally

important function of the skin is the ability to secrete mucus, which acts as both a physical barrier, by trapping pathogens, and a chemical barrier [16]. Mucus from teleost fish contains a combination of lectins, lysozymes, complement proteins, and antimicrobial peptides (AMPs), all of which play a critical role in neutralizing pathogens [16,21]. While it is likely that skin mucus from cartilaginous fish and lobe-finned fish contains these compounds as well, it has not been as extensively explored as in teleost fish. However, a transcript for a lectin, pentraxin, was observed in the skin mucus of the common skate (*Raja kenojei*), while AMPs, including histones and S100 proteins, were found in the skin mucus of the African Lungfish (*Protopterus dolloi*) [20,22].

In addition to being involved in osmotic balance and gas exchange, the gills are also an important physical barrier, having both innate and adaptive immune components. The physical barrier of the gills consists of the gill epithelium, a glycocalyx layer, and a mucus layer. In teleost fish, the interbranchial septum is reduced and contains a single caudal opening of the operculum, rather than multiple openings while in cartilaginous fish, the gills are supported for almost their entire length by an interbranchial septum with multiple branchial slits or gill openings [23]. Immune cells, including macrophages, neutrophils and eosinophilic granulocytes have been observed in the gill associated lymphoid tissues (GIALT) of teleost fish [24] . Lymphocytes have been identified in the gills of several teleost species [25,26] and of the nurse shark (*Ginglymostoma cirratum*) [27]. For example, B cells and T cells have been identified in the gills of rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) while a specific B cell Ig transcript was observed in the gills of nurse shark (see adaptive immune section for a discussion on B cells, Ig, and T cells). Microbes present in the mucosal surface of the GIALT have been found to induce specific immunoglobulin producing B cells [28].

The gastrointestinal (GI) tract facilitates the absorption of nutrients, while preventing pathogen invasion through its epithelium. If a pathogen is ingested, it will encounter the gastrointestinal tract, which, like the skin and gills, contains both innate and adaptive immune cellular components. Gut associated lymphoid tissue (GALT) can be found in both bony and cartilaginous fish; however, unlike in mammals, it is not highly organized but is composed of a diffuse network of myeloid and lymphoid cells. The intestine of teleost fish, especially the posterior segment, contains both innate and adaptive immune cells including macrophages, mast/eosinophilic granule cells, dendritic cells, B cells and T cells [24,29]. Anal administration of *Vibrio anguillarum* to carp (*Cyprinus carpio*) and intraperitoneal injection of *V. anguillarum* to sea bass (*Dicentrarchus labrax*) resulted in the production of B-cells and Igs in the gut [30,31]. T cells have also been identified in the GALT of several teleost species [30,32,33]. In teleost fish, as in mammals, the gut microbiota plays a major role in the development and maturation of the GALT, which in turn mediates its immune response [34,35]. For example, resident microbiota stimulates intestinal epithelial cell proliferation in the developing zebrafish intestine, while absence of microbiota prevents differentiation of the GI tract [36,37]. Dietary administration of probiotics to the gilthead seabream (*Sparua aurata*) enhanced the intestinal microbiota and increased expression of various immune genes in the intestine including MHCII and TNF-α while administration of probiotics to the Nile Tilapia (*Oreochromis niloticus*) and rainbow trout promoted greater development of the intestine, as measured by villous height, and increased the population of intestinal granulocytes [38–40]. Lymphoid aggregates, as well as macrophages and granular cells, have been found in the spiral valve of various shark and ray species [41,42]. Lymphocytes and macrophages appear in the gut of the Dogfish shark at hatching and their numbers increase with age, as determined by histological analysis [43]. In

addition, cytoplasmic Ig has been identified in some intraepithelial lymphoid cells of the shark gut and two Igs (one of high molecular weight and one of low molecular weight) were observed in the intestinal mucosa of the skate (*Raja kenojei*), although the exact Igs are unknown without the development of antibodies specific to detect cartilaginous fish Igs/proteins [42,44]. Large accumulations of lymphoid cells have been found in the gut of the Australian lungfish (*Neoceratodus forsteri*); although the cellular and molecular composition of these lymphoid masses is currently unknown [45]. While there has been extensive research on the GALT of teleost fish, likely due to their economic importance, there are very few recent studies on the GALT of cartilaginous and lobe-finned fish and most are histological studies. It is unknown how the GALT in these species respond to infection and if it is in a similar manner as teleost fish and mammals. In addition, while the gut microbiome of some shark species has been identified [46], it is unknown how the microbiota effects the development of the GALT and its immune response in both cartilaginous fish and lobe-finned fish.

2.3.2. Cellular components

If a pathogen passes through the physical barriers, it will encounter the cellular and humoral aspects of the innate immune system. The cellular components of the fish innate immune system consist of many different cell types such as monocytes/macrophages, granulocytes such as mast/eosinophilic granule cells and neutrophils, dendritic cells, and natural killer cells. In bony fish, the primary sites for leukocyte production are the anterior (or head) kidney and thymus, while in cartilaginous fish, the primary sites include the epigonal organ, Leydig organ, thymus and spleen (Figure 2.2) [47–50]. Recently, a new organ, the salmonid bursa, a thick lymphoepithelial formation that is dominated by CD8+ T cells, but also includes some CD4+ T cells, was discovered in the anal region of Atlantic salmon [51]. Analysis of

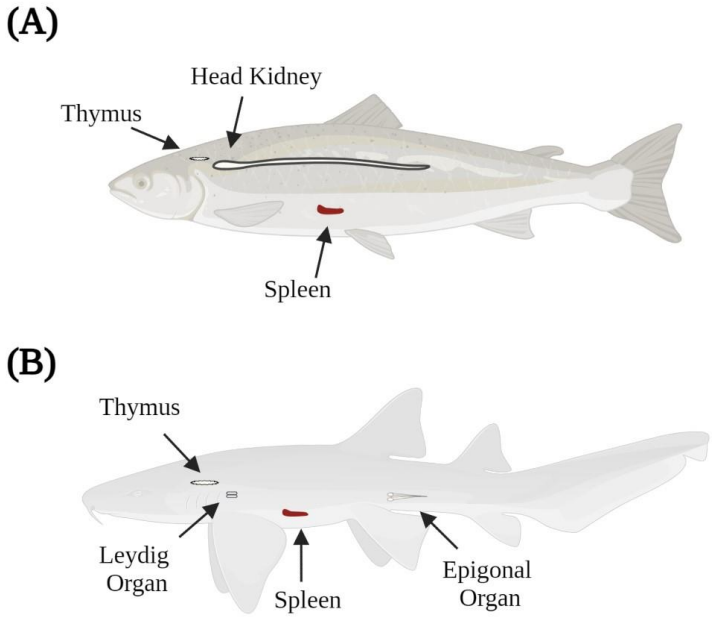

Figure 2.2. Site of leukocyte production in (A) ray-finned fish and (B) cartilaginous fish.

The site of leukocyte production has not yet been studied in lobe-finned fish. Modified from [48–50]. Image created using BioRender.com.

possible sites of leukocyte production (such as the kidney and/or gonads) have yet to be studied in lobe-finned fish [52]. Knowing the site of hematopoiesis in lobe-finned fish would allow for isolation of these cells and experiments that would lead to a better understanding of immune cells in these species.

When an innate immune cell encounters a pathogen, it will recognize a pathogen-associated molecular pattern (PAMP) found on the pathogen. Once recognized, the innate immune cell will become activated and can participate in several responses depending on their cell subtype including, but not limited to, phagocytosis and subsequent destruction of the pathogen, production of various cytokines and activation of the adaptive immune system via antigen presentation along with cytokine stimulation.

2.3.2.1. Monocytes/macrophages and neutrophils

Monocytes/macrophages and neutrophils are the first to arrive and respond to initial infection. Macrophages are derived from hematopoietic progenitors which differentiate via circulating monocytes or via tissue resident macrophages. Differentiation of vertebrate macrophages is controlled by engagement of the colony-stimulating factor 1 receptor (CSF1R) [53]. CSF1R has been characterized in several teleost species, and has been identified in the elephant shark (*Callorhinchus milii*) genome [54–57]. Macrophages play a role in both the innate and adaptive immune systems and are key players during inflammation and pathogen infection, as well as in tissue homeostasis. In the innate immune system, macrophages of several teleost fish species have been demonstrated to destroy pathogens through phagocytosis, the production of reactive oxygen species (ROS) and nitric oxide (NO), and the release of several inflammatory cytokines and chemokines, similar to mammalian macrophages (reviewed in [58–60]). In the

adaptive immune system, macrophages are one type of professional antigen presenting cell (pAPC) that can present phagocytosed materials to the T lymphocytes of the adaptive immune system through a process termed antigen presentation. Macrophages in cartilaginous fish have not been studied as in depth as in teleost fish, however, it is known that nurse shark macrophages exhibit spontaneous cytotoxicity [61]. Lungfish macrophages are described to have typical vertebrate macrophage morphology [62,63]. Very few functional studies have been completed in lungfish, however, one study found that injection of lipopolysaccharide did not change the number of macrophages in the coelomic cavity, as was expected [62]. Similar to mammals, functionally distinct subpopulations of macrophages exist in bony fish. M1 (classically activated macrophages) are characterized by production of pro-inflammatory cytokines such as TNFα and IL-1β and production of ROS and NO, whereas M2 (alternatively activated macrophages) are linked to immunosuppression, wound repair and increased levels of arginase and anti-inflammatory cytokines such as interleukin (IL)-10 [59,60,64]. The best characterized macrophage phenotype in teleost fish is comparable to M1 macrophages where they can destroy pathogens via acidification, nutrient restriction, production of reactive intermediates and various cytokines and chemokines [58–60]. Macrophages, as well as virtually all immune cells, are able to communicate with each other via cell-derived extracellular vesicles (EVs) which contain and deliver messenger RNA (mRNAs), microRNA (miRNAs) and proteins [65,66]. While in recent years, EVs have been extensively studied in mammals, very few studies exist in fish. In one fish study, Atlantic salmon (*Salmo salar*) head kidney leukocytes were stimulated with CpG oligonucleotides which caused the release of EVs that contained mRNA and miRNA, as well as a protein composition similar to mammals including MHC I and MHC II molecules [67]. The secretion of EVs was not induced by CpG in a splenocyte culture (containing mostly B cells)

suggesting that the EVs were likely produced by macrophages or dendritic cells in the head kidney leukocyte culture [67]. The existence of M1 and M2 cell populations, as well as EVs, have yet to be examined in cartilaginous and lobe-finned fish.

The most abundant granulocytes in bony fish are neutrophils, and like macrophages, neutrophils are critical to the innate defense against pathogens [68]. Neutrophils exhibit potent antimicrobial responses through various intracellular and extracellular mechanisms including the release of granules containing cytotoxic and antimicrobial enzymes, the release of neutrophil extracellular traps (NETs), phagocytosis and the production of ROS and NO (reviewed in [60,68]. Some bony fish granulocytes have a similar appearance to that of mammalian cells (neutrophils) or avian cells (heterophils). Fish granulocytes exhibit a wide variation in morphology, numbers and types of cells between species causing much confusion regarding their nomenclature. For example, a study by Tavares-Dias et al. [69] identified only one type of neutrophil in channel catfish, while a study by Cannon et al. [70] reported heterophils instead of neutrophils. Granulocytes in cartilaginous fish are classified in three types based on size, shape and staining properties. G1 granulocytes, referred to as heterophils or fine eosinophilic granulocytes, are often the most common granulocyte in cartilaginous fish. Their numbers can range from 20-50% of the total leukocytes in the blood, depending on species. G2 granulocytes resemble mammalian neutrophils, while G3 are referred to as coarse eosinophilic granulocytes [71,72]. G3 is more commonly seen in cartilaginous fish, compared to bony fish. Not all species of cartilaginous fish exhibit all three types of granulocytes; for example, only G1 and G3 granulocytes have been found in Thornback rays (*Raya clavate*) and small eyed rays (*Raja microcellata*) [72]. In the African lungfish (*Protopterus dolloi*), two types of granulocytes were identified in the South American lungfish (*Lepidosiren paradoxa*), three granulocyte types were

identified based on Giemsa-staining and granule size (eosinophilic I, eosinophilic II and basophilic type) [73] and in the Australian lungfish (*Neoceratodus forsteri*) four types of granulocytes have been described (basophil, neutrophils, large eosinophils and small eosinophils) [74].

2.3.2.2. Recognition of non-self

Initiation of the innate immune response begins when germline-encoded intracellular or extracellular pattern recognition receptors (PRRs) of an immune cell bind to a PAMP found on a pathogen, such as bacteria-derived lipopolysaccharides (LPS), viral RNA, bacterial DNA, or a danger-associated molecular pattern (DAMP) found on proteins or other biomolecules that are released from stressed cells or injured cells. All PRRs have a domain for recognizing the PAMP that is coupled to a domain that interacts with downstream signalling molecules [75]. In mammals, PRRs can be classified into at least five major groups: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), C-type lectins (CLRs), the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs) and absent in melanoma (AIM)-like receptors [76]. Many homologues of mammalian PRRs have been identified in fish.

TLRs were the first PRRs to be discovered in fish and therefore have been the most extensively studied. To date there have been thirteen TLRs identified in mammals, whereas over twenty have been identified in different fish species [76–79]. A comparison of the TLRs found in mammals, cartilaginous fish, ray-finned fish and lobe-finned fish, as well as their ligands (in mammals and when known in bony fish) can be found in Table 1. Some mammalian orthologues of TLRs have not been identified in fish, whereas some TLRs, including soluble TLR5 (sTLR5),

TLR13, TLR14, and TLR18-28 are “fish-specific” [80]. For example, a sTLR5 has been identified in bony fish, including rainbow trout, and Atlantic salmon, whereas no no sTLR5 has been found in mammalian genomes [81–83]. Interestingly, TLR5, as well as TLR1, TLR2 and TLR6, are missing from the Atlantic cod (*Gadus morhua*) genome [84,85]. Some bony fish, including the zebrafish (*Danio rerio*), the Dabry’s sturgeon (*Acipenser dabryanus*) and the yellow catfish (*Pelteobagrus hydrophila*), possess TLR4-like genes, while TLR4 is absent in other bony fish species, as well as absent in coelacanths and cartilaginous fish [59,86,87]. TLR4 in fish, however, does not possess the ability to recognize LPS as it does in mammals [59]. TLR27 was first identified and thought to only be found in the coelacanth genome but has since been identified in the spotted gar (*Lepisosteus oculatus*) and elephant shark [88,89]. TLR2, TLR3, TLR6, and TLR9 have been identified in the gray bamboo shark (*Chiloscyllium griseum*) genome whereas no TLR6 or TLR10 homolog has been identified in teleost fish. In addition, a novel TLR with sequence similarity to TLR4 and TLR13 in mammals, and TLR21 in teleost fish, has been identified in the whale shark (*Rhincodon typus*) [90,91].

Due to genome duplication events, several paralogues of various TLRs exist in fish. Two rounds of genome duplication (1R and 2R) are thought to have occurred early in vertebrate evolution, one before and one after the divergence of the lamprey (jawless fish) lineage, before the Cyclostome/Gnathostome divergence, approximately 500-800 MYA (Figure 2.1) [13]. Evidence, such as an increase in the number of Hox gene clusters, indicates that an additional genome duplication event (3R) occurred early in the teleost lineage, after it split from the lobe-finned lineage 325-350 MYA, while an additional round of genome duplication (4R) occurred in salmonids, thus leading to several paralogues of genes, including TLRs [92,93]. Paralogous TLR4 and TLR8 genes have been identified in zebrafish (*Danio rerio*) [94,95], TLR8 in rainbow

trout [79] and TLR3 and TLR7 in common carp (*Cyprinus carpio*) [96], while multiple copies of TLR7, TLR8, TLR9, TLR22 and TLR25 have been identified in the Atlantic cod [84]. The high number and large diversity of fish TLRs is likely derived from their distinct and diverse evolutionary history and environments that they occupy (reviewed in [80]).

In addition to TLRs, differences in several other PRRs between ray-finned, lobe-finned and cartilaginous fish have been noted. While AIM has not been identified in teleost or cartilaginous fish, two HIN200 domains, a PAMP-recognizing protein domain characteristic of AIM in mammals, were discovered in the coelacanth genome [88,97]. A group of unique NLRs possessing a C-terminal B30.2 domain has been identified in teleost fish, but is missing from the coelacanth genome [88]. Additionally, novel immune-type receptors (NITRs) which have been studied extensively in ray-finned fishes are missing from the coelacanth genome [88]. While all three RIG-I-like receptors have been characterized in teleost fish, only RIG-I and MDA5 have been identified in the elephant shark and coelacanth genomes [57,88]. However, as more high quality, well-assembled, and annotated genomes become available for additional cartilaginous and lobe-finned fish, additional NITRs may be identified. These differences indicate that not only is pathogen recognition quite diverse in fish, it can also be lineage-specific.

2.3.2.3. Phagocytosis

Phagocytosis is one of the most ancient and universal tools of defense against foreign material. This mechanism of defense is observed even in unicellular eukaryotes, predating complex multicellular life [60,98–101]. Binding of a pathogen to a PRR triggers phagocytosis in cells termed phagocytes. These include macrophages, monocytes, neutrophils and dendritic cells and are found in both bony and cartilaginous fish [60,98–101]. Recently, the existence of B cells

Table 2.1. TLRs present in mammals, ray-finned fish, lobe-finned fish, and cartilaginous fish.

TLR	Ligand	Cartilaginous fish	Ray-finned fish	Lobe-finned fish	Mammals
TLR1[57,78,80-82]	Lipopeptide/Peptidoglycan (m)	-	+	+	+
TLR2[57,78,80,81,83,84]	Lipopeptide/Peptidoglycan	+	+	+	+
TLR3[57,82,83,85-87]	dsRNA	+	+	+	+
TLR4[78,82,84,88]	LPS (m)	-	+*	-	+
sTLR5[60,78,82,85]	Flagellin	-	+	+	-
mTLR5[78,81]	Flagellin	-	+	+	+
TLR6[80-82,86]	dsRNA	+	-	-	+
TLR7[57,78,80,82,86,87,89]	ssRNA/dsRNA	+	+	+	+
TLR8[62,78,82-84,86]	ssRNA/dsRNA	+	+	-	+
TLR9[62,78,80-82,86]	CpG, IFN-γ	+	+	+	+
TLR10[81]	ND	-	-	-	+
TLR11[82]	Profilin (m)	-	-	-	+
TLR12[76]	Profilin (m)	-	-	-	+
TLR13[78,80,81,83]	Bacterial RNA	+	+	+	+
TLR14[78,80-82]	ND	-	+	+	-
TLR18[78,80,81]	ND	-	+	-	-
TLR19[78,80,82]	dsRNA	-	+	-	-
TLR20[78,82]	ND	-	+	-	-
TLR21[78,81-83]	CpG DNA	+	+	+	-
TLR22[78,80-82,90,91]	dsRNA/Bacterial PAMPs	-	+	+	-
TLR23[78,82]	ND	-	+	-	-
TLR24[78]	ND	-	-	-	-
TLR25[78,80,90]	ND	-	+	-	-
TLR26[78]	ND	-	+	-	-
TLR27[80,81,92]	LPS/poly (I:C)	+	+	+	-
TLR28[80]	LPS/poly (I:C)	-	+	-	-

(m) represents ligand known in mammals but not fish; (+) represents identified; (-) represents not identified; ND represents not determined; * only found in zebrafish (*Danio rerio*) and Chinese rare minnow (*Gobiocypris rarus*). It is important to note that ligands may be fish species specific.

with phagocytic ability was discovered in various teleost fish species including rainbow trout, Atlantic salmon, and Atlantic cod [102,103]. It is unknown if cartilaginous fish and lobe-finned fish have phagocytic B cells. After engulfment, the phagosome, containing the pathogen, binds to a lysosome, forming a phagolysosome, where the pathogen is killed by various means including the production of ROS and NO [60]. Studies in shark, skate, lungfish and teleost fish have demonstrated both ROS and NO production in various leukocytes [68,104].

2.3.3. Humoral responses

Humoral responses are mediated by macromolecules produced by cells and released into the extracellular fluids following infection by a pathogen. Some of the most studied humoral components in fish include the complement system, lysozyme, antimicrobial peptides, and acute phase proteins. These components have many different functions including the promotion of inflammation and phagocytosis and direct bactericidal effects.

2.3.3.1. Complement system

The complement system is a cascade of serum proteins that act cooperatively to mediate defense mechanisms including the elimination of pathogens through opsonisation and phagocytosis and the promotion of the inflammatory response. The mammalian complement system is composed of approximately 30 proteins that make up three activation pathways: the classical pathway, activated by antibody-antigen complexes and thus a bridge between innate and adaptive immunity; the alternative pathway, which is independent of antibodies and activated directly by pathogens; and the lectin pathway which is activated by the binding of the mannose-binding lectin (MBL), or ficolin, to mannose (or other sugar) residues present on the pathogen surface [105]. Figure 2.3 illustrates these three pathways, along with some of the associated proteins. Ultimately, these pathways induce activation of the C3 convertase, which

cleaves inactive C3 into C3a, an anaphylatoxin that acts as a chemotactic factor and aids in inflammation, and C3b, which acts as an opsonin, as well as an activator of downstream complement proteins leading to the formation of the membrane attack complex [106,107].

Most of the mammalian complement components have homologues in various teleost species, including rainbow trout [108], zebrafish [113] and channel catfish [114], among many others, and their functions have been well characterized (reviewed in [60,115]). Similarly, components of all three pathways have been characterized in several cartilaginous fish species, where they have been found to have haemolytic properties [116–119]. Furthermore, genes encoding complement components have been identified in lungfish [120,121] and in the coelacanth genome [88]. These studies in different fish classes/subclasses suggest that some components of the complement system are evolutionarily conserved and similar to that of higher vertebrates.

However, not all fish species contain all three pathways. MBL and ficolin genes have not been identified in any cartilaginous fish studied to date, while MASP2 transcripts are lacking in the elephant shark, little skate (*Leucoraja erinacea*) and catshark (*Scyliorhinus canicular*) [57,109,112]. In addition, the hammerhead shark contains a MASP2 transcript that contains no serine protease domain, which is necessary to initiate the lectin pathway. This data suggests that the lectin pathway may not be present in cartilaginous fish [109].

Furthermore, some fish species contain multiple forms of various complement factors. Multiple C3 forms have been identified in teleost fish and cartilaginous fish. For example, rainbow trout have three C3 forms, common carp have eight, and gilthead seabream (*Sparus aurata*) have five [110,111,122], with each form demonstrating different binding efficiencies and functions. Two C3 variants have been described in the nurse shark and the small-spotted catshark,

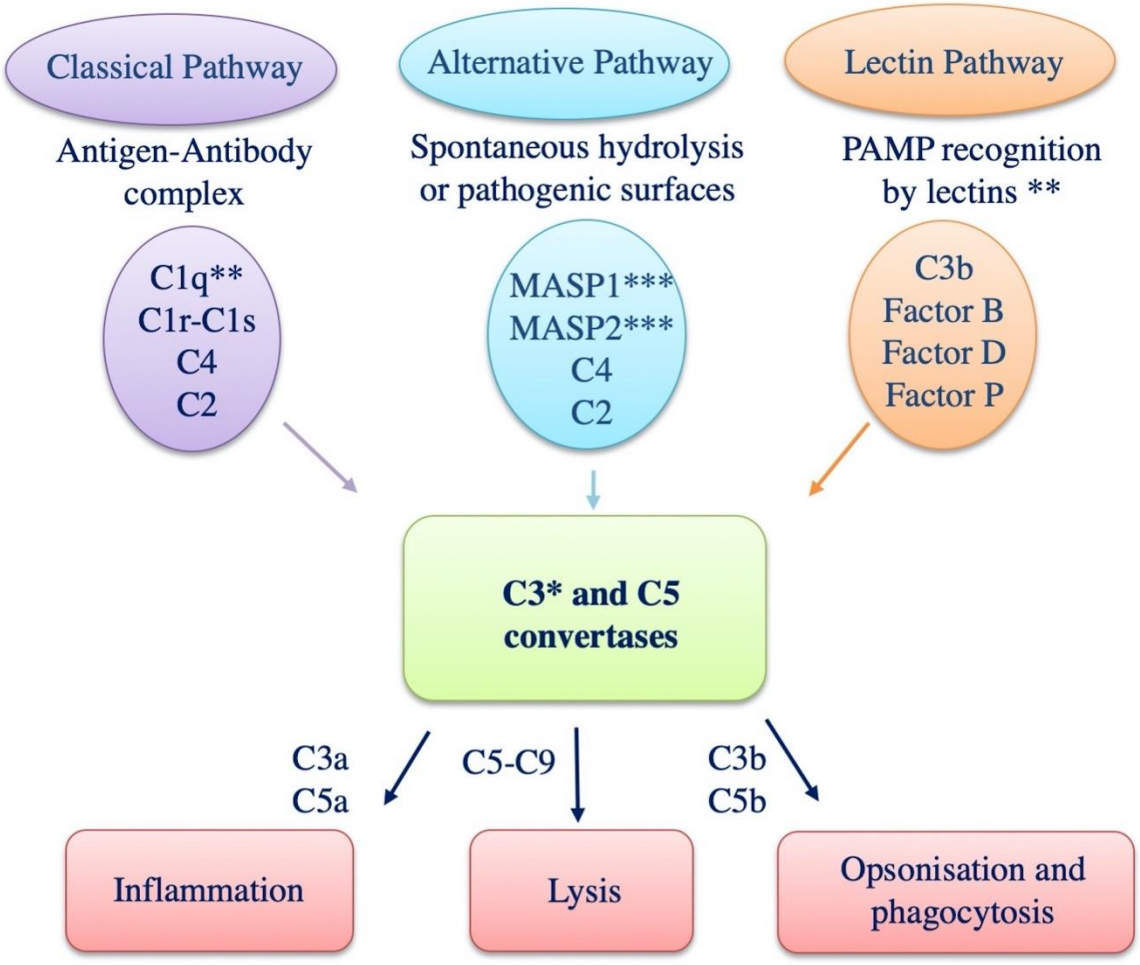

Figure 2.3. The three complement pathways with associated proteins. *Multiple C3 and/or C4 isoforms in some teleost and cartilaginous fish species. **Absences of mannose-binding lectin (MBL), ficolin, C1qA and C1qC from genome of any cartilaginous fish studied to date ***MASP2 transcript with no serine protease domain in hammerhead shark genome; MASP2 missing from elephant shark, little skate and catshark genome [105-112].

while two C4 gene haven been identified in the elephant shark and hammerhead shark [57,109,112]. This structural and functional diversity suggests that these fish may have an increased capacity to recognize and destroy a broader range of pathogens compared to those with fewer forms, although this remains to be demonstrated.

2.3.3.2. Lysozyme

Lysozyme is a lytic enzyme that acts on the peptidoglycan layer of bacterial cell walls by hydrolysing 1-4 β-linked glycoside bonds resulting in lysis of the bacterium. It is also involved in other defenses such as opsonisation and phagocytosis and activation of the complement system [123–125]. Two types of lysozyme have been described in vertebrates: (chicken) c-type and (goose) g-type.

Lysozyme is one of the most studied innate immune components in fish. C-type and g-type lysozymes have been reported in several teleost species where they are found in neutrophils, monocytes and to a lesser extent in macrophages of several tissues (e.g. liver, kidney, spleen, gills) and in mucus [123,126,127]. Recombinant (r-) c-type and g-type lysozymes have been found to have high bacteriolytic activity against a variety of pathogens of teleost fish such as *Vibrio anguillarum*, *Aeromonas hydrophila* and *Micrococcus lysodeikticu*s [128,129]. A sequence homology search of the Atlantic cod genome revealed an absence of c-type lysozyme genes; however, four g-type lysozyme genes were identified in several different tissues [130]. Intraperitoneal injection of *Francisella noatunensis*, an intracellular bacterium that commonly infects cod, stimulated the expression of two of the g-type lysozyme genes in the head kidney [130] . The presence of multiple g-type lysozymes may compensate for the lack of c-type lysozymes in the Atlantic cod [130]. The presence of lysozyme in the lymphomyeloid tissues of several cartilaginous fish was first discovered in 1979 [131]. A recent genomic investigation by

Venkatesh et al. failed to identify g-type lysozyme in the elephant shark genome, however c-type lysozyme was identified [57]. This c-type lysozyme was characterized in the nurse shark and found to hydrolyze the cell wall of *M. lysodeikticus* and inhibit the growth of Gram-positive bacteria, suggesting a similar function for lysozyme as in teleost fish and higher vertebrates [132]. In addition, two g-type lysozyme genes were discovered in the coelacanth genome, although no functional studies on lysozymes have been completed in coelacanth or lungfish to date [133]. Collectively, these studies suggest that the function of lysozyme is similar in both bony and cartilaginous fish.

2.3.3.3. Antimicrobial peptides (AMPs)

AMPs, also known as host defence peptides are oligopeptides with a varying number of amino acids that generally positively charged and play a major role in the innate immune system. AMPs protect against a variety of pathogens via disruptive or pore-forming actions against bacterial membranes. Over 90 fish AMPs have been identified and are characterized as β-defensins, cathelicidins, hepcidins, histone-derived peptides and fish-specific piscidins. Several of these AMPs have been cloned and subsequent functional studies have demonstrated antiviral and antibacterial activities against a variety of pathogens, demonstrating that AMPs from teleost fish exhibit many if not all of the characteristics of other vertebrate AMPs [134–137]. For example, β-defensin has been characterized in gilthead seabream, where it demonstrated antimicrobial activity against *V. anguillarum*, while in Nile tilapia (*Oreochromis niloticus*) β-defensin has shown an inhibitory effect on the growth of *Escherichia coli* DH5α and *Streptococcus agalactiae* [138]. Two cathelicidin genes have been identified in rainbow trout where it displayed activities against bacteria including *V. anguillarum* and *P. damselae* [139] while in Atlantic salmon, cathelicidin has demonstrated microbicidal properties against *V.*

anguillarum [140]. Unlike the comprehensive studies conducted on AMPs in teleost fish, research into shark and lobe-finned fish AMPs has not been as extensive. Two AMPs have been isolated from the dogfish shark (*Squalus acanthias*), transferrin [141] and squalamine [142], which were found to have potent bactericidal activity against both Gram-negative and Gram-positive bacteria. In addition, the AMP Kenojeninin I, has been isolated from the skin of fermented skate (*Raja kenojei*) and was found to have inhibitory effects on *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* [143]. A recent study by Heimroth et al. [20] identified an increase in proteins with known antimicrobial function including histones and S100 proteins in skin mucus of the lungfish *Protopterus dollo* during terrestrialization.

2.3.3.4. Acute phase proteins

In both fish and mammals, tissue injury, infection and inflammation, induces immune cells, such as macrophages, to secrete various cytokines into the bloodstream, which stimulate hepatocytes to produce and release acute phase proteins (APPs) [144,145]. APPs are classified based on the extent to which their concentrations change (minor, intermediate or major) and the direction of change (positive or negative). They are involved in a variety of defense activities and include coagulation factors, such as fibrinogen and prothrombin, transport proteins such as ferritin, complement components, C-reactive protein (CRP) and serum amyloid proteins (SAP) (reviewed in [146]). APPs are well conserved in arthropods, fish, amphibians, and mammals [147]. CRP and SAP are considered major APPs (e.g. their concentrations may increase up to 1000-fold) and are the most extensively studied APPs in fish. They are members of the pentraxin family of APPs, are present in the body fluids of vertebrates and invertebrates, and are commonly associated with the acute phase response of inflammation [146]. In addition to

inflammation, CRP and SAP have been shown to activate the complement pathways and play a role in the clearance of apoptotic cells [146,148].

Both CRP and SAP have been identified in several teleost species [149–151] where their levels in the serum have been shown to increase in response to various inflammation-inducing stimuli [152–155]. For example, CRP and SAP expression in Atlantic salmon head kidney leukocytes is upregulated in response to r-IL-Iβ and r-IFNγ, two cytokines that stimulate acute phase protein production in mammals, suggesting that the acute phase response is evolutionarily conserved [154]. Both CRP and SAP have also been identified in several different cartilaginous fish [156–158]. CRP and SAP isolated from the serum of iridescent shark (*Pangasianodon hypophthalmus*) was found to agglutinate *Edwardsiella ictaluri* and *A. hydrophila* [159]. Moreover, increased levels of CRP were found in the serum of sharks inhabiting a highly industrialized harbour estuary where exposure to polycyclic aromatic hydrocarbons (PAHs) and other contaminates was likely to lead to an inflammatory response [157]. As well, transcriptome analysis of the Indonesian coelacanth, *Latimeria menadoensis,* genome identified SAP encoding transcripts [160], however, to our knowledge, no other studies examining CRP or SAP in coelacanths or lungfish have been reported.

2.4. The adaptive immune system

If a pathogen persists, despite the innate immune defenses, the adaptive immune system will be activated. While jawless fish have an adaptive immune system based on VLRs, B-like and T-like cells, specific components of the adaptive immune system, including immunoglobulins (also known as antibodies (Ab)), T cell receptors (TCR) and major histocompatibility complex (MHC), are believed to have arisen in the first jawed vertebrates [3].

Like the innate immune system, the adaptive immune system includes both humoral and cellular components. B cells are key elements of the humoral adaptive immune response. The main role of B cells is to produce high affinity Ig against foreign antigen, and to act as a pAPC to present processed antigen to activate T cells. Abs occur in two forms: a soluble form that is secreted from the cell and a membrane-bound form that, in combination with the signalling molecules Igα/Igβ, forms the B cell receptor (BCR). T cells are key elements of cellular adaptive immunity. The T cell receptor (TCR) is always membrane bound and once stimulated via interaction with antigen presented by the pAPC, in the presence of co-stimulation, the T cell can be activated to function as a helper (CD4+) T cell, a regulatory (CD4+) T cell or a cytotoxic (CD8+) T cell.

Antigen-specificity of B cells and T cells is determined by their BCR or TCR, respectively, which are formed from somatic recombination of variable (V), diversity (D) and joining (J) gene segments (Figure 2.4A), produced by the DNA-recombination ability of the RAG 1 and 2 enzymes and TdT [161,162]. RAG 1/2 and TdT enzymes, as well as the gene segments V, D and J are present in all classes of jawed vertebrates (reviewed in [163,164]). This results in a highly diverse repertoire of BCRs and TCRs able to recognize innumerable different specific antigens and is unique to the adaptive immune system. Due to the random nature of the VDJ recombination, some BCRs and TCRs produced may recognize self-antigens as foreign. Therefore, developing B and T cells will also undergo negative and positive selection to ensure only cells that recognize foreign antigen survive. Negative selection occurs when a B cell

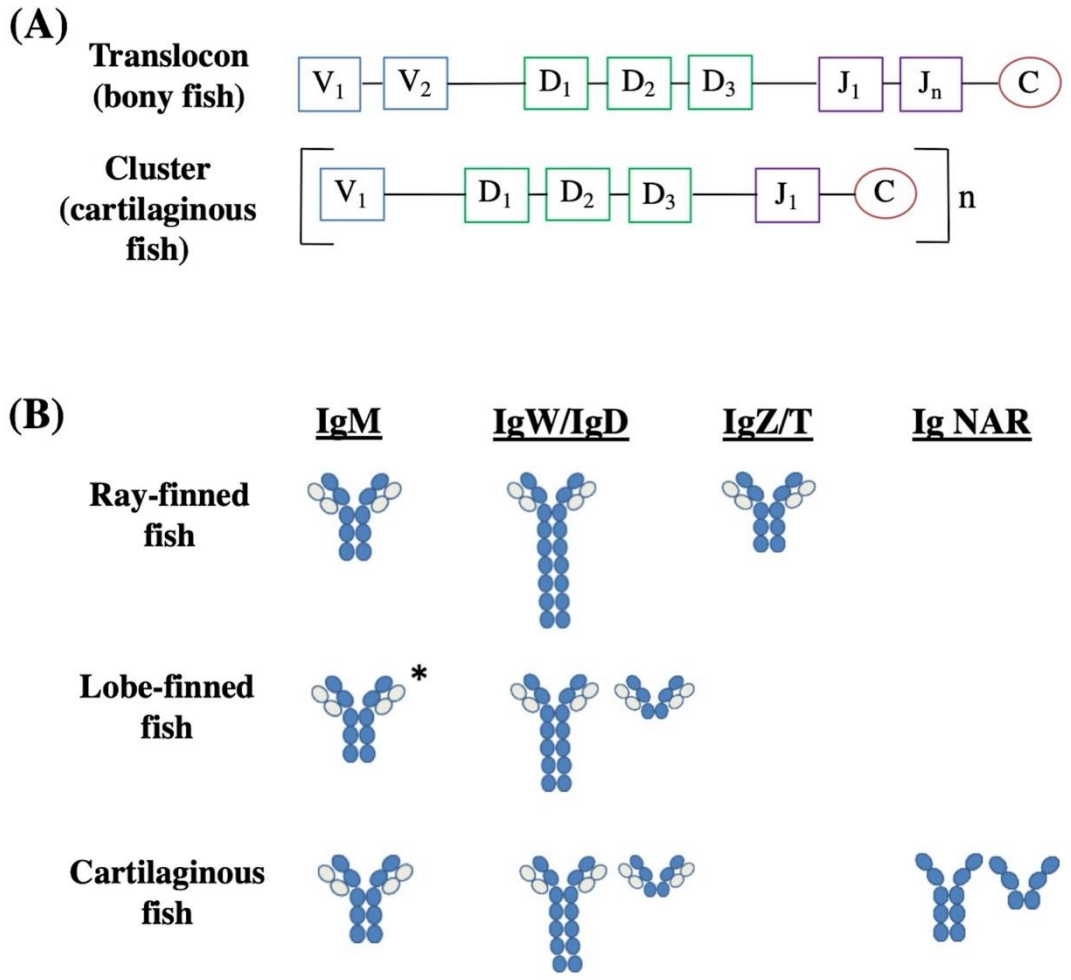

Figure 2.4. Antibody diversity and isotypes is divergent in fish. **A.** Arrangement of the heavy chain loci in bony fish and cartilaginous fish. V represents variable segments, D represents diversity segments, J represents joining segments and C represents constant domains. **B.** Examples of the immunoglobulin isotypes in fish. Dark blue circles represent heavy chain domains, light blue circles represent light chain domains [8,163,165-168]. * IgM for lungfish only, no IgM in the coelacanth.

recognizes self-antigen, inducing apoptosis or receptor editing, while positive selection occurs through antigen-independent signalling involving the BCR. In the case of T cells, a double positive T cell (CD4+ and CD8+) must bind MHC I or MHC II complex to be positively selected, which will induce the surviving T cell to become a CD8+ or CD4+ T cell, respectively. Negative selection occurs when a double positive T cells binds to MHC I or II with a high enough affinity to receive an apoptosis signal. While VDJ recombination has been characterized in fish (reviewed in [163,164]), the process of negative and positive selection has not been fully elucidated, although these processes likely occur in a similar manner as mammals. For example, double positive T cells were observed in the thymic cortex of sea bass, while single CD4+ or CD8 α+ cells were found in the thymic medulla, similar to that of mammals [169]. In addition, the absence of autoimmunity indicates negative selection of B cells in fish. The development of antibodies that specifically detect fish proteins, such as CD4 and CD8, is necessary to fully understand the homing and recirculation of B and T cells in fish.

2.4.1. The adaptive humoral response: B cells

The BCR includes the membrane-bound Ab and the Ig-α/Ig-β (CD79a/b) heterodimer, which is involved in signal transduction. Ab proteins are comprised of two heavy chains (IgH) and two light chains (IgL) held together by disulfide bonds forming a "Y" shaped quaternary structure [161]. Both IgH and IgL chains contain one N-terminal variable domain (VH and VL) and one or more C-terminal constant domains (CH and CL). The arms of the "Y" are composed of one constant and one variable domain from each heavy and light chain and are the site of antigen binding, called the Fab region (fragment, antigen-binding). The base of the "Y" is composed of two heavy chain constant domains and is referred to as the Fc (fragment,

crystallizable) region. The Fc region mediates the effector functions of the antibody by binding to a specific class of Fc receptors (and other molecules such as complement proteins) with the IgH categorizing them into specific isotypes. The variable regions of the heavy and light chain loci are assembled via somatic gene rearrangement from an array of multiple V, D and J segments during B cell development, allowing each B cell to produce a unique Ab. In response to antigen, in combination with interactions with helper T cells, B cells will secrete antigen–specific Abs. Three classes of Ab have been identified in both teleost fish and cartilaginous fish: IgM, IgD and IgZ/T in teleost fish, and IgM, IgW and IgNAR in cartilaginous fish, presumably each with different effector functions. In lungfish, IgM, IgW and IgN have been identified, while in coelacanths two forms of IgW has been discovered (Figure 2.4B) (reviewed in [161]).

Similar to all vertebrates (except cartilaginous fish), the IgH genes of teleost fish are arranged in a translocon configuration of which multiple V segments are found upstream of several D and J segments, followed by C segments (Vn-Dn-Jn-C) (Figure 2.4A) [162]. Depending on the species, differences may occur such as duplication of individual V, D or J segments, or tandem duplication of C domain exons such as that found in Atlantic salmon and zebrafish [166,170]. Instead of the single translocon locus, the IgH loci of cartilaginous fish adopt a multiple mini-cluster organization, with each cluster consisting of one V, two or three Ds and one J, followed by one set of C region exons for a specific isotype (Figure 2.4A)[171]. The clusters can be repeated as many as 100 times in the genome, depending on the species. While most clusters are capable of rearrangement, some clusters are partially (VD-J) or fully recombined (VDJ or VJ) in the germline, a rearrangement that is unique to cartilaginous fish [172]. IgH genes in lungfish are organized in a transiting form, having both cluster (like cartilaginous fish) and translocon (like teleost fish) configurations [163].

2.4.1.1. IgM

IgM is the most ancient antibody class found in all jawed vertebrates; with the exception of coelacanths, which is the only known jawed vertebrate that does not contain IgM in the genome [173–175]. IgM is the most prevalent Ab in both bony and cartilaginous fish plasma and can be found in both secreted and transmembrane forms. It shares a similar function in all jawed vertebrates, which includes mediating opsonisation, antibody-dependent cell-mediated cytotoxicity, and complement activation, and thus contributes to both innate and adaptive immune responses [59,105,174,175].

In teleost fish, IgM is multimerized into a tetrameric form, although there have been reports of a monomeric IgM form in some teleost species [176,177]. Due to an alternative splicing pathway, the transmembrane form of IgM is one domain shorter than the secreted form in teleost fish, resulting in a shortened IgM receptor on the B cell surface [178]. The lack of this domain does not interfere with the ability to interact with Igα/Igβ signalling molecules [179]. The J chain, which is required for IgM polymerization and secretion into the mucosa, has not been found in teleost fish, and therefore, tetrameric IgM is polymerized by interchain disulfide bonds [180]. IgM is the only teleost isotype for which sub-isotypes have been identified. Two sub-isotypes of IgM have been identified in Atlantic salmon and brown trout (*Salmo trutta*), reflecting the pseudotetraploid state of salmonid genomes [167,181].

In cartilaginous fish, IgM accounts for more than 50% of serum protein [161]. Both the secreted and transmembrane forms of IgM contain four C domains, except in the neonatal nurse shark, where a subclass of IgM ($IgM1_{gj}$) found in high amounts in the serum has only 3 C domains [171]. IgM in the serum of cartilaginous fish is found in two different states, a monomeric 7S and pentameric 19S, which are present in approximately equal amounts [182].

Pentameric IgM serves as the first line of defense, while 7S is produced later [182]. Both 7S and 19S IgM play a role in cytotoxicity reactions via phagocytosis [61]. In some cartilaginous fish species, such as the nurse shark, the J chain is present in pentameric IgM, although it may not be involved with IgM secretion, unlike the J chain in mammalian IgM [48,164].

In contrast to the coelacanth, which does not contain IgM in the genome, lungfish species express multiple diverse IgM genes which vary among species [163,183]. For example, the West African lungfish has three IgM isotypes, while the spotted lungfish (*Protopterus dolloi*) has two. Recently the J chain was identified in the spotted lungfish [184].

2.4.1.2. IgD/IgW

IgD is found in many vertebrate classes, including teleost fish and acipenseriformes (a group of fish that phylogenetically links elasmobranches, teleosts and sturgeons). It is orthologous to IgW (also known as IgX, IgNARC or IgR depending on the species), which is found only in cartilaginous fish [185,186], lungfish, and coelacanths [174,175,183], suggesting that IgD/IgW is as phylogenetically old as IgM [187,188]. The function of IgW and IgD, however, is poorly understood in both fish and mammals.

Teleost fish contain many forms of IgD, with constant domains ranging from 2-16 [189–191]. IgD has only been found in a transmembrane form, with the exception of the channel catfish and the Japanese puffer (*Takifugu rubripes*), which contains both membrane and secretory forms [162]. Teleost IgD is unique in that it is a hybrid of the CHμ1 domain followed by a varying number of CH-δ domains, depending on the species [192–195]. The IgD heavy chain has not been identified in any teleost fish without the CHμ1 domain [193,196,197]. IgD is co-expressed with IgM in most teleost fish, with the exception of channel catfish and rainbow trout. Three different types of IgD+ cells have been identified in catfish: small IgM+/IgD+ B

cells, larger IgM-/IgD+ B cells and granular cells containing exogenous IgD via a putative IgD-receptor. In rainbow trout, the ratio of IgD to IgM in the gills is much higher than other tissues. As well, an IgM-/IgD+ B cell subset has been found mainly expressed in the gills, indicating a role for IgD in the gills [189–191].

IgW in cartilaginous fish contains six to eight C domain exons, in addition to the V, D and J segments. Multiple splice forms of IgW exist in cartilaginous fish other than the original six C domains (IgW-long), including a two C domain (IgW-short) form and a four C domain form [186,198,199]. A V-less form of IgW has been identified in both the spiny dogfish (*Squalus acanthias*) and the nurse shark but represents only 8% of the IgW transcripts analyzed [198].

Two IgW transcripts have been identified in the African lungfish [163]. Similar to cartilaginous fish, lungfish IgW can be found in a long form, consisting of seven C domains (homologous to IgW-long) or a short form, consisting of two C domains [163,183]. Two distinct loci for IgW have also been discovered in the Indonesian and African coelacanth (*Latimeria chalumnae)* [175]. It remains unknown if the short and long forms of IgW found in cartilaginous fish and in lungfish have different effector functions and if the functions of IgD/IgW are species specific.

2.4.1.3. Species specific Igs: IgNAR, IgZ/T, IgQ

IgNAR (new/nurse shark antigen receptor) is a heavy-chain only Ig found only in sharks. Each chain of IgNAR contains a single-domain V region that can bind to antigen independently [200]. IgNAR exists in both long and short forms, which can vary between species [182]. The long transmembrane and secreted forms consist of five C domains while the short transmembrane form consists of three C domains [48,201]. Serum levels of IgNAR are much lower than IgM and it is unknown if the J chain is required for IgNAR multimer formation [8].

The immunoglobulin IgT/Z is produced only in bony fish and was first identified in rainbow trout (IgT) and zebrafish (IgZ) [170,197]. In most bony fish characterized to date, IgT/Z contain four C domains, although this is known to vary in a number of species [202–204]. While only a few studies have been performed, it is thought that IgT is specialized for mucosal immunity and functions analogously to mammalian IgA. While the concentration of IgT/Z in the serum of rainbow trout is much lower than that of IgM, the ratio of IgT/Z:IgM is 63 times higher in the gut than in the serum [205]. This study also demonstrated that following intestinal parasitic infection, the number of IgT+ B cells increased in the gut, but the number of IgM+ B cells in the gut did not change [205]. In addition, IgT+ B cells are also found in teleost skin associated lymphoid tissue (SALT) where they secrete IgT into skin mucus [17].

High-throughput sequencing of two species of African lungfish (*P. dolloi* and *P. annectens*), followed by Southern blot, identified two unique Ig isotypes in lungfish; these include 3 IgN isotypes (IgN1 found only in *P. dolloi* while IgN2 and IgN3 found only in *P. annectens*) and IgQ (found only in *P. annectens*) [163]. Both IgN and IgQ are thought to originate from the IgW lineage [163].

2.4.1.4. B cell response and immunity

Both bony and cartilaginous fish lack bone marrow, the main site of haematopoiesis in mammals, and germinal centers (GC), specialized sites where mature B cells proliferate, differentiate, and selection of high affinity BCR occurs in mammals. Instead, in teleost fish, the main site of haematopoiesis is the anterior (or head) kidney. Progenitor B cells and plasma cells are found in the anterior kidney, while mature B cells and plasma blasts are found in the posterior kidney and in the spleen [206,207]. Evidence for B cell development in the anterior kidney is supported by expression of RAG-1/2 and terminal deoxynucleotidyl transferase (TdT),

and the resulting development of immature B cells with membrane Ig on their surface. It is proposed that mature B cells are released into the blood where they encounter antigen and mature into plasmablasts or plasma cells. Plasma cells then migrate back to the anterior kidney where they may become long-lived plasma cells, supporting the storage of Ig-secreting cells [207,208]. However, more work is required to fully elucidate the mechanisms regulating homing of B cells in fish. The spleen is considered the only secondary lymphoid organ (SLO) in teleost fish, where expression of AID (see below) has been exclusively observed, suggesting that the spleen is the site for antigen stimulation [209].

In cartilaginous fish, the Leydig organ, a gland-like structure associated with the esophagus, and the epigonal organ, a structure physically attached to the gonads with a similar structure and organization as the Leydig organ, are the main sites of hematopoiesis and B cell production [47]. Lymphocytes of various sizes are abundant in these organs and form a loose follicle-like aggregate with scattered plasma cells [210]. While most cartilaginous species have both organs, some species only have one, such as the nurse shark, which only has an epigonal organ [47]. Like bony fish, RAG1 and TdT expression in the epigonal organ provides evidence that it is a site of B cell development [211]. Additionally, hematopoietic transcription factors important in B and T cell development are expressed in the Leydig and epigonal organ of the embryonic clearnose skate (*Raja eglanteria*) [212]. The spleen of cartilaginous fish contains well defined white pulp (WP) and red pulp (RP) regions and is considered a SLO. The WP consists of lymphocytes and mature and developing plasma cells, while the RP consists of macrophages, erythrocytes and plasma cells [211,213]. Antigen stimulation, leading to Ab synthesis, has been described in the cartilaginous fish spleen [211,213]. As previously stated, analysis of possible hematopoietic organs (kidney and/or gonads) in lobe-finned fish has yet to be completed [52].

Structural analysis of the African lungfish spleen identified characteristics of a secondary lymphoid organ; the red pulp is likely the site erythropoiesis, as well as site of plasma cell differentiation, similar to cartilaginous fish, as evidenced by mature and immature plasma cells [52]. The WP appears to be involved in immune responses [52].

Both bony and cartilaginous fish have been shown to develop immunological memory (i.e. the ability to respond more rapidly and effectively to a pathogen that has been previously encountered). One of the first studies to identify immunological memory in fish was in rainbow trout where it was demonstrated that the secondary response to trinitrophenylated-keyhole limpet hemocyannin (TNP-KLH) was faster and of a larger magnitude than the primary response, as determined by ELISA [214]. Several other studies in fish, including rainbow trout and turbot (*Psetta maxima*), have since shown that neutralizing Ab can be induced against a variety of viral, bacterial and parasitic pathogens and vaccines [215,216]. However, the response time of teleost IgM is much slower than in mammals, taking 3-4 weeks after immunization before specific titers are detected. Interestingly, some fish species, such as the Atlantic cod, do not appear to produce a specific antibody response upon immunization, despite high levels of serum Abs [217]. This is likely due to the lack of MHC II molecules in the Atlantic cod [84,218].

Similar to teleost fish, the immune response time of IgM in cartilaginous fish is much longer than in mammals. Dooley and Flajnik (2005) completed a three year-long immunization study in the nurse shark [182]. The results demonstrated that, following immunization, pentameric IgM, which localizes mainly in the plasma, was induced before other isotypes, but with a low-affinity interaction with antigens. The results also demonstrated that monomeric IgM, which is capable of entering tissues, appeared after pentameric IgM and was the main Ig involved in antigen-specific responses. A significant increase in antigen-specific IgNAR titers

was also observed with a high specificity to antigen following immunization. It can take up to 28 months before the antigen-specific titer levels return to pre-immunization levels once the Ig response has reached a plateau [182]. Memory was demonstrated for both monomeric IgM and IgNAR as re-immunization after a decrease in titer induced a quicker response than the primary immunization [182].

2.4.1.5. AID and affinity maturation

Activation-induced cytidine deaminase (AID) is an enzyme that mediates somatic hypermutation (SHM) (i.e. a process that fine tunes the Ig, increasing its affinity (affinity maturation)), and mediates class switch recombination (CSR) (i.e. a process whereby the constant region of an antibody heavy chain is changed to a different isotype, thus changing its effector function) [219]. AID was first reported in channel catfish, and has since been reported in many other fish species [220,221]. Teleost fish AID differs from mammals in that it has a longer cytindine deaminase motif and substitutions in its carboxy-terminal region [222]. Catfish and zebrafish AID has been demonstrated to mediate SHM in mouse fibroblasts (NIH3T3PI19) [223], while zebrafish AID was shown to be capable of deaminating methylated deoxycytidines [224]. In addition, the biochemical properties of AID from the sea lamprey, nurse shark, tetraodon, and coelacanth were recently characterized where it was found that these AIDs exhibit unique substrate specificities and optimal temperature tolerances while the lethargic enzymatic rate and affinity for ssDNA of AID are conserved [225]. However, a search of the African lungfish mucosal lymphoid tissue transcriptome for AID found no evidence of expression using cartilaginous fish, teleost fish, or tetrapod AID sequences for comparison suggesting that the African lungfish may have lost AID expression in its genome [226]. In addition, no AID was found using RT-qPCR [226]. However, other members of the apolipoprotein B mRNA-editing

catalytic polypeptide (APOBEC) family (to which AID belongs) were found to be expressed in the African lungfish [226].

Affinity maturation is generated during immune responses in bony fish, as evidenced by the replacement of low-affinity Ab by intermediate-affinity Ab and eventually by high-affinity Ab in rainbow trout [227]. The affinity maturation response in fish is much less efficient than mammals, likely due to the absence of GCs. Affinity maturation was also reported in the nurse shark, where purified monomeric IgM showed an increase in the intrinsic association constant to a ^{3}H-ε-DNP-l-lysine ligand over a 20 month period [228]. IgNAR also exhibits affinity maturation, as demonstrated by a correlation between somatic mutations and increased binding affinity in IgNAR clones from immune tissues of a hyperimmunized nurse shark [229]. The affinity of pentameric IgM, however, does not increase during an immune response [182].

Although teleost fish express AID, they lack class switch recombination (CSR), likely due in part to the structure of the IgH gene [223]. However, AID from teleost fish, specifically zebrafish, Japanese puffer, and catfish can catalyze CSR in mammalian AID -/- lymphocytes suggesting that AID in fish has the potential to catalyse the class-switching reaction [223,230]. Although it was once thought that cartilaginous fish were also incapable of CSR due to the cluster organization of their genes, it is now known that they can undergo an “unconventional” type of CSR among different IgM clusters and between IgW and IgM clusters [231].

2.4.1.6. Major histocompatibility complex (MHC) and antigen presentation

A major function of B cells, as well as other pAPCs such as macrophages and dendritic cells, is to process and present antigen to activate T cells. T cells, however, will only recognize antigen fragments that are bound to MHC I or MHC II, cell surface proteins, found on pAPCs. While the structure of MHC is conserved over various species, the genes encoding MHC

demonstrate a high degree of polymorphism in mammals, lobe-finned fish, and ray-finned fish and cartilaginous fish, allowing different repertoires of peptides to be presented [232–234]. In most teleost fish, MHC class I and II reside on different chromosomes, while in cartilaginous fish, and all other vertebrates, MHC I and II are found on the same chromosome [235–237]. Interestingly, while MHC I and II are conserved in most jawed vertebrates, Gadiformes, such as the Atlantic cod, have lost the genes for MHC II and CD4, a co-receptor on T cells that interacts with MHC II [84,85,218,238]. The Atlantic cod does, however, contain more genes related to the MHC I component of the immune system, as well as the expansion of some TLR clades, compared to other vertebrates, which may help compensate for the missing MHC II and CD4 [84,218].

Antigens that are to be presented by MHC I are processed via the immunoproteasome and transferred to the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP) where they associate with MHC I and are eventually transported to the cell membrane. MHC I is ubiquitously expressed in various tissues in teleost and cartilaginous fish including spleen and head kidney [237,239,240]. In addition, β_2 microglobulin, which is associated with MHC I, has been isolated in several teleost fish, as well as the nurse shark and sandbar shark (*Carcharhinus plumbeus*) [241–243]. MHC-I related immunoproteasomes, as well as TAP genes, have also been identified in both bony and cartilaginous fish [232]. While there have only been a few studies examining MHC I in lobe-finned fishes, MHC class I genes, including α1, α2 and α3, have been sequenced from blood of the African lungfish and muscle and skin of the West Indian Ocean coelacanth (*Latimeria chalumnae*) [244,245]. Additionally, lmp1 and lmp2, catalytic subunits of the immunoproteasome, have been characterized in the African lungfish and

were found to be induced in primary lung and kidney cell cultures by the synthetic dsRNA polyinosinic-polycytidylic acid (poly (I:C)) [246].

Antigens that are to be presented by MHC II are endocytosed, digested in lysosomes and loaded onto MHC II molecules prior to their migration to the cell surface. MHC II genes have been identified in teleost fish, cartilaginous fish, and the African coelacanth [175,247,248]. Teleost MHC class II genes can be organized into three groups based on sequence features such as insertions and deletions [248]. Several studies have concluded that MHC class II affects resistance to bacterial pathogens, including *Aeromonas salmonicida* in Atlantic salmon [249]. Likewise, challenge with *Vibrio harveyi* increased expression of MHC II B mRNA in the gill, liver, and spleen of the white bamboo shark (*Chiloscyllium plagiosum*), similar to teleost fish [250]. The identification and characterization of MHC I and II genes in both bony and cartilaginous fish, with the exception of the Gadiformes lineage, suggests that MHC is generally well conserved in these species.

2.4.2. The adaptive cellular response: T cells

T cells possess a T cell receptor (TCR) which recognizes a specific antigen and is formed using RAG-mediated V(D)J rearrangement for the development of diverse repertoires. However, unlike the BCR, the TCR is always membrane bound and only recognizes antigen when presented in the context of MHC I or II [3]. T cells are classified into 2 main populations: CD8+ cytotoxic T-cells (Tc) which interact with MHC class I and CD4+ helper T cells (Th) which interact with MHC class II. In addition to MHC, all TCR possess a CD3 complex and recognize co-stimulatory (e.g. CD28) and co-inhibitory (e.g. CTLA-4) molecules. In both bony and cartilaginous fish, and similar to mammals, T cells are produced in the thymus. Research in sea

bass detected T cells in the developing gut at the same time as in the thymus, suggesting that the gut may also be a primary lymphoid organ for T cells in bony fish [32].

2.4.2.1. T cell receptor

TCRs are type I transmembrane glycoproteins with extracellular V and C Ig domains and a short cytoplasmic tail (Figure 2.5). This structure is conserved in almost all vertebrates [251]. The TCR is found in two forms: a heterodimer of α and β chains (αβ-TCR) or a heterodimer of γ and δ chains (γδ-TCR), linked by disulphide bonds. Most T cells contain the αβ-TCR, while γδ-T cells account for 1-10% of T cells in the blood of mammals, and 8-20% of total lymphocytes in various tissues of the zebrafish [252]. *In situ* hybridization experiments in the nurse shark identified higher levels of TCR α and β in the central cortex of the thymus but weaker expression in the medulla and subcapsular region. Expression of TCR γ and δ were also high in central cortical cells but were most highly expressed in the subcapsular region. TCR δ was the most highly expressed TCR chain in the medulla [27].

The genes for TCR-α, β, γ, and δ are diversified using V(D)J rearrangement and have been identified in teleost fish, cartilaginous fish, and coelacanths [168,175,251,253]. In both bony fish and cartilaginous fish, the TCR gene segments are in the translocon arrangement, similar to mammals [27,256]. While the structure of TCR is generally well conserved among all vertebrates, there are several unusual aspects of the cartilaginous fish TCR. Two forms of TCR-δ have been identified in cartilaginous fish, one form having an extra V domain that is closely related to IgNAR (and thus given the name NARTCR) [169,254]. It is hypothesized that the NARTCR-δ chain dimerizes with a TCR-γ chain that lacks the additional domain and therefore NARTCR-δ V domain does not have a binding partner [255]. Another unique aspect of shark TCR is that some TCR-δ chains may be formed from a trans-rearrangement of Ig heavy chain V

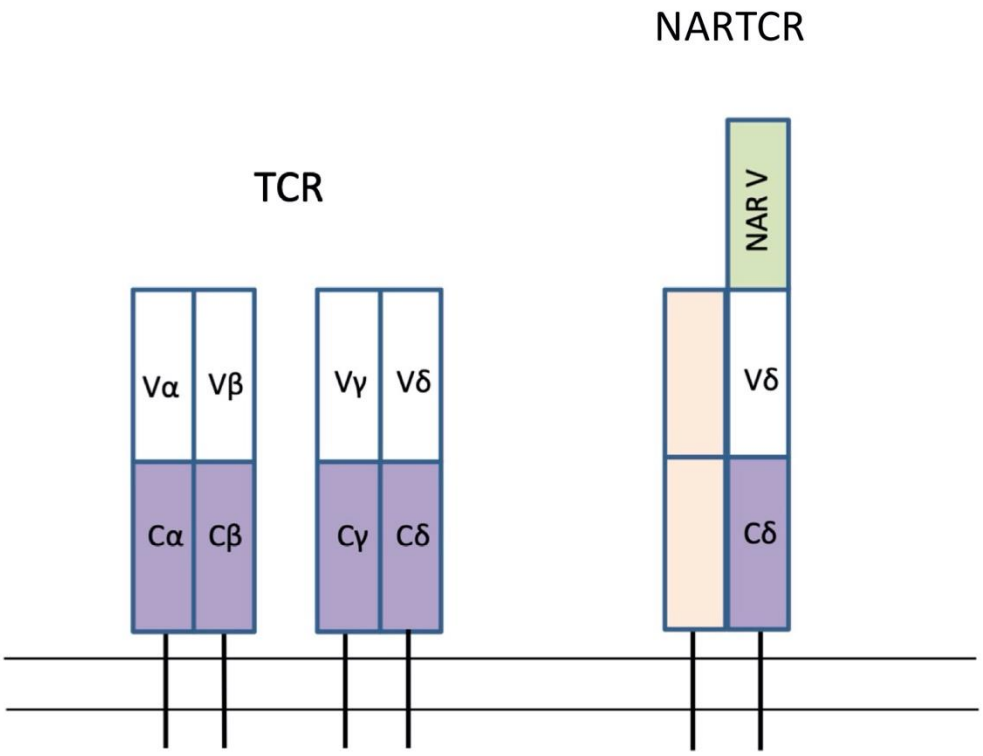

Figure 2.5. Schematic of the conventional TCR forms found in all Gnathostomes and NARTCR found in cartilaginous fish. Rectangles represent Ig super-family domains; V represent variable domains (white), C represents constant domains (purple) and NAR V represents extra variable domain in NARTCR (green). Modified from Criscitiello et al. [169], Roux et al. [254] and Criscitiello [255].

segments with D, J and C segments of TCR-δ [169]. Finally, sharks use AID and somatic hypermutation (SHM) to diversify the shark TCR-γ and -α chains [27,256]. SHM is not known to diversify the TCR in any other vertebrates [27,256]. Figure 2.5 depicts a schematic of a conventional TCR found in gnathostomes and a NARTCR found in cartilaginous fish.

2.4.2.2. TCR co-receptors

The αβ subtypes can be further divided into helper CD4+ cells (Th) or cytotoxic CD8+ cells (Tc). CD4+ T cells are stimulated by peptides presented via MHC-II molecules and, when activated, CD4+ T cells release cytokines that can activate and regulate responses elicited by the antigen [257]. The CD4 molecule is a single protein with four extracellular Ig-like domains and a cytoplasmic tail containing a CxC motif which interacts with the tyrosine kinase Lck, initiating intracellular signalling [258]. While tetrapods contain a single CD4 molecule with four Ig domains, two types of CD4 molecules have been described in bony fish: CD4-1 containing four Ig domains and CD4-2 which contains either two or three Ig domains, depending on the species [251]. In addition, salmonids contain two CD4-2 molecules (CD4-2a and CD4-2b) [259]. An early study of the elephant shark genome suggested that CD4, as well as CD4 associated genes involved in the differentiation (RORC, FOXP3) and function (IL-4, IL-5, IL-13, IL-9, IL-21) of CD4+ cell lineages were missing from the genome [57]. Although this study identified several CD4/Lag3-like molecules, they lacked the C-terminal intracellular CxC motif required for interacting with Lcks suggesting that CD4 is absent or not functional in the cartilaginous fish genome. However, a more recent genomic study by Redmond et al. [260] in the Small-spotted catshark used newly available sequence datasets and found putative sequences for CD4 T-cell associated genes including IL-4/IL-13, IL-21, IL-23, IL-27, IL-6Ra, IL-12R and FOXP3, suggesting that cartilaginous fish do in fact have CD4 T-cell subsets, although more work is still required to fully understand the T cell subsets present in cartilaginous fish, as well as their biological roles [260].

CD8+ T cells are activated by peptides presented via MHC-I molecules and secrete cytotoxins such as perforin and granzymes that initiate apoptosis in the target cells. The CD8 molecule can be in one of two forms: a homodimer formed from two α-chains (CD8αα) or a heterodimer formed from one α- and one β- chain (CD8αβ) [261]. Both CD8 chains have been characterized in multiple teleost fish and cartilaginous fish [27,57,262,263]. Teleost and cartilaginous fish CD8 exhibit an extracellular Ig-like domain, but the domain has a CxH motif in the cytoplasmic tail, instead of the CxC motif found in mammals, suggesting that CxH represents a primordial Lck binding site [169,264].

T-cell activation is triggered via antigen:MHC recognition by the TCR and mediated via CD3. All TCR have a short cytoplasmic tail and therefore need to partner with CD3, a complex of transmembrane proteins with intracellular domains containing the conserved motif known as immunoreceptor tyrosine-based activation motif (ITAM). Characterization studies of CD3 in teleost fish have identified a conserved structure of CD3 between teleost fish and mammals [265,266]. Genes encoding the CD3 chains have been annotated in the elephant shark genome and were recently cloned in the small-spotted catshark (*Scyliorhinus canicula*) where two copies of CD3 were observed [57,267]. Three CD3 chains have also been identified in the coelacanth genome [175]. The sequence homology of all 3 chains encoded in the coelacanth genome were distinct from other fishes but grouped together with the corresponding molecules found in avians and mammals [175].

The initial interaction of TCR/MHC/peptide is not sufficient to fully induce activation of naïve T cells and therefore T cells require additional co-stimulatory signals. This is provided by the interactions between CD28, a co-stimulatory factor expressed on T cells, and B7.1 (CD80) and B7.2 (CD86) ligands on the APC. In contrast, binding of B7.1 and B7.2 to CTLA4, a powerful negative regulator of T cell activation, exerts an inhibitory effect on T cell activation. Both CD28 and CLTA4, as well as orthologues of B7.1 and B7.2, have been identified in several teleost species [268–270].

The binding sites for B7.1 and B7.2 are conserved in teleost fish CD28 and CTLA4, indicating that CD28 and CTLA4 recognize a B7-like receptor [268]. In addition, viral infection in rainbow trout increases CTLA-4 expression, while CD28 remains constitutively expressed, similar to mammals, suggesting that these molecules may have similar roles as their mammalian orthologues [268]. Putative CD28, CLA-4, and B7 genes have been annotated in the elephant shark genome, while CD28 has been identified in the coelacanth genome, however the function of these co-receptors in many fish species remains to be fully investigated [57,175].

2.4.2.3. The T-cell effector response

Upon activation of CD4+ cells, naïve cells can differentiate into specific subsets including Th1, Th2, Th17, and inducible T-regulatory (Treg) cells; each subset defined by their cytokine production [271]. Activation of CD8+ cells induces differentiation into cytotoxic effector cells which release cytotoxins that induce apoptosis of the target cell.

2.4.2.4. CD4+ Th cells

The structures of several orthologues and paralogues of Th cytokines, as well as their functions, have been characterized in both teleost fish and cartilaginous fish and are reviewed in Secombes et al. [272,273] and Secombes and Wang [274]. In brief, two forms of IFNγ, produced by Th1 cells, IFNγ, and IFNγ rel, have been identified in teleost fish including Atlantic salmon, rainbow trout, and ginbuna crucian carp (*Carassius carassius*), while one form has been identified in fugu [275–277]. Recombinant IFNγ (r-IFNγ) was found to increase the expression of anti-viral and inflammation-relevant genes, as well as increase ROS and NO production in zebrafish, rainbow trout and goldfish macrophages, indicating a similar function as mammalian IFNγ [278,279]. A single copy IFNγ has been identified in the Elephant shark genome [57]. Three Il-4/13 genes (IL-4/13A, IL-4/13B1 and IL-4/13B2), produced by Th cells, have been

characterized in salmonids [272,280,281]. Intraperitoneal injection of r-IL-4/13A in zebrafish increased the number of IgZ+ B cells circulating in the blood, compared to a PBS control injection [282], while r-IL-4/13A in rainbow trout modulates the expression of a number of Th2 genes [283]. While Venkatesh et al. [57] found no IL-4/13 genes in the elephant shark genome, subsequent interrogation of the genome by Dijkstra [284] found three putative IL-4/13 genes. In addition, Redmond et al. identified a IL-4/IL-13 gene in the small spotted catshark genome [260]. Analysis of the coelacanth genome failed to identify Il-4 [175]. The IL-17 family in teleost fish, produced by Th17 cells, has several members (A-F) which are structurally related to orthologous proteins in mammals [285,286]. Two homologues of the IL-17 family, IL-17B and IL-17D have been identified in teleost fish, as well as several isoforms of molecules termed IL-17A/F1-3, IL-17C and IL-17E [287]. r-IL-17A/F2 induced the expression of antibacterial peptide β-defensin-3 and the pro-inflammatory cytokines IL-6 and IL-8 in rainbow trout splenocytes, suggesting its role in antibacterial defenses [286]. Several IL-17 family members have been found in a cartilaginous fish genome (*C. milii*) including IL-17A/F, IL-17B, IL-17C and IL-17D [273]. One copy of the IL-10 gene, produced by Treg cells, is found in most species of teleost fish, with the exception of rainbow trout and carp, where two genes have been identified [288,289]. Sequences with homology to IL-10 were found in the spiny dogfish (*Squalus acanthias*), elephant shark and coelacanth genomes [175,273]. These studies, among many other fish cytokine studies, indicate that the structure of cytokines released from Th cells is relatively conserved between ray-finned fish, lobe-finned fish, and cartilaginous fish.

2.4.2.5. CD8+ cytotoxic cells

Cytotoxic T cells kill their targets via two mechanisms: the secretory and non-secretory pathways, both of which induce apoptosis. The secretory pathway releases granular toxins such

as perforin and serine proteases called granzymes which work together to induce apoptosis [290]. The non-secretory pathway involves the engagement of target-cell death receptors, such as Fas, located on the cell surface of the cytotoxic T cells, which results in caspase-dependent apoptosis [291].

The secretory pathway has been identified in many different fish species. A perforin-like molecule has been characterized in several teleost species [292,293]. The killing function of α/β TCR alloantigen specific cytotoxic clones was inhibited in channel catfish by treatment with concanamycin A, a perforin inhibitor [294]. Similarly, treatment of ginbuna crucian carp CD8α+ lymphocytes with concanamycin A partially inhibited their function in a dose dependent manner, suggesting that the perforin-mediated pathway in teleost fish is similar to that of higher vertebrates [295]. Granzyme has also been recently identified in ginbuna crucian carp (gcGzm) and has a similar primary structure to that of mammals [296]. Expression of gcGzm mRNA was greatly enhanced by allo-sensitization and infection with *Edwardsiella tarda*, indicating that gcGzm is involved in cell mediated immunity [296]. In spite of the absence of CD4 and associated CD4 genes, many cytotoxic T cell related genes, including perforin and granzyme, have been identified in the elephant shark genome, suggesting that such cell types are present in cartilaginous fish [57].

While the non-secretory pathway has not been as thoroughly studied in fish as the secretory pathway, the FasL protein has been identified in channel catfish, tilapia (*Oreochromis niloticus*), and gilthead sea bream [297–299]. Recombinant FasL protein from Japanese flounder (*Paralichthys olivaceus*) induced apoptosis in a flounder cell line, indicating that fish possess a similar Fas ligand system [300]. FasL has yet to be identified in cartilaginous and lobe-finned fish.

2.5. Conclusion and future directions

Comparative studies in fish help in uncovering the evolutionary history of the immune system. Whereas innate immunity is present in all multicellular organisms, an adaptive immune system, based on VLRs, B-like and T-like cells is found in jawless vertebrate, while an adaptive immune system, based on an Ig/TCR/MHC system, evolved with the appearance of jawed vertebrates. Research on the fish immune system is continuously on the rise, however there is still much to be discovered. For example, there is limited information on TLR ligands, especially in cartilaginous and lobe-finned fish, as well as limited information on complement proteins in lobe-finned fish. In order to gain a better understanding of the lobe-finned fish immune system, the site(s) of haematopoiesis needs to be determined. There is also limited knowledge, compared to mammals, on the homing and recirculation of B and T cells in ray-finned fish, cartilaginous fish and lobe-finned fish. Much of this knowledge will only be gained with the development of the appropriate reagents and techniques. The development of cell lines for cartilaginous and lobe-finned fish will aid in determining basic cell biology, one of the first steps in understanding the immune system. Many comparative fish immunology studies are genome-based, and fish genomes are often not well-assembled and/or annotated. The development of high-quality, well-assembled, and annotated genomes in fish species will allow the identification of more immune-relevant transcripts, such as NITRs. In addition, the lack of protein-specific antibodies for fish is hindering many research avenues, such as flow cytometry and cell-specific analyses. A comprehensive understanding of the evolution of the immune system will continue to develop as more comparative research on cartilaginous fish, lobed-finned fish, and ray-fined fish is completed.

Table 2.2. Overview of the Innate Immune System highlighted in this chapter.

Innate Immune System			
	Ray-finned	**Lobe-finned**	**Cartilaginous**
Physical Barriers			
Skin	Contains multiple cell types including secretory cells, lymphocytes, granulocytes, macrophages	Contains multiple cell types including keratinocytes, granulocytes, B cells	Contains multiple cell types melanocytes, lymphocytes, macrophages, granular leucocytes
Mucus	Contains lectins, lysozymes, complement proteins, AMPs	Likely present; not as extensively investigated. Histones and S100 proteins (AMPs) identified	Likely present; not as extensively investigated. Transcript for pentraxin (a lectin) identified
Cellular Components			
Site of leukocyte production	Anterior (or head) kidney and thymus	Unknown	Epigonal organ, Leydig organ, thymus and spleen
Recognition of non-self (TLRs)	Some contain a TLR4-like gene Absence of TLR6 TLR8 present TLR14 present TLR18-TLR20 present TLR22 present TLR23 present TLR25/26/28 present	Absence of TLR4-like gene Absence of TLR6 Absence of TLR8 TLR14 present Absence of TLR18-TLR20 TLR22 present Absence of TLR23 Absence of TLR25/26/28	Absence of TLR4-like gene TLR6 present TLR8 present Absence of TLR14 Absence of TLR18-TLR20 Absence of TLR22, TLR23 Absence of TLR25/26/28
Humoral Responses			
Complement System	Contains most components of mammalian system; Multiple C3 forms	Genes encoding some complement components have been identified	Contains most components of mammalian system; Absence of MBL, ficolin, C1qA and C1qC; MASP2 transcript with no serine protease domain identified; Multiple C3 and C4 forms
Lysozyme	c-type and g-type present; Absence of c-type gene in Atlantic cod but 4 g-type genes identified	Two g-type genes identified	c-type transcript identified; absence of g-type transcript
Antimicrobial peptides	Over 90 AMPs identified and characterized	Unknown	Not extensively investigated; Three AMPs identified: transferrin, squalamine, Kenojeninin I
Acute phase proteins	CRP, SAP identified and characterized	SAP transcript identified	CRP, SAP identified and characterized

Table 2.3. Overview of the Adaptive Immune System highlighted in this chapter.

Adaptive Immune System			
	Ray-finned	**Lobe-finned**	**Cartilaginous**
B cells			
IgH configuration	Translocon (Vn-Dn-Jn-C)	Transiting form: both translocon and cluster	Multiple mini-clusters $(V_1\text{-}D_{2\text{ or }3}\text{-}J_1\text{-}C)_n$
IgM	IgM present	IgM absent in coelacanth; multiple IgM isotypes present in lungfish	IgM present
IgD/IgW	Multiple forms of IgD present	Two IgW transcripts present	Multiple forms of IgW present
Species specific IgNAR, IgT/Z, IgQ	IgT/Z present	IgN and IgQ preent in lungfish	IgNAR present
Site of B cell development	Progenitor B cells in anterior kidney; mature B cells in posterior kidney and spleen	Unknown	Epigonal organ; Leydig and epigonal organ in skate
AID	Present	Present in coelacanth; absent in lungfish	Present
MHC I/II	MHC I and MHC II present. No MHC II in Gadiformes	MHC I associated genes (α1, α2, α3) present. MHC II present.	MHC I and MHC II present
T cells			
Site of production	Thymus	Thymus?	Thymus
TCR gene arrangement	Translocon	Translocon	Translocon
TCR	TCR-α, β, γ, and δ present	TCR-α, β, γ, and δ present	TCR-α, β, γ, and δ present Two forms of TCR-δ
CD4+ Th cells	IL-4/13 present Members of IL-17 family present IL-10 present	IL-4 absent Members of IL-17 family unknown Sequence with homology to IL-10 present	IL-4/13 present Members of IL-17 family present Sequence with homology to IL-10 present
CD8+ cytotoxic cells	Perforin-like molecule characterized Granzyme identified FasL identified	Perforin, Granzyme, FasL unknown	Gene for perforin and granzyme identified FasL unknown

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CHAPTER 3

Characterization of miRNAs in cultured Atlantic salmon head kidney monocyte-like and macrophage-like cells

3.1. Abstract

Macrophages are among the first cells to respond to infection and disease. While microRNAs (miRNAs) are involved in the process of monocyte-to-macrophage differentiation in mammals, less is known in teleost fish. Here, Atlantic salmon head kidney leukocytes (HKLs) were used to study expression of miRNAs in response to in vitro culture. The morphological analysis of cultures showed predominantly monocyte-like cells at Day 1 and macrophage-like cells at Day 5, suggesting that the HKLs had differentiated from monocytes to macrophages. Day 5 HKLs also contained a higher percentage of phagocytic cells. Small RNA sequencing and RT-qPCR analysis were applied to examine the miRNA diversity and expression. There were 370 known mature Atlantic salmon miRNAs in HKLs. Twenty-two miRNAs (15 families) were downregulated while 44 miRNAs (25 families) were upregulated at Day 5 vs. Day 1. Mammalian orthologs of many of the differentially expressed (DE) miRNAs are known to regulate macrophage activation and differentiation, while the teleost-specific miR-2188, miR-462 and miR-731 were also DE and are associated with immune responses in fish. *In silico* predictions identified several putative target genes of RT-qPCR-validated miRNAs associated with vertebrate macrophage differentiation. This study identified Atlantic salmon miRNAs likely to influence macrophage differentiation, providing important knowledge for future functional studies.

3.2. Introduction

Macrophages are some of the first cells that respond to infection and disease. They are critical in mounting and resolving an immune response during tissue injury and/or pathogen infection [1]. Macrophages are derived from hematopoietic progenitors which differentiate from their precursor cells, monocytes [2]. Macrophage differentiation is tightly controlled via a multitude of cytokines, growth factors and transcription factors such as colony stimulating factor 1 (CSF-1) and the transcription factors PU.1 and Runx1 [2,3]. In mammals, two distinct subsets of macrophages have been described: M1 (classically activated macrophages) and M2 (alternatively activated macrophages) [4]. M1 macrophages are induced by cytokines primarily secreted by Th1 cells, including IFN-γ and TNF-α, and are involved in the inflammatory response, producing reactive oxygen species (ROS) and inflammatory cytokines. M2 macrophages are induced by cytokines including IL-4, IL-13 and TGF-β and are involved in wound healing, repair and immune suppression [4–6]. M2 macrophages can be further divided into various subtypes (M2a, M2b and M2c), based on their activation and function [6]. Much of our knowledge of fish macrophage differentiation and activation comes from zebrafish (*Danio rerio*), ginbuna carp (*Carassius auratus langsdorfii*) and goldfish (*C. auratus*, L.) models [7,8]. Important factors in mammalian macrophage differentiation, such as CSF-1, CSF-1R, and Toll-like receptors, as well as factors involved in M1/M2 macrophage activation, such as TNF-α, Interferons, IL-4/13 and Arginase, have been identified and characterized in fish studies (reviewed in [7,9,10]). While our knowledge of fish macrophage biology is expanding, macrophage differentiation and activation across all teleost species, including the Atlantic salmon, an economically important farmed fish, remain to be adequately described.

MicroRNAs (miRNAs) are short, non-coding RNAs (ncRNAs) that play roles in controlling many biological processes through post-transcriptional regulation of gene expression [11–13]. Following transcription, the primary miRNA transcript is cleaved by Drosha into precursor miRNAs, followed by exportation of the precursor miRNA out of the nucleus by Exportin 5. The precursor miRNA is then processed further by Dicer to produce mature miRNAs (5p or 3p) that are 20-24 nucleotides long, which are then incorporated into the miRNA induced silencing complex (miRISC) [12,13]. As part of the miRISC, the miRNA can then bind to its partially complementary sequence, usually located in the 3'-untranslated region (3'-UTRs), of its target mRNA leading to mRNA degradation or the prevention of translation. miRNAs regulate several biological processes including, but not limited to, cell differentiation, cell development, apoptosis and immune response [14,15].

Mammalian studies have demonstrated that several miRNAs mediate the activation and differentiation of macrophages (reviewed in [16,17]). For example, a study in murine bone marrow-derived macrophages found that the expression levels of 109 miRNAs were altered between M1 and M2 conditions [18]. Functional studies have demonstrated that miRNAs (e.g. miR-9, miR-127, miR-155, miR-125b) promote the activation of M1 macrophages and the pro-inflammatory response, while other miRNAs (e.g. miR-124, miR-34a, let-7c, miR-132, miR-146a and miR-125a-5p) promote the activation of M2 macrophages and the anti-inflammatory response [16,17]. Several miRNAs are also capable of regulating myeloid cell development, including monocyte to macrophage differentiation. For instance, miR-15, miR-20a and miR-106a target the 3'-UTR of Runx1, a transcription factor that controls expression of colony-stimulating factor-1R (CSF-1R) which promotes differentiation and maturation to the monocyte lineage [19]. Small RNA profiling has identified miRNAs involved in the immune response of fish, such as differential

miRNA expression in immune tissues following pathogen exposure (reviewed in [20]). However, few studies have examined miRNAs involved in fish macrophage differentiation, activation and/or function [21–25].

In fish, leukocytes can be isolated from the anterior (or head) kidney (HK), the main hematopoietic organ in fish, equivalent to the mammalian bone marrow [26–28]. The adhered HK leukocytes (HKLs; which consists of mostly monocytes and macrophages, as opposed to the non-adherent leukocytes which consists of mostly B cells and T cells) are frequently used in fish immunological studies as a monocyte/macrophage model ([26,29–32], to name a few), yet their transcriptome and function during culture remain poorly characterized. Our results show that the morphology and phagocytic ability of adherent HKLs change during culture time, for up to 5 days of culture. Five days of culture was chosen as the HKLs started to form multinucleated bodies beyond this time point and no longer represented macrophage morphology. To more thoroughly characterize this change at the molecular level, we have examined the miRNA expression profile in adherent HKLs from Atlantic salmon as they differentiate during culture time.

3.3. Materials and Methods

3.3.1. Animals

Atlantic salmon were reared in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre in 3,800 L tanks and kept at 12˚C with 95-110% oxygen saturation, using a flow-through seawater system. All procedures in this experiment were approved by Memorial University of Newfoundland's Institutional Animal Care Committee (protocols 18-01-MR and 14-02-MR), based on the guidelines of the Canadian Council of Animal Care. A total of 19 salmon (1.8 kg +/- 0.5 kg SD) were used in this study as follows: 4 individuals were used for morphology analysis and phagocytosis assays (section 3.3.3 and 3.3.4), 4 individuals were used

for both respiratory burst assays (section 3.3.5), 6 individuals were used for miRNA sequencing with 1 individual excluded from analysis (see section 3.3.6 for explanation), and 5 individuals were used for RT-qPCR validation of miRNA results (section 3.3.9). Three of the 5 individuals used for RT-qPCR validation of miRNA sequencing results, were used to examine *marco* and *mhc ii* mRNA expression (methods in Supplementary File 3.1).

3.3.2. Adherent HKL isolation and culture

Adherent HKLs were isolated as previously described, with some modifications [30,33]. Briefly, the head kidney was removed and placed in isolation media: 500 ml of Leibovitz-15 medium (L-15 Gibco, Carlsbad, CA, USA) supplemented with 2.5% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco) and 27.5 mg of heparin (Sigma-Aldrich, St. Louis, MO, USA). The head kidney cells were forced through a 100 µM nylon cell strainer (Thermo-Fisher Scientific, Waltham, MA, USA), placed on a 34/51% Percoll (GE Healthcare, Uppsala, Sweden) gradient (prepared with H_2O and 10X Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich) to ensure an isotonic solution), and centrifuged at 500 x g for 30 min at 4˚C. Following centrifugation, the interface between the 34% and 51% gradient, which contains leukocytes, was collected and washed twice in isolation media at 500 x g for 5 min at 4˚C. The cells were re-suspended in culture media (L-15 supplemented with 5% FBS and 1% penicillin/streptomycin), and viable cells were counted on a hemocytometer using Trypan Blue (Sigma-Aldrich) exclusion. The cells were then seeded in 6-well culture plates (Corning, Corning, NY, USA) at 1 x 10^7 cells (for Giemsa staining) or 3 x 10^7 cells (for RNA extraction) in 2 mL of culture media per well and incubated at 15˚C for 24 h to allow cell adherence. Following the 24 h incubation, cells were washed twice in culture media to remove non-adherent cells, and the media was

replaced with fresh culture media. Media was changed every 48 h thereafter. The cells were cultured for up to 5 days.

3.3.3. Morphology analysis

Twenty-four hours (Day 1) and 120 h (Day 5) after seeding, cells were washed twice with PBS then 1.0 mL of Giemsa stain (Thermo Fisher Scientific) was added directly to the culture plate for 3 minutes. The Giemsa stain was then removed and replaced with 2.0 mL of PBS for 6 min. The cells were then rinsed with PBS until the edges of the well were slightly pink and excess stain was removed. The cells were then air dried and images were taken on an Eclipse Ti-S inverted microscope immediately following air drying. The morphology of the cells was analyzed by counting approximately 200 cells from at least 3 fields of view from each fish and defining the cells as either round (non-spread; no pseudopodia visible) or spread (cells with pseudopodia present). A Chi-Square test was performed to determine if changes in the proportion of round and spread cells in Day 1 vs. the proportion of round and spread cells in Day 5 were significant using GraphPad Prism v 8.0 (GraphPad Software Inc., La Jolla, CA).

3.3.4. Phagocytosis assay

Twenty-four hours (Day 1), 72 h (Day 3) and 120 h (Day 5) after seeding, cells were washed twice in culture media, and 1 µm Fluoresbrite YG microspheres (Polysciences, Warrington, PA, USA) were added at a ratio of approximately 1:30 macrophage:microsphere [29,34]. Twenty-four hours after microsphere addition, the cells were washed twice with culture media, followed by removal from the plate using trypsin-EDTA (0.25%) (Thermo Fisher Scientific) and then re-suspended in 500 µl of fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS) + 1% FBS). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analyzed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA,

USA). FITC positive cells, based on gating using the negative/unstained cells, were identified and the percentage of FITC-positive cells were determined. A one-way ANOVA was performed to determine significant differences between the percentage of phagocytic cells between Day 1, Day 3 and Day 5 cells using GraphPad Prism v 8.0.

3.3.5. Respiratory burst assays

A change in morphology between Day 1 and Day 5 cells was confirmed visually before sampling. To examine the respiratory burst response using flow cytometry, on Day 1 and Day 5 of culture, the culture media was removed and replaced with 500 µl of respiratory burst assay buffer (L-15 media + 1% BSA + 1 mM CaCl2). One microliter of dihydrorhodamine 123 (DHR) (5 mg/ml) was diluted in 1 ml of PBS and 50 µl of the dilution was added to the cells for 15 min. Following DHR addition, 1 µl of 1 mM phorbol myristate acetate (PMA) dissolved in dimethyl sulfoxide (DMSO) was diluted in 1 ml of respiratory burst assay buffer and 125 µl of this solution (or an equal volume of respiratory burst assay buffer containing 0.1% DMSO for a negative control) was added to the cells (i.e. final concentration of 0.185 µM PMA) for 45 min to stimulate ROS production [35]. Cells were removed from the plate using trypsin-EDTA (0.25%), centrifuged for 5 min, 500 x g, at 4 °C and re-suspended in FACS buffer. Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analyzed using BD FACS Diva v7.0 software. The DMSO vehicle control cells were used to define the region of ROS negative cells and based on this gating the FITC positive cells were identified.

To examine the respiratory burst response using the Oroboros (Oroboros Instruments, Innsbruck, Austria), on Day 1 and Day 5, cells were removed from the plate using trypsin-EDTA (0.25%), centrifuged for 5 min, 500 x g, at 4 °C, counted and 2 x 10^6 cells were re-suspended in 50 µL of L-15+FBS. Cells were placed in the 2 ml Oroboros chamber containing L-15+1% FBS

at 15°C for 20-30 min to equilibrate. The rate of ROS production was estimated by measuring extramitochondrial H_2O_2 detected by the green fluorescence sensor of the O2k-Fluo LED2 module (with gain and LED intensity set to 1000 and 500 mV, respectively) with Amplex® UltraRed (10 μmol l^{-1}), horseradish peroxidase (3 U ml^{-1}), and SOD (U ml^{-1}). The ROS signal was calibrated by the addition of H_2O_2 (0.1 μmol l^{-1}) before and after adding cells to the chamber. After 20-30 min, ROS was stimulated by adding 4 μl of 0.1 mM PMA (or the equivalent amount of DMSO for a negative control) to the chamber and the rate of ROS production was recorded in real time using DatLab 7 software (Oroboros Instruments) to obtain the maximum rate (3-15 min) of ROS production in each sample.

Paired Student's T-test was used to determine statistical differences between Day 1 and Day 5 control DMSO samples, between Day 1 and Day 5 PMA samples and between control DMSO and PMA samples in each day, using GraphPad Prism v 8.0.

3.3.6. Total RNA extraction for sequencing

A change in morphology between adherent HKLs at Day 1 and Day 5 in culture was confirmed visually before sampling. Total RNAs were extracted using the mirVana miRNA isolation kit according to the manufacturer's instructions. All RNAs had a 260/280 ratio and a 260/230 ratio greater than 1.8, as determined by NanoDrop spectrophotometry, and tight 18S and 28S ribosomal RNA bands, as determined by a 1% agarose gel electrophoresis. The RNA concentration from Fish 2 was too low to proceed with RNA sequencing and therefore it was excluded from sequencing.

3.3.7. Library preparation and sequencing

Library construction and sequencing analyses were carried out at the Norwegian High-Throughput Sequencing Centre (NSC; Oslo, Norway). The Illumina NEBNext Multiplex Small RNA Library Preparation Kit (New England Biolabs, Inc. Ipswich, MA, USA) was used to construct 10 libraries (five Day 1 samples and five Day 5 samples) with 1 μg total RNA input, according to the manufacturers' protocols. RNAs isolated from the same 10 samples were ligated with 3' and 5' RNA adapters, followed by reverse transcription and PCR enrichment using barcoded RT-primers. The cDNA products were purified using 6% polyacrylamide gels, and size selection of fragments (approximately 145-160 bp) was carried out to enrich for small RNAs. Sequencing was performed on a NextSeq 500 from Illumina, producing 75 bp single-end reads.

3.3.8. Data processing, differential expression analysis and miRNA diversity estimation

FASTQC software was used to check the raw sequence reads to ensure the data was of good quality and size for downstream analysis. The adapter sequences were then removed (trimmed) and size-filtered using the Cutadapt Python Package (v.1.13) to discard reads shorter than 18 nucleotides (nts) or longer than 25 nts (i.e. outside the size range of mature miRNAs) [36]. A second FastQC analysis was performed to assess the quality of the adapter-trimmed and size filtered sequence reads.

The sequence reads were mapped to a reference index consisting of all known mature miRNAs in Atlantic salmon using STAR aligner software (v2.4.2b) [37,38]. The alignment files (BAM format) were further processed in R using the feature Counts function from the Rsubread package to produce count matrices [39]. These count tables were used as input in the R package DESeq2 to test for differential expression of miRNAs [40]. Differentially expressed miRNAs were identified by comparing the Day 1 group (control) to the Day 5 group (n=5 from each

experimental condition). miRNAs were considered to be statistically differentially expressed if they had Benjamini-Hochberg adjusted p-value of ≤ 0.05, basemean readcounts ≥ 20 and $\log_2$ fold-change of ≥ 1 or ≤ -1.

To estimate the miRNA diversity in Day 1 and Day 5 samples, the normalized read counts from the DESeq2 analysis (370 miRNAs) were exported and processed in Microsoft Excel. MicroRNAs with an average normalized read count in Day 1 cells and Day 5 cells of less than 20 were filtered out. The remaining miRNAs were used to develop a pie-chart of the miRNA diversity in Day 1 cells and Day 5 cells.

The normalized data of differentially expressed miRNAs were hierarchically clustered using the Pearson correlation and complete linkage clustering function in Genesis software (Rockville, Maryland, USA). Principal components were calculated using the Singular Value Decomposition method and ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap [41].

3.3.9. RT-qPCR analysis of miRNA expression

A change in morphology between Day 1 and Day 5 adherent HKLs was confirmed visually before sampling. Total RNA was isolated using mirVana miRNA isolation kit, as described in section 4.6. cDNA was synthesized using the miScript II RT Kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions, with 400 ng of total RNA in 20 µl reactions. Each RT-qPCR reaction was composed of 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10× miScript Universal Primer (1 µM final concentration), 2.5 µl specific forward primer (1 µM final concentration), 5 µl RNase-free water (Thermo Fisher Scientific) and 2.5 µl of diluted cDNA template representing 5 ng of input total RNA. The sequences of the mature miRNAs of interest were used as the forward specific primer, while a universal primer, provided by the miScript

SYBR Green PCR Kit (Qiagen), was used as the reverse primer. Three-fold, 5-point standard curves of pooled cDNA were used to assess the quality of all primers, with the exception of miR-155-5p and miR-146a-5p, where a 4-point standard curve was used. Primer sequences, R^2 and amplification efficiencies (how efficiently a template doubles per PCR cycle) can be found in Table 3.1.

To select the normalizers used in this study, several miRNAs that demonstrated stable expression in the RNA sequencing results were tested for stability between Day 1 and Day 5 samples using RT-qPCR [42]. The selected normalizers were expressed stably in our RT-qPCR study (i.e. geometric mean of normalizers' Ct less than 0.1 cycle different for Day 1 and Day 5 groups). RT-qPCR assays for normalizers and miRNAs of interest included a no-template control and were performed in duplicate using a ViiA7 Real-Time PCR system (Applied Biosystems). The PCR program consisted of one cycle of 95 °C for 15 min, and 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s, followed by a final melting point analysis.

Excel was used to determine the relative quantity (RQ) values of each miRNA relative to a calibrator (i.e. the Day 1 sample that showed the lowest expression (highest normalized Ct value: RQ=1) of a given miRNA of interest compared to other Day 1 samples) [43]. Student's T-test was used to determine statistically significant differences between Day 1 and Day 5 samples using GraphPad Prism v 8.0.

3.3.10. In silico predictions of target genes

Target gene predictions were carried out using the target gene prediction software RNAhybrid version 2.2.1 [44]. The mature sequences of the nine differentially expressed miRNAs analyzed by RT-qPCR were tested against a 3´UTR dataset for 3448 genes obtained

Table 3.1. RT-qPCR Primers

miRNA	Primer sequence 5' to 3'[a]	R^2	Amplification efficiency (%)
miR-155-5p[b]	TTAATGCTAATCGTGATAGGGGT	0.999	81.3
miR-146b-5p	TGAGAACTGAAGTCCATAGATGG	0.986	104.6
miR-146a-5p[b]	TGAGAACTGAATTCCATAGATGG	0.989	115.9
miR-126-3p	TCGTACCGTGAGTAATAATGCA	0.984	107.2
miR-150-5p	TCTCCCAATCCTTGTACCAGTG	0.992	113.9
miR-2188-3p	GCTGTGTGAGGTCAGACCTATC	0.982	116.5
miR-139-5p	TCTACAGTGCATGTGTCTCCAGT	0.974	100.9
miR-221-5p	ACCTAGCATACAATGTAGATTTC	0.984	115.4
miR-200ae-3p	TAATACTGCCTGGTAATGATGAT	0.952	82.3
Normalizers			
miR-125a-5p	TCCCTGAGACCCTAACTTGTGA	0.994	115.1
miR-19c-3p	TGTGCAAATCCATGCAAAACTG	0.990	104.1

[a] Mature miRNA sequences were used as forward specific primer, whereas a universal primer was used as a reverse primer. All primers showed no amplification in the no-template controls and generated an amplicon with a single melting peak.
[b] 4-point serial dilution curve was used.

from the Refseq database of GenBank by RNAhybrid. The following parameters were used in the analysis: Helix constraint 2–8, no G:U in seed, and a minimum free energy threshold of ≤−18 kcal/mol. These parameters allowed for only target genes with perfect seed complementarity and high stability site matches from RNA hybrids to be detected.

3.4. Results

3.4.1. Influence of culture time on the morphology, phagocytic ability, reactive oxygen species (ROS) production and macrophage markers in Atlantic salmon adherent HKLs

On Day 1 and Day 5 of culture, Atlantic salmon adherent HKLs were stained with Giemsa and imaged to observe cell morphology. On Day 1, the majority of cells were round (R) with no pseudopodia (i.e. non-spread; Figure 3.1A), while on Day 5, the majority of the cells had pseudopodia present (i.e. spread (S); Figure 3.1B). On Day 1, the cell population consisted of 97.8% round, non-spread cells (range of 97.1% - 98.8%) and 2.2% spread cells (range of 1.2% – 2.9%) while on Day 5, 14.9% of the cells were round and non-spread (range of 10.5% - 21.1%) and 85.1% of the cells were spread (range of 79.0% - 89.5%) (Figure 3.1C). There was a significant change in the porportion of round and spread cells in Day 1 vs. the proportion of round and spread cells in Day 5 ($p<0.0001$).

To determine how the phagocytic ability of HKLs changes from Day 1 to Day 5 of culture, cells were incubated with fluorescent (FITC) beads and, 24 h later, FITC fluorescence was analyzed via flow cytometry. On Day 1, 21.4 ± 3.1% (SE) of the cells were phagocytic; on Day 3, 26.9 ± 2.9% of the cells were phagocytic; and on Day 5, 53.9 ± 6.1% of the cells were phagocytic (Figure 3.1D). There was a significant increase in the percentage of phagocytic cells in cells cultured for 5 days compared to cells cultured for 1 (Tukey's post hoc $p=0.048$) and 3 days

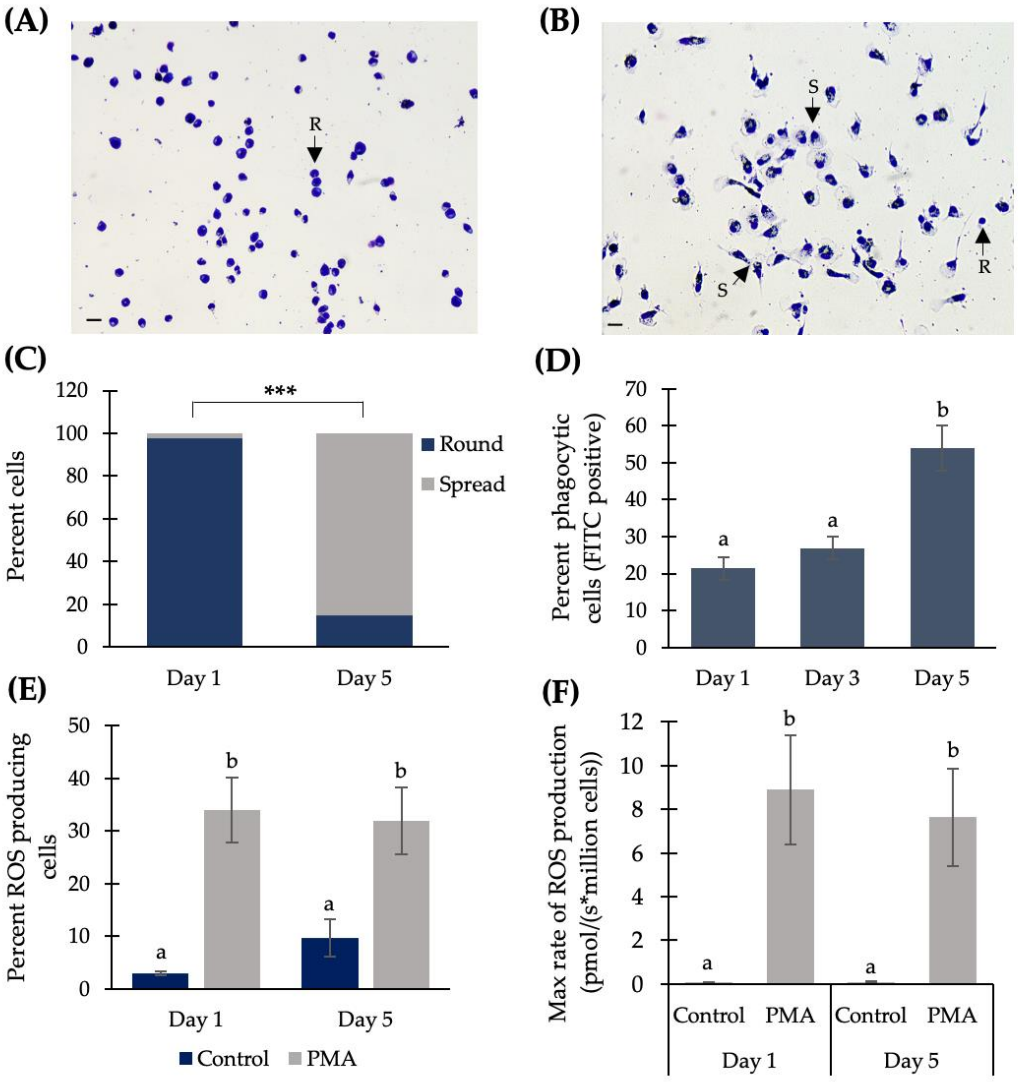

Figure 3.1. Influence of culture time on the morphology and function of Atlantic salmon adherent HKLs. Representative images of Giemsa-stained HKLs cultured for (**A)** 1 day and (**B)** 5 days. Arrows indicate spread cells (S; cells with pseudopodia present) and non-spread, round cells (R; cells with no pseudopodia present) Scale bar on the bottom left of panel A. and panel B. is equal to 20 μm. (**C)** Mean percentage of round vs. spread cells in Day 1 and Day 5 cultures where *** indicates a significant difference of $p<0.0001$ by Chi-square test. (**D)** Percentage of phagocytic HKLs at Day 1 and Day 5 of culture. E. Percentage of HKLs producing reactive oxygen

species (ROS), as determined by flow cytometry. **(F)** Maximum rate of ROS production (pmol/(s*million cells)), as determined by Oroboros respirometry. Day 1 Control value: 0.089 pmol/(s*million cells); Day 5 Control value: 0.105 pmol/(s*million cells). Data shown as mean +/- SE; different lowercase letters indicate a significant difference of $p<0.05$ as determined by a repeated measures one-way ANOVA for phagocytosis data and a paired Student's T-test for ROS data, n=4. PMA: phorbol myristate acetate.

(Tukey's post hoc p=0.015). There was no significant difference in the percentage of phagocytic cells in Day 1 and Day 3 samples (p=0.442).

To determine if the percentage of HKLs producing ROS changed during culture duration, on Day 1 and Day 5, cells were treated with phorbol myristate acetate (PMA) to stimulate ROS production, or dimethylsulfoxide (DMSO) as a negative control, and analyzed via flow cytometry (Figure 3.1E). On Day 1, 2.95 ± 0.39% (SE) of the DMSO negative control cells produced ROS, while 33.98 ± 6.09% of the PMA stimulated cells produced ROS. On Day 5, 9.73 ± 3.52% of the DMSO negative control cells produced ROS, while 32.0 ± 6.35% of the PMA stimulated cells produced ROS (Figure 3.1E). There was a significant increase in the percentage of ROS producing cells between Day 1 DMSO negative control cells and Day 1 PMA stimulated cells (p=0.013), and a significant increase between Day 5 DMSO negative control cells and Day 5 PMA stimulated cells (p=0.008). While there was an overall ~3-fold increase in the percentage of ROS producing cells between the Day 1 (2.95%) and Day 5 (9.73%) for the DMSO negative control conditions, it was not significant (p=0.171). There was also no significant difference in the percentage of ROS producing cells between the Day 1 and Day 5 PMA stimulated conditions (p=0.879).

To determine if the maximum rate of ROS production changes during culture duration, on Day 1 and Day 5, cells were treated with PMA or DMSO, and analyzed via Oroboros respirometry (Figure 3.1F). On Day 1, the maximum rate of ROS production was 0.09 ± 0.02 pmol/(s*million cells) in the DMSO negative control cells, and 8.90 ± 2.51 pmol/(s*million cells) in the PMA stimulated cells. On Day 5, the maximum rate of ROS production was 0.10 ± 0.01 pmol/(s*million cells) in the DMSO negative control cells, and 7.65 ± 2.22 pmol/(s*million cells) in the PMA stimulated cells (Figure 3.1F). There was a significant increase in the maximum rate of ROS

production between Day 1 DMSO negative control cells and Day 1 PMA stimulated cells (p=0.039), and a significant increase between the Day 5 DMSO negative control and Day 5 PMA stimulated cells (p=0.042). There was no significant difference in the maximum rate of ROS production between Day 1 DMSO negative control and Day 5 DMSO negative control cells (p=0.175) and no significant difference in the maximum rate of ROS production between Day 1 PMA stimulated and Day 5 PMA stimulated cells (p=0.097).

To provide further confirmation that the HKLs become more macrophage-like during culture time, we examined changes in mRNA expression of two macrophage markers, macrophage receptor with collagenous structure (*marco*) and major histocompatibility complex II (*mhc ii*) [10,45–50] in the samples used for miRNA RT-qPCR validation. There was a significant increase in the mRNA expression of both marco and MHC II in Day 5 HKLs compared to Day 1 HKLs (see Supplementary File 3.1 for information on this experiment).

3.4.2. Library preparation, deep sequencing, miRNA diversity estimation and differential expression analysis of small RNA sequence data

The total number of reads obtained from sequencing the small RNA libraries ranged from 8.3 million to 29.3 million. Following trimming and size filtering, the percentage of reads that mapped to the Atlantic salmon miRNAome ranged from 76.6% to 95.4% (5.5 million reads to 7.2 million reads) (Figure 3.2; Table 3.2) [37]. All deep sequencing reads have been submitted to the NCBI Sequence Read Archive (SRA) database (see accession numbers in Table 3.2).

Analysis of miRNA diversity and abundance showed that 370 out of 589 known mature Atlantic salmon miRNAs [37] are expressed in the Day 1 monocyte-like HKLs and Day 5 macrophage-like HKLs (Figure 3.3A). None of these were exclusively expressed in Day 1 cells or Day 5 cells. The top 20 most abundant miRNAs in both Day 1 and Day 5 HKLs are shown in

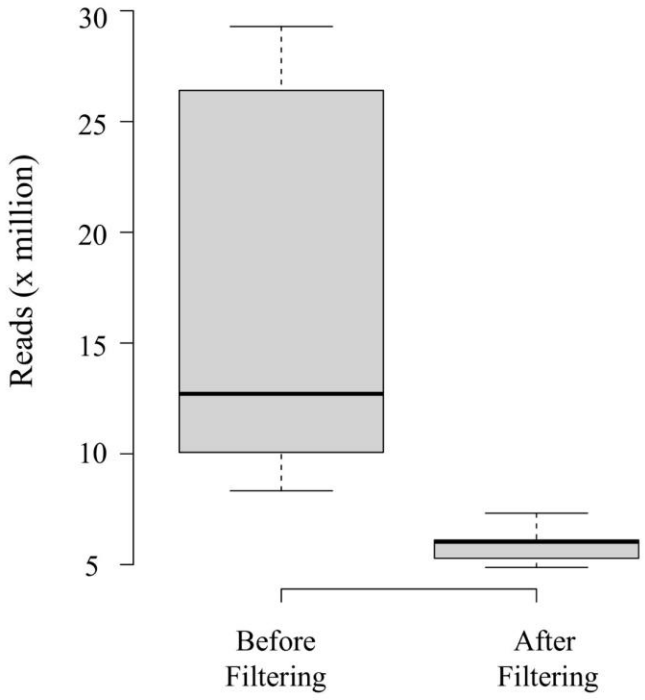

Figure 3.2. Total number of reads before and after trimming and size filtering. Box plot created using BoxPlotR: a web-tool for generation of box plots [51].

Table 3.2. Sequencing results

Sample	Total number of reads [a]	Trimmed and filtered reads [b]	Reads mapped to miRNA (%) [c]	Accession number [d]
Fish 1 Day 1	10,954,203	6,089,270	87.9%	SRR9710703
Fish 1 Day 5	14,469,109	7,317,802	95.4%	SRR9710704
Fish 3 Day 1[e]	27,215,751	5,019,027	76.6%	SRR9710705
Fish 3 Day 5	19,158,159	6,104,257	88.3%	SRR9710706
Fish 4 Day 1	29,288,867	5,521,671	77.6%	SRR9710709
Fish 4 Day 5	26,403,552	6,365,057	81.9%	SRR9710710
Fish 5 Day 1	10,711,291	6,013,861	78.8%	SRR9710711
Fish 5 Day 5	8,325,813	4,870,035	79.6%	SRR9710712
Fish 6 Day 1	10,064,941	5,282,051	80.5%	SRR9710707
Fish 6 Day 5	8,760,222	6,032,420	87.1%	SRR9710708

[a] Total number of reads in raw fastq file.
[b] Total number of reads after removing adaptors and filtering reads by size.
[c] Reads mapped to reference index (known miRNAs of Atlantic salmon) [37].
[d] Accession number of sequencing results for each sample submitted to NCBI's SRA database.
[e] See Materials and Methods (section 4.6) for an explanation for the exclusion of Fish 2.

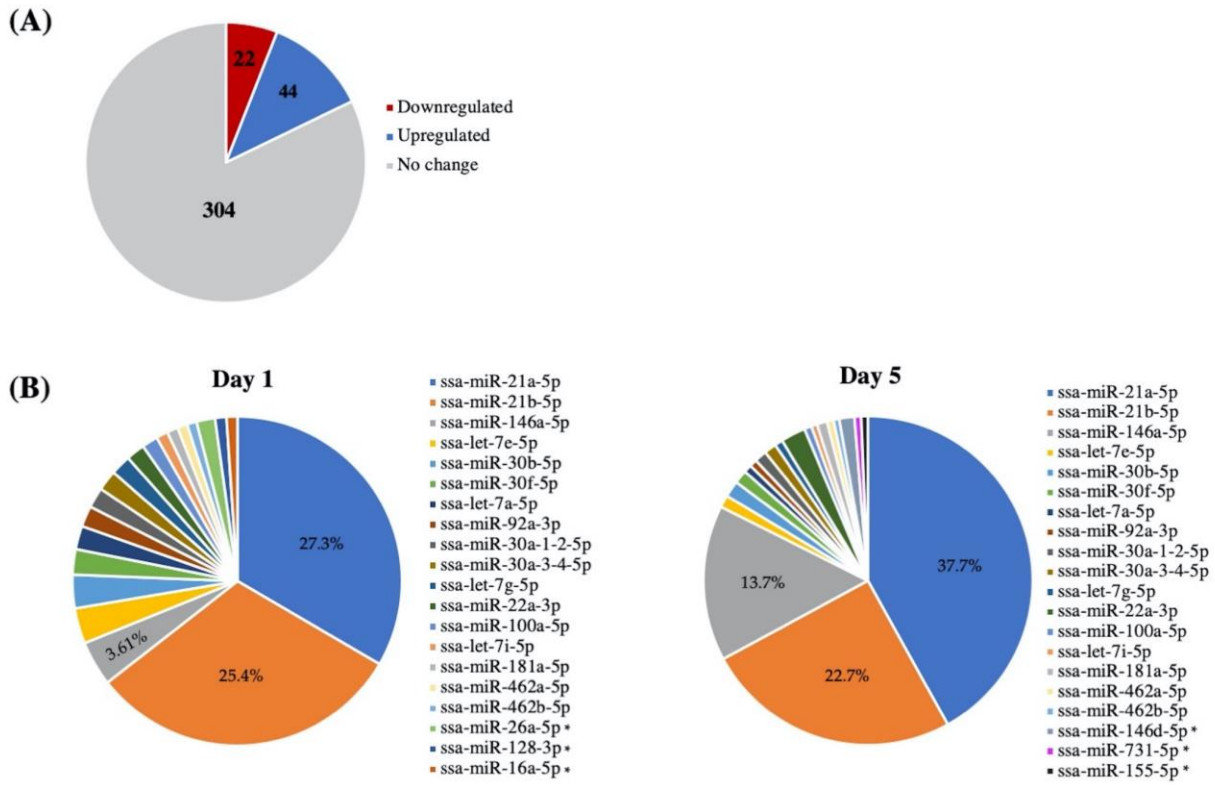

Figure 3.3. miRNA expression in Day 1 and Day 5 Atlantic salmon adherent HKLs. (A) Total number of miRNAs with downregulated expression, upregulated expression or no change in expression in Day 5 HKLs compared to Day 1 HKLs. **(B)** miRNA expression and diversity (average normalized read counts of 370 miRNAs). The top 20 most abundant miRNAs in Day 1 and Day 5 are indicated. * indicates miRNAs present in the top 20 of one day but not the other day.

Figure 3.3B, while the abundance of all 370 miRNAs can be found in Supplementary Table 3.1. Three mature miRNAs, miR-21a-5p, miR-21b-5p and miR-146 a-5p, predominated in both Day 1 and Day 5 HKLs, composing 56.3% of all miRNAs expressed in Day 1 cells and 74.1% of all miRNAs in Day 5 cells. While miR-21a-5p and miR-146a-5p were among the highest expressed miRNAs in both days, there was an obvious increase in the proportion of both miRNAs in Day 5 cells compared to Day 1 cells. To further investigate changes in expression of any of the identified miRNAs we carried out differential expression analysis of the small RNA sequenced samples from Day 1 vs. Day 5 cells.

Sixty-six miRNAs were found to be differentially expressed (DE) between Day 1 and Day 5 cells. However, a large number of these DE miRNAs were major and minor mature miRNAs from the same precursor or miRNAs from the same families. Thus, there were only 40 different miRNA families represented in the DE miRNAs. Twenty-two miRNAs from 15 miRNA families were downregulated in Day 5 HKLs compared to Day 1 HKLs while 44 miRNAs from 25 families were upregulated in Day 5 HKLs compared to Day 1 HKLs (Figure 3.3A). miR-21a-5p, miR-146a-5p, miR-22a-3p, miR-181a-5p, miR-26a-5p, miR-462a-5p and miR-462b-5p were among the top 20 most abundant miRNAs that also demonstrated differential expression between Day 1 and Day 5 cells. Principal component analysis (PCA) of the DE miRNAs grouped Day 1 samples and Day 5 samples separately (Figure 3.4A). PC1 accounted for 60.3% of the variance while PC2 accounted for 16.1% of the variance. Day 1 samples showed a positive loading on PC1, where are Day 5 samples showed a negative loading on PC1. There was a near split between positive and negative loading on PC2 for both Day 1 and Day 5 samples. Similarly, hierarchical clustering of the DE miRNAs showed that all Day 5 samples clustered separately from all Day 1 samples (Figure 3.4B).

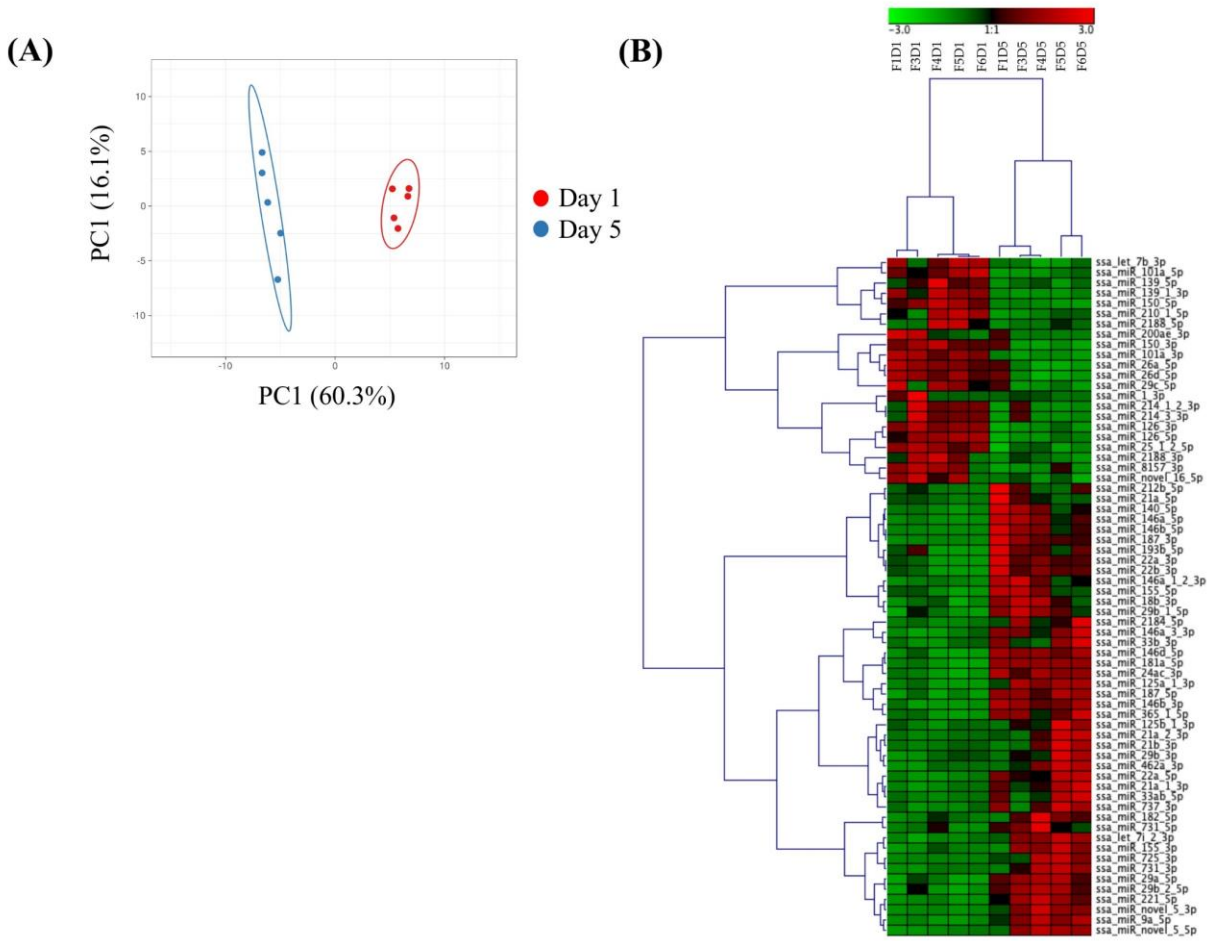

Figure 3.4. Results from (A) Principal component analysis (PCA) and (B) hierarchical clustering of DE miRNAs from Day 1 and Day 5 HKLs. (A) PCA analysis based on DE miRNAs. Day 5 samples are represented by blue, Day 1 samples are represented by red. The X and Y axis show principal component 1 (PC1) and principal component 2 (PC2) that explain 60.3% and 16.1% of the total variance, respectively. **(B)** DE miRNAs counts per million were normalized and clustered using Pearson correlation and complete linkage hierarchical clustering and shown as a heatmap. F indicates fish number; D indicates Day 1 or Day 5 (for example F1D1 is Fish 1 Day 1).

Supplementary Table 3.2 gives a complete summary of the DESeq2 analysis results including the fold-change and mature sequence of each DE miRNA.

3.4.3. RT-qPCR validation of DESeq2-identified miRNAs

Nine DE miRNAs that were the major expressed mature miRNA in their families were chosen for RT-qPCR validation. The chosen miRNAs were a combination of upregulated and downregulated miRNAs in the DESeq2 analysis and are known immune or macrophage related miRNAs in mammalian and/or fish literature [16,17,20,52,53]. Three miRNAs were validated as significantly upregulated in Day 5 cells compared to Day 1 cells (Figure 3.5A-C), while four miRNAs were validated as significantly downregulated in Day 5 cells compared to Day 1 cells (Figure 3.5E-H). The expression levels of two miRNAs (miR-221, p=0.67; miR-200ae, p=0.06) were not found to be significantly different by RT-qPCR analysis, however, they followed the same trend as the sequencing results (Figure 3.5D, 3.5I). Table 3.3 shows a comparison of the sequencing and the RT-qPCR results of the significantly DE miRNAs. In summary, the RT-qPCR experiment (which analyzed HKL RNA from a different set of Atlantic salmon than the sequencing experiment) supported the differential expression analysis findings of the small RNA sequencing study.

3.4.4. In silico target gene predictions

The nine RT-qPCR validated miRNAs, representing the major expressed mature miRNAs from their families, were used as input for target gene predication by in silico analysis against the 3'UTRs from all Atlantic salmon transcripts in the NCBI Reference Sequence database (https://www.ncbi.nlm.nih.gov/refseq). A total of 771 genes were predicted as putative targets of one or more of the DE miRNAs (Supplementary Table 3.3). Predicted targets selected for discussion can be found in Table 3.4. miR-126-3p had the lowest number of predicted targets (4),

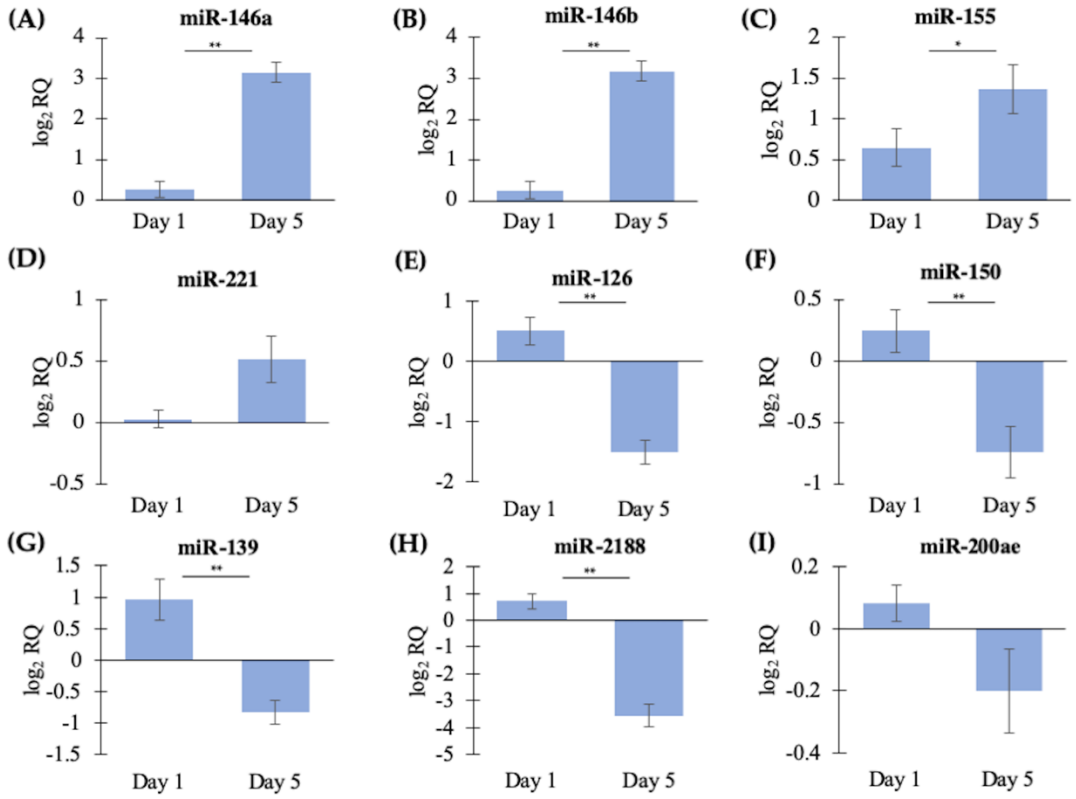

Figure 3.5. RT-qPCR analysis of DE miRNAs identified by small RNA sequencing. Data shown as mean $\log_2$ relative quantity (RQ) ± SE., n=5. Significance determined by a Student's T-test, * $p<0.05$, ** $p<0.01$. **(A)** miR-146a **(B)** miR-146b **(C)** miR-155 **(D)** miR-221 **(E)** miR-126 **(F)** miR-150 **(G)** miR-139 **(H)** miR-2188 **(I)** miR-200ae

Table 3.3. Differentially expressed miRNAs identified by sequencing and validated by RT-qPCR

	Sequencing			RT-qPCR	
	Base mean[a]	log_2 FC[b]	Adjusted p-value[c]	log_2 FC[d]	p-value[e]
miR-146a-5p	737973.84	2.91	1.02e-11	10.99	2.00e-04
miR-155-5p	38126.56	1.39	5.81e-03	2.09	3.01e-02
miR-146b-5p	15429.78	4.03	3.76e-17	12.88	1.00e-04
miR-126-3p	11661.74	-1.52	3.51e-06	-2.99	2.00e-04
miR-150-5p	3140.46	-1.52	1.28e-08	-2.97	2.00e-04
miR-2188-3p	1269.71	-1.49	8.23e-03	-5.00	2.00e-04
miR-139-5p	643.30	-1.00	1.78e-02	-0.87	5.30e-03

[a] The mean of normalized read counts for all of the samples.

[b] Log_2 transformed fold-change (FC) (Day 5/Day 1) as determined by DESeq2 analysis.

[c] Benjamini-Hochberg p-value.

[d] Log_2 fold-change (FC).

[f] Student's paired T-test p-value.

Table 3.4. Selected predicted miRNA targets

	miRNA	Predicted target mRNA (gene symbol)*	Predicted target mRNA (gene name)
Lipid-related	ssa-miR-139-5p	*srebf1*	Sterol regulatory element-binding protein 1
	ssa-miR-139-5p	*srebf2*	Sterol regulatory element-binding protein 2
	ssa-miR-139-5p	*elvol5a*	Elongation of very long chain fatty acids protein 5
	ssa-miR-155-5p	*elvol5a*	Elongation of very long chain fatty acids protein 5
	ssa-miR-200ae-3p	*elovl7*	Elongation of very long chain fatty acids protein 7
	ssa-miR-221-5p	*elovl5*	Elongation of very long chain fatty acids protein 5
	ssa-miR-221-5p	*facr1*	Fatty acyl-CoA reductase
Transcription Factors	ssa-miR-146a-5p	*gata3*	transcription factor GATA-3
	ssa-miR-146b-5p	*gata3*	transcription factor GATA-3
	ssa-miR-200ae-3p	*irf5*	Interferon regulatory factor 5
	ssa-miR-200ae-3p	*cebpb*	CCAAT/enhancer-binding protein beta
	ssa-miR-2188-3p	*cebpa*	CCAAT/enhancer-binding protein alpha
Immune/ macrophage related	ssa-miR-126-3p	*i13r2*	Interleukin-13 receptor alpha-2 chain
	ssa-miR-150-5p	*lgmn*	Legumain
	ssa-miR-139-5p	*tnr1a*	Tumor necrosis factor receptor superfamily member 1A
	ssa-miR-155-5p	*tnr1a*	Tumor necrosis factor receptor superfamily member 1A
	ssa-miR-155-5p	*grn*	Granulin
	ssa-miR-155-5p	*ccl25*	C-C motif chemokine 25
	ssa-miR-200ae-3p	*ccr6*	C-C chemokine receptor type 6
	ssa-miR-2188-3p	*tnr1a*	Tumor necrosis factor receptor superfamily member 1A

*Full details found in Supplementary Table 3.3

while miR-2188-3p had the highest number of predicted targets (239). Many of the potential targets are known to play a role in macrophage differentiation and/or function in other species. For example, transcripts encoding transcription factors (e.g. SREBPs, IRF5) and cytokines (e.g. TNF-α) were identified as predicted targets of one or more of the DE miRNAs (see section 3.3 for a more thorough discussion on the potential targets).

3.5. Discussion

Aquaculture production in Canada has increased four-fold since the early 1990s. Atlantic salmon is the top aquaculture export in Canada and is of significant economic value. Therefore, elucidating how their immune cells develop and function is a key step toward better understanding their immune system and, therefore, improving our ability to maintain healthy farmed salmon. The current study identified a change in the morphology of Atlantic salmon adherent HKLs from Day 1 (predominantly rounded, i.e. monocyte-like) to Day 5 (predominantly spread with pseudopodia present, i.e. macrophage-like) in culture, suggesting that the cells are differentiating over time. In addition to analyzing the morphology of Day 1 and Day 5 HKLs, we also analyzed phagocytosis and ROS production, both functions of macrophages. There was a higher percentage of phagocytic cells in the Day 5 culture compared to the Day 1 culture, supporting the hypothesis that these cells are differentiating into macrophages during culture duration. A change in morphology and phagocytic ability has also been observed in common carp (*Cyprinus carpio* L.) and goldfish HKLs during culture duration [26,54]. However, there was no change in ROS production between Day 1 and Day 5 HKLs. It is important to note that PMA is used to differentiate monocytes to macrophages in several mammalian models [55–57] and is a strong inducer of ROS production. Thus, it is possible that using such a strong inducer may have masked any differences in ROS production between the two cell populations. Finally, there was a significant increase in mRNA

expression of two macrophage markers, *marco* and *mhc ii* in Day 5 HKLs compared to Day 1 HKLs (Supplementary File 3.1).

Studies in other species have shown that miRNAs are important regulators of macrophage differentiation and polarization [58]. We hypothesized that Atlantic salmon monocyte-like and macrophage-like HKLs would have differences in their miRNA complement and expression which likely have important roles in macrophage differentiation; we used RNA-seq and RT-qPCR based miRNA identification and expression analyses of cultured salmon HK monocytes/macrophages to test this hypothesis. We sought to validate the sequencing results by using a different miRNA detection method (RT-qPCR) and new sample material. In general, there was good agreement between the smaller set of 9 miRNAs analyzed by RT-qPCR and the sequencing for those miRNAs, which provided support for the sequencing results and DESeq2 analysis. Identifying changes in miRNA expression throughout culture time will help reveal what miRNAs may have important functions in these cells, as well as what miRNAs are likely involved in regulating their differentiation and function.

3.5.1. miRNA diversity and abundance in Atlantic salmon adherent HKLs

miRNA diversity and abundance in Atlantic salmon adherent HKLs have never been reported. MicroRNAs with high abundance and ubiquitous expression in a cell population may play a role in function and in maintaining important lineage-specific gene regulation. This study identified 370 miRNAs in Atlantic salmon adherent HKLs (Figure 3.3 and Supplementary Table 3.1). Two miRNAs of the miR-21 family (ssa-miR-21a-5p and ssa-miR-21b-5p) were found to be the top two most abundant miRNAs in both Day 1 (27.3% and 25.4%) and Day 5 (37.7% and 22.7%) HKLs, while ssa-miR-146a-5p was the third most abundant miRNA in both Day 1 (3.61%) and Day 5 (13.7%) cells. Together, these miRNAs compose over half of all miRNAs in both Day 1

and Day 5 cells, suggesting that they have important functions in Atlantic salmon monocytes and macrophages.

A study by Woldemariam et al. (2019) identified the top 10 most abundant miRNAs across several tissues in Atlantic salmon, which accounted for more than 30% of the miRNAs expressed in any tissue [37]. Two of the top 20 most abundant miRNAs identified in this current study (ssa-miR-21b-5p, ssa-miR-26a-5p) were among the top 10 most abundant miRNAs in the ten tissues investigated, suggesting that these miRNAs are constitutively highly expressed and may have housekeeping functions. In addition, ssa-miR-21b-5p, ssa-miR-21a-5p and ssa-miR-146a-5p were among the most abundant miRNAs in HK tissue [37]. Interestingly, the proportion of these miRNAs was more pronounced in the isolated adherent HK cells of this current study, compared to the HK tissue in Woldemariam et al. (2019) [37]. This is likely due to the higher number of different cell types within the HK organ, compared to the isolated and cultured HKLs. The miR-21 family was also found to be among the most abundant miRNAs in the HK and spleen of Atlantic salmon that were infected with *Piscirickettsia salmonis*, as well as the control group that was not infected [59].

In other species, miR-21 is involved in macrophage differentiation, immune response and polarization suggesting that this miRNA may have more than just housekeeping roles in leukocytes. For example, in both humans and mice, miR-21 promotes the differentiation of granulocyte-macrophage progenitors (GMP) into monocytes [58,60]. Barnett et al. (2016) found that overexpression of miR-21 in mouse LPS treated peritoneal macrophages decreased expression of the inflammatory cytokine TNF-α, while suppression of miR-21 increased TNF-α and IL-6 [61]. Wang et al. (2015) found that miR-21 impaired the expression of the M2 macrophage markers arginase and resistin-like molecule alpha (*Retnla*) while enhancing the expression of the M1

markers TNF-α and IL-1β [62]. Interestingly, there is an IRF8-transcription factor binding motif upstream of all of the Atlantic salmon miRNA-21 family genes [52]. This transcription factor is known to induce transcription of genes important in murine monocyte/macrophage differentiation [63]. Similarly, miR-146a, the third most abundant miRNA in this study, is also involved in macrophage differentiation and function in other vertebrates [17,64–67] (see section 3.2 for discussion on miR-146a).

3.5.2. Expression analysis identified DE miRNAs known to be involved in macrophage function or differentiation in other species and/or immune function in Atlantic salmon

In addition to characterizing the miRNAs in Day 1 and Day 5 adherent HKLs, this study identified and confirmed miRNAs that are differentially expressed in these cell populations. MicroRNAs that are differentially expressed between Day 1 cells (monocyte-like cells) and Day 5 cells (macrophage-like) are likely involved in HK monocyte-macrophage differentiation and/or function via gene regulation. Many of the miRNAs identified in this study are known to be involved in macrophage function and/or differentiation in other species. In addition, several miRNAs only identified in Atlantic salmon [37], were also identified as differentially expressed between Day 1 and Day 5 cells.

With respect to ssa-miR-146a-5p, along with being the third most abundant miRNA in both Day 1 and Day 5 cells, there was also a large increase in the proportion of ssa-miR-146a-5p in Day 5 cells (13.7%) compared to Day 1 cells (3.6%). The increase in expression of ssa-miR-146a-5p in Day 5 cells was identified by both the miRNA-seq and the RT-qPCR analyses. Ssa-miR-146a and ssa-miR-146b (also differentially expressed in this study) are involved in macrophage differentiation, polarization and inflammatory response in other species. For example, ectopic expression of miR-146a can drive maturation of human hematopoietic stem cells (HSCs) to

peritoneal macrophages during adult hematopoiesis in vivo, while knockdown of miR-146a diminished macrophage formation in early zebrafish (*Danio rerio*) embryos [64]. In addition, PU.1, a transcription factor that favors differentiation of GMPs to the monocyte lineage, induces the expression of a subset of miRNAs, including miR-146a. miR146a may also have a role in macrophage polarization; downregulation of miR-146a was found in M1-polarized murine bone marrow derived macrophages (BMDMs) compared to M2-polarized macrophages, while in human peripheral blood mononuclear cells (PBMCs), miR146a was upregulated in M1 macrophages compared to M2 macrophages [18,53]. miR-146b was found to be upregulated in M2c human PBMCs [53]. In addition, both miR-146a and miR-146b have been identified as inflammation-sensitive miRNAs as both are LPS-responsive [66]. Inhibition of miR-146a upregulates M1 associated genes in LPS-stimulated RAW macrophages and decreases M2 associated genes, while over-expression of miR-146a increases the expression of anti-inflammatory IL-10 and Arginase-1, markers of M2 macrophages [67]. In several fish species, miR-146 expression changes in various tissues in response to bacterial and viral challenge. For example, both ssa-miR-146a and ssa-miR-146b expression increased in cardiac tissue of Atlantic salmon following salmonid alphavirus (SAV) infection [52]. Similarly, ssa-miR-146a expression increased in the head kidney of Atlantic salmon intraperitoneally injected with poly(I:C) or formalin-killed *Aeromonas salmonicida* compared to a saline-injected control group [68]. In grouper macrophages, miR-146a was significantly upregulated following infection with Singapore grouper iridovirus (SGIV), while inhibition of miR146a in these macrophages had an anti-viral effect, decreasing SGIV replication [24]. The results presented here show an increase in the proportion of miR-146a and miR-146b in Day 5 macrophage-like cells compared to Day 1 monocyte-like cells, suggesting that these cells are differentiating (or have differentiated) toward the macrophage lineage. In addition, the data

presented here also suggest that ssa-miR-146a and ssa-miR-146b may have similar functions in Atlantic salmon as other species; however, functional studies, as well as specific M1/M2 gene expression studies, are required to test this hypothesis.

miR-155 is also involved in macrophage differentiation and function in other species. For example, miR-155 is, like miR-146a and miR-146b, induced by PU.1, suggesting it has a role in myeloid cell differentiation [64]. In addition, a study by Mann et al. (2010) demonstrated that commitment of the mouse monocyte cell line RAW264.7 to active macrophages involves the upregulation of miR-155 expression [69]. Infection of mammalian macrophages with *Listeria monocytogenes* or *Mycobacterium avium* upregulated miR-155 expression, as well as miR-146a and miR-146b expression, suggesting their involvement in regulating the response to bacterial infections [70,71]. Likewise, miR-155 expression increased in response to both LPS and poly(I:C) [66,72]. An in vitro study in the teleost fish species ayu (*Plecoglossus altivelis*) found that infection of ayu macrophages with *Vibrio anguillarum* increased the levels of miR-155. Furthermore, overexpression of miR-155 in the *V. anguillarum* infected macrophages enhanced the expression of pro-inflammatory cytokines (IL-1β, TNF-α) and decreased the expression of anti-inflammatory cytokines (IL-10, TGF-β) compared to the control, while inhibition of miR155 had the opposite effect [22]. In addition, miR-155 promoted M1-type polarization and inhibited M2-type polarization, suggesting that, similar to higher vertebrates, miR-155 may play a role in macrophage function and polarization in this fish species [22].

There is little known of the involvement of miR-150 and miR-126 inmonocyte/macrophage differentiation and function. In mammals, miR-150 plays a central role in B-cell development, where it is expressed in mature lymphocytes but not their progenitors [73]. miR-150 was identified as differentially regulated during the maturation of human

monocytes into macrophages, where it was downregulated in macrophages compared to monocytes [53]. miR-150 expression was also decreased in LPS stimulated murine BMDMs, which negatively correlated with PU.1 transcript expression, suggesting that miR-150 is inflammation responsive and may also interact with PU.1 [74]. Luciferase assays confirmed that miR-150 directly targeted the PU.1 transcript. Furthermore, overexpression of miR-150 in murine BMDMs significantly reduced PU.1 transcript expression and shifted polarization away from M1 [74]. These results suggest that miR-150 can alter macrophage activation and inflammatory response. The role and function of miR-150 in teleost macrophages remains to be determined. We found a decrease in ssa-miR-150 expression in Day 5 macrophage-like HKLs compared to Day 1 monocyte-like cells, suggesting that if ssa-miR-150 has the same role in Atlantic salmon as it does in other vertebrates, then both the expression changes and the morphology analysis indicate that the HKLs become more macrophage-like and less monocyte-like during culture time. miR-126, along with a subset of miRNAs, is enriched in human and murine HSCs, compared to other cells in the bone marrow, and its expression is decreased in differentiated cells of the lymphoid and myeloid lineages, as determined by a bidirectional miR-126 reporter vector [75]. Likewise, knockdown of miR-126 increased mouse and human HSC proliferation, while overexpression of miR-126 increased HSC quiescence [76]. In fish, miR-126 is involved with vascular, oocyte and early embryo development, however it is unknown if it plays a role in monocyte/macrophage differentiation and/or function [77–80]. The results of the current study found higher expression of ssa-miR-126 in Day 1 cells compared to Day 5 cells, suggesting that, like ssa-miR-150, if ssa-miR-126 plays a similar role in Atlantic salmon as it does it mammals, then the Day 1 HKL population consists mostly of early, undifferentiated

myeloid cells, such as monocytes, while the Day 5 HKL population consists mostly of differentiated myeloid cells, such as macrophages. The
role of both ssa-miR-150 and ssa-miR-126 in Atlantic salmon monocyte/macrophage differentiation and function warrants further investigation.

miRNA-2188, miRNA-462 and miR-731 are teleost-specific miRNAs that have not yet been discovered in mammals and are associated with immune responses in fish including Atlantic salmon [20,52]. In the olive flounder and in the Atlantic salmon, miR-2188 expression decreased in the head kidney following viral hemorrhagic septicemia infection and in cardiac tissue following salmonid alphavirus infection, respectively [52,81]. Conversely, miR-2188 expression increased in Atlantic cod macrophages in response to poly(I:C) stimulation at 48 h and 72 h post-stimulation [23]. Interestingly, miR-2188 was significantly downregulated in unstimulated cod macrophages at 72 hours compared to cells cultured for 12 and 24 hours [23]. Similar to the cod macrophages, we saw a decrease in miR-2188 expression after 5 days of culture. Therefore, it is possible that miR-2188 plays a conserved role in teleost fish monocyte-like and macrophage-like cells. DESeq2 analysis of miRNA-seq data herein showed that three novel Atlantic salmon miRNAs [37] were differentially expressed between Day 5 and Day 1 cells (e.g. ssa-miR-novel-5-3p and ssa-miR-novel-5-5p were upregulated in Day 5 cells; ssa-miR-novel-16-5p was downregulated in Day 5 cells). Novel miRNAs may be species-specific as they are absent or have not been identified in other species including higher vertebrates; this suggests that some aspects of miRNA roles in Atlantic salmon macrophage differentiation may be species-specific.

3.5.3. In silico target prediction identified potential targets involved in macrophage differentiation and function in other species

The potential target genes of the nine RT-qPCR validated DE miRNAs were identified as they represent the major expressed mature miRNAs from eight of the 40 different families. Applying such predictions on these validated DE miRNAs could reveal whether they could target genes relevant to macrophage function and differentiation. A total of 771 genes were predicted as putative targets for one or more of the DE miRNAs, with a range of 4 to 239 target genes per miRNA (Supplementary Figure 3.3). Several of the targets are known to be involved in macrophage activation, immune response and cell differentiation. In mammals, the lipidomic and transcriptomic profiles change profoundly during macrophage differentiation and lipid metabolism plays a key role in macrophage activation and function [82,83]. Potential targets of the DE miRNAs identified and validated in this study include various lipid related transcripts such as sterol regulatory element binding proteins 1 and 2 (SCREBP1 and SCREBP2 (also known as SCREBF1 and SCREBF2); potential targets of ssa-miR-139-5p and ssa-miR-200ae-3p), and elongation of very long chain fatty acids protein 5 and 7 (ELOVL5 (also known as ELVOL5a), ELVOL7; potential targets of ssa-miR-139-5p, ssa-miR-155-5p, ssa-miR-200ae-3p and ssa-miR-221-5p). SREBPs are key transcription factors in the synthesis of fatty acids and cholesterol. An increased expression of srebp-1a following LPS simulation was demonstrated in mouse macrophages, while macrophages from mice with a SREBP-1a deficiency were unable to induce lipid biosynthesis in response to LPS. They also displayed a decreased level of Il-1β cytokine secretion [84]. In addition, Ecker et al. (2010), demonstrated that a SREBP1 dependent induction of human monocyte fatty acid synthesis is vital for monocyte-macrophage differentiation, while Lee et al. (2018) determined that phagocytosis is impaired in cells that lack a key SREBP isoform [85,86]. In monocyte-macrophage differentiation of a human cell line,

elovl5 mRNA expression was strongly induced while fatty acyl-CoA reductase (a potential target of ssa-miR-221-5p) mRNA expression was downregulated [82].

Several transcription factors with known functions in macrophage M1/M2 polarization were identified as potential miRNA targets. Interferon regulatory factor 5 (IRF5) was identified as a putative target for ssa-miR-200ae-3p. In mammalian macrophages, IRF5 is a regulator of M1 macrophage polarization; M1 macrophages have higher mRNA and protein expression of IRF5 compared to M2 macrophages [87,88]. Forced expression of IRF5 in human M2 macrophages strongly induced the mRNA expression of M1-specific cytokines, while reducing the mRNA expression of the anti-inflammatory cytokine IL-10. Conversely, knockdown of IRF5 in M1 macrophages inhibited the LPS induced expression of pro-inflammatory cytokines [87]. In common carp, *irf5* mRNA expression increased in several immune tissues, including the HK, following poly(I:C) challenge, suggesting it plays a role in regulating immune response in fish [89]. C/EBPα (a potential target of ssa-miR-2188-3p) and C/EBPβ (a potential target of ssa-miR-200ae-ep) are also important transcription factors in regulating polarization of M1/M2 macrophages [90,91]. Macrophages from C/EBPα deficient mice exhibited a decreased expression of M1 and M2 markers following LPS and IL-4 stimulation, respectively, suggesting that C/EBPα plays a role in both M1 and M2 polarization [92]. Similarly, in LPS/IFN-γ stimulated mouse macrophages, impaired C/EBPβ expression was associated with the suppression of M2 markers, while M1 markers were unaffected [93]. In mammalian macrophages, C/EBPβ is a direct target of mir-155 [94]. GATA3, which was identified as a target of both ssa-miR-146a-5p and ssa-mir-146b-5p in the current study, has been associated with M2 polarization in mouse macrophages [95]. Treatment of mouse monocytes with gata3 shRNA decreased the expression of M2 markers (Arg-1, IL-4), while forced expression of gata3

downregulated the expression of M1 markers (TNF-α, MCP-1, CD206) but induced the expression of M2 markers (Arg1, IL-1, iNOS) [95].

Transcripts encoding several cytokines, chemokines and other inflammatory and macrophage related proteins were also identified as potential miRNA targets in the current study. For example, transcript encoding tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) was identified as a potential target of ssa-miR-139-5p, ssa-miR-155-5p and ssa-miR-2188-3p. TNFRSF1A is one of the major receptors for TNFα (a potential target of ssa-miR139-5p) which is produced primarily by monocytes and macrophages and plays a role in critical cell processes including inflammation and differentiation [96–98]. In fish, *in vitro* treatment of primary rainbow trout (*Oncorhynchus mykiss*) HKLs with TNFα induced expression of several inflammatory genes, including *il1b*, *il8*, *il17c*, *tnfa* and *cox2* and enhanced phagocytic activity [99]. TNF-α also plays a role in macrophage differentiation. An increase in tnfa gene expression is observed during differentiation of BMDMs [100]. Blocking the increase in tnfa expression by using antisense oligomers prevented macrophage differentiation, causing the cells to proliferate instead [100]. In the current study, the transcript encoding the protease Legumain was identified as a potential target of ssa-miR-150-5p. Legumain expression and secretion is increased during human monocyte-macrophage differentiation, with M2 macrophages expressing significantly higher mRNA levels and secretion of Legumain than M1 macrophages [101]. In goldfish HKLs, the mRNA expression of legumain was highest in mature macrophages, compared to early progenitor and monocyte populations [102]. In this study, ssa-miR-150 expression was lower in Day 5 cells compared to Day 1 cells, which would be expected if Legumain transcript is a target of miR-150. Also in goldfish HKLs, Granulin transcript, a potential target of ssa-miR-155-5p in the current study, is more highly expressed in monocytes

compared to early progenitor cells and mature macrophages [102]. Macrophages isolated from CCR6 (a potential target of ssa-miR-200ae-3p) deficient mice had lower levels of inflammatory cytokines following LPS stimulation, compared to WT macrophages [103]. In addition, there was significantly higher mRNA expression of CCR6 in human M1 macrophages than in M2 macrophages, while CCL25 (a target of ssa-miR-155-5p) induced chemotaxis of M1 macrophages [104].

Taken together, the results of this *in silico* target prediction analysis with the RT-qPCR validated miRNAs suggest that many of the DE miRNAs in this study may target genes that are involved in macrophage differentiation, function and immune response, similar to other species. It is important to note that there are often many false positives identified during target prediction. This is in part due to the lack of consistency in target prediction tools, and that the rules for governing miRNA target recognition are not fully understood, and so can vary for each miRNA-target prediction [20,105,106]. In addition, we only examined target miRNAs from a small subset of DE miRNAs. Validation studies, including overexpression and/or knockdown studies, are required to confirm that a miRNA-target interaction is authentic.

3.6. Conclusions

This present study identified changes in miRNA expression in Atlantic salmon adherent HKLs that were cultured for 1 day or 5 days. As these cells are often used in fish in vitro immunology research, it is important to characterize how the cells change throughout culture time. Morphology and phagocytosis analyses, as well as marco and MHC II mRNA expression, suggested that the adherent HKLs studied differentiated from monocyte-like to macrophage-like over 5 days in culture. Sequencing analysis identified several differentially expressed miRNAs that are associated with macrophage differentiation and function in other vertebrates, indicating

that the role of these miRNAs may be similar in Atlantic salmon as in other species. This is the first study of to examine potential miRNAs involved in macrophage differentiation in Atlantic salmon. Future functional studies, e.g. by manipulating expression of certain DE miRNAs in cells cultured over 5 days, are required to further elucidate and fully understand the roles of the identified miRNAs in Atlantic salmon HK cell differentiation and function.

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CHAPTER 4

Characterization of miRNAs in extracellular vesicles released from Atlantic salmon monocyte-like and macrophage-like cells

4.1. Abstract

Cell-derived extracellular vesicles (EVs) participate in cell-cell communication via transfer of molecular cargo including genetic material like miRNAs. In mammals, it has previously been established that EV-mediated transfer of miRNAs can alter the development or function of immune cells, such as macrophages. Our previous research revealed that Atlantic salmon head kidney leukocytes (HKLs) change their morphology, phagocytic ability and miRNA profile from primarily "monocyte-like" at Day 1 to primarily "macrophage-like" at Day 5 of culture. Therefore, we aimed to characterize the miRNA cargo packaged in EVs released from these two cell populations. We successfully isolated EVs from Atlantic salmon HKL culture supernatants using the established Vn96 peptide-based pull-down. Isolation was validated using transmission electron microscopy, nanoparticle tracking analysis and Western blotting. RNA-sequencing identified 19 differentially enriched (DE) miRNAs packaged in Day 1versus Day 5 EVs. Several of the highly abundant miRNAs, including those that were DE (e.g. ssa-miR-146a, ssa-miR-155 and ssa-miR-731), were also identified as DE in HKLs and are associated with macrophage differentiation and immune response in other species. Interestingly, the abundance of the miRNAs in EVs, including the most abundant miRNA (ssa-miR-125b), was different than the miRNA abundance in HKLs, indicating selective packaging of miRNAs in EVs. Further study of the miRNA cargo in EVs derived from fish immune cells will be an important next step in identifying EV biomarkers useful for evaluating immune cell function, fish health or response to disease.

4.2. Introduction

Extracellular vesicles (EVs) are cell-derived, lipid bilayer-enclosed particles that are secreted from many, if not all, cell types, including immune cells [1–3]. Three categories of EVs have been described: exosomes (30-100 nm in diameter), which are formed when multivesicular bodies fuse with the plasma membrane to release intraluminal vesicles; microvesicles (100-1000 nm in diameter), which are formed from direct budding of the plasma membrane; and apoptotic bodies (>1 μM in diameter), which are formed from the blebbing membrane of an apoptotic cell [4,5]. For the purpose of this study, the term EV will refer to exosomes and microvesicles since, due to our isolation methods, large apoptotic bodies are unlikely to represent a major contribution to the observed results. EVs share some common characteristics which enable their identification from cells and other particles. Among the most robustly validated canonical markers are membrane-associated proteins such as heat shock proteins (HSP70, HSP90) and certain members of the tetraspanin superfamily of proteins (CD9, CD63, CD37, CD81, CD82) [4,6]. While EVs have been widely studied in mammals, there are only a few studies that examine EVs in teleost fish, which will be discussed below [7–12].

EVs participate in cell-cell communication via transfer of their molecular cargo, which can include messenger RNA (mRNA), microRNA (miRNA), DNA, and protein [2,13]. In mammals, EVs have been implicated in many physiological and pathological processes, including immune cell regulation and host-pathogen interactions [14,15]. Phagocytic immune cells have been shown to use EVs as a mechanism to regulate neighbouring cells within their environment. For example, pathogen-challenged macrophages release EVs containing pathogen associated molecular patterns (PAMPs) that stimulate recipient cells to produce cytokines including IL-10, IFNγ, TNFα, and IL-1β [16–19]. Similarly, B cells and dendritic cells can use

EVs carrying surface-bound MHC I and II molecules to present antigens and stimulate T cell activation [14,20,21]. EVs are derived from cells under both normal and pathological conditions, and their molecular cargo is reflective of their cell of origin. For instance, tumour cells have been shown to release EVs containing tumour specific miRNAs [22]. Therefore, EVs can also serve as biomarkers for health and disease [23].

Mature miRNAs are short (~22 nucleotides), non-coding RNAs that play a key role in the regulation of biological processes via post-transcriptional regulation of gene expression [24–26]. As part of the RNA induced silencing complex (RISC) the mature guide miRNAs downregulate gene expression by binding to partially complementary mRNA sequences to either block their translation or induce their degradation [25]. EVs can transfer miRNAs between cells where they can regulate the expression of various genes, including those relevant for cell differentiation and immune response [27–29]. In teleost fish, miRNAs have been reported to be involved in cell differentiation, growth, reproduction and regulation of immune responses [30,31]. For example, miR-21 modulates the inflammatory response in miiuy croacker (*Miichthys miiuy)* and grass carp (*Ctenopharyngodon idella*) following *Vibrio anguillarum* and *Aeromonas hydrophila* infection, respectively, and miR-155 is associated with the immune response of several fish species following viral challenge [31–33]. Additionally, small RNA deep sequencing has identified differential miRNA expression in multiple tissues of various teleost fish species following pathogen exposure [30,31,34–36]. Some miRNAs involved in the teleost immune response are also associated with the immune response of mammals, suggesting the function of these miRNAs may be evolutionarily conserved [31,37]. However, putative fish-specific miRNAs, such as miR-2188 and miR-731, play a role in the immune response of several fish species, but have not been described in mammals [34,38–41].

Macrophages play a critical role in initiating an immune response through several processes including phagocytosis, production of reactive intermediates, and production of cytokines and other pro- and anti-inflammatory proteins [42,43]. Two major types of macrophages have been characterized depending on their activation and cytokines produced: M1 (pro-inflammatory) and M2 (anti-inflammatory) [42,43]. While M1 macrophages are involved in the ability to respond to pathogenic challenge, M2 macrophages are involved in processes such as tissue remodeling, fibrosis and wound repair [42,43]. In fish, an adherent population of leukocytes, consisting of multiple cell types including macrophages and their precursors monocytes, can be isolated and cultured from the anterior (or head) kidney, which is the main site of hematopoiesis in fish and equivalent to the mammalian bone marrow [44–46]. Based on morphology, phagocytic ability and miRNA profile, our previous research suggested that Atlantic salmon head kidney leukocytes (HKLs) change *in vitro* from primarily monocyte-like at Day 1 of culture to primarily macrophage-like at Day 5 of culture [47]. Therefore, we analyzed the miRNA profile of EVs released from these two cell populations. If differentially packaged miRNAs are present in the two populations, they may help distinguish EVs released by monocytes or progenitor cells (Day 1) from EVs released from macrophages (Day 5). This is particularly relevant for health and disease monitoring. Monocytes represent a comparatively naïve, steady-state cell type whereas their differentiation into macrophages is associated with active immunity, response to pathogenic conditions, and antigen presentation [48–50]. As such, identifying EVs with differences in miRNA abundance between monocytes and macrophages could provide a means for quantifying the activity of the immune system.

Studying EVs and their packaged cargo in teleost fish may aid in the identification of biomarkers of health, disease and/or response to environmental stressors. Using Q-TOF mass

spectrometry (MS), proteins including MHCIIB, HSP70 and HSP90 were identified in EVs derived from Atlantic salmon leukocytes that were stimulated with cytosine–phosphate–guanosine (CpG) oligonucleotides, an established PAMP analogue [7,12]. Similarly, proteins including granulins, MHCI, MHCII and proteasome subunits were identified in serum-derived EVs from Atlantic salmon infected with *Piscirickettsia salmonis* [8]. In rainbow trout (*Oncorhynchus mykiss*), it was demonstrated that heat shock induced the release of HSP70 enriched exosomes *in vivo* isolated from plasma, and *in vitro* isolated from cultured hepatocytes [10]. The differential loading of EV cargo molecules, including miRNAs between physiological states, has been established in mammals [22,23]. If EVs have similar characteristic differences in miRNA profiles in teleost fish they may serve as molecular signatures for fish physiological state. For example, Atlantic cod (*Gadus morhua*) reared in elevated water temperature were found to have serum EVs with different protein and miRNA cargo than control Atlantic cod reared in optimal water temperature [9]. Additionally, signature miRNAs corresponding to sex differentiation were identified in serum EVs of tongue sole (*Cynoglossus semilaevis)*, allowing early detection of sex differentiation, which may enhance the efficiency of reproduction and cultivation [11]. Studying the miRNA cargo of fish EVs is, therefore, of considerable interest in understanding how they may be related to fish health and response to disease. The use of EVs from blood samples, for example, as opposed to more invasive biopsies, or sacrificed animals, may be used for responsive, potentially non-lethal, and timely monitoring of health in both wild and farmed fish. Characterizing EVs and their cargo derived from immune cells is a key first step in determining immune-related EV specific biomarkers.

4.3. Materials and Methods

4.3.1. Animals

The Atlantic salmon (1.5 kg +/- 0.3 kg SD) used for this experiment were reared in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre and kept at 12˚C with 95-110% oxygen saturation, using a flow-through seawater system. All procedures in this experiment were approved by Memorial University of Newfoundland's Institutional Animal Care Committee (18-01-MR; 14-02-MR), following the guidelines from the Canadian Council on Animal Care. Due to the limiting number of HKLs isolated per fish, and the low amount of RNA available in EVs, a total of 16 Atlantic salmon were used in this study: 5 individuals for RNA-seq (1 individual was excluded from RNA-seq due to low RNA yield), 5 individuals for reverse transcriptase (RT)-qPCR and nanoparticle tracking analysis (NTA), 3 individuals for Western blot, and 3 individuals for transmission electron microscopy (TEM).

4.3.2. Head kidney leukocyte isolation

HKLs have been used in several fish immunology studies (e.g. 44,51–53). In this study, HKLs were isolated as previously described in Smith et al. [47]. Briefly, the HK was removed and placed in isolation media: Leibovitz-15 medium (L-15 Gibco, Carlsbad, CA, USA) supplemented with 2.5% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco) and 27.5 mg of heparin (Sigma-Aldrich, St. Louis, MO, USA). The HK was forced through a 100 µm nylon cell strainer (Thermo-Fisher Scientific, Waltham, MA, USA) to generate a single-cell suspension, which was then loaded onto a 34/51% Percoll (GE Healthcare, Uppsala, Sweden) gradient (prepared with H_2O and10X Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich) and centrifuged at 500 x g for 30 min at 4˚C. Following centrifugation, the interface between the 34% and 51% gradient, which consists of leukocytes, was collected and washed

twice in isolation media at 500 x g for 5 min at 4˚C. The cells were re-suspended in culture media (L-15 supplemented with 5% FBS and 1% penicillin/streptomycin; held on ice), and viable cells were counted on a haemocytometer using Trypan Blue (Sigma-Aldrich) dead-cell exclusion. The cells were then seeded in 6-well culture plates (Corning, Corning, NY, USA) at 3 x 10^7 cells in 2 mL of culture media per well and incubated at 15˚C. Six hours after plating, the cells were washed twice in culture media, leaving only the adhered cells. The media of the cells to be sampled on Day 1 was replaced with vesicle-free culture media, while the media of the remaining cells was replaced with regular culture media. Vesicle-free culture media was made as follows: culture media was prepared as described above, except with double the amount of FBS (10% FBS). The media was centrifuged at 100,000 x g for 16 h at 4˚C. The supernatant was sterilized through a 0.22 μm filter and then diluted with depleted culture media (media without FBS) to reach a final concentration of 5% FBS [54]. Twenty-four hours later, the media from Day 1 cells was collected and centrifuged at 1800 x g for 5 min at 4°C, followed by 17,000 x g for 15 min at 4°C, to eliminate cells and debris. The media was stored at -80°C until further processing. This procedure was then repeated for Day 5 cells where the media was replaced with vesicle-free media 24 h before sampling. In the current study's Day 5 cultures, macrophage-like cell morphology as seen in [47] was confirmed by eye. In addition, viability was assessed by lack of cell debris for each experiment.

4.3.3. Transmission electron microscopy (TEM)

The morphology of HKL-derived EVs was analyzed using TEM. Five microlitres of culture media containing EVs were placed on a copper formvar/carbon grid (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with 2% Uranyl Acetate for 1 min, followed by a 1 min wash in phosphate buffered saline (PBS; Thermo-Fisher Scientific) at room

temperature. Imaging was performed using a Tecnai Spirit Transmission Electron Microscope, equipped with a 4 megapixel AMG digital camera.

4.3.4. Nanoparticle tracking analysis (NTA)

Culture media containing EVs released from Day 1 HKLs and culture media containing EVs released from Day 5 HKLs was diluted 1:10 in 0.1 μM filtered PBS, and the concentration and size of the EVs were analyzed using a NanoSight NS300 (Malvern Panalytical, St-Laurent, Quebec, CA). Samples were applied to the Nanosight using a continuous syringe pump. The number of particles in the window was kept at 40-100 per frame. The screen gain was set to 3.0 and the camera level to 13. Five videos were recorded per sample at 60 s per video.

4.3.5. Extracellular vesicle isolation

EVs were isolated using the Vn96 peptide (New England Peptide, Gardner, MA, USA) following the manufacturer's instructions. Vn96 binds to at least 5 unique HSPs secreted by a variety of different cell types [55,56]. In addition, Vn96 isolates EVs with reduced contamination from protein aggregates or lipoproteins, compared to other methods of EV isolation (i.e. ultracentrifugation) [57]. Briefly, 1 ml of EV-containing media was incubated with 40 μl (2.5 mg/ml) of Vn96 for 1 h, rotating, at room temperature. Following the 1 h incubation, the EV-containing media was centrifuged at 17,000 g for 15 min at 4°C. The pellet was washed 3 times in 0.1 μm filtered PBS at 15,000 x g for 10 min at 4°C and resuspended in the appropriate buffer: 100 μl of mirVana lysis buffer for RNA isolation or 30 μl of radioimmunoprecipitation assay buffer (RIPA: 50 nM Tris-HCl, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150 mM NaCl) for Western blot.

4.3.6. Western blot

All samples (Atlantic salmon head kidney and liver, Vn96 isolated EVs and murine Wehi-231 B-cells) were lysed in RIPA buffer supplemented with 1:100 of 10 mg/ml PMSF (Sigma), 1 μM aprotinin (Sigma) and 1X HALT protease inhibitor cocktail (Thermo-Fisher). Protein content of the head kidney and liver samples was determined using the bicinchoninic acid (BCA; Thermo-Fisher Scientific) assay following the manufacturer's protocol. One microgram, 5 μg and 10 μg of head kidney and liver lysate and all of the Wehi-231 lysate (from 5.0 x 10^5 cells) or 10 μl of EV lysate were run on 10% SDS-PAGE gels followed by transfer to nitrocellulose membranes. Blocking was performed for using 5% (w/v) skim milk in tris buffered saline plus tween (TBST) for 1 h at room temperature. Anti-mouse HCS 70 (B-6) (sc-7298; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and HSP 90 (4F10) (sc-69703; Santa Cruz Biotechnology Inc.) antibodies were used at 1:400 diluted in TBST + 5% skim milk, while the secondary goat-anti-mouse IgG-HSP antibody (sc-2005; Santa Cruz Biotechnology Inc.) was diluted at 1:1000. Primary antibodies were incubated overnight at 4°C and the secondary antibody was incubated for 1 h at room temperature. Immobilon Western Chemiluminescent HRP Substrate (Millipore, Oakville, Ontario, Canada) was used for signal detection. Images were acquired using an AlphaImager Gel Documentation system with FluorChem HD2 software, version 3.4.0. Image manipulation was limited to adjustments to brightness and contrast of the entire image.

4.3.7. Total RNA isolation

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Thirty microlitres of elution solution were used to resuspend the pellet and quantity was determined by

nanodrop spectrophotometry. Similar quantities of RNA were isolated from Day 1 and Day 5 HKL EVs, indicating that both populations of cells secrete similar amounts of EV cargo. Samples were sent to the Atlantic Cancer Research Institute (Moncton, New Brunswick, Canada) for library preparation and sequencing.

4.3.8. Library preparation and miRNA sequencing

RNA quality was assessed on a 5200 fragment analyzer (Agilent Technologies, Santa Clara, CA, USA) using the HS RNA assay and the HS small RNA assay (Agilent Technologies). Eight small RNA libraries were prepared using the Clean Tag Small RNA library prep kit following manufacturer recommended conditions (TriLink Biotech, San Diego, CA, USA). Ion Torrent specific RT primer and barcodes were used during the library construction. Barcoded cDNA libraries were subjected to double size selection (150-200 bp) using Ampure XP beads (Beckman Coulter, Mississauga, Ontario, CA) to enrich for miRNA transcripts. The quality of the library was analyzed using a D1000 assay on TapeStation 2200 (Agilent Technologies, Mississauga, Ontario, Canada). Libraries were equally pooled at a loading concentration of 7 pM and amplified onto Ion Sphere™ Particles (ISP) using the Ion PI Hi-Q™ OT2 kit (Life Technologies). The ISP enriched library was sequenced using the Ion Proton (ThermoFisher).

4.3.9. Data processing

The raw sequencing fastq files are deposited in NCBI's Gene Expression Omnibus (GEO) under the identifier GSE143360 (accession numbers can be found in Supplementary Table 4.1). The adapter sequences were trimmed and size filtered (to remove reads shorter than 18 nucleotides (nts) or longer than 25 nts) using the Cutadapt Python Package (v.1.13). The sequence reads were mapped to a reference index consisting of all known mature miRNAs in

Atlantic salmon (including the Atlantic salmon miRNAs in miRbase) [58,59] using STAR aligner software (v2.4.2b). A complete overview of the unique mature Atlantic salmon miRNA sequences in this reference index can be found in Woldemariam et al. [58]. The reference index is the current updated version of the Atlantic mature miRNAome previously provided to miRbase in 2013 [59]. The alignment files were further processed in R using featureCounts [60] to produce count matrices that were used as input in the R package DESeq2 to determine miRNAs that were significantly differently enriched (DE) in Day 1 and Day 5 [61].

4.4.10. RT-qPCR analysis of miRNA expression

To validate the miRNA sequencing results, the experiment was repeated with a different group of Atlantic salmon. Total RNA was isolated using the mirVana kit, as above. cDNA was synthesized using the miScript II RT Kit (Qiagen, Hilden, Germany), as per the manufacturer's instructions, with 100 ng of total RNA in 20 µl reactions. The sequences of the mature miRNAs of interest were used as forward specific primers (Supplementary Table 4.2) while a universal primer, provided by the miScript SYBR Green PCR Kit (Qiagen), was used as a reverse primer. Three-fold, 5-point standard curves of pooled cDNA from cultured HKLs were used to assess the quality of all miRNA primers, with the exception of ssa-miR-155-5p and ssa-miR-146a-5p, where a 4-point standard curve was used. RNA from cultured HKLs was used for primer quality control instead of RNA from HKL EVs due to the very low amount of RNA obtained from isolated EVs. The efficiencies of the primers ranged from 78.3% to 116.5%. As the miRNA primer is the same size of the miRNA, there is no way to improve the efficiency of the primer. The geometric mean of the two chosen normalizers (ssa-miR-30b-5p and ssa-miR-142-3p) showed stability between the two sample groups (i.e. average geometric mean of normalizers Ct less than 0.25 between the two groups). Mature miRNAs are extremely robust and the common

methods to measure RNA quality cannot be used to judge the degree of degradation of mature miRNAs with an average size of 22 nts [62]. However, the fact that the miRNAs applied as normalizers showed good agreement between the Day 1 group and the Day 5 group suggests that the miRNA was not degraded. Each reaction was run in duplicate and was composed of 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10× miScript Universal Primer, 2.5 µl specific forward primer (10 µM), 5 µl RNase-free water (Qiagen), and 2.5 µl of diluted cDNA template representing 5 ng of input total RNA. The PCR program consisted of one cycle of 95 °C for 15 min, and 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s, followed by a final melting point analysis, on a 7500 Fast Real-Time PCR System (Applied Biosystems). Microsoft Excel was used to determine the relative quantity (RQ) values of each miRNA relative to the average delta Ct of the control miRNA (Day 1 samples) using the comparative Ct method [63], with the assumption of 100% efficiency of the primers.

4.4.11. Statistical analysis

Differentially expressed miRNAs were identified by comparing the Day 1 group to the Day 5 group (n=4 from each experimental condition) applying DESeq2 as described in 2.9. For RNA-seq, miRNAs were considered to be statistically DE if they had a Benjamini-Hochberg adjusted p-value of <0.05, base mean read counts >20 and $\log_2$ fold-change of >1. The average normalized read count in Day 1 cells and Day 5 cells from the DESeq2 analysis was used to reveal the miRNA diversity and abundance in EVs released from Day 1 and Day 5 HKLs. A paired Student's T-test was used to determine statistically significant differences between Day 1 and Day 5 RT-qPCR samples using the Prism package v 8.0 (GraphPad Software Inc., La Jolla, CA). Genesis software (Rockville, Maryland, USA) was used for the hierarchical clustering of

median centred normalized counts of DE miRNAs using the Pearson correlation and complete linkage clustering.

4.4.12. In silico prediction of target genes and gene pathway enrichment analysis

The putative target genes of the DE miRNAs were predicted using the target prediction tool RNAhybrid 2.2 [64]. The parameters applied in the *in silico* prediction ensured that only matches with perfect seed complementarity and high base-pairing stability were returned to minimize false positives. The settings were: helix constraint 2-8, no G: U in seed and minimum free energy threshold -18 kcal/mol as described in Andreassen et al. [38] and the predictions were against 3'UTRs from all Atlantic salmon mRNA transcripts (NM entries) in the Refseq database in GenBank (https:// www.ncbi.nlm.nih.gov/). Gene symbols and gene IDs of the predicted target transcripts were extracted from the Universal Protein Resource (UniProt) database https://www.uniprot.org/ [65]. The gene pathway enrichment analysis was carried out as in Woldemariam et al. [66] using Enrichr [67,68] to identify significantly enriched pathways and gene ontology (GO) terms using the predicted miRNA targets as input. GO terms and pathways with adjusted p-value < 0.05 were considered as significantly enriched.

4.4. Results

4.4.1. Atlantic salmon adherent HKLs release extracellular vesicles (EVs) during in vitro culturing

EVs were characterized according to the Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) guidelines using TEM, NTA, and Western blotting for the EV protein HSP90 [69]. We recognize that detection of additional protein markers is suggested in the MISEV2018 guidelines; however, the wide testing of available antibodies for the canonical EV markers that cross-react with Atlantic salmon proteins is beyond our present

capacity. The workflow for this experiment can be found in Figure 4.1A. To confirm that putative EVs were released from HKLs into the culture media, the culture media was analyzed by TEM. Round, double-membraned structures of variable sizes were observed in the culture media (Figure 4.1B), confirming that the HKLs secrete EVs.

Our previous research determined that Day 1 HKLs are primarily "monocyte-like" while Day 5 HKLs are primarily "macrophage-like" [47]. Therefore, the concentration and size of EVs released from Day 1 and Day 5 HKLs were analyzed by NTA (Figure 4.2); an accurate and precise method to measure both size and concentration, as opposed to TEM. EVs from Day 1 HKLs had a mean size of 122.9 nm, a mode size of 109.5 nm and a range of 109.0 – 137.6 nm. EVs from Day 5 HKLs had a mean size of 118.2 nm, a mode size of 108.8 nm and a range of 107.6 – 127.4 nm (Figure 4.2B, C). The average concentration of EVs from Day 1 HKLs was $2.23x10^8 \pm 1.08x10^7$ EVs/mL, while the average concentration of EVs from Day 5 HKLs was $2.06x10^8 \pm 8.73x10^6$ EVs/mL (Figure 4.2D). Overall, there was no significant difference in the size ($p=0.6363$) or concentration ($p=0.8162$) of EVs released from Day 1 HKLs compared to EVs released from Day 5 HKLs, as determined by a paired Student's T-test.

4.4.2. Confirmation of EVs derived from Atlantic salmon adherent HKLs by Vn96 isolation

In this study, the Vn96 peptide was used to isolate EVs for small RNA-sequencing (RNA-seq). Vn96 can bind to several distinct HSPs, found on the exterior of the EV, from multiple species including human, canine, rodents (mouse and hamster), bovine, and Chinook salmon [56]. However, it was not previously demonstrated that Vn96 can bind to HSPs on EVs from Atlantic salmon. Therefore, we first confirmed that Vn96 binds to EVs derived from Atlantic salmon HKLs based on the detection of HSPs using Western blotting. Due to the limited

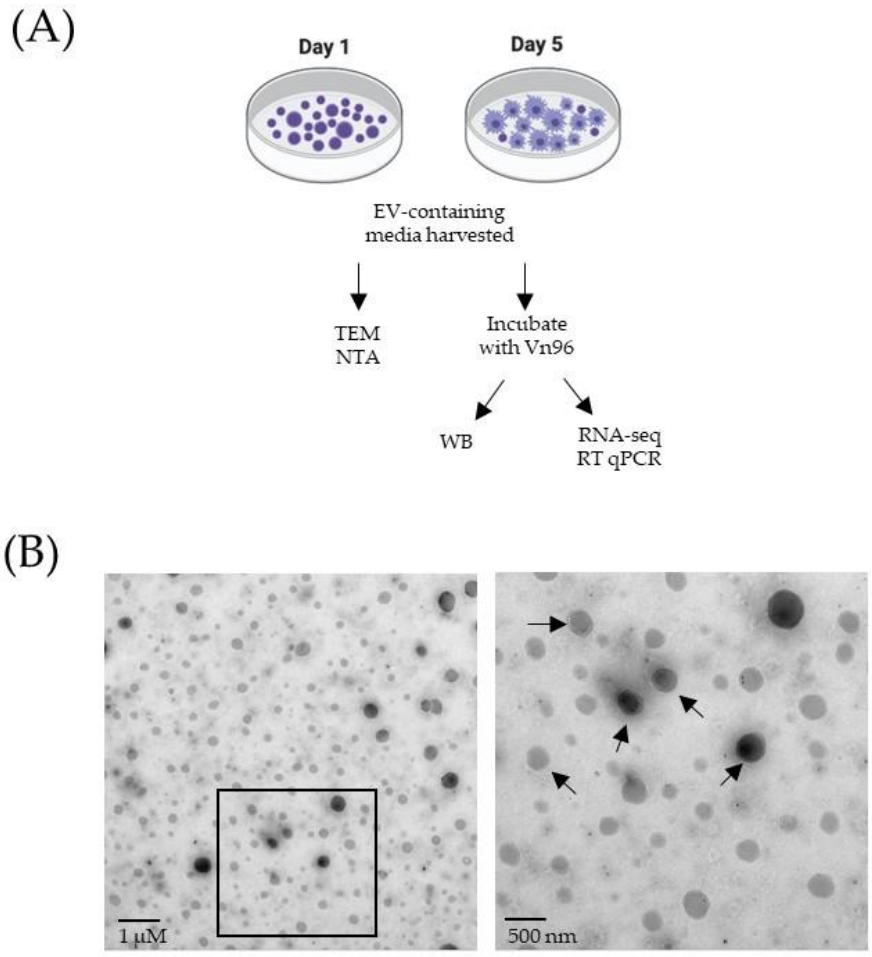

Figure 4.1. Confirmation of extracellular vesicle (EV) release from Atlantic salmon HKLs. **(A)** Diagram of experimental workflow. **(B)** Transmission electron microscopy (TEM) images of EVs released into cell culture media by Day 1 HKLs (magnification 2700x and 6500x respectively; size of scale bar indicated on image). Area within the square of the left image is magnified in the right image. Arrows are pointing to double membranes. TEM images representative of n=3. NTA: Nanoparticle tracking analysis; WB: Western blot; RNA-seq: RNA-sequencing; RT-qPCR: reverse-transcriptase quantitative polymerase chain reaction.

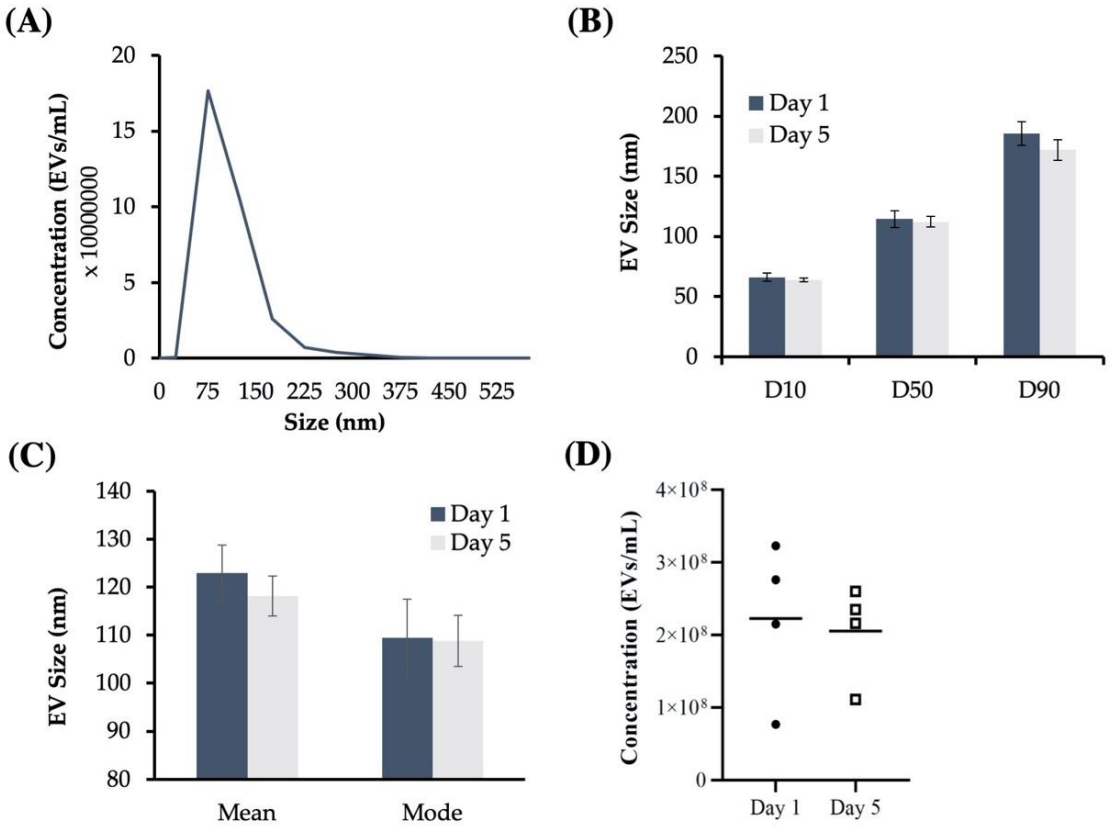

Figure 4.2. Characterization of Atlantic salmon extracellular vesicle (EV) size and quantity. Cell culture media containing EVs released from adherent Atlantic salmon HKLs was analyzed using nanoparticle tracking analysis (NTA). Five videos were captured per sample and results were reported as an average of the 5 videos. **(A)** Representative histogram of EV size profile **(B)** EV size distribution D10 (diameter where 10% of the population lies below the D10), D50 (diameter where 50% of the population lies below D50) and D90 (diameter where 90% of the population lies below D90) for EVs released at Day 1 and Day 5. Data reported as average mean +/- SE. **(C)** Mean size and mode size (+/- SE) of EVs released from Day 1 and Day 5 HKLs. **(D)** Concentration of EVs released from Day 1 and Day 5 HKLs. Scatterplots show data from individual fish (average of 5 videos); n=4; no statistical differences were observed as determined by a paired Student's T-test.

availability of commercial Atlantic salmon antibodies (Abs), we first sought to confirm cross-reactivity of anti-mouse Hsc70 and anti-mouse HSP90 Abs with Atlantic salmon head kidney and liver lysates, using the Wehi-231 murine B-cell line as a positive control [70]. We found that the anti-mouse Hsc70 Ab did not cross-react with lysates from Atlantic salmon (data not shown). However, the anti-mouse HSP90 Ab detected a protein of the same molecular weight in both Atlantic salmon and mouse (Figure 4.3A). Using the anti-HSP90 Ab, we confirmed that the Vn96 peptide successfully enriched HSP90-positive EVs from Atlantic salmon HKL culture media since one protein species at the expected size of 90 kDa was detected (Figure 4.3B).

4.4.3. RNA-seq identified 19 differentially packaged miRNAs in Day 1 and Day 5 HKL EVs

RNA-seq was used to examine the miRNAs packaged in Vn96-isolated EVs released from Day 1 and Day 5 HKLs. The number of reads mapped to miRNAs ranged from 22,364 to 61,094 (Figure 4.4A; Supplementary Table 4.1). A total of 479 miRNAs were detected in either Day 1 or Day 5 HKL EVs (Supplementary Table 4.3). However, most of these consisted of very low counts. Sixty-one and sixty-five miRNAs with an abundance of more than 0.1% were identified in Day 1 and Day 5 groups, respectively, twenty-two and thirty miRNAs with an abundance of more than 0.5% were identified in Day 1 and Day 5 groups, respectively, while six and ten miRNAs had an abundance of more than 2% in Day 1 and Day 5 groups, respectively (Supplementary Table 4.4). Interestingly, ssa-miR-125b-1-3p was the most abundant mature miRNA in both groups representing 45% and 14% of all mature miRNAs in Day 1 and Day 5 EVs, respectively (Supplementary Table 4.4), while representing 52.7% and 16.9% of the top 20 most abundant miRNAs in Day 1 and Day 5 EVs, respectively (Figure 4.5).

Differentially enriched (DE) miRNAs between EVs released from Day 1 HKLs compared with EVs released from Day 5 HKLs were analysed by DESeq2. There were 19 DE

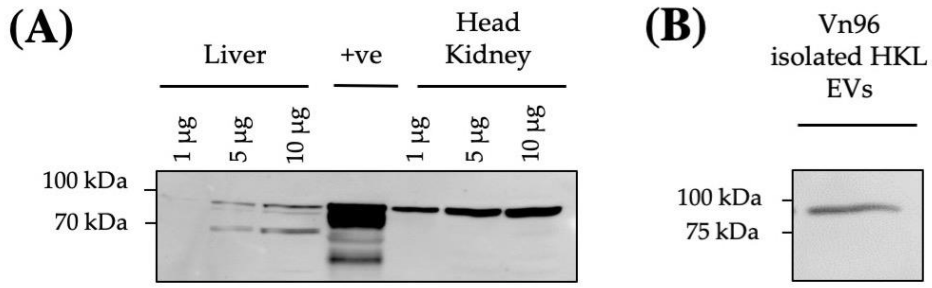

Figure 4.3. HSP90 protein expression in Atlantic salmon HK, liver and HKL derived extracellular vesicles (EVs). (A) Protein lysates from Atlantic salmon liver and head kidney tissue at 1 µg, 5 µg and 10 µg were tested for cross-reactivity with anti-mouse HSP90. Wehi-231 murine B cells were used as a positive control (+ve). **(B)** HSP90 expression in Vn96 isolated EVs derived from Day 1 HKL culture media.

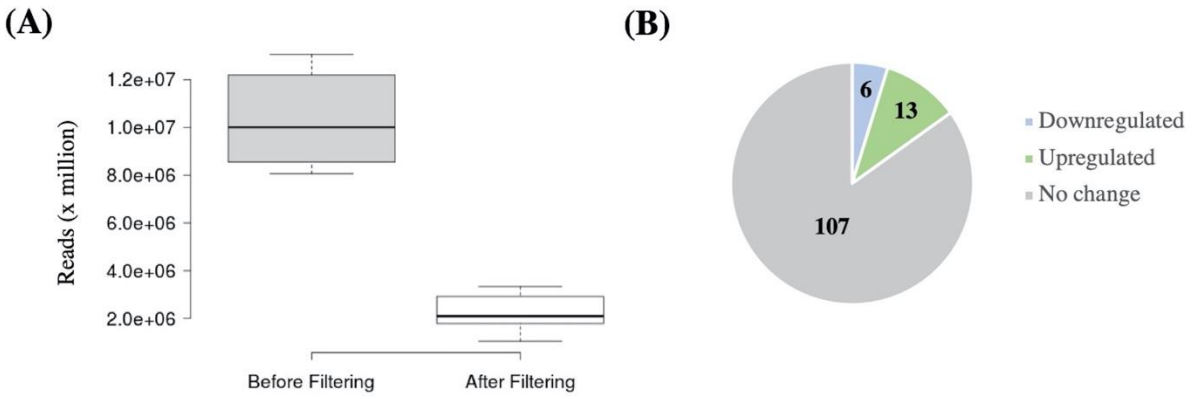

Figure 4.4. RNA-sequencing results. (A) Total number of reads before and after trimming and size filtering. Box plot developed using BoxPlotR: a web-tool for generation of box plots [71]. **(B)** The number of miRNAs that with downregulated expression, upregulated expression and no change in EVs released from Day 5 HKLs compared to EVs released from Day 1 HKLs. Only miRNAs with greater than 0.1% abundance in either Day 1 or Day 5 EVs were included in this figure.

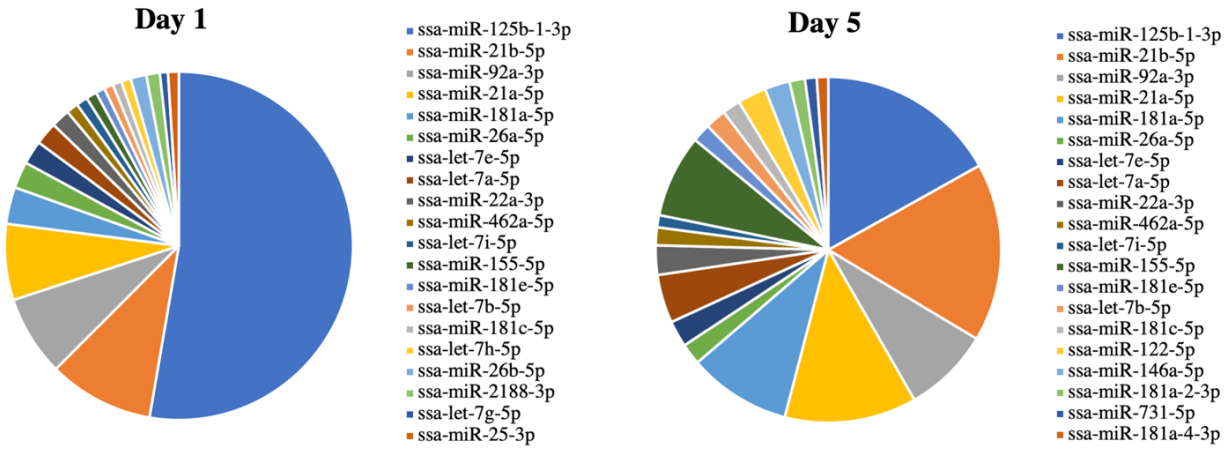

Figure 4.5. miRNA abundance (average normalized read counts) in extracellular vesicles (EVs) released from Day 1 and Day 5 Atlantic salmon HKLs. The top 20 most abundant miRNAs in Day 1 and Day 5 EVs are shown.

Table 4.1. Differentially enriched miRNAs in Day 5 compared with Day 1 extracellular vesicles (EVs) released from Atlantic salmon HKLs

miRNA[a]	baseMean	log_2 Fold-change[b]	Adjusted p-value	Mature sequence 5-3'
Upregulated				
ssa-miR-122-5p	392.55	4.95	0.001	TGGAGTGTGACAATGGTGTTTG
ssa-miR-155-5p	1235.36	2.89	< 0.001	TTAATGCTAATCGTGATAGGGGT
ssa-miR-146a-5p	384.47	2.63	< 0.001	TGAGAACTGAATTCCATAGATGG
ssa-miR-148a-3p	47.68	2.46	0.011	AAGTTCTGTGATACACTTCGACT
ssa-miR-27d-2-5p	30.70	1.80	0.006	AGGACTTAGCACACATGTGAACA
ssa-miR-731-5p	207.06	1.54	< 0.001	AATGACACGTTTTCTCCCGGATT
ssa-miR-10d-5p	119.34	1.47	0.027	CACCCTGTAGAACCGAATTTGT
ssa-miR-10b-5p	122.16	1.46	0.032	TACCCTGTAGAACCGAATTTGT
ssa-miR-181a-5p	1866.45	1.41	0.026	AACATTCAACGCTGTCGGTGAGT
ssa-miR-27b-3p	28.37	1.37	0.027	TTCACAGTGGCTAAGTTCTGC
ssa-miR-221-3p	204.17	1.34	0.006	ACCTAGCATACAATGTAGATTTC
ssa-miR-222cd-3p	93.62	1.18	0.009	AGCTACATCTGATTACTGGGTCA
ssa-let-7a-5p	950.56	1.02	0.030	TGGAAGACTAGTGATTTTGTTGT
Downregulated				
ssa-miR-16a-5p	143.42	-1.12	0.053	TAGCAGCACGTAAATATTGGAG
ssa-miR-210-1-3p	35.34	-1.47	0.038	CTGTGCGTGTGACAGCGGCT
ssa-miR-1338-3p	88.38	-1.52	0.011	ATCTCAGGTTCGTCAGCCCATG
ssa-miR-7a-5p	35.86	-1.52	0.009	TGGAAGACTAGTGATTTTGTTGT
ssa-miR-125b-1-3p	10213.94	-1.72	0.005	ACAGGTGAGGTCCTCGGGAA
ssa-miR-8156-5p	61.01	-2.64	0.001	GTCCTGACTGTCCTGACTGTC

[a] The names are in a few cases with different lettered/numbered suffixes than in miRBase as several mature family members are identical

[b] Negative fold-change values are down-regulated in Day 5 compared with Day 1; positive fold-change values are up-regulated in Day 5 compared with Day 1

miRNAs in Day 1 HKL EVs compared with Day 5 HKL EVs (Figure 4.4B; Table 4.1). Thirteen miRNAs were more abundant in EVs released from Day 5 HKLs, while six miRNAs were less abundant in EVs released from Day 5 HKLs (Table 4.1). Several of the most abundant miRNAs were also DE including ssa-miR-125b-3p, ssa-miR181a-5p and ssa-miR-155-5p (Table 4.1), while the highly abundant ssa-miR-21a and ssa-miR-21b were not significantly DE despite a rather large difference in percentage of these miRNAs between Day 1 and Day 5 EVs (Figure 4.5). Hierarchical clustering analyses of the DE miRNA based on normalized counts showed that all samples from Day 1 HKL EVs clustered together and all samples from Day 5 HKL EVs clustered together, indicating the two groups represent distinct sub-populations (Figure 4.6). However, we also observed a large variation in normalized read counts within groups for several miRNAs (e.g. ssa-miR-148a-3p, Supplementary Table 4.3) indicating that there was substantial variability in abundance of certain miRNAs within each of the two groups. Given the relative paucity of available data involving investigations of fish-derived EVs and their molecular cargo, considerable potential remains for optimizing future studies.

4.4.4. RT-qPCR analysis confirmed miRNA abundance in Day 1 and Day 5 HKL EVs

Five new Atlantic salmon (i.e. different from the salmon used for RNA-seq) were used for the RT-qPCR analysis. Nine miRNAs were chosen for RT-qPCR validation, along with two normalizer miRNAs (ssa-miR-30-5p and ssa-miR-142a-3p) (Figure 4.7). A combination of upregulated and downregulated miRNAs was chosen for RT-qPCR, as well as miRNAs involved in vertebrate immune responses and macrophage function [31,72,73]. In addition, we examined immune-relevant miRNAs that were identified by RNA-seq but not DE to confirm their presence

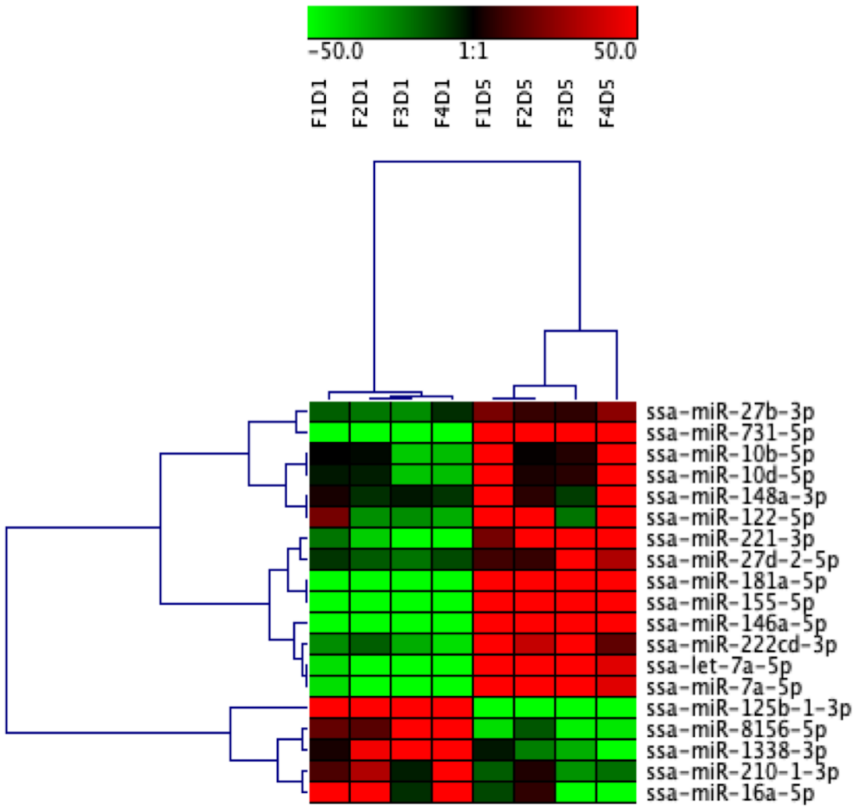

Figure 4.6. Heatmap illustration and hierarchical clustering analyses of differentially expressed miRNAs packaged in extracellular vesicles (EVs) released from Day 1 and Day 5 HKLs. The heatmap represents the normalized counts of DE miRNAs in EVs released from Day 1 HKLs and EVs released from Day 5 HKLs in each individual fish. miRNA normalized counts were median centred and clustered using Pearson correlation and complete linkage hierarchical clustering. Red indicates higher counts and green indicates lower counts. Integer adjusted to a maximum of 50 and a minimum of -50. F indicates fish number; D indicates Day 1 or Day 5 (i.e. F1D1 is Fish 1 Day 1).

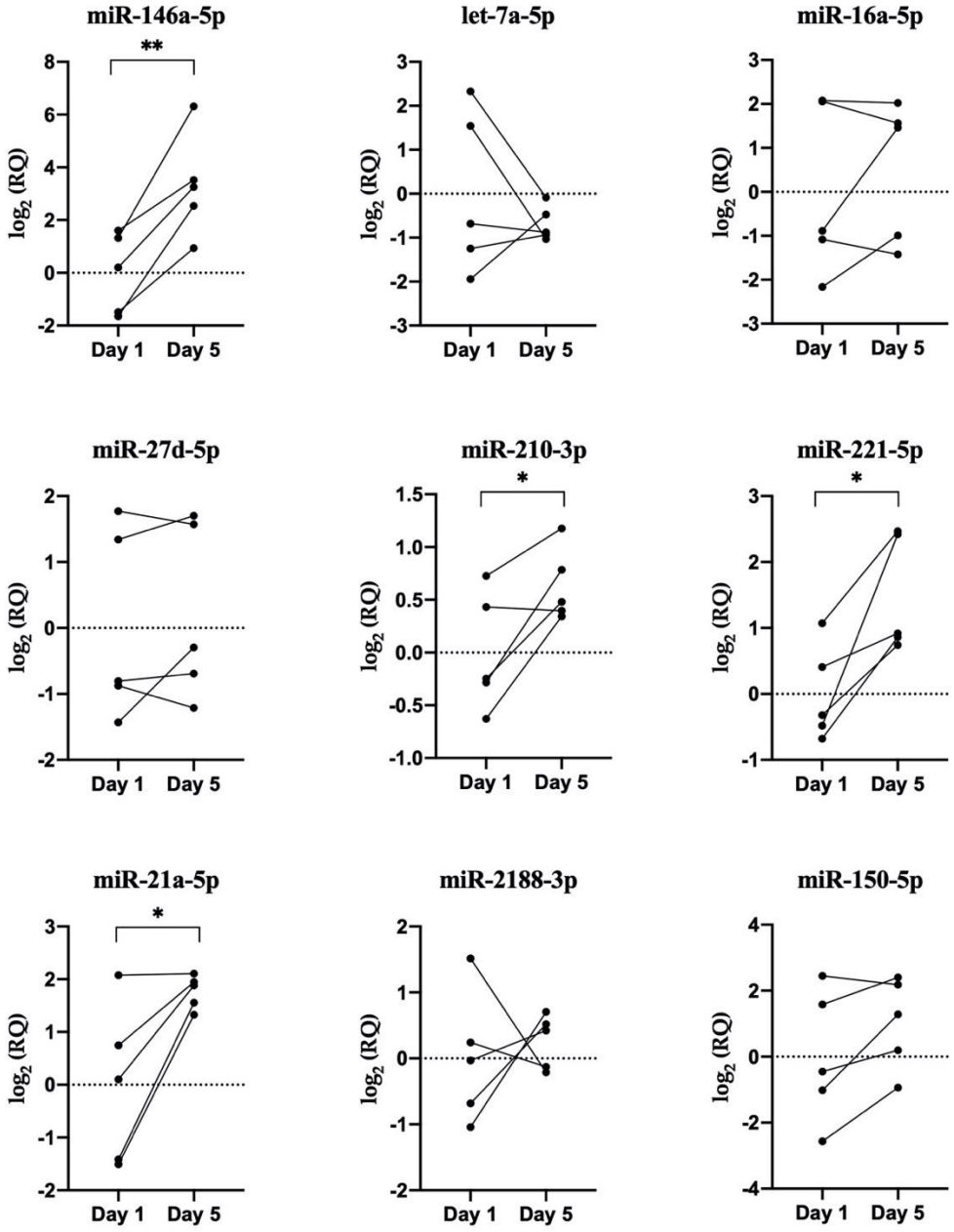

Figure 4.7. RT-qPCR results. Scatterplots of the $\log_2$ relative quantity (RQ) values of miRNAs determined by RNA sequencing to be DE between EVs released from Day 1 and Day 5 HKLs. Scatterplots show individual data with lines connecting data point from each individual fish, n=5, * $p<0.05$. **(A)** miR-146a-5p **(B)** let-7a-5p **(C)** miR-16a-5p **(D)** miR-27s-5p **(E)** miR-210-3p **(F)** miR-221-5p **(G)** miR-21a-5p **(H)** miR-2188-3p **(I)** miR150-5p

in fish EVs: the fish-specific ssa-miR-2188-3p, as well as ssa-miR-21a-5p, ssa-miR-150-5p and ssa-miR-221-5p, are all virus responsive miRNAs in teleost fish, and involved in mammalian macrophage activation and/or differentiation [37,74–77]. All nine miRNAs that were detected by RNA-seq were also detected by RT-qPCR. However, only one miRNA, ssa-miR-146a-5p, showed the same differential expression found by RNA-seq in both direction and significance. One miRNA that was not identified as DE by RNA-seq (ssa-miR-21a-5p) was found to be DE by RT-qPCR. While ssa-miR-221-5p could not be analysed by RNA-seq method due to low read numbers, the qPCR-method, being more sensitive, detected a significant increase in the low abundant 5p mature ssa-miR-221. A comparison of the sequencing and RT-qPCR results can be found in Table 4.2. The RT-qPCR results showed considerable biological variability between fish and miRNA expression. This variability is clear from the high standard error (SE) in many of the miRNAs examined via RT-qPCR and RNA-seq (Table 4.2) and is illustrated in the heat map of the RNA-seq data (Figure 4.6).

4.4.5. Target gene prediction and gene pathway enrichment analysis

The *in silico* prediction of target genes showed that the 19 DE miRNAs could potentially target between 39 to 225 mRNA transcripts each. In total, there were 2873 potential targets, however, as several DE miRNAs targeted the same transcripts, there were only 1556 unique transcripts that were putative targets. The results from the *in silico* target prediction analysis for each of the DE miRNAs is given in Supplementary Table 4.5. Subsequent pathway analysis and GO term enrichment analysis did not result in any significant findings (Supplementary Table 4.6).

Table 4.2. Comparison of sequencing and RT-qPCR results[a]

	RNA-seq						RT-qPCR				
	Average Normalized Count[b]		Standard Error		p-value[b]	p-value (padj)[c]	Average Log_2 RQ[d]		Standard Error		p-value[e]
miRNA	**Day 1**	**Day 5**	**Day 1**	**Day 5**			**Day 1**	**Day 5**	**Day 1**	**Day 5**	
miR-146a-5p	106.66	662.28	3.86	100.28	<0.001	<0.001	0.00	3.31	0.68	0.87	0.004
let7a-5p	627.33	1273.78	109.71	636.89	0.003	0.030	0.00	-0.68	0.82	0.18	0.437
miR-16a-5p	196.71	90.13	27.66	19.54	0.007	0.053	0.00	0.53	0.87	0.72	0.387
miR-27d-5p	13.62	47.79	2.31	11.63	0.001	0.006	0.00	0.22	0.62	0.61	0.455
miR-210-3p	51.95	18.72	11.67	5.29	0.005	0.038	0.00	0.64	0.25	0.16	0.033
miR-221-5p	0.00	3.77	0.00	1.89	0.531	n/a	0.00	1.48	0.32	0.39	0.033
miR-21a-5p	2096.99	3480.17	143.06	615.90	0.033	0.136	0.00	1.76	0.68	0.14	0.032
miR-2188-3p	372.12	112.00	147.83	46.94	0.017	0.377	0.00	0.26	0.43	0.15	0.691
miR-150-5p	22.30	13.69	4.60	0.98	0.015	0.355	0.00	1.03	0.90	0.63	0.079

[a] RNA-seq and RT-qPCR experiments were completed with two different groups of fish.
[b] As determined by a paired Student's T-test.
[b] As determined by a paired Student's T-test adjusted using the Benjamini-Hochberg method.
[c] Mean log_2 relative quantity (RQ).
[d] As determined by a paired Student's T-Test.

4.5. Discussion

This study examined the miRNA cargo in EVs released from Atlantic salmon HKLs as they differentiated *in vitro*. Verification of EV isolation for study remains an evolving topic, and we have used the gold-standard approaches of TEM, NTA and Western blot to verify the identity, isolation and quantity of these structures. By TEM we identified round, double-membraned structures consistent with previous reports on EV structure [78–80]. Next, the size distribution and the concentration of EVs released from Day 1 and Day 5 HKLs were quantified and found to be consistent across time points *in vitro*. Using Western blot for HSP90, we then confirmed the presence of a canonical mammalian EV protein marker is also associated within Atlantic salmon EVs. Finally, we report the presence and potential differential packaging of miRNAs, including immune-related miRNAs, into EVs released from Day 1 HKLs and Day 5 HKLs using small RNA-seq and RT-qPCR. These studies provide some of the first evidence for the isolation and validation of EVs from Atlantic salmon, and therefore provide a starting point for future studies aimed at examining these EV cargos, such as miRNA, in greater detail.

4.5.1. The Abundance profile of the EV miRNAs and the difference between Day 1 and Day 5 suggest they have a role in macrophage differentiation

The abundance of miRNAs in Day 1 EVs and Day 5 EVs show some striking differences to the abundance in monocyte-like HKLs (Day 1) and macrophage-like HKLs (Day 5) [47]. The mature ssa-miR-125b-1-3p is the most abundant miRNA in both groups of EVs, while it is less than 0.01% of mature miRNAs in the cells. Likewise, ssa-miR-92a-3p, ssa-miR-181a-5p and ssa-miR155-5p are much more abundant in EVs compared to their relative abundance in the cells at same developmental stage (Supplementary Table 4 and Supplementary File 1 in [47]). This indicates that they are selectively enriched in EVs as their abundance is not reflecting the general

abundance in the cells. In addition, ssa-miR-125b-1-3p, ssa-miR-181a-5p and ssa-miR155-5p also showed significant differences when comparing Day 1 EVs to Day 5 EVs. Together, this suggests that the miRNAs in the EVs serve particular functions. Altogether, the RNA-seq analysis identified 19 DE miRNAs packaged in Day 1 versus Day 5 HKL EVs. Most of these are conserved miRNAs (identical "seed" and very similar mature sequences in most vertebrates), and studies of miRNAs associated with macrophage differentiation and immune responses in other species may, therefore, shed light on their putative functions in Atlantic salmon.

The most abundant mature miRNA ssa-miR-125b-1-3p showed a large decrease from Day 1 EVs to Day 5 EVs. Interestingly, the miRNA-125 family is involved with immune system development and host defense (reviewed in [81]). In particular, miR-125b expression is enriched in murine macrophages, compared to T-cells and B-cells [82]. Overexpression of miR-125b in murine bone marrow cells induced a spread-like morphology with pseudopods and increased the protein expression of MHCII and the co-stimulatory molecules CD40, CD86 and CD80, indicating that miR-125b potentiates macrophage activation [83]. Similarly, a study by Zhang et al. (2013) identified a decrease in miR-125b expression in M1 macrophages compared to M2 macrophages [75]. In addition to identifying a decrease in miR-125b in Day 5 cells compared to Day 1 cells, our results identified monocyte to macrophage differentiation protein (*paqrb)* as a potential target of miR-125b. It is possible that the role of miR-125b in macrophage differentiation and function may be species specific. However, further experiments are required to determine this.

The sequencing results of this study found an increase in ssa-let-7a in Day 5 EVs. However, the RT-qPCR results did not find a significant difference in let-7a incorporation between Day 1 and Day 5 EVs which may in part be due to a different group of salmon being

used for RNA-seq and RT-qPCR. Let-7a miRNA expression is induced by LPS stimulation in human primary macrophages [84]. In addition, overexpression of let-7a in human THP-1 macrophages attenuated the increase of pro-inflammatory TNF-α and IL-6 mRNA levels following LPS stimulation [85]. These studies suggest a role for let-7a in macrophage function and inflammation.

RNA-seq identified increased incorporation of ssa-miR-155-5p in Day 5 EVs compared to Day 1 EVs. While we did not include ssa-miR-155-5p in the RT-qPCR study, our previous work found an increase in ssa-miR-155-5p in Day 5 HKLs compared to Day 1 HKLs by RNA-seq and RT-qPCR [47]. In several other species, miR-155 is involved in macrophage differentiation and function. The addition of exosomes loaded with miR-155 inhibitor to murine RAW macrophages resulted in decreased LPS-induced TNF-α protein levels [86]. In mammalian macrophages, miR-155 expression is increased following infection with *Listeria monocytogenes* or *Mycobacterium avium* and stimulation with both LPS and poly(I:C) [87,88]. Similarly, stimulation of macrophages isolated from the fish species ayu (*Plecoglossus altivelis*) with *Vibrio anguillarum* increased miR-155 expression while overexpression of miR-155 increased the expression of pro-inflammatory cytokines and decreased the expression of anti-inflammatory cytokines [89]. The results of these studies, in addition with the results of this current study, suggest that miR-155 may be a marker of immune response in both EVs and miRNAs.

4.5.2. Increased ssa-miR-146a incorporation in Day 5 HKL EVs compared to Day 1 HKL EVs

Despite substantial biological variability between individual fish, this study validated the increased packaging of ssa-miR-146a-5p in EVs derived from Day 5 HKLs compared to EVs from Day 1 HKLs by both RNA-seq and RT-qPCR. MiR-146a plays a role in macrophage differentiation, activation and function in several species including some fish species [76,90–94].

However, to date, no studies have identified the presence of miR-146a in fish EVs. A study by Song et al. [95] found that human mesenchymal stem cells (MSCs) stimulated with the pro-inflammatory cytokine Il-1β produced exosomes that transferred miR-146a to macrophages where it induced the downregulation of M1 markers and upregulation of M2 markers, suggesting it has a role in macrophage polarization [95]. In other studies, miR-146a -/- knockout mice injected with miR-146a-containing exosomes had reduced TNF-α and Il-6 serum levels following LPS injection, compared to mice injected with miR-146a deficient exosomes, demonstrating that exosomal miR-146a can also play a role in moderating the inflammatory response [29].

At the cellular level, miR-146a is induced by PU.1, a transcription factor that stimulates the differentiation of hematopoietic stem cells (HSCs) into lymphoid-myeloid progenitors [93]. In a mouse transplant model, forced expression of miR-146a directed the differentiation of HSCs into peritoneal macrophages. Congruently, preventing miR-146a function in a zebrafish model inhibited the formation of macrophages [93]. Infection of human primary monocytes and the human monocytic cell line THP-1 with bacterial pathogens such as *Salmonella* serovar Typhimurium DT104 and *Mycobacterium avium*, and infection of murine bone marrow-derived macrophages (BMDMs) with *Listeria monocytogenes* increased miR146a expression, suggesting that miR-146a is involved in regulating macrophage response to infection, as well as their differentiation [87,88,96]. In addition, its expression is increased in the Atlantic salmon head kidney following formalin-killed typical *Aeromonas salmonicida* or poly(I:C) injection [97]. Overexpression of miR-146a in grouper macrophages promoted Singapore grouper iridovirus (SGIV) propagation while inhibition of miR-146a decreased virus production [91]. In our previous study, miR-146a was upregulated in Day 5 HKLs compared to Day 1 HKLs [47].

Therefore, we propose that since miR-146a is DE in Day 1 and Day 5 EVs and HKLs, it may be an indicator for the presence of macrophage cells and/or macrophage activation and function.

4.5.3. The highly abundant miR-21a is associated with macrophage activation in vertebrates

While RNA-seq did not identify a significant difference in the incorporation of ssa-miR-21a-5p, RT-qPCR analysis identified a significant increase in ssa-miR-21a-5p in Day 5 HKL EVs. Again, this may in part be due to a different group of fish being used for RNA-seq and RT-qPCR. At the cellular level, miR-21a is involved in macrophage activation and immune response in both mammals and fish [33,72,73,]. MiR-21 deficient mice had decreased expression of M1 macrophage markers and enhanced expression of M2 markers, while transfection of a miR-21 mimic enhanced M1 markers and decreased M2 markers [99]. Stimulation with LPS, poly(I:C) or *V. anguillarum* upregulated expression of miR-21 in cultured macrophages from miiuy croaker [33,98]. Inhibiting miR-21 in miiuy croaker macrophages increased the expression of inflammatory cytokines (*tnfa, il6)* and antiviral genes (*mx1, isg15*), suggesting a role for miR-21 in fish macrophage function [98]. Our previous work identified miR-21a as the most abundant miRNA in both Day 1 and Day 5 HKLs, as well as upregulated in Day 5 HKLs compared to Day 1 HKLs [47].

M1 macrophages are broadly considered to be pro-inflammatory, whereas M2 macrophages are considered anti-inflammatory [42,43]. During an immune response, there is an increase in macrophage number and activity [100]. The enrichment of miR-21a in Day 5 HKL EVs is, therefore, suggestive of differentiation associated with M1 polarization (unlike miR-146a) and may be useful as an EV biomarker for evaluating pro-inflammatory vs. anti-inflammatory responses in Atlantic salmon. Future studies examining the miRNA packaged in

EVs following pathogen exposure, or stimulation with M1 (i.e. IFNγ) or M2 (i.e. IL-4 or IL-13) cytokines, are required to test this hypothesis.

Interestingly, it has been previously reported that the abundance of miR-21 in Atlantic cod EVs is responsive to changing environmental conditions. Exosomes isolated from Atlantic cod sera contained higher levels of miR-21 in fish reared at 9°C compared to fish reared at 4°C, suggesting that EV miR-21 may be a biomarker for exposure to environmental stress [9]. In addition, there were significantly less EVs in the serum of cod reared at 9°C further suggesting EV biogenesis is linked to environmental conditions [9].

4.5.4. Teleost- specific ssa-miR-2188 and ssa-miR-731 are present in HKL EVs

MiR-731 and miR-2188 are teleost-specific, immune-responsive miRNAs [31,38,39]. This study is the first to identify miR-731 and mir-2188 in fish EVs. The sequencing results of this study found a significant increase of miR-731 in Day 5 EVs compared to Day 1 EVs and a decrease of miR-2188 in Day 5 EVs, although the decrease of miR-2188 was not significant. In several fish species including Atlantic salmon, miR-731 is upregulated in response to both viral and bacterial challenges [31,36,38,41]. Loss of the miR-462-731 cluster in zebrafish decreased erythroid cell numbers, and myeloid cell expansion, suggesting a role for miR-731 in regulating hematopoiesis [101]. Interestingly, the PU.1 motif and several IRF-transcription binding motifs are upstream of the miR-462/731 miRNA gene indicating that these mature miRNAs are important in hematopoietic stem cell differentiation and immune response [38]. In Atlantic salmon and in olive flounder (*Paralichthys olivaceus*), miR-2188 expression decreased in cardiac tissue following salmonid alphavirus (SAV) infection and decreased in the head kidney following viral hemorrhagic septicemia virus (VHSV) infection, respectively [34,38]. Conversely, miR-2188 expression increased in Atlantic cod HKLs following 48 h and 72 h of

poly (I:C) stimulation, while its expression was significantly downregulated in unstimulated cod HKLs at 72 h compared to cells cultured for 12 and 24 h.

4.5.5. Discrepancies between RNA-seq and RT-qPCR data

EV biogenesis is known to generate a diverse, heterogeneous population of vesicles. Previous studies have shown that individual EVs may vary considerably with respect to the biomolecules they incorporate, but that the population as a whole may be representative of a particular cell type or physiological state [102,103]. However, validation of results using complementary techniques remains technically challenging. EVs have a small internal lumen space and packing volume [102,104,105] and therefore a limited number of molecules are available for analysis in any given EV isolation. Due to the low concentration of RNA that could be isolated from the EVs, our RNA-seq and RT-qPCR analyses were performed on independent biological samples (i.e. different groups of Atlantic salmon). While our RT-qPCR analysis validated the presence of all miRNAs identified by RNA-seq, we could only corroborate a significant change of one of the miRNAs (ssa-miR-146a-5p).The use of a different group of fish for RNA-seq and for RT-qPCR may in part account for the variability between sequencing and RT-qPCR results. Despite these limitations, it is particularly noteworthy that two independent groups of fish showed the inclusion of the same miRNA species, suggesting that these miRNAs reflect an accurate depiction of the underlying cellular biology. In addition, several of the miRNAs identified in this study are immune-relevant in both mammals and fish. The RT-qPCR results exhibited high biological variability as observed in RNA-seq, but together these data suggest that differential packaging of miRNAs into EVs, including miRNAs involved in the immune response and macrophage activation, is a feature of Atlantic salmon HKL differentiation. Future studies will seek to improve power by performing validation experiments

on a larger cohort of specimens or minimize intra-assay biological variability by using pooled populations of EV material.

HSPs appear to be the best conserved markers of EVs [106] and are present essentially ubiquitously. A study in rainbow trout found increased HSP70 protein expression in exosomes released from hepatocytes following a 1 hour heat shock [10]. However, cortisol treatment significantly reduced the expression of HSP70 in hepatocyte released exosomes [10]. Nevertheless, since Vn96 binds to multiple HSPs, changes to individual proteins will not likely impact the overall number of EVs isolated when cells are similarly healthy. We found that cells in both Day1 and Day 5 HKL cultures are equally healthy based on visual observations of adherent cultures where there was no change in cell debris seen, the observation that EV concentration and size are similar, and similar amounts of RNA were extracted. In addition, our previous study found that was similar amounts of RNA were extracted from the cells and there was an increase in phagocytic ability at Day 5 [47]. Therefore, we conclude that Vn96-based isolation is a sound approach. In addition, previous experiments in our lab have found that Vn96 can extract EVs in multiple sequential incubations which suggests that the one incubation performed here is not enough to saturate the capacity of the Vn96. It remains to be determined if different quantities of EVs from healthy compared to highly stressed cells are isolated by Vn96.

4.5.6. In silico analysis of DE miRNAs identified potential targets that are macrophage and immune relevant

We performed *in silico* target prediction analysis to identify putative targets of the DE miRNAs in Day 1 and Day 5 HKL EVs. The identified putative targets included transcription factors, lipid-related genes and immune-related genes that are associated with macrophage function and immune response. For example, the transcription factors *klf2* (a putative target of

miR-222cd-3p and miR-221-3p) and *gata3* (a putative target of miR-146a-5p and miR-125b-1-3p). *Klf2* is a negative regulator of monocyte activation and function while *gata3* is involved with the regulation of M2 macrophages [107–109]. Lipid-related genes such as fatty acyl-CoA reductase (*facr1*; miR-27b-3p, miR-221-3p, miR-222cd-3p), sterol regulatory element-binding proteins (*srebps*; miR-731-5p, miR-27b-3p) and delta-6 fatty acyl desaturase (*d6fadc* or *fads2;* miR-181a-5p) were also identified as putative targets. The lipid-related transcriptome changes dramatically during monocyte to macrophage differentiation, including the levels of *facr1, srebps* and *fads2,* suggesting they play a role in macrophage differentiation and/or function [110,111]. Finally, several immune and inflammation-related genes were also potential targets, including *tnfa* (miR-16a-5p), viperin (alias *rsad2*; miR-222cd-3p and miR-221-3p), granulin (miR-155-5p), *irfg* (miR-27b-3p) and the transcription factors *irf4*, *irf7* (miR-731-5p) and *irf9* (miR-8156-5p). In addition, monocyte to macrophage differentiation protein (*paqrb*) was a target of the most abundant miRNA (ssa-miR-125b-3p) which was also significantly DE in Day 1 vs Day 5 HKL EVs. It is important to note that many of these targets may likely be false positives [112] and functional assays, such as manipulation of the miRNA of interest, are necessary to prove a potential target.

4.5.7. Further studies are needed to elucidate the function of miRNAs in fish EVs

Several studies have demonstrated the transfer and uptake of miRNA-containing EVs from multiple cells types, including immune cells (reviewed in [3,113]). EV transfer of miRNAs regulates gene expression in the target cell, thereby modulating its function [113]. For instance, human monocytes release miR-150 containing EVs which are taken up by endothelial cells prompting cell migration; macrophage-derived EVs transfer miR-233 to monocytes inducing cell differentiation; and T cells release EVs containing miR-335 are taken up by APCs and modulate

gene expression [28,114,115]. The target cells and subsequent effects of HKL-derived EVs on these cells remain unexplored. We identified several immune-related miRNAs packaged in EVs released from HKLs. It is possible that these EVs target other immune cells to regulate their response during both health and disease. However, future studies are required to elucidate the function of fish HKL EVs.

4.6. Conclusion

Very little is known about the biology of fish EVs, their molecular profile or their function. Our previous work identified changes in miRNA expression in Atlantic salmon HKLs as they differentiated during *in vitro* culture [47]. Several of the miRNAs identified as being upregulated in Day 5 HKLs (e.g. ssa-miR-146a, ssa-miR-155 and ssa-miR-731) [47] were also identified as upregulated within the EVs derived from Day 5 HKLs by RNA-seq. Then again, the abundance of some miRNAs in EVs were very different to the abundance of miRNAs in HKLs. Together, this indicates that profiling a selection of these miRNAs could both confirm that they originate from HKL EVs (e.g. sssa-miR-125b) and provide useful information about HKL maturation (e.g. expression of ssa-miR-146a, ssa-miR-155 and ssa-miR-731). Used in such a manner, these miRNAs may be useful biomarkers of fish macrophages. Many of the identified miRNAs are also involved in macrophage differentiation and function in both mammals and fish, including ssa-miR-146a and ssa-miR-21a, further suggesting that these miRNAs are involved in immune response and/or macrophage activation. Future studies should focus on functional studies required to test this hypothesis. Thus, our study provides a suitable foundation for future studies on the ability of EVs to serve as indicators of fish immune cell differentiation, activity and their response to stress or disease.

4.7. References

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Chapter 5

Transcriptome profiling of Atlantic salmon adherent head kidney leukocytes reveals that macrophages are selectively enriched during culture

5.1. Abstract

The Atlantic salmon (*Salmo salar*) is an economically important fish, both in aquaculture and in the wild. In vertebrates, macrophages are some of the first cell types to respond to pathogen infection and disease. While macrophage biology has been characterized in mammals, less is known in fish. Our previous work identified changes in the morphology, phagocytic ability, and miRNA profile of Atlantic salmon adherent head kidney leukocytes (HKLs) from predominantly "monocyte-like" at Day 1 of *in vitro* culture to predominantly "macrophage-like" at Day 5 of culture. Therefore, to further characterize these two cell populations, we examined the mRNA transcriptome profile in Day 1 and Day 5 HKLs using a 44K oligonucleotide microarray. Large changes in the transcriptome were revealed, including changes in the expression of macrophage and immune-related transcripts (e.g. *csf1r, arg1, tnfa, mx2),* lipid-related transcripts (e.g. *fasn, dhcr7, fabp6*), and transcription factors involved in macrophage differentiation and function (e.g. *klf2, klf9, irf7, irf8, stat1*). The *in silico* target prediction analysis of differentially expressed genes (DEGs) using miRNAs known to change expression in Day 5 HKLs, followed by gene pathway enrichment analysis, supported that these miRNAs may be involved in macrophage maturation by targeting specific DEGs. Elucidating how immune cells, such as macrophages, develop and function is a key step in understanding the Atlantic salmon immune system. Overall, the results indicate that, without the addition of exogenous factors, the adherent HKL cell population differentiates *in vitro* to become macrophage-like.

5.2. Introduction

Macrophages are white blood cells, found in all vertebrate species, that play a role in both the innate and adaptive immune systems [1]. In innate immunity, macrophages provide some of the first lines of defense against infections and diseases, where they act as phagocytic cells to destroy foreign pathogens [2]. In the adaptive immune system, macrophages function as a bridge between the innate and adaptive immune responses, acting as antigen-presenting cells to activate T lymphocytes [2,3]. Much of our knowledge of macrophage biology, such as macrophage differentiation and polarization, comes from mammalian models, while macrophages remain to be fully characterized across all fish species. However, using the mammalian model system as a platform and through various fish models, including zebrafish (*Danio rerio*), ginbuna crucian carp (*Carassius langsdorfii*) and goldfish (*Carassius auratus*), our knowledge of fish macrophage differentiation and activation is starting to expand (reviewed in [4]).

The ways in which macrophages respond to infections and diseases have been well-characterized in mammals: by producing cytokines and other inflammation-related proteins, by engulfing foreign pathogens through phagocytosis, and by destroying foreign pathogens by producing reactive oxygen species (ROS) and nitric oxide (NO), among other responses [2,5]. Macrophages demonstrate a high degree of plasticity, with the ability to generate different subtypes (also fully described in mammals): M1 macrophages (or classically activated) and M2 macrophages (or alternatively activated) [6]. M2 macrophages can be further separated into distinct sub-populations, based on their activation and function (M2a, M2b, M2c) [6]. M1 macrophages are considered pro-inflammatory; they are activated by cytokines including IFN-γ and TNF-α and produce pro-inflammatory cytokines and ROS to protect against pathogens [7]. Similar to mammals, IFN-γ and TNF-α have been described in several fish species, where they

induce pro-inflammatory effects including increased phagocytosis, increased ROS and NO production, and enhanced expression of inflammatory cytokines [8–16]. On the other hand, M2 macrophages are considered anti-inflammatory and are linked to immunosuppression and wound repair. M2 macrophages are activated by cytokines such as IL-4 and IL-13 (M2a), immune complexes or apoptotic cells (M2b) and IL-10, TGF-β or glucocorticoids (M2c) and are characterized by increased arginase activity, decreased microbicidal activity, and increased production of collagen and polyamines necessary for cell growth and wound-healing [3,7,17,18]. Teleost fish *il-4/13A* and *il-4/13B* genes have been identified and have similar functions as their mammalian counterparts; stimulation of macrophages from various teleost species with recombinant (r-) IL-4/13A and r-IL-4/13B increased the expression of immunosuppressive genes such as *tgf-β, il-10* and *socs3*, increased arginase activity, and decreased the expression of pro-inflammatory genes and NO production [3,10,18–21].

Hematopoiesis, the process of blood cell formation, begins when a self-renewing hematopoietic stem cell (HSC) commits to a multipotent progenitor (MPP), which then gives rise to a common myeloid progenitor (CMP) cell. The CMP will then differentiate into either a megakaryocyte/erythroid progenitor (MEP) or a granulocyte/macrophage progenitor (GMP), which gives rise to erythrocytes/platelets or granulocytes/monocytes, respectively [4,22]. This process is tightly controlled by a multitude of cytokines, growth factors, and transcription factors and has been extensively studied in mammals. In particular, monocyte-to-macrophage differentiation, as well as macrophage polarization, are regulated by multiple factors including the growth factor colony-stimulating factor 1 (CSF1) and its receptor, CSF1R, the transcription factor PU.1, and members of the CCAAT/enhancer-binding proteins (C/EBP), interferon regulatory factor (IRF) and signal transducer and activator of transcription (STAT) families,

among many others (reviewed in [23]). One of the first studies to investigate fish macrophage differentiation examined goldfish primary kidney macrophages and identified three sub-populations that were characterized as progenitor cells, monocytes, and macrophages, with each population expressing differentiation markers including *c-kit* (early progenitors), *granulin* (monocytes) and *legumain* (mature macrophages) [24]. It is now well-known that CSF1 and CSF1R are required for both mammalian and teleost myeloid cell differentiation [3]. While our knowledge of fish macrophage biology is advancing, macrophage differentiation and polarization across all teleost species, including the Atlantic salmon (*Salmo salar*), remain to be described. The Atlantic salmon is an economically important farmed fish species in several countries including Canada, Norway and Chile. Given the essential role of macrophages in defense against pathogens, investigation into the genes and molecular pathways involved in Atlantic salmon macrophage differentiation and function is central to fully understanding the fish immune response and will aid in developing methods of disease prevention, therefore improving the health of farmed fish.

In mammals, HSCs originate from the bone marrow, while in fish, the primary hematopoietic organ is the anterior (or head) kidney. A heterogeneous population of adherent leukocytes, containing monocytes and macrophages, amongst other cells, can be isolated from the head kidney using Percoll density gradient centrifugation [21,25,26]. Head kidney leukocytes (HKLs) are frequently used as a macrophage-like model in fish immunological studies ([27–31], and many others); however, many of these studies use HKLs from different culture times, which may produce data that are from different cell populations. Our previous work observed a change in the morphology, phagocytic ability, and miRNA profile of Atlantic salmon HKLs *in vitro*, suggesting that the cells differentiate from predominantly “monocyte-like” at Day 1 of culture to

predominantly “macrophage-like” at Day 5 of culture [32]. Microarrays are powerful tools that have been used to identify changes in gene expression profiles during fish immune responses (reviewed in [33,34]). Therefore, to further characterize the HKLs *in vitro,* we used 44K salmonid oligonucleotide microarrays [35] to examine the global transcript expression profiles of Atlantic salmon adherent Day 1 HKLs versus Day 5 HKLs.

5.3. Materials and Methods

5.3.1. Animals

The Atlantic salmon (1.2 kg ± 0.3 kg SD) used in this experiment were held in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre in a 3,800 L tank and kept at 12˚C with 95-110% oxygen saturation, using a flow-through seawater system. All procedures in this experiment were approved by Memorial University of Newfoundland’s Institutional Animal Care Committee (protocols: 18-01-MR and 14-02-MR) based on the guidelines of the Canadian Council of Animal Care. Five animals were used for the microarray experiment (one animal was removed following array hybridizations due to a technical error in labelling, therefore 4 animals were used for subsequent analysis), and 5 different animals were used for RT-qPCR analysis.

5.3.2. Macrophage isolation and culture

HKLs were isolated as previously described [32,36]. Atlantic salmon were euthanized with an overdose of MS222 (0.4 g/L, Syndel Laboratories, Vancouver, BC, Canada). The head kidney was removed and placed in isolation media: 500 mL of Leibovitz-15 medium (L-15, Gibco, Carlsbad, CA, USA) supplemented with 2.5% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco), and 27.5 mg of heparin (Sigma-Aldrich, St. Louis, MO, USA). The head kidney was pushed through a 100 µM nylon cell strainer (Thermo-Fisher Scientific,

Waltham, MA, USA), then placed on a 34/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) prepared with 5% Hank's buffered salt solution (HBSS; Sigma-Aldrich) to ensure an isotonic solution, and centrifuged at 500 x g for 30 min at 4˚C. Following centrifugation, the interface between the 34% and 51% gradient, which contains leukocytes, was collected and washed twice in isolation media at 500 x g for 5 min at 4˚C. The cells were then re-suspended in culture media (L-15 supplemented with 5% FBS and 1% penicillin/streptomycin), and viable cells were counted on a haemocytometer using the Trypan Blue (Sigma-Aldrich) exclusion method. The cells were then seeded in 6-well culture plates (Corning Inc., Corning, NY, USA) at 3 x 10^7 cells in 2 mL of culture media and incubated at 15˚C for 24 h to allow cell adherence. Cells were then washed twice in culture media to remove non-adherent cells, and the media was replaced with fresh culture media. Media was changed every 48 h thereafter for up to 5 days.

5.3.3. Sampling of head kidney cells for RNA extraction

Twenty-four hours (Day 1) and 120 h (Day 5) after seeding, cells were washed twice in cell culture media then lysed in 500 μL of TRIzol (Invitrogen, Burlington, ON, Canada) and immediately placed at -80°C until RNA extraction.

5.3.4. RNA extraction

Total RNA was extracted from the TRIzol-lysed samples following the manufacturer's protocol, and RNA pellets were dissolved in DNase/RNase-free water (Gibco). The RNA samples were treated with 6.8 Kunitz units of DNase I (Qiagen, Mississauga, ON, Canada) to degrade residual genomic DNA, followed by purification using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was measured using NanoDrop spectrophotometry, and RNA integrity was checked by 1% agarose gel

electrophoresis. All column-purified RNA samples had A260/280 and A260/230 ratios above 1.8.

5.3.5. Microarray hybridization

Day 1 (24 h) and Day 5 (120 h) samples were subjected to microarray analysis using the consortium for Genomic Research on All Salmonids Project (cGRASP)-designed Agilent 44K salmonid oligonucleotide microarray [35]. The microarray experiment was based on a common reference design, where the differences among Day 1 and Day 5 HKL samples were determined by comparing individual samples against a common reference pool consisting of equal quantities from all samples.

Five hundred nanograms of each sample of DNase-treated, column purified RNA were *in vitro* transcribed into antisense amplified RNA (aRNA) using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and quantity of the aRNAs were checked by agarose gel electrophoresis and NanoDrop spectrophotometry, respectively. Amplified RNA from all samples was pooled and used as a common reference. Twenty micrograms of aRNA were ethanol precipitated overnight and re-suspended in coupling buffer. The experimental samples were then labelled with Cy5 (GE Healthcare Life Sciences, Buckinghamshire, UK), while the common reference was labelled with Cy3 (GE Healthcare Life Sciences), following the manufacturer's instructions. The efficiency of labelling and aRNA concentration were assessed using the "microarray" function of the NanoDrop spectrophotometer. The Cy5-labelled aRNA (825 ng) from each experimental sample was mixed with an equal quantity of Cy3-labelled aRNA from the common reference, and the resulting pool was fragmented using the Gene Expression Hybridization Kit, following the manufacturer's instructions (Agilent, Mississauga, ON, Canada). Each labelled aRNA pool was co-hybridized to

the microarray (8 arrays final in total, Figure 5.1A) for 17 h at 65°C with 10 rpm rotation using an Agilent hybridization oven. The array slides were washed immediately following hybridization as per the manufacturer's instructions.

5.3.6. Microarray data acquisition and analysis

The microarray slides were scanned at 5 µm resolution and 90% laser power using a ScanArray Gx Plus scanner and ScanExpress v4.0 software (Perkin Elmer, Waltham, MA, USA), and the Cy3 and Cy5 channel photomultiplier tube (PMT) settings were adjusted to balance the fluorescence signal. The raw data were saved as TIFF images, and the signal intensity data were extracted using Imagene 9.0 (BioDiscovery, El Segundo, CA, USA). R and the Bioconductor package 'marray' were used for background correction, removal of low-quality spots on the microarray and to $\log_2$-transform and Loess-normalize the data [37]. Probes with more than 25% missing values were omitted from the dataset, and the missing values were imputed using the least square methods ('EM_array') and the 'LSimpute' package [37–39]. The final dataset that was used for statistical analyses consisted of 18,108 probes for all arrays. The data have been submitted to NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE173493.

A two-class paired Significance Analysis of Microarrays (SAM) [40] with a false discovery rate (FDR) of 0.05 was used to determine the differentially expressed probes (DEPs) between Day 1 and Day 5 groups, using R and the SAM project GitHub repository (https://github.com/MikeJSeo/SAM) [41]. The resulting significant transcript lists were annotated using the contiguous sequences that were used to design the 60mer oligonucleotide probes of the array [35]. Annotation was carried out with BLASTx searches against the NCBI non-redundant (nr) amino acid sequence database using an E-value threshold of 10^{-5} [42].

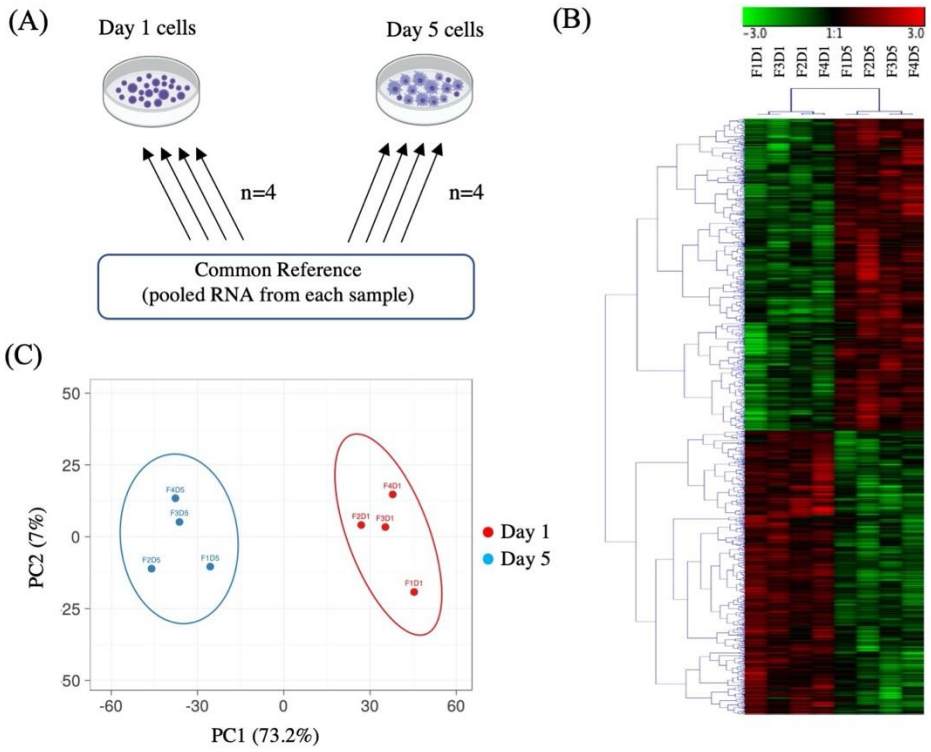

Figure 5.1. Overview of microarray experimental design and global gene expression profiles. (A) Common reference-based microarray experimental design. Each arrow represents one array and identifies the samples co-hybridized on that array; the base of the arrow identifies the Cy3-labeled sample and the head of the arrow identifies the Cy5-labeled sample. **(B)** Hierarchical clustering analysis of 2140 DEPs in Day 1 and Day 5 HKLs identified by paired SAM (FDR 0.05). Complete linkage was performed on median-centred genes using a Pearson correlation. Green represents downregulation and red represents upregulation. F represents fish; D represents Day (i.e. F1D1 is Fish 1 Day 1). **(C)** Principal component analysis (PCA) of Day 1 and Day 5 samples based on DEPs identified by paired SAM (FDR 0.05). Day 5 samples are represented by blue, Day 1 samples are represented by red. The X and Y axis show principal component 1 (PC1) and principal component 2 (PC2) that explain 73.2% and 7% of the total variance, respectively.

5.3.7. GO term enrichment and network analysis, hierarchical clustering analysis and principal coordinate analysis

Gene Ontology (GO) term enrichment analyses for all (both upregulated and downregulated) differentially expressed genes (DEGs; the distinction between DEPs and DEGs is explained in section 3.1), with a fold-change > |2| were performed using ClueGO plugin, available at the Cytoscape software (version 3.8.2). The ClueGO plug-in identifies and integrates significant GO terms from large gene lists and generates a functionally grouped GO term network [43]. In this study, the GO database (30.03.2021) for the categories biological process (BP) and cellular component (CC) was used for analysis. The enrichment/depletion analysis was performed using a two-sided hypergeometric test after its adjustment by the Bonferroni step-down procedure. The kappa-statistics score threshold was set to 0.4 and GO pathways/terms with a p-value <0.05, corrected with the Bonferroni step-down procedure, were considered significant.

Genesis software (Rockville, MD, USA) was used for the hierarchical clustering and heatmap visualization of median centred data of DEPs (for analysis of the entire experiment) and of DEGs (for analysis of selected significant GO terms identified using ClueGO; see section 3.2) using Pearson correlation and complete linkage clustering. Hierarchical clustering analysis of all DEPs grouped Day 1 samples together and Day 5 samples together, with the exception of 1 fish (Fish 5), which we eliminated from further analysis. Principal components were calculated using the Singular Value Decomposition method and ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap [44].

5.3.8. cDNA synthesis

Five hundred nanograms of purified RNA were reverse transcribed to cDNA in 20 µL reactions consisting of random primers (250 ng; Invitrogen) and MMLV-reverse transcriptase (200

U; Invitrogen) with the manufacturer's first-strand buffer (1x final concentration), DTT (10 mM final concentration), 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP) and RNase OUT (40 Units; Invitrogen) at 37°C for 50 min.

5.3.9. Reverse transcription quantitative PCR (RT-qPCR)

For RT-qPCR validation, HKLs from 5 additional Atlantic salmon (i.e. different from those used in the microarray experiment) were harvested, RNA isolated, and cDNA synthesized as stated in sections 2.2-2.4 and section 2.8. All primer sets used for RT-qPCR analysis were quality-tested according to MIQE guidelines [45]. For each primer set, amplification efficiencies were determined by a 5-point standard curve using pooled cDNA from 5 fish, starting at 10 ng of input RNA, diluted in DNAse/RNAse-free water (Thermo Fisher Scientific) [46]. Only primer pairs generating an amplicon with a single melting peak and no primer-dimer present in the no-template control (NTC) were used for RT-qPCR analysis. Primer sequences, amplification efficiencies, R^2, and amplicon sizes for each assay can be found in Supplementary Table 5.1.

Five candidate normalizer genes were tested with cDNA from all experimental samples to determine the 2 most stable normalizer transcripts (i.e. with lowest M-value) using GeNorm software [47]. The candidate normalizer genes tested were *60S ribosomal protein 32 (rpl32), elongation factor 1 alpha-1 (ef1a1), RNA polymerase 2 (polr2), polyadenylate-binding protein 1 (pabpc1) and elongation factor 1-alpha-2 (ef1a2).* The 3 most stable genes were *ef1a2* (M-value 0.180), *ef1a1* (M-value 0.187) and *rpl32* (M-value 0.198). The normalizers chosen for this study were *ef1a2* and *rpl32*. The geometric mean of *ef1a2* and *rpl32* was calculated for each sample and was used as normalizer value in the relative quantity (RQ) calculations stated below.

For each reaction, 50 nM of both the forward and reverse primers and cDNA template representing 5 ng of input RNA were mixed with Power SYBR Green Master Mix (Thermo

Fisher Scientific) for a total reaction volume of 13 µl. The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min, with fluorescence detection at the end of each 60°C step. All reactions were run in triplicate in a ViiA 7 Real-Time PCR System (382-well format) (Applied Biosystems/Life Technologies). The RQ values of a given mRNA of interest were calculated using Excel, and relative to a calibrator (i.e. the Day 1 sample with the lowest expression (i.e. assigned a RQ value = 1.0)) taking into account the amplification efficiencies [46]. A paired Student's T-test was used to determine statistical differences. All statistical analyses were performed using GraphPad Prism v 8.0 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

5.3.10. In silico prediction of putative miRNA target genes and target gene pathway analysis

The miRNA target prediction tool RNAhybrid (v.2.2) [48] was used to determine if any of the DEGs identified in this study could be potential targets of the miRNAs identified as significantly differentially expressed (DE) in Day 1 monocyte-like cells compared with Day 5 macrophage-like cells in Smith et al. [32]. The mature miRNAs analyzed were selected from those DE in Smith et al. [32], but in cases where both mature miRNAs from the same precursor were DE then only the most abundant (which is most likely to be the guide miRNA) was used. The 36 miRNAs used, along with their mature sequences [49] are given in Supplementary Table 5.2. The parameters applied in the RNA hybrid analysis were: No G:U in seed, helix constraint 2–8, loop constraints 5–5 and a minimum free energy threshold of -20 kcal/mol. These parameters allowed RNAhybrid to detect only candidate genes with perfect seed complementarity and high base-pairing stability.

The input sequences for target genes were those DEGs from this study with 3' untranslated region (UTR) information, found using the ExUTR pipeline [50]; i.e. a total of 1234

out of the 1477 DEGs. The predicted target genes from the *in silico* target gene prediction analysis were used as input in a gene pathway enrichment analysis [51] against the bioplanet database of all known biological pathways [52]. Gene pathways are poorly described in Atlantic salmon, therefore the gene symbols for putative human orthologs were used against the human database. The significance level for enrichment was set as p-adjusted (Q-value) less than 0.05.

5.4. Results

5.4.1. Global transcriptomic changes in Atlantic salmon HKLs in response to culture period

Our previous work identified a change in the morphology, phagocytic ability, miRNA profile, and mRNA expression of two macrophage markers (*mhc ii* and *marco*), in Day 1 and Day 5 adherent HKLs [32]. To explore changes in the mRNA transcriptome between these two cell populations, the DEPs between Day 1 (i.e. predominantly monocyte-like) and Day 5 (i.e. predominantly macrophage-like) HKLs were identified using a 44K salmonid microarray platform [35]. The design for this microarray study is illustrated in Figure 5.1A. Using paired Significance Analysis of Microarrays (SAM) and a false discovery rate (FDR) of 0.05, 2140 DEPs were identified; 1123 DEPs were identified as upregulated in Day 5 HKLs compared to Day 1 HKLs while 1017 DEPs were downregulated in Day 5 HKLs compared to Day 1 HKLs. Using BLASTn/BLASTx searches against NCBI nr/nt databases, putative identities were determined for 2034 of the 2140 DEPs (1076 upregulated DEPs, 958 downregulated DEPs). The 44K platform contains some redundancies (i.e. multiple probes for one gene). Therefore, taking the redundancy into account, 1477 differentially expressed genes (DEGs) with known putative identities were identified (797 upregulated DEGs in Day 5 and 680 downregulated DEGs in Day 5). Selected DEPs for discussion can be found in Table 1, and complete information on the DEPs and paired SAM results can be found in Supplementary Tables 5.3 and 5.4.

Hierarchical clustering analysis of median-centered DEPs grouped Day 1 samples and Day 5 samples separately (Figure 5.1B). Similarly, principal component analysis (PCA) also grouped Day 1 samples separately from Day 5 samples together (Figure 5.1C). PC1 and PC2 accounted for 73.2% and 7.0% of the variation, respectively. Day 1 samples showed a positive loading on PC1, whereas Day 5 samples showed a negative loading on PC1. There was a near spilt between positive/negative loading on PC2 with both Day 1 and Day 5 samples. These data indicate that Day 1 HKLs and Day 5 HKLs represent two separate groups of cells with distinct molecular phenotypes.

5.4.2. GO term network analysis identified immune-related and lipid-related terms

To further understand the biological relevance of the identified DEGs, gene ontology (GO) term enrichment analyses, followed by network analysis, were performed on all DEGs with a fold-change greater than |2| (FDR = 0.05). GO terms with p-values less than 0.05 were considered statistically significant. The analysis resulted in 111 significant GO terms divided into 19 groups. The top GO term group (i.e. lowest individual term p-value) was “leukocyte activation” (GO:0045321; p-value 1.34e-15) which was the leading term of two groups, group 17 of which 36 GO terms belong and group 18, of which 55 GO terms belong, followed by “myeloid cell activation involved in immune response” (GO:0002275; p-value 2.02e-09) of which 13 GO terms belong, followed by “extracellular exosome (GO:0070062; p-value 2.00e-

Table 5.1. Selected[a] probes differentially expressed between Day 1 and Day 5 HKLs

	Probe ID[b]	Gene symbol	Gene description[c]	Log_2 fold-change[d]
Upregulated in Day 5 HKLs				
Immune-related				
	C228R013	*tlr3*	Toll-like receptor 3_1*	4.04
	C157R134	*csf1r*	Macrophage colony-stimulating factor 1 receptor$_4$	3.28
	C095R005	*il12b*	Interleukin-12 subunit beta$_2$	3.12
	C040R101	*ifit5*	Interferon-induced protein with tetratricopeptide repeats 5_4	2.86
	C163R118	*mrc1*	Macrophage mannose receptor 1_5	2.71
	C236R043	*mx2*	Interferon-induced GTP-binding protein $Mx2_2$	2.57
	C237R068	*tnfa*	Tumor necrosis factor (TNF-alpha)$_2$	2.45
	C041R022	*mx3*	Interferon-induced GTP-binding protein $Mx3_1$	2.28
	C022R023	*socs1*	Suppressor of cytokine signaling 1_1	2.18
	C139R032	*rsad2*	Radical S-adenosyl methionine domain-containing protein 2 (alias viperin)$_1$	2.13
	C029R132	*ifng1*	Interferon gamma 1_2	2.06
	C198R010	*hamp*	Hepcidin-1_2	1.94
	C063R127	*ddx58*	Probable ATP-dependent RNA helicase $DDX58_2$	1.59
	C174R152	*cd83*	CD83 antigen$_5$	1.30
Lipid-related				
	C066R040	*fadsd5*	Delta-5 fatty acyl desaturase$_1$	5.78
	C227R073	*lpl*	Lipoprotein lipase$_2$	4.45
	C193R045	*elovl6*	Elongation of very long chain fatty acids protein 6_1	4.19
	C180R145	*lipe*	Lipase, hormone-sensitive$_3$*	3.99
	C038R110	*fadsd6*	Delta-6 fatty acyl desaturase (alias fatty acid desaturase 2 (*fads2*))$_1$*	3.79
	C119R039	*dhcr7*	7-dehydrocholesterol reductase$_2$	3.26
	C004R046	*fasn*	Fatty acid synthase$_3$	3.11
Transcription factors	C143R078	*irf7*	Signal transducer and activator of transcription 1-alpha/beta$_3$*	2.73
	C261R073	*stat1*	Interferon regulatory factor 7_3[e]	2.70
	C169R001	*irf3*	Interferon regulatory factor 3_1[e]	2.30
	C169R089	*irf8*	Interferon regulatory factor 8_3[e]	1.26
Downregulated in Day 5 HKLs				
Immune-related				
	C056R147	*tnfrsf6b*	Tumor necrosis factor receptor superfamily member $6B_2$	-3.95
	C233R142	*cfd*	Complement factor D_2	-3.20
	C157R080	*cd79a*	B-cell antigen receptor complex-associated protein alpha chain$_1$	-3.06
	C249R147	*cd28*	T-cell-specific surface glycoprotein $CD28_1$	-2.84
	C158R168	*btla*	B- and T-lymphocyte attenuator$_1$	-2.76
	C121R047	*tnfrsf11b*	Tumor necrosis factor receptor superfamily member $11B_2$	-2.56
	C252R066	*cxcr4*	C-X-C chemokine receptor type $4\text{-}A_2$	-2.46
	C017R011	*csf3r*	Granulocyte colony-stimulating factor receptor$_1$	-2.43
	C162R124	*cxcr1*	C-X-C chemokine receptor type 1-like$_2$*	-2.42
	C203R099	*ighm*	Ig heavy chain $Mem5_{15}$	-2.37
	C206R019	*tlr9*	Toll-like receptor 9_1	-2.10

	C249R147	*cd28*	T-cell-specific surface glycoprotein CD28$_5$	-2.00
	C241R142	*arg1*	Arginase-1$_2$*	-1.99
	C230R100	*il1b*	Interleukin 1 beta$_1$	-1.56
	C251R068	*tgfb1*	Transforming growth factor beta-1 proprotein$_1$	-1.21
Lipid-related				
	C211R005	*fabp6*	Fatty acid binding protein 6 (alias gastrotropin)$_3$	-4.75
	C043R091	*alox5ap*	Arachidonate 5-lipoxygenase-activating protein$_1$	-1.40
Transcription Factors				
	C259R111	*klf2*	Krueppel-like factor 2$_2$	-4.02
	C055R098	*jun*	Transcription factor AP-1 (alias jun proto-oncogene)$_1$	-3.60
	C142R114	*klf9*	Krueppel-like factor 9$_1$	-2.18
	C088R028	*runx3*	Runt-related transcription factor 3-like$_1$*	-1.61

[a] Probes were selected based on their known immune-related function and/or immune response in both fish and mammalian literature. See Supplementary Table 5.3 for complete list of differentially expressed probes.

[b] 44K microarray identifier. When multiple probes share the same annotation, the probe ID with the largest $\log_2$ fold-change was indicated.

[c] Taken from the most significant (lowest E-value) BLASTx hit in the Blast2GO annotation. If no reliable BLASTx hits were found, the best BLASTn hit was chosen instead and is represented by an asterisk (*). If BLASTn and BLASTx analyses for a given probe showed different results, then the best BLASTn hit was reported. The subscript after the BLASTx hit's name represents the number of differentially expressed probes sharing the same annotation.

[d] Log_2 fold-change (Day 5/Day 1) for differentially expressed probes (FDR <0.05) as determined by SAM analysis. An average $\log_2$ fold-change was taken when multiple probes with the same annotation were differentially expressed.

[e] Transcription factors that are also immune-relevant

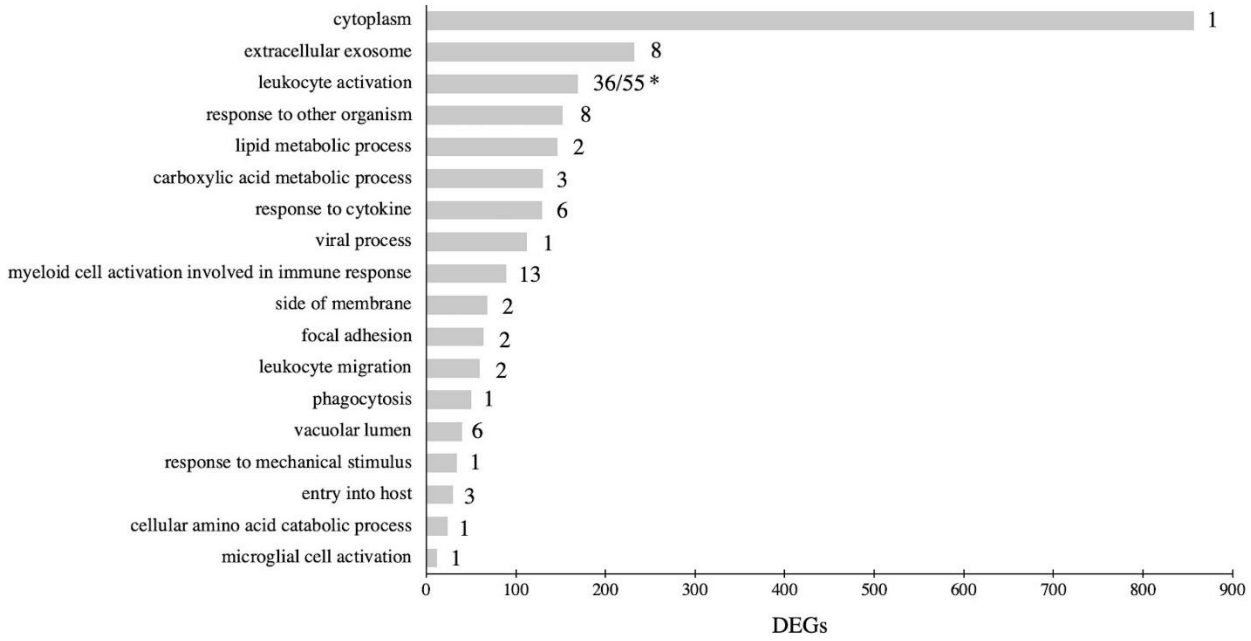

Figure 5.2. Gene Ontology (GO) term enrichment analysis of differentially expressed genes (DEGs) between Day 1 and Day 5 HKLs with a fold-change > |2|. The leading term of each identified group is shown. The bars represent the number of DEGs associated with the term while the number after each bar represents the number of GO terms associated with that group. * "Leukocyte activation" is the leading term for two groups: Group 17 (consisting of 36 GO terms) and Group 18 (consisting of 55 GO terms). GO terms from the Biological Process database are identified by the subscript "bp", while GO terms from Cellular Component are identified by "cc".

08) of which 8 GO terms belong. The leading term of all 19 groups can be found in Figure 5.2 and full details of the GO term analysis can be found in Supplementary Table 5.5. The results of the network analysis showed that the significant GO terms form a dense integrated network of functional groups (Figure 5.3). Notable transcripts related to macrophage differentiation and/or function, that were DE in Day 1 and Day 5 HKLs and appeared in multiple GO terms, include *irf7* and *irf8* (both upregulated in Day 5 HKLs compared to Day 1 HKLs), *klf2* (downregulated in Day 5 HKLs compared to Day 1 HKLs), *csf1r* (upregulated in Day 5 HKLs compared to Day 1 HKLs), *arg1* (downregulated in Day 5 HKLs compared to Day 1 HKLs) and *fasn* (upregulated in Day 5 HKLs compared to Day 1 HKLs). The appearance of these DE transcripts in multiple GO terms that are associated with leukocyte differentiation and function (e.g. "innate immune response", "leukocyte activation", "hemopoiesis", to name a few) provides evidence that these transcripts are important for these processes in Atlantic salmon adherent HKLs.

A total of 54 DEGs contributing to the GO term "mononuclear cell differentiation" (GO:1903131) were used for hierarchical clustering and displayed using a heat map (Figure 5.4). Similar to the clustering of all DEPs (Figure 5.1B), within the transcripts associated with the GO term "mononuclear cell differentiation", all Day 1 samples clustered together, and Day 5 samples clustered together, indicating Day 1 and Day 5 samples consist of two groups of cells with distinct molecular phenotypes. Of the DE transcripts annotated with the GO term "mononuclear cell differentiation", 44% were downregulated (e.g. *il1b, jun, cd28, cd4)* and 56% were upregulated (e.g. *csf1r, irf7, ifng1, fasn)* in Day 5 HKLs compared to Day 1 HKLs, suggesting that these transcripts are likely important in mononuclear cell differentiation in Atlantic salmon HKLs.

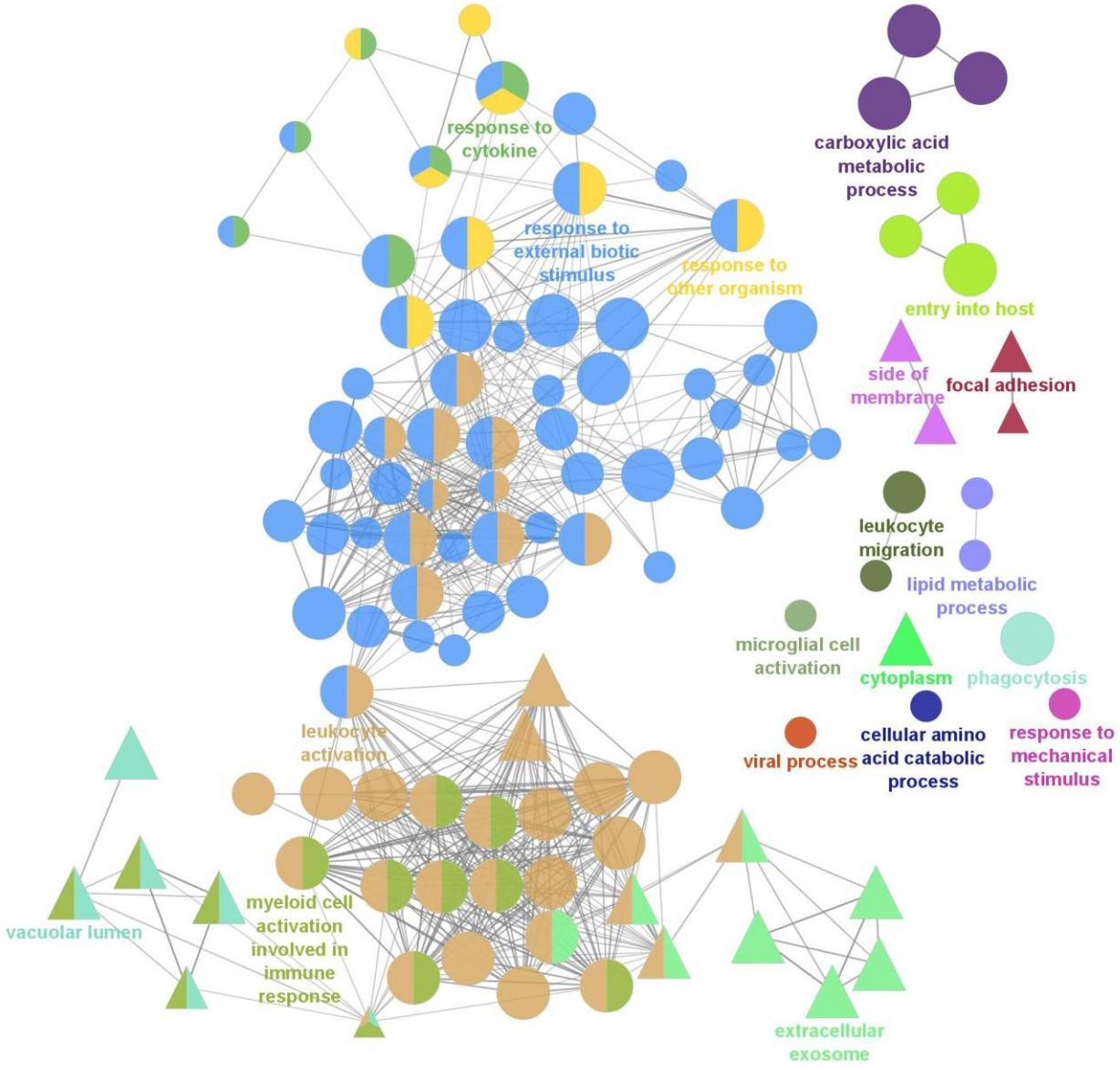

Figure 5.3. Gene Ontology (GO) term enrichment and network analysis DEGs between Day 1 and Day 5 HKLs. Two GO databases were used, Biological Process (BP; represented by circles) and Cellular Component (CC; represented by triangles) and each node represents a significantly enriched GO term ($p<0.05$, corrected with the Bonferroni step-down procedure). Related GO terms are labelled with the same colour and, when a term is shared by two or more

GO cluster groups, the node is illustrated by multiple colours. The most significant terms unique to BP and CC are labelled. The size of the node represents the enrichment significance of the terms, and the thickness of edges indicates the kappa score.

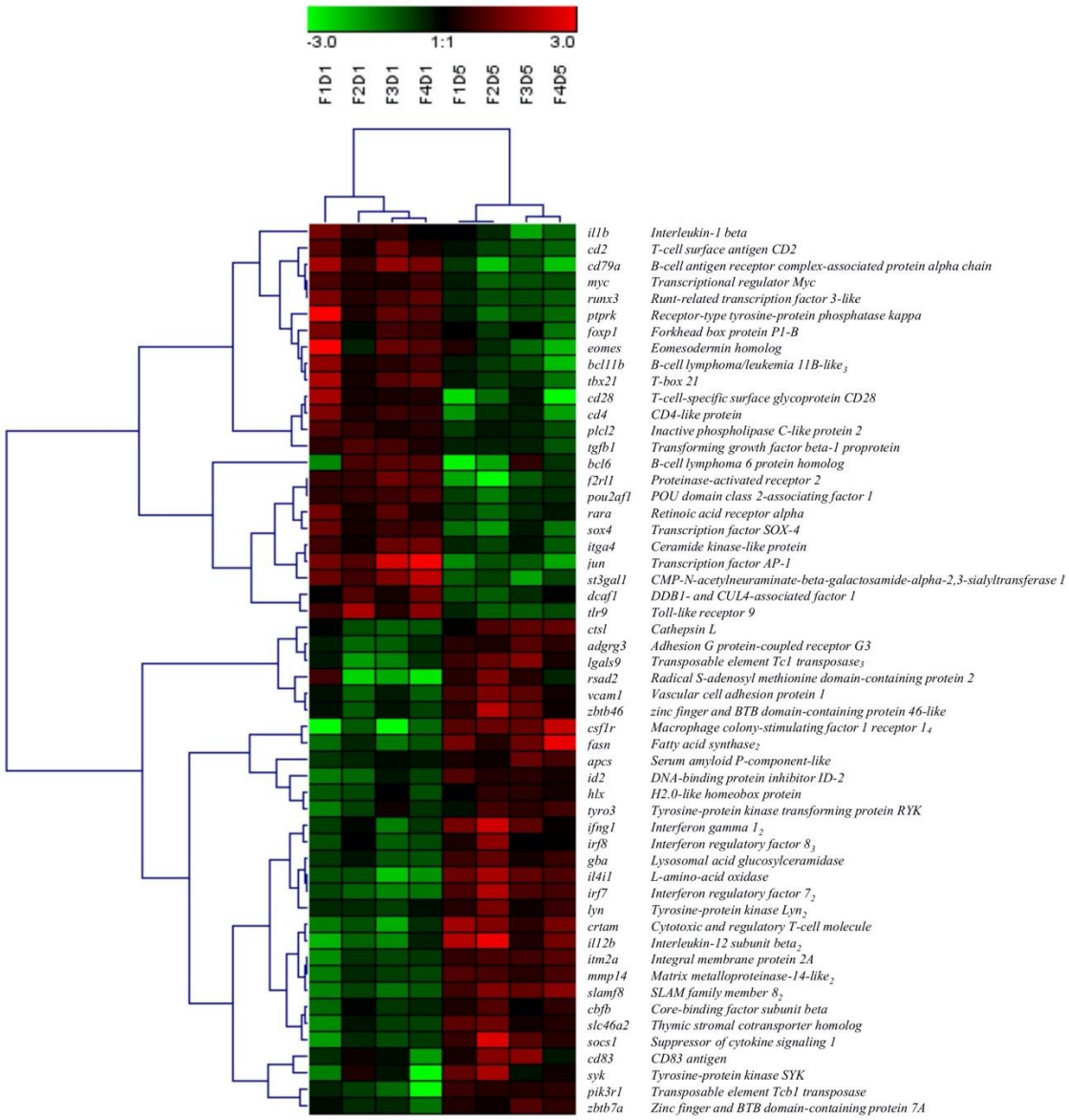

Figure 5.4. Hierarchical clustering analysis of DEGs associated with "mononuclear cell differentiation" (GO:1903131), shown as a heatmap. DEGs were median-centred and clustered using Pearson correlation and complete linkage hierarchical clustering. An average expression is shown when multiple probes were identified for one gene, and the subscript after the gene description indicates the number of probes. F indicates fish number; D indicates Day 1 or Day 5 (i.e. F1D1 is Fish 1 Day 1).

5.4.3. RT-qPCR of DE transcripts validated microarray results

Sixteen DE transcripts identified by the microarray were chosen for RT-qPCR validation. Transcripts were selected for RT-qPCR backed on functional categories: macrophage-related transcripts, anti-bacterial/anti-viral-related transcripts, lipid-related transcripts and transcription factors (Figure 5.5).

All transcripts examined validated the microarray results, with the exception of *irf8*, which followed the same upregulated trend, but was not significant (p=0.058) and *jun* which followed the same downregulated trend but was not significant (p=0.164). In addition, using the same group of Atlantic salmon used in this RT-qPCR experiment, we previously confirmed a significant upregulation in Day 5 cells compared with Day 1 cells of two macrophage-related transcripts that were not identified as DE by the microarray but are known macrophage markers in the literature (*marco* and *MHC II*) [32]. Of the transcripts examined by RT-qPCR, *rsad2* had the largest significant upregulated fold change (FC) (FC = 31.38) in Day 5 HKLs, while *irf7* had the smallest significant upregulated FC (FC = 5.01). *Fabp6* had the largest significant downregulated FC (FC = 0.03) in Day 5 HKLs and *cxcr4* had the smallest significant downregulated FC (FC = 0.47).

5.4.4. In silico miRNA target gene predictions and target gene pathway enrichment analysis

Out of the 1477 DEGs identified in this current study, 1234 (84%) had 3'UTR information and could be included in the target prediction analysis. The analysis identified 680 of them to be potential targets of one, or more, of the 36 DE miRNAs selected from our previous comparison of miRNA expression in Day 1 monocyte-like cells and Day 5 macrophage-like cells

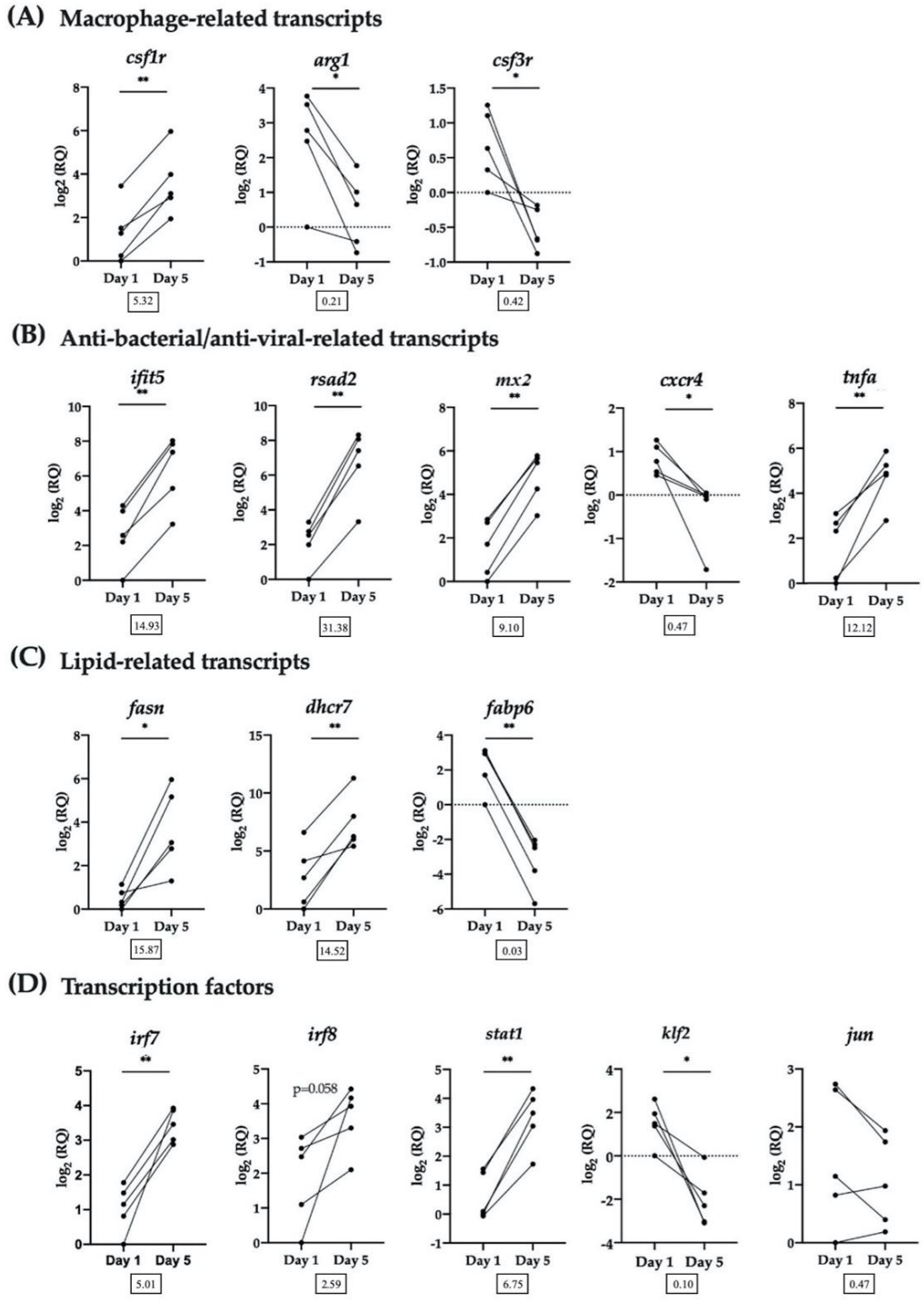

Figure 5.5. RT-qPCR validation of selected transcripts. (A) Macrophage-related transcripts. Colony-stimulating factor 1 receptor (*csf1r*), arginase-1 (*arg1*), granulocyte colony-stimulating factor receptor (alias colony stimulating factor 3 receptor (*csf3r)).* **(B)** Anti-bacterial/anti-viral-

related transcripts. Interferon-induced protein with tetratricopeptide repeats 5 (*ifit5)*, radical SAM domain-containing 2 (*rsad2,* alias viperin), interferon-induced GTP-binding protein Mx (*mx2)*, C-X-C chemokine receptor type 4 (*cxcr4*), tumor necrosis factor alpha (*tnfa*). **(C)** Lipid related transcripts. Fatty acid synthase (*fasn*), 7-dehydrocholesterol reductase (*dhcr7*), gastrotropin (alias fatty acid binding protein 6 (*fabp6)*). **(D)** Transcription factors. Interferon regulatory factor 7 (*irf7*), interferon regulatory factor 8 (*irf8*), signal transducer and activator of transcription 1 (*stat1*), krueppel-like factor 2 (*klf2*), transcription factor AP-1 (alias jun proto-oncogene (*jun)*). Data from each individual fish shown as $\log_2$(RQ), n=5, * $p<0.05$; **$p<0.01$. The number under each figure represents the average fold-change in Day 5 HKLs compared to Day 1 HKLs.

in [32] (Supplementary Table 5.6). The gene pathway enrichment analysis shown in Supplementary Table 5.7 identified gene pathways that were more likely to be regulated by miRNAs including interleukin-3, interleukin-5, and GM-CSF signaling; Fc gamma receptor-mediated phagocytosis; hematopoietic cell lineage; and lipid and lipoprotein metabolism. The complete overview of all pathways, p-values, and target genes participating in each pathway is given in Supplementary Table 5.7.

5.5. Discussion

The aquaculture sector in Canada generates $5.4 billion CAD in economic activity annually [53]. The Atlantic salmon is Canada's top aquaculture product (by volume) and is therefore of high economic importance. Identifying how their immune cells develop and function is necessary to fully understand the fish immune system. HKLs have been used in many *in vitro* immunology studies involving several fish species ([27–31], among many others), but remain to be fully characterized. Our previous work observed a change in morphology, phagocytic ability, and miRNA profile of HKLs cultured for 5 days, from predominantly monocyte-like at Day 1 of culture to predominantly macrophage-like at Day 5 of culture [32]. Several mammalian studies have observed large numbers of differentially expressed transcripts during monocyte-to-macrophage differentiation and/or macrophage polarization using high-throughput profiling methods, such as microarrays, many of which were identified in this current study and are discussed below [54–58]. Therefore, we used a 44K microarray to examine changes in transcript expression profiles between Day 1 monocyte-like HKLs and Day 5 macrophage-like HKLs. Changes in the transcript expression of immune related genes, lipid-related genes, and genes encoding transcription factors that are involved with macrophage differentiation, polarization, and function in other vertebrates were identified. In addition, GO term analyses identified

biological processes including leukocyte differentiation, hematopoiesis, innate immune response and lipid metabolic process.

5.5.1. Transcriptional changes associated with macrophage differentiation, polarization, and immune response in Atlantic salmon HKLs

The results of this study identified several macrophage and immune-related transcripts in both Day 1 and Day 5 HKLs. As the sample materials used in this study were immune cells, some of the identified transcripts were not unexpected. The paired SAM analysis identified differentially expressed transcripts between Day 1 and Day 5 HKLs that are involved in macrophage differentiation (including *csf1r* and *csf3r*), polarization of M1/M2 macrophages (including *arg1* and *ifng1*), and macrophage function (including *mx1, mx2* and *tlr3*).

The differentiation, proliferation, and survival of myeloid cells depends on signals derived from CSF1 upon binding with its receptor CSF1R [59–61]. In humans and mice, CSF1R increases during macrophage differentiation, with CMPs expressing the lowest levels of CSF1R, monocytes expressing significantly more CSF1R and macrophages expressing the highest levels of CSF1R [reviewed in 52]. On the other hand, signaling through the granulocyte colony-stimulating factor 3 receptor (CSF3R, also known as GCSFR) is important for the proliferation, differentiation, and activation of neutrophils [62–64]. Both *csf1r* and *csf3r* sequences have been identified in multiple fish species, and studies have indicated a conserved function for both receptors [4]. As in mammals, *csf1r* has been identified as a marker of monocytes and macrophages in fish, and its expression is increased with macrophage differentiation [62,65,66]. Similarily, *csf3r,* has been demonstrated to be necessary for neutrophil development in several fish species [67–69]. In the current study, *csf1r* was significantly increased in Day 5 HKLs compared to Day 1 HKLs, while *csf3r* was significantly decreased in Day 5 HKLs compared to

Day 1 HKLs, suggesting that, without the addition of exogenous factors, such as M1 (i.e. IFN-γ) and M2 (i.e. IL-4) activation stimuli, HKLs differentiate along the monocyte/macrophage lineage and not toward the granulocyte lineage during *in vitro* culturing. However, the downregulation of *csf3r* may also indicate that neutrophils were present at Day 1 of culture but had died off by Day 5. Several other transcripts related to different immune cells, including B cells (*cd79a, ighm, igha2, cxcr3*) and T cells (*cd2, cd4, cd8b, cd28, cd96*), were also downregulated in Day 5 cells compared to Day 1 cells [70–72]. These results suggest that the Day 1 culture contained a heterogeneous mixture of several cell types but by Day 5 most of these cells were no longer present, leaving the Day 5 culture with a more homogenous population of cells (i.e. macrophages).

M1 "pro-inflammatory" macrophages and M2 "anti-inflammatory" macrophages can be defined based on their gene and protein expression profiles. Arginase enzyme activity and mRNA expression are hallmarks of M2 macrophages in both mammals and fish (reviewed in [3,73]). Like mammals, fish possess two arginase genes, *arginase-1* (*arg1)* and *arginase-2* (*arg2*) [3]. While *arg1* is a marker of M2 macrophages in mammals, results have shown that *arg2* expression is a marker for the M2 phenotype in fish [19,21,74]. Similarily, the chemokine receptors *cxcr1* and *cxcr4* are upregulated following M2 stimulation and are potential markers of M2 macrophages in mammals (*cxcr1* and *cxcr4)* and fish (*cxcr1)* [21,54]. This current study revealed a decrease in *arg1*, *cxcr1*, and *cxcr4* expression in Day 5 HKLs compared to Day 1 HKLs. Interestingly, we found a decrease in *arg1* expression (similar to mammals) and not *arg2* expression (similar to fish) in Day 5 HKLs, suggesting that the role of the arginase genes in macrophage differentiation and function may be species-specific. However, an examination of

both *arg1* and *arg2* expression in Atlantic salmon, along with arginase enzyme activity in response to M2 stimulation, will be required to determine this.

Several markers of M1 macrophages, such as *tnfa, il12b,* and *ifng1* were upregulated in unstimulated Day 5 HKLs. These genes have been identified in different fish species and their role in the fish macrophage immune response and M1 polarization are conserved with other vertebrates [1,3,9,75–77]. On the other hand, markers of M2 macrophages in mammals, including *mrc1, socs1, and tgm,* were also upregulated in Day 5 HKLs. While these genes are present in fish, they have yet to be characterized as teleost M2 markers, unlike the M1 markers identified here [1,3]. It is interesting to find an upregulation of both M1 and M2 markers in non-stimulated cells. These results may indicate that during the culture, adherent HKLs become primed to develop into M1 or M2 macrophages upon stimulation. Future research, using functional studies with M1 and M2 activating stimuli, protein expression data and assays to determine arginase activity, would help to determine if the transcripts identified here are in fact M2 markers in teleost fish, as they are in mammals, and if the HKLs cells become primed to develop into the M1 or M2 phenotype during culture time.

In addition to the classic markers of macrophages, this study showed the differential expression of several virus-responsive, bacteria-responsive and inflammation-related genes in the two cell populations including Toll-like receptor 3 *(tlr3),* interferon-induced GTP-binding proteins *mx1* and *mx2,* radical SAM domain-containing 2 *(rsad2*), interferon-induced protein with tetratricopeptide repeats 5 *(ifit5b),* DExD/H-box helicase 58 (*ddx58*; also known as RIG-I), granulin (*grn*), hepcidin *(hamp),* and legumain (*lgmn*). These genes have been described in many fish species and have similar immune-related functions as their mammalian counterparts [24,27,78–83]. In mammals, *Tlr3* levels are highest in macrophages, compared to other

mononuclear cells, and is not detected in neutrophils [84–86]. While *tlr3* has been described in several fish species, it is unknown if *tlr3* is involved in HKL differentiation in fish. However, our results showed an upregulation of *tlr3* in Day 5 HKLs, suggesting that *tlr3* could be a novel marker of macrophages in fish. Legumain (*LGMN*) is associated with M2 macrophages [87–89] and its expression and activity is increased during monocyte-to-macrophage differentiation in both human THP-2 cells and murine RAW264.7 cells [88,89]. In goldfish, *lgmn* expression is highest in macrophages, compared to monocytes and progenitor cells, suggesting that *lgmn* may be a marker for macrophages in fish [24]. Similarily, granulin may play a role in fish myeloid cell differentiation; in mutant zebrafish that do not express granulin, decreased differentiation of myeloid precursors into neutrophils and macrophages was observed, while adult mutants developed a head kidney with increased progenitors and decreased mature myeloid cells [90,91]. The upregulation of *lgmn* and *grn* in Day 5 HKLs, like the transcripts discussed thus far, point to the differentiation of HKLs into macrophages. In addition, the upregulation of virus-related and bacteria-related transcripts may indicate that Day 5 HKLs are more prepared to combat pathogen infection than Day 1 HKLs.

While we observed upregulation of several immune-relevant transcripts in Day 5 HKLs, there were also some immune-related genes (e.g. virus-responsive and bacteria-responsive) that were downregulated in Day 5 compared with Day 1 HKLs. For example, *il1b, tnfrsf6b, tnfrsf11b* and *tlr9* were downregulated in Day 5 HKLs compared to Day 1 HKLs. These genes have been demonstrated to be responsive to bacterial and viral challenges in various fish species [92–94]. The upregulation of some pathogen-responsive genes, and the downregulation of others, in Day 5 HKLs compared with Day 1 HKLs, suggests that these cells are likely changing in their

responsiveness to pathogens over time in culture. Future research should use live pathogen challenges at different time points during differentiation to test this hypothesis.

5.5.2. Transcriptional changes associated with lipid metabolism observed in Atlantic salmon HKLs

Lipids play a major role in regulating many biological processes including cell growth, proliferation, and function. Lipids and fatty acids are required for a cell to grow and proliferate and, therefore, the enzymes involved in the formation of fatty acids are necessary for the development and differentiation of macrophages [95]. Significant changes in the lipid-related transcriptome occur during mammalian monocyte-to-macrophage differentiation and M1/M2 polarization. [57,58,96]. Transcripts involved with fatty acid synthesis, elongation and desaturation, and cholesterol production, utilization and export are differentially expressed between mammalian monocytes and macrophages, as well as between M1 and M2 macrophages [57,58,96]. In this study, transcripts related to the synthesis of fatty acids (e.g. fatty acid synthase (*fasn*) and long-chain fatty acid elongase 6 (*elovl6*)), transcripts involved in hydrolyzing triglycerides into free fatty acids (e.g. lipoprotein lipase (*lpl*)), and transcripts involved in fatty acid desaturation (e.g. fatty acid desaturase 2, *fads2*, alias delta-6 fatty acyl desaturase, *fadsd6;* and *fads1*, alias delta-5 fatty acyl desaturase, *fadsd5*)) were upregulated in Day 5 HKLs compared to Day 1 HKLs. In addition, GO term analysis identified lipid-related GO terms including lipid biosynthetic process (GO:0008610), neutral lipid catabolic process (GO:0046461) and cholesterol metabolic process (GO:0008203).

FASN is necessary for macrophage function in humans and the expression of both *FASN* and *Elovl6* is upregulated in human and mouse macrophages, respectively, upon differentiation from monocytyes [57,58,96]. While both *fasn* and *elovl6* have been described in numerous fish

species, their role in macrophage differentiation in fish is unknown. In several fish studies, liver *fasn* and *elovl6* expression were found to be responsive to diet [97–100]. In white Pacific shrimp (*Litopenaeus vannamei*), *fasn* expression was increased in the gills and hemocytes (immune cells of shrimp) following *V. parahaemolyticus* infection and knockdown of *fasn* increased morbidity, suggesting that *fasn* may have a role in immune cell response in some aquatic species; however, this requires further investigation [101].

Lipoprotein lipase is an enzyme that hydrolyzes triglycerides in lipoproteins found in chylomicrons and very low-density lipoproteins (VLDLs) into free fatty acids. A dramatic upregulation of *LPL* was observed in human macrophages differentiated with M-CSF, as well as without exogenous factors [58,96]. Futhermore, differentiation of bone marrow cells from LPL-deficient mice had 40% less differentiated macrophages than control mice, suggesting that LPL is necessary for macrophage differentiation [102]. Like *fasn* and *elovl6, lpl* expression in fish has been reported to be modified by diet [103–105], however, its role in HKLs differentiation and/or function is unknown. The increased expression of *fasn*, *elovl6,* and *lpl* suggests the need for macrophages to access fatty acids for inflammatory functions and this need is conserved in fish and mammals. Addionally, the high expression level of these transcripts may serve as novel markers of macrophages in fish.

Fatty acid desaturases are enzymes required for the synthesis of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) through the formation of double bonds between fatty acyl chain carbons. Fatty acid desaturase 2 (*fads2,* alias *fadsd6*) and fatty acid desaturase 1 (*fads1,* alias *fadsd5)* were upregulated in Day 5 HKLs compared to Day 1 HKLs. While most studies to date have examined *fads2* and *fads1* expression in organs with high fatty acid turnover, such as the liver, there are data suggesting that they play a role in myeloid cells [106,107]. In human

macrophages, *FADS2* expression increased during monocyte-to-macrophage differentiation and inhibition of FADS2 in human peripheral blood mononuclear cells (PBMCs) decreased the number of proliferating cells. Similar to *fasn, elovl6,* and *lpl, fads2* and *fads1* have been described in fish species [108], and their expression level in HKLs is regulated by nutrition and diet [109–113]. The role of *fads2* and *fads1* in HKL differentiation and function has not been investigated, however, the results of this study suggest that, along with *fasn and lpl*, *fads2* and *fads1* may be conserved markers of macrophages and macrophage function.

7-dehydrocholesterol reductase (DHCR7) is an enzyme that catalyzes the production of cholesterol in the final step of cholesterol biogenesis [114]. A significant increase in *dhcr7* was observed in Day 5 HKLs compared to Day 1 HKLs, suggesting an increase in cholesterol biosynthesis in Day 5 HKLs. Ecker et al. [57] observed an increase in *DHCR7* expression in primary human monocytes undergoing macrophage differentiation for 4 days. Interestingly, the increase in *DHCR7* expression at 4 days, decreased to below baseline (day 1) values following 6 days of macrophage differentiation. Similar to the lipid-related transcripts discussed here, liver, muscle, and gut *dhcr7* is responsive to diet in several fish species, but the role of *dhcr7* in macrophage differentiation and/or function in fish has yet to be investigated [115–117].

5.5.3. Transcription factors involved in mammalian macrophage differentiation were DE in Atlantic salmon HKLs

Macrophage differentiation and polarization are tightly regulated by transcription factors (TFs) and are associated with large changes in transcriptional programming. The TFs that regulate myeloid cell differentiation and macrophage polarization have been extensively studied and characterized in mammals, while this area of research is expanding in teleost fish [4,23,118]. Transcripts encoding several TFs involved in mammalian macrophage biology were

differentially expressed in Day 1 and Day 5 HKLs in the current study, suggesting possible conserved roles for these TFs. In the present study, members of the Krueppel-like factors (KLF) family (i.e. *klf2*, *klf9*) were downregulated in Day 5 HKLs compared to Day 1 HKLs , while members of the interferon regulatory factor (IRF) family (i.e. *irf3*, *irf7*, *irf8*), as well as signal transducer and activator of transcription 1 (*stat1*), were upregulated in Day 5 HKLs compared to Day 1 HKLs (Supplementary Table 5.4).

KLFs are members of the zinc-finger family of TFs which play roles in many biological processes including cell proliferation, differentiation, growth, apoptosis, and inflammation [119,120]. In primary human monocytes, *KLF2* expression is reduced upon differentiation into macrophages and its overexpression in the THP-1 human cell line inhibited LPS-induced cytokine secretion and decreased phagocytic ability, indicating that the suppression of KLF2 is necessary for macrophage differentiation and function [121]. Similarly, KLF9 overexpression in RAW264.7 murine cell line reduced LPS-induced inflammatory cytokine release [122]. While KLF9 is mostly known for its involvement in B-cell differentiation [123], these studies suggest that KLF2 and KLF9 have a role in monocyte maintenance and their downregulation is necessary for macrophage differentiation.This current study found a decrease of both *klf2* and *klf9* in Day 5 HKLs compared to Day 1 HKLs. There is very little information on fish KLFs, however, there are recent studies that provide evidence for a role of KLF2 and KLF9 in the immune response [124,125]. For example, KLF2 expression was found to be highest in PBMCs of ayu (*Plecoglossus altivelis*) compared to other tissues (liver, spleen, brain, gill, head kidney) and its expression increased with *L. anguillarum* infection. Furthermore, siRNA knockdown of KLF2 increased *il1b* and *tnfa* expression in both resting and *L. anguillarum* infected head kidney monocytes/macrophages, suggesting that, similar to mammalian cells, KLF2 suppresses ayu

monocyte/macrophage activation [124]. While the role of KLF2 and KLF9 in macrophage differentiation and polarization is unknown in fish, the results of this study suggest that, as in mammals, these TFs are involved in regulating myeloid cell differentiation in fish. It is possible that KLF2 and/or KLF9 play a role in maintaining the monocyte or precursor population and their decrease in expression is necessary for macrophage differentiation and function.

Members of both the IRF and STAT TF families have been implicated in a wide range of cellular events, including cell growth, proliferation, survival, and immune responses and each has members that are important mediators of macrophage polarization and/or differentiation [126]. IRF3, IRF7 and IRF8 are involved in mammalian macrophage differentiation, polarization and/or function [127]. The expression of both IRF8 and IRF7 increases during macrophage differentiation, while the expression of IRF8 declines upon granulocytic differentiation [128–130]. Furthernore, IRF8 is necessary for the formation of mature, functional macrophages while the expression of IRF7 is both necessary and sufficient to induce monocyte-to-macrophage differentiation in U937 monocytic cell line [128–130]. In mammals, IRF3 is associated with M1 polarization [131,132]. In fish, *irf8* is specifically associated with primary macrophages during zebrafish embryogenesis [90]. While *irf8* null mutants have decreased macrophage development and enhanced neutrophil production, overexpression of *irf8* in the mutants could partially recover this effect [90]. Similar to mammals, both *irf3* and *irf7* are responsive to viral infection in a fish monocyte/macrophage cell line (RTS11), as well as primary fish macrophages, suggesting that *irf3* and *irf7* have a role in the immune response of fish macrophages [133,134]. The increase in *irf3, irf7* and *irf8* expression in Day 5 HKLs compared to Day 1 HKLs observed in the current study may indicate that, if the functions of these genes are the same in fish as they are in

mammals, then the Day 5 culture is composed more of macrophages compared to the Day 1 culture.

In primary human monocytes, STAT1 activity increased as monocytes differentiated into macrophages [135]. Moreover, STAT1 binding was detected in the promotor of genes important for macrophage differentiation and function, such as *FcyRI*, *ICAM-1* and *IRF1* [135]. In several fish species, *stat1* expression and/or signalling, as well as M1 markers, are increased in head kidney leukocytes following IRF-γ stimulation [115,116,136]. Here we found an upregulation of *stat1* in Day 5 HKLs compared to Day 1 HKLs, suggesting an increase in *stat1* is indicative of macrophage differentiation in the Day 5 culture.

5.5.4. DE miRNAs are predicted to target DE transcripts and are associated with macrophage immune function gene pathways

miRNAs are short, non-coding RNAs that play a role in regulating gene expression by binding to a partially complementary sequence in the (usually) 3' UTR of their target mRNA, leading to mRNA degradation or the prevention of translation [137]. miRNAs regulate several biological processes including cell differentiation and immune response, among many others (reviewed in [138,139]). Work in mammals has demonstrated that miRNAs can mediate the differentiation and activation of macrophages [140,141]. Our previous work identified 66 DE miRNAs when comparing Day 1 and Day 5 HKLs (22 miRNAs downregulated and 44 miRNAs upregulated in Day 5 HKLs, compared to Day 1 HKLs), including many that are involved in mammalian macrophage function (e.g. miR-146a, miR-155 and miR-21)[142–144], as well as teleost fish immune response (e.g. miR-146a, miR-462, miR-2188 and miR-731) [145,146]. The 36 major expressed DE miRNAs, likely to be the biologically relevant guide-miRNAs, were used as input against the 3'UTRs from the DEGs identified in this study. This targeted approach

could identify whether any of the DEGs are potential targets of the DE miRNAs in [32]. This is a first step to determine which miRNAs may be involved in monocyte-to-macrophage differentiation by targeting DEGs for post-transcriptional regulation by the RISC-complex.

The results from the *in silico* target prediction applying the selected DE miRNAs from [32] and all DEGs with 3'UTR information revealed that 660 of the DE transcripts identified in the current study were potential targets. It is unlikely that more than half of the DEGs are true targets as there are usually a large percentage of false positives for several reasons in such predictions [147]. However, such in silico predictions are still used as a first means to single out which DEGs that may be true miRNA targets. Among the interesting putative targets with known roles in macrophage differentiation and/or function with predicted miRNA response elements for particular DE miRNAs were *tnfa* (ssa-miR-214-1-3p and ssa-miR-139-5p), *fadsd5* (of ssa-miR-21a-5p), and *ifit5* (ssa-miR210-1-5p and ssa-miR-22a-3p), all of which showed increased expression in Day 5 cells. Other interesting predicted targets like *arg1* (ssa-miR-214-3-3p and ssa-miR-2188-3p), *cxcr4* (ssa-miR-214-3-3p), *klf2* (-miR-181a-5p, ssa-miR-29b-3p and ssa-miR-novel-16-5p), *klf9* (ssa-miR-155-5p, ssa-miR-214-3-3p and ssa-miR-210-1-5p), and *il1b* (ssa-miR-139-5p, ssa-miR-24ac-3p and ssa-miR-725-3p) all showed decreased expression in Day 5 cells. The traditionally acknowledged function of miRNAs is to downregulate gene expression which would lead to a decrease of target transcripts if the miRNA expression increases [137]. Such inverse relationships were not always the case between a miRNA and its predicted target from our in silico analysis. However, the function of most cellular miRNAs is to maintain equilibrium of the target transcripts, which is regulated positively by the rate of transcription and negatively by miRNAs. Differentiation of a cell type that is dependent on an increased level of a given transcript can be triggered by transcriptional activation. However, the

miRNAs that contribute to maintaining this transcript in equilibrium would also increase in order to maintain the higher expression level of this transcript in balance. Such relationships between a miRNA and its target, also referred to as feed forward loops [148], lead to increases of both the targets and their miRNAs as they are (often) activated by the same transcription factors. Similar dynamics have been proposed for miRNAs associated with immune responses and their targets [147], and many of the DE miRNA genes changing expression in Day 5 HKLs have upstream transcription binding motifs of *irf8*, *irf1*, and *irf3* [146] that are increased in Day 5 HKLs in this study. Future functional studies, using knock-out or overexpression models, are required to fully determine if a DE gene identified in this study is the target of a certain DE miRNA identified in our previous work [32].

The DEGs found in this current study that were identified as potential targets (see Supplementary Table 5.6) of the DE miRNAs [32] were used for pathway enrichment analysis. The results showed that the putative target genes were significantly enriched in pathways associated with macrophage immune function, such as interleukin-3, interleukin-5, and Fc gamma receptor-mediated phagocytosis, pathways associated with macrophage differentiation, such as GM-CSF signaling and hematopoietic cell lineage, and lipid-related pathways such as lipid and lipoprotein metabolism. Although not proving certain miRNA-target interactions, the enrichment of these gene pathways further suggests that the miRNAs are involved in macrophage maturation.

5.6. Conclusion

The aim of the current study was to build on our previous work [32] and examine changes in gene expression of Atlantic salmon HKLs *in vitro*. We identified immune-related transcripts, lipid-related transcripts, and transcripts encoding TFs that were differentially

expressed between Day 5 and Day 1 HKL populations. Many of the identified transcripts are markers of macrophages, involved in M1/M2 polarization and/or involved in macrophage function in other species, suggesting a conserved function for some of the transcripts, as well as the possibility of using these transcripts as macrophage markers, although future functional studies are required to confirm this. Overall, the results indicate that, without the addition of exogenous factors, the HKL cell population differentiates *in vitro* to become macrophage-like, and this dynamic change in cell population is an important consideration when working with Atlantic salmon HKLs *in vitro*.

5.7. References

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Chapter 6: General Discussion

6.1. Summary of results

The second chapter of this thesis, published in *Frontiers in Immunology* [1], was a comprehensive review of the innate and adaptive immune systems of the key branches of Gnathostomata: Chondrichthyes (i.e. cartilaginous fishes) and Osteichthyes (i.e. bony fishes: Actinopterygii (ray-finned fish, focusing on Teleost fish) and Sarcopterygii (lobe-finned fish, focusing on coelacanths and lungfish). In this chapter, I examined, in detail, aspects of the innate immune system, such as physical barriers (e.g. skin, mucus), cellular components (e.g. Toll-like receptors, phagocytosis) and humoral responses (e.g. complement system, lysozyme and acute phase proteins), as well as aspects of the adaptive immune system such as B cells (e.g. immunoglobulins, B cell response and activation-induced cytidine deaminase) and T cells (e.g. T cell receptor and co-receptors, CD4+ T cells and CD8+ T cells). While many of these components are similar among these fish species, there are sizable differences as well. Highlighting not only what is known about the immune systems of fishes, but also what is still unknown, is important in directing comparative research. Comparative research, that includes species where the adaptive immune system (based on Igs, TCR and MHC) first appears, such as cartilaginious fish, will help us gain a comprehensive understanding of the evolution and functionality of the immune system in fish.

The third chapter on this thesis, published in *The International Journal of Molecular Sciences* [2], examined changes in the morphology (via Giemsa staining), function (via phagocytosis and respiratory burst assays) and microRNA (miRNA) profile (via RNA-sequencing) of Atlantic salmon adherent head kidney leukocytes (HKLs) during culture. This study found that the morphology of adherent HKLs changed from predominantly round, non-

spread cells at Day 1 of culture to predominantly spread cells with pseudopodia present at Day 5 of culture. Examination of phagocytosis revealed that the percentage of phagocytic cells more than doubled from Day 1 (21.4% phagocytic cells) to Day 5 (53.9% phagocytic cells), while examination of the respiratory burst response found no changes in reactive oxygen species (ROS) production between Day 1 and Day 5 cells. It is important to note that the use of a strong ROS inducer (PMA) may have masked any differences in ROS production between the Day 1 and Day 5 HKL populations. Nevertheless, these experiments suggest that during culture, adherent HKLs differentiate to become more "macrophage-like". To provide further confirmation of differentiation into macrophages, I examined the mRNA expression of two macrophage markers, *macrophage receptor with collagenous structure* (*marco*) and *major histocompatibility complex II* (*mhc II*), and found that both were significantly increased in Day 5 HKLs compared to Day 1 HKLs. MiRNAs are involved in regulating many biological processes, including macrophage differentiation, polarization and function [3,4]. Based on the HKL morphology, functional and gene expression data, I then determined changes in the miRNA profile between Day 1 and Day 5 HKLs with the hypothesis that if the expression of a miRNA changes during culture, then it may play a role in HKL differentiation. The three most abundant miRNAs (ssa-mir-21b, ssa-miR-21a and ssa-miR-146a) in both Day 1 and Day 5 HKLs, are involved in macrophage differentiation, polarization and function in other species. These miRNAs comprised over half of all miRNAs in both Day 1 and Day 5 HKLs suggesting they may also have important functions in Atlantic salmon monocytes and macrophages. Similarly, many of the differentially expressed (DE) miRNAs identified between Day 1 and Day 5 HKLs (e.g. ssa-miR-146a, ssa-miR-146b, ssa-miR-155) also play a role in macrophage differentiation in other species, suggesting that these miRNAs may also be important in HKL differentiation in

Atlantic salmon. However, to determine if these miRNAs play a role in HKL differentiation in Atlantic salmon, functional assays (knock-down or gain of function) are required (see section 6.2). This work is the first to identify miRNAs in Atlantic salmon HKLs and suggests miRNAs that may be involved in Atlantic salmon HKL differentiation and/or function.

The fourth chapter of this thesis, published in *Frontiers in Immunology* [5], characterized extracellular vesicles (EVs) released from Day 1 and Day 5 Atlantic salmon adherent HKLs. Using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA), I confirmed that EVs are released from Atlantic salmon adherent HKLs into the supernatant during culture. Through NTA, I determined that the size and the quantity of the EVs released from Day 1 and Day 5 HKLs were similar. I then used the Vn96 peptide to isolate EVs released from Day 1 and Day 5 HKLs (the first time this approach has been used to isolate EVs from fish cells) and examined the miRNA profile of Day 1 and Day 5 HKL EVs via RNA-sequencing. The results revealed 19 differentially packaged miRNAs between EVs released from the two cell populations, some of which were also identified as DE in Day 1 and Day 5 HKLs in Chapter 3 (e.g. ssa-miR-146a, ssa-miR-155, ssa-miR-731). On the other hand, the relative abundance of some miRNAs (i.e. ssa-miR125b, ssa-miR-92a, ssa-miR-181a) was much higher in EVs than in HKLs, suggesting that these miRNAs were selectively enriched in EVs, but not in HKLs, and may serve particular functions. While the RT-qPCR results of this study confirmed the presence of EVs, it did not corroborate the DE of many of the miRNAs found in RNA-sequencing results. Additionally, there was high variation in both the RNA-sequencing results and the RT-qPCR results. Given that this area of research in fish is relatively new, potential remains for optimization in future studies which should include more animals. Nevertheless, this study was a first step in characterizing EVs released from Atlantic salmon adherent HKLs,identifying

miRNA biomarkers for EVs that are derived from HKLs, as well as biomarkers for the stage of HKL differentiation and provides a foundation for future work on the ability of EVs to serve as indicators of fish immune cell activity.

The fifth chapter of this thesis, published in *Frontiers in Immunology* [6], examined changes in the mRNA transcriptome between Day 1 and Day 5 Atlantic salmon adherent HKLs. My previous work observed changes in morphology, function, and miRNA profile in adherent HKLs during culture, therefore, I next wanted to examine changes in the mRNA profile of Day 1 and Day 5 HKLs. A 44K oligonucleotide microarray experiment revealed over 1400 differentially expressed genes (DEGs) with known putative identities between Day 1 and Day 5 HKLs. Many of the DEGs were macrophage/immune-related transcripts (e.g. *csf1r, csf3r arg1, tnfa, cxcr4*), lipid-related transcripts (e.g. *fasn, elvol6, lpl, fads1, fads2*) and transcripts encoding transcription factors (e.g. *klf2, klf9, irf7, irf8, stat1*) that play a role in macrophage differentiation and/or function in other species, suggesting that these genes may also play a role in Atlantic salmon HKL differentiation. Furthermore, the downregulation of transcripts related to other immune cells, such as neutrophils, B cells and T cells, indicates that the Day 1 culture is composed of a heterogeneous population of cells, while the Day 5 culture is a more homogenous population of cells (i.e. macrophages) and this should be noted when using HKLs in *in vitro* fish immunology studies. In addition, GO term analysis identified several macrophage and immune-related GO terms where several DEGs appear, suggesting that these DEGs may be important in the respective processes. This work demonstrated that the mRNA transcriptome of cultured adherent HKLs changed dramatically from Day 1 to Day 5 becoming selectively enriched with macrophages in Day 5 of culture and is a first step in identifying biomarkers for specific cell types within the HKL population.

6.2. Future directions

Although the culture of adherent HKLs is commonly used in fish *in vitro* studies, the changes in the cell population that occur during culture have never been investigated using transcriptomics and complementary methods. The work in this thesis sets the groundwork for more fully characterizing the Atlantic salmon adherent HKLs on a molecular level, however, there is much about this cell population that still remains unknown. There are several areas of future research that can be derived from this work: investigating the role miRNAs play in HKL differentiation and/or function, determining biomarkers for different cell types (e.g. monocytes and macrophages) within the HKL population, as well as the EVs derived from them, and investigating the M1 and M2 phenotype, and their biomarkers, in Atlantic salmon adherent HKLs.

Chapter 2 of this thesis identified miRNAs that change expression during HKL culture time, suggesting that these miRNAs may play a role in HKL differentiation. However, to investigate the potential roles of these miRNAs in Atlantic salmon macrophage differentiation, functional assays (knock-down or gain of function) are required. For example, the large increase of ssa-miR146a (a miRNA known to be involved in mammalian macrophage differentiation), observed in Day 5 HKLs, could be inhibited using small-interfering RNAs (siRNAs), and its effect on HKL differentiation determined via functional assays (e.g. phagocytosis), morphology analysis (e.g. Giemsa staining and light microscopy, electron microscopy), and mRNA expression of macrophage markers (including M1 and M2 markers) identified by the microarray (e.g. *csf1r, csf3r, arg1, tnfa, marco*). Conversely, gain of function assays, where the expression of a miRNA that was decreased in Day 5 HKLs is increased or the decrease is prevented, could be used and the change in HKL morphology, function and mRNA expression observed. In

Chapter 5, I identified DEGs in Day 1 and Day 5 HKLs that were predicted targets of the DE miRNAs in Chapter 2 via *in silico* analysis. If the increase of a miRNA was inhibited (e.g. through siRNAs), and no decrease in mRNA expression of its predicted target was observed (if the expression decreased in Day 5 HKLs compared to Day 1 HKLs), then this would provide evidence that the target was predicted correctly. Further evidence could then be gathered via direct miRNA:mRNA binding using a luciferase reporter assay. Luciferase reporter assays are commonly used in mammals to detect miRNA:mRNA binding (reviewed in [7]) and while I have not found any studies where a luciferase assay was used to detect miRNA:mRNA binding in Atlantic salmon, it has been successful in validating miRNA:mRNA binding in other fish species including zebrafish (*Danio rerio*), Atlantic halibut (*Hippoglossus hippoglossus*) and blunt snout bream (*Megalobrama amblycephala*) [8–10].

The work in Chapter 5 suggests possible biomarkers for monocytes (Day 1 HKLs) and macrophages (Day 5 HKLs). In order to confirm specific biomarkers, analysis at a single cell level is required. While in mammals, biomarkers of specific immune cells have been well documented through the use of protein-detecting antibodies, the lack of commercially available antibodies specific for fish proteins has limited this area of research. To address this, a single cell assay, such as the PrimeFlow RNA assay, which detects RNA targets on single cells via flow cytometry, could be used. After choosing specific cell targets based on the microarray data, the PrimeFlow RNA assay would be used to sort each cell type, followed by morphology analysis via Giemsa staining and light microscopy and/or electron microscopy to determine the cell type. Furthermore, single cell RNA-sequencing, similar to what has been done in zebrafish and Atlantic cod (*Gadus morhua*) [11,12], could then be applied to the isolated cells to determine a distinct RNA profile, including rare transcripts, for each cell type in the HKL culture.

While no exogenous factors were used in this work to stimulate the M1 or M2 phenotype, both M1 and M2 markers were present in Day 5 HKLs. It is unknown if there is a combination of M1 and M2 cells or if these cells have become primed to develop into M1 or M2 cells. To examine if the cells become more primed to develop into M1 or M2 cells during culture, M1 and M2-activating stimuli (for example LPS or CSF-1 (M1) or cAMP (M2), as demonstrated in European common carp (*Cyprinus carpio carpio* L.) and goldfish (*Carassius auratus* L.) [13,14]) could be used to polarize the cells at Day 1 and at Day 5, followed by analysis of M1 and M2 markers. Ideally protein expression would be analyzed, but with the limited availability of fish specific protein-detecting antibodies, the PrimeFlow RNA assay could be used, which would allow for quantification of cells expressing M1 or M2 markers. If there is a higher expression and/or more cells expressing the M1 or M2 markers following stimulation in the Day 5 HKLs compared to the Day 1 HKLs, then that would suggest that the Day 5 cells are more primed to become M1 or M2 macrophages compared to Day 1 HKLs.

The study of EVs in fish is a relatively new area of research. Previous work in mammals has shown that the biomolecules packaged in EVs can vary individually, but overall can represent a particular physiological state or cell type [15,16]. Therefore, future research examining EVs in Atlantic salmon, and any other fish species, should use more animals. Nonetheless, very little is known about fish EVs and their molecular profile, and there remains huge potential for fish EV research. Future work could focus on identifying biomarkers (e.g. mRNA, miRNA) of EVs released from bacterial or viral infected cells (e.g. HKLs), as well as EVs released into the blood of pathogen infected Atlantic salmon, as well as other pathogen infected fish speices, to determine speices specific differences. Determining biomarkers of pathological EVs in the blood could allow for early, non-invasive, detection of health and disease

in both fish in aquaculture and fish in the wild. Furthermore, while it is known that EV transfer and uptake of miRNAs regulate gene expression of the target cells, including immune cells, in mammals, the function of fish EVs is unknown. By isolating specific miRNA containing EVs in fish and transferring them to other cells, we could start to determine their effect on gene expression and function.

6.3. Conclusion

HKLs are commonly used in fish immunology research, yet they have not been fully characterized. Using transcriptomics and complementary techniques, the results of this thesis showed that the Atlantic salmon adherent HKL population changes during culture time. At Day 1 of culture, the results suggest that adherent HKLs are a heterogeneous population of cells, but by Day 5 of culture, the cells become more homogenous selectively enriched with macrophages. This is something that should be kept in mind when using HKLs for *in vitro* fish immunology studies as the cells will likely respond differently to experimental conditions depending on the time of culture. This work also highlights both mRNAs and miRNAs that may be involved in macrophage differentiation and/or function, many of which are similar to other species, and also suggests specific biomarkers for macrophages, providing the groundwork for future research into the biology of Atlantic salmon HKLs.

6.4. References

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