University of Alberta

Characterization of a novel soluble CSF-1 receptor in teleost fish

by

Johanna Maria Lund

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Physiology, Cell and Developmental Biology

Department of Biological Sciences

Fall 2012
Edmonton, Alberta

ABSTRACT

Teleosts rely on innate immunity to protect themselves from pathogens. Colony stimulating factor-1 (CSF-1) and its cognate receptor CSF-1R control survival, proliferation, differentiation and function of macrophages. Recently, a novel soluble form of CSF-1R (sCSF-1R) was identified in goldfish. My studies on characterization of sCSF-1R in goldfish and zebrafish assessed the contributions to development and inflammation.

In goldfish and zebrafish, sCSF-1R and CSF-1R are not confined to the hematopoietic compartment and show broad expression. The differential expression of these transcripts showcases heterogeneity in immune responses between outbred individuals. Zebrafish sCSF-1R is expressed early in development, suggesting a role during organism and macrophage development. Finally, CSF-1R and sCSF-1R appear to contribute to inflammation, showing expression changes as peritonitis progresses and resolves.

My results should contribute to an increased understanding of the regulation of macrophage development and function in teleosts, and allow for characterization of analogous systems in other vertebrate species.

ACKNOWLEDGMENTS

It is a pleasure to thank those who have made this thesis possible. Foremost, I would like to thank my supervisors, Dr. Daniel Barreda and Dr. Ted Allison, whose encouragement, guidance and support have allowed for me to come this far. I am grateful to my supervisory committee, Dr. Andrew Waskiewicz, for valuable feedback and suggestions throughout my program.

I am obliged to many of my colleagues who have assisted and taught me, including Ms. Aja Rieger, Ms. Adina Bujold, Dr. Valerie Fleisch, Ms. Laura Pillay, Mr. Jeff Konowalchuk, and Ms. Moira Kiemele. Many thanks to the members of both of my labs, past and present, for their continued support and friendship. I am grateful to the Department of Biological Sciences and the University of Alberta for their financial support.

I owe my deepest gratitude to my family for their love and support throughout my entire education, and to Adam for his understanding and giving me the encouragement I needed.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

AML1	acute myeloid leukemia 1 protein
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCM	cell conditioned medium
cDNA	complementary DNA
C/EBP	ccaat-enhancer-binding protein
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
DNA	deoxyribonucleic acid
dpf	days post fertilization
Epo	erythropoietin
FITC	fluorescein isothiocyanate
Flt	fms-like tyrosine kinase
Fms	feline sarcoma virus
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grb2	growth factor receptor-bound protein 2
hpf	hours post ferilization
IFN	interferon
IL	interleukin
JAK	janus kinase
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MAF	macrophage activating factor
MeOH	methanol
MGF	macrophage growth factor
MITF	microphthalmia-associated transcription factor

MPS	mononuclear phagocyte system
mRNA	messenger RNA
Mϕ	macrophage
NBT	nitro-blue tetrazolium chloride
NK	natural killer
P56^{dok-2}	docking protein 2
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PKM	primary kidney macrophage
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
sCSF-1R	soluble form of CSF-1 receptor
SH2	Src-homology 2
SHIP	Src-homology 2-containing inositol phosphatase
SHP-1	Src-homology-2-domain-containing protein tyrosine phosphatase 1
SSC	saline sodium citrate
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

Teleost fish rely strongly on the innate immune response to protect themselves from pathogens. The innate immune system, which is highly conserved throughout evolution, is the first line of defence to prevent infection. One of the key cells of the innate immune response is the macrophage, which acts as a central effector of innate defence mechanisms and as a regulator of innate and adaptive responses. In addition, the macrophage acts as a regulator of hematopoiesis and homeostatic events in the host, revealing a wide-spread role throughout an organism. As macrophages are crucial to the well-being of an organism, macrophage function and development needs to be tightly regulated.

A major regulator of macrophages in vertebrates is a cytokine, colony stimulating factor-1 (CSF-1), which directly controls the survival, proliferation, differentiation and function of macrophages and their progenitors by signalling through its receptor, colony stimulating factor-1 receptor (CSF-1R) (1). While much is known about the CSF-1 system in mammals, there is a lack of knowledge pertaining to teleost fish. Previous work in our group and others has shown CSF-1 and CSF-1R are conserved in teleost fish, but little is known about their full function and what they regulate (2,3). Recently, a novel soluble form of CSF-1R (sCSF-1R) was identified in goldfish primary kidney macrophages (3). It is hypothesized to function by competing with CSF-1R for binding of CSF-1, thereby inhibiting macrophage survival, proliferation and differentiation. However, to date sCSF-1R has only been identified in goldfish. As such, it is unknown if this serves as a conserved mechanism of immune regulation throughout vertebrates.

In this first chapter of my thesis, I review information pertinent to CSF-1 biology as well as the relevance of teleost fish as model organisms to study this cytokine system. In particular, I review information regarding the structure and

function of CSF-1 and CSF-1R, the regulation of the CSF-1 system, and the generation of soluble cytokines as a mechanism of control. I also focus on the use of teleost goldfish and zebrafish as model systems to study the immune response. Chapter II provides a description of materials and techniques utilized during this thesis to characterize CSF-1R and sCSF-1R in goldfish and zebrafish. In Chapter III, I expand on prior characterizations of CSF-1R and sCSF-1R in goldfish. Specifically, this chapter focuses on determining the tissue expression of goldfish to elucidate function and characterization of the role CSF-1R and sCSF-1R play during inflammatory responses in goldfish. In Chapter IV, I focus on the identification of sCSF-1R in zebrafish by taking advantage of developmental and genetic tools available for this model organism. The identification of sCSF-1R in zebrafish would hint at the possibility of a conserved mechanism of hematopoietic control in lower vertebrates, and allow for characterization of this molecule before assessing the presence and function across evolution. Additionally, I determine the tissue expression of CSF-1R and sCSF-1R to assess the potential contributions of these genes to hematopoiesis, host and macrophage development, and immune function with focus on macrophage activity. Finally, in Chapter V, I provide a general discussion of the findings generated in this thesis and their implications to innate immunity in vertebrates in the context of current literature. In addition, I discuss future studies that would further understanding of sCSF-1R and its role in conserved innate immune responses in vertebrates.

2. OBJECTIVES OF THESIS

The main objective of my thesis was to expand our understanding of the role of the novel sCSF-1R in the regulation of CSF-1 activity in goldfish and zebrafish. The regulation of the CSF-1 system by CSF-1R has been well characterized in mammals. However, novel sCSF-1R has only been identified and minimally characterized in goldfish (3). As such, it is not known if this novel sCSF-1R is conserved in other vertebrates. By further characterizing sCSF-1R in goldfish and identifying sCSF-1R in zebrafish, I will be able to elucidate the roles

this molecule plays in teleosts and postulate as to the role it may play in higher vertebrates. The specific aims of my project were as follows:

1. Expand on sCSF-1R and CSF-1R characterization in goldfish. This will provide:
    a. Tissue expression patterns of sCSF-1R and CSF-1R.
    b. Potential contributions of sCSF-1R and CSF-1R to peritoneal inflammation.
2. Identify and characterize sCSF-1R and CSF-1R in zebrafish during adulthood and development. This will provide:
    a. Tissue expression patterns of sCSF-1R and CSF-1R.
    b. Potential contributions of sCSF-1R and CSF-1R during development of immune system and host.
    c. Potential contributions of sCSF-1R and CSF-1R to function and activity of macrophages.

3. LITERATURE REVIEW

3.1. The macrophage

Macrophages play a key role in clearance of spent cells, capable of clearing approximately $2x10^{11}$ erythrocytes per day (4). A normal function of these cells is the removal of cellular debris and apoptotic cells during homeostatic tissue remodelling, mediated by a variety of receptors, including scavenger receptor, phospatidylserine receptor, thrombospondin receptor, integrins and complement receptors (4,5). Upon removal of necrotic cells however, the physiology of a macrophage is altered, resulting in different surface protein expression and production of cytokines and pro-inflammatory mediators (4). This response is mediated by Toll-like receptors (TLRs), pattern recognition receptors, and interleukin 1 receptor (IL-1R) (4,5). By responding to an assortment of factors including endogenous stimuli from injuries, antigen-specific immune cells and self-produced factors (4) and being capable of diverse gene expression (6-8), macrophages represent a vast and heterogeneous population of cells. Furthermore, tissue-resident macrophages have unique functions based on their

localization, including microglia in the central nervous system, alveolar macrophages in the lung, Kupffer cells in the liver, as well as four types of macrophages in the spleen (4,7).

Several classifications of macrophages exist depending on activation and function, as well as expressed gene products. These consist of classically activated macrophages, wound healing macrophages (also known as alternatively activated macrophages), and regulatory macrophages. Classically activated macrophages provide cell-mediated immune responses to intracellular pathogens due to enhanced microbicidal and tumoricidal capacity (8,9,11,12). Activation of these macrophages is mediated through interferon-gamma (IFN-γ) production from helper T-lymphocytes and natural killer (NK) cells, as well as production of tumor necrosis factor (TNF) (4,12,13). This results in the production of IL-1, IL-6, IL-17 and IL-23, which need to be tightly controlled to prevent excessive host damage (4,12). Wound healing macrophages respond to IL-4, typically produced by basophils and mast cells, as well as in response to injury (4,11,12). This leads to increased arginase production, leading to formation of extracellular matrix (11,12,14). Wound healing macrophages also indirectly affect cytokine production and clonal expansion of nearby lymphocytes (4,12). Finally, regulatory macrophages are produced in response to an assortment of factors, including IL-10 production from regulatory T-lymphocytes, immune complexes, prostaglandins, glucocorticoids and apoptotic cells (4,11). These macrophages are capable of inhibiting macrophage-mediated host defense and inflammatory functions, as well as transcription of pro-inflammatory cytokines (4).

Macrophages are dynamic cells capable of phagocytosis, endocytosis, extensive trafficking, as well as homeostatic and inflammatory processes depending upon activation state (4,6). As they function during development and adult life, and are able to fulfill functions including acute inflammation, vascular changes, trophic roles, tissue turnover and clearance of pathogens (6,12), they are essential to proper functioning of the host. Due to this importance, their development, survival and function need to be tightly controlled.

3.2. Structure of CSF-1

The main regulator of macrophage control is colony stimulating factor-1 (CSF-1), which is conserved in vertebrates. CSF-1 was initially isolated from murine serum and human urine, as well as various other tissues and culture supernatants (1,15). The production and secretion of CSF-1 varies amongst organisms based on specific cell types showing expression, including mesothelial cells, endothelial cells, keratinocytes, fibroblasts, thymic epithelial cells, bone marrow stromal cells, osteoblasts, astrocytes, myoblasts, endometrial gland cells and placental trophoblast support cells (16-18). As CSF-1 is expressed in such a wide range of cell types and tissues, the responses mediated by CSF-1 are pleiotropic and vary depending on cell type and the presence of co-stimulating factors (2). However, CSF-1 mediates the proliferation, differentiation, survival and function of cells of the macrophage lineage.

CSF-1 is a homodimeric sialoglycoprotein cytokine that is capable of binding high-affinity receptors on various cell types (19-21). CSF-1 mRNA is encoded from a single 21 kb gene with 10 exons and 9 introns, which is utilized for translation of a precursor protein of 522 amino acids in humans (18,22). This protein in humans consists of a 32 amino acid signal sequence, 4 possible N-glycosylation sites, a conserved residue (Ser-277) for glycosaminoglycan, a hydrophobic transmembrane domain (residues 464-486), and a stop transfer sequence consisting of charged amino acids (RWRRR) (18). CSF-1 functions as a disulfide-linked dimer with each monomer composed of four alpha-helical bundles and an anti-parallel beta sheet (19). Mouse CSF-1 shows high identity to human CSF-1 (60% identity) with the highest conservation in the N-terminal region which is essential for biological activity (18,23).

Mammalian CSF-1 can be generated in three specific isoforms: a secreted glycoprotein, a secreted proteoglycan, and a membrane-bound glycoprotein that is biologically active (19,22). These three isoforms arise from differential splicing in coding or non-coding regions. Alternative splicing in the 3' non-coding region of Exons 9 and 10 directly affects the stability of the CSF-1 transcripts (16-20). The secreted glycoprotein or proteoglycan arises from alternative splicing in the

coding region of Exon 6 which affects the structural characteristics and protein fate of CSF-1 (19,28,29). Finally, alternative splicing in the 5' terminus of Exon 6 results in the formation of the stable membrane-bound glycoprotein (19). The production of these isoforms allow for differential modes of regulation by CSF-1. Secreted isoforms play a role in the humoral response of cellular targets, while the membrane-bound form is involved in local regulation (19). As such, CSF-1 can regulate cell function in a direct cell-cell contact manner, as well as autocrine, paracrine, juxtacrine or endocrine mechanisms (20).

CSF-1 is produced in steady state conditions and is seen to rapidly increase in response to stimuli (3). The production of CSF-1 increases upon stimulation from a variety of activated cell types, including monocytes, macrophages, microglia, T-lymphocytes, B-lymphocytes, fibroblasts, chondrocytes, mesangial cells and endothelial cells (16-18). In addition, pro-inflammatory cytokines including GM-CSF (30), IL-1 (31-33), TNF-α (34) and IFN-γ (35,36) can increase the expression of CSF-1 in macrophages.

3.3. Structure of CSF-1R

The mediator of CSF-1 activity is its receptor, CSF-1R. CSF-1R is primarily responsible for promoting the proliferation, differentiation, survival and function of macrophages and their progenitors. However, the role of CSF-1R is inferred to be more broad based on endogenous expression in various cell types including osteoclasts, placental trophoblasts and mammary epithelial cells (37-39), as well as expression following injury on astrocytes and neurons and on cancerous or leukemic cells (40-42).

CSF-1R is an integral membrane glycoprotein that belongs to the class III receptor tyrosine kinase family (43-46). Other members of this family include critical regulators of hematopoiesis including c-kit (47), Fms-like tyrosine kinase 3 (Flt3) (48,49) and platelet-derived growth factors alpha and beta (PDGFα and β) (50,51). CSF-1R in humans is encoded by a single 58 kb gene (c-*fms*) with 22 exons and 21 introns, which generates a 972 amino acid protein (52,53). The protein structure of CSF-1R consists of an N-terminal glycosylated extracellular

protein that contains 5 repeated immunoglobulin domains, a short transmembrane domain, an intracellular kinase domain divided into two parts by a unique kinase insert, and the C-terminal domain (43-46). The production of CSF-1R in all species is controlled by extracellular and intracellular stimuli that alter the level of transcription. CSF-1R is transcribed and translated from two tissue-specific promoters (39,54-56). The upstream promoter is located 350 bp from the start of non-coding Exon 1 and responds to sex steroid hormones to regulate placental trophoblasts and mammary epithelial cells. The downstream promoter mediates transcription at multiple sites upstream of coding Exon 2 to regulate macrophages, their progenitors and other cell types that express CSF-1R.

As an effector of CSF-1, CSF-1R can be activated by picomolar levels of its ligand which results in the recruitment and activation of cytoplasmic molecules to initiate specific signalling cascades to mediate a cellular response. Following CSF-1 binding to the ligand-binding domain, CSF-1R undergoes dimerization, activates its tyrosine kinase activity and transphosphorylates conserved tyrosine residues (57,58). This results in generation of binding site for intracellular signaling molecules with Src-homology 2 domains (SH2), including phosphoinositide 3-kinase (PI3K), growth factor receptor-bound protein 2 (Grb2), signal transducer and activator of transcription 1 (STAT1) and Src. This then allows for a variety of signal cascades to be controlled by one regulator.

3.4. Function of the CSF-1 system

As mentioned above, the responses mediated by CSF-1 are pleiotropic and varied however they are largely confined to regulating proliferation, differentiation, survival and function. The responses also depend on cell type as well as the presence of any co-stimulatory factors. In its classical role, CSF-1 regulates macrophages and their progenitors, controlling their survival, proliferation, differentiation and function from early progenitors to monocytes to macrophages. In addition, CSF-1 stimulates proliferation, differentiation and survival of multipotent, bipotent and unipotent hematopoietic precursors (18). Intravenous injections of CSF-1 have previously been shown to increase

circulating monocytes in the blood from 3% to 30% and increase macrophages in the periphery (19). Furthermore, CSF-1 has been shown to increase chemotactic, phagocytic and killing mechanisms by regulating the activation of monocytes and macrophages (54-60). It does so by increasing production of various cytokines including G-CSF, GM-CSF, IL-1, IL-6, IL-8, TNFα and IFN (66-73), as well as increasing reactive oxygen and nitrogen processes (74-78).

CSF-1 has been shown to be essential for many other developmental processes as shown by CSF-1 null mice. These mice possess a thymidine insertion in the coding region of CSF-1 (base pair 262), resulting in the generation of a biologically inactive truncated protein of only 63 amino acids (19,79). This null mutation results in a deficiency in osteoclasts resulting in abnormal bone remodelling/metabolism and osteopetrosis, a lack of teeth, low weight, shortened lifespan and decreased fertility in both male and female mice. In addition, these mice display a partial or complete deficiency of macrophages in specific tissues at birth and throughout development, indicating a role for CSF-1 in both prenatal and postnatal development (19). Furthermore, CSF-1 has been shown to be an important regulator of female fertility and pregnancy, having roles in blastocyst attachment, trophoblast outgrowth, implantation and proliferation of the placental tissue (80).

The primary role of CSF-1R is to promote proliferation, differentiation, survival and function of macrophages. CSF-1R null mice possess phenotypes very similar to CSF-1 null mice (discussed in detail above); however the phenotypes of CSF-1R present as more severe which may be explained by CSF-1 independent activation of CSF-1R (81). In addition, CSF-1R null mice have 20-fold increased levels of circulating CSF-1, which has previously been implicated in causing myeloproliferative diseases including myeloid metaplasia and peripheral bone marrow extension (18).

Several orthologues of CSF-1R have been examined in teleost fish, including gilthead seabream, rainbow trout and zebrafish. In gilthead seabream, the CSF-1R orthologue has been shown to be expressed exclusively in cells of the monocyte/macrophage lineage in immunologically-relevant tissues including the

spleen, thymus, head kidney, liver, blood and gills (82). This is similar to that of rainbow trout, which express high levels of CSF-1R in the head kidney, kidney, intestine, spleen, ovary and blood, with minimal expression in other tissues including the liver (83). Zebrafish also possess an orthologue of CSF-1R which is conserved in cells of the macrophage and osteoclast lineages (84). Zebrafish CSF-1R has been previously studied in its role in regulating macrophage development. Zebrafish possessing loss-of-function mutations in CSF-1R show normal macrophage development and can phagocytose apoptotic bodies, however early macrophages fail to exit the yolk sac and do not invade other tissues of the embryo (84,85). Detailed analysis of the full range of function and activity of these mutant macrophages during development has not to date been performed. Furthermore, characterization of the function and activity of macrophages in adults deficient in CSF-1R has not been examined. Zebrafish CSF-1R also plays a unique role due to its expression in neural crest-derived cells. CSF-1R has been shown to play a direct role in pigmentation in the zebrafish, a previously unattributed function of CSF-1R, affecting both xanthophore and melanocyte development (yellow and black pigmentation, respectively) in embryonic and adult zebrafish (84).

3.4.1. IL-34 activation of CSF-1R

In addition to CSF-1, IL-34 also functions as a ligand of CSF-1R, capable of initiating downstream signalling pathways. IL-34 is a homodimeric glycoprotein of 241 amino acids (86). While it shares very minimal identity with CSF-1, IL-34 also possesses four alpha-helical bundles similar to that of CSF-1. However while the general structure is conserved, it has been shown that IL-34 binds to a separate site at the junction between the third and fourth immunoglobulin domains of CSF-1R (86). In addition, IL-34 binds to CSF-1R at two- to five-fold lower affinity than that of CSF-1 (87).

IL-34 has been identified from numerous tissues including heart, brain, lung, liver, kidney, thymus, testes, ovary, small intestine, colon and prostate, with highest levels in the spleen (88). Compared to CSF-1, IL-34 shows higher

expression in embryonic and adult brain and heart, but lower expression in the pregnant uterus and osteoblasts (87,89). While IL-34 and CSF-1 show differential spatiotemporal expression, they are believed to have complementary and non-redundant roles. Similar to that of CSF-1, IL-34 is capable of stimulating monocyte viability and proliferation, CSF-1R tyrosine phosphorylation, signalling, and synergizing with other cytokines, including SCF, IL-6 and IL-3, to generate macrophages and osteoclasts from progenitors (87,88). In addition, transgenic expression of IL-34 from a CSF-1 promoter in $Csf1^{op/op}$ mice can rescue fertility, osteoporotic, and macrophage phenotypes (87). However, CSF-1 and IL-34 expressing macrophages have distinct phenotypes, with different expression of chemokines MCP-1 and eotaxin-2, as well as differential signal activation of ribosomal S6 kinases and STAT proteins, and a greater susceptibility to West Nile Virus in IL-34 macrophages (90,91). IL-34 also shows preferential expression in keratinocytes, neurons, microglia and Langerhans cells, and specifically directs differentiation and development of myeloid cells in the skin epidermis and central nervous system (91,92). As such, IL-34 is hypothesized to function in a trophic role by regulating macrophage functions in homeostasis and development, as it is not expressed in cells of the innate immune system as is CSF-1 (86).

3.5. Regulation of the CSF-1 system

The CSF-1 system is crucial to the organism, resulting in the need for tight regulation of its functions. This occurs both at the level of CSF-1, as well as CSF-1R. Circulating CSF-1 has a very short life span of 10 minutes and is effectively cleared by the Kuppfer cells in the liver, which clear approximately 95% of CSF-1, with the spleen clearing the remaining CSF-1 (19,93). This clearance is attributable to receptor-mediated internalization and intracellular destruction which is partially mediated by c-Cbl (93-96). Following activation by CSF-1 and downstream signalling events, CSF-1R undergoes covalent dimerization through its disulphide bonds (97). This further leads to polyubiquitination of the cytoplasmic domain of CSF-1R, inactivation of the

kinase domain, dephosphorylation of the phosphotyrosine residues, internalization of the receptor-ligand complex, lysosome targeting and destruction of the complex (18,57).

CSF-1R levels can be regulated by a variety of mechanisms. These include transcriptional attenuation, DNA methylation and regulation by lineage-specific transcription factors including Ccaat-enhancer-binding proteins alpha and beta (C/EBPα and β), microphthalmia-associated transcription factor (MITF), PU.1, Ets1, Ets2, acute myeloid leukemia 1 protein (AML1), and E12 (98-110). In addition, CSF-1R signalling itself can be down-regulated by binding of negative regulators to its phosphorylated binding site, including Src-homology 2-containing inositol phosphatase (SHIP), Src-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP-1), PTPase, suppressor of cytokine signaling 1 (SOCS1), and docking protein 2 ($P56^{dok-2}$)(111-120).

The synthesis and release of soluble cytokine receptors to regulate cytokine function is a common occurrence. Two main types of this mechanism are i) production of a soluble receptor agonist that binds to the cytokine receptor; and ii) generation of a soluble receptor that binds to the cytokine ligand. Only one example of the first type of mechanism exists, which is the human IL-1 receptor agonist, IL-1ra (121,122), which is responsible for the regulation of inflammation, sepsis and fever. There are numerous examples of soluble receptors that bind to the ligand, including G-CSF, GM-CSF, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, type I and II interferons, TNF, leukemia inhibitory factor (LIF), growth hormone and erythropoietin (Epo) (123-130). Of these soluble receptors, there are two modes of generation: i) alternative splicing of the mRNA species to retain ligand-binding domains but not the transmembrane domains (seen in GM-CSFRα, IL-4Rα, IL-5Rα, IL-6Rα, IL-7Rα and IFNαRβ); or ii) proteolytic cleavage of the full-length receptor to release it from the membrane (seen in GM-CSFRα, IL-2Rα, TNFRI and TNFRII).

3.5.1. Soluble CSF-1R

Until recently, a soluble CSF-1 receptor had yet to be identified in any species. However, it was identified in 2005 from goldfish primary kidney macrophage (PKM) cultures during senescence phase, which is characterized by a cessation of proliferation and differentiation, debris formation and cell clumping and cellular death through apoptosis (3,131). sCSF-1R was found to be confined to a unique population of macrophages which appear to bypass the typical monocytic stage of macrophage differentiation (termed alternative pathway (AP) macrophages) based on semi-quantitative reverse-transcriptase PCR expression in early progenitors and mature macrophages (3).

The transcript of sCSF-1R is believed to be derived via alternative splicing from the membrane CSF-1R, a phenomenon that is common with other cytokines and their regulators as discussed above. This transcript contains a start codon, a predicted signal peptide (16 amino acids), Exons 2 through 4 of CSF-1R, a stop codon and a polyadenylation sequence (3). Alignment of CSF-1R and sCSF-1R indicated 100% identity from the start codon to residue 619, which corresponded to the Exon4/Intron4 boundary. It is believed generation of sCSF-1R results in part by deviations in the donor splice-site consensus recognition sequence resulting in inefficient splicing (3). The transcript encodes a product with 3 putative N-glycosylation sites, but only two of the five N-terminal immunoglobulin domains of CSF-1R which correspond to the ligand-binding domain. These two immunoglobulin domains are sufficient for high-affinity binding of CSF-1 as previous studies have shown that binding affinity increases with each immunoglobulin domain to a maximum of three from the N-terminus (44).

sCSF-1R was identified as a native soluble protein, with low levels during lag phase, a sharp decrease during early proliferation, and a progressive increase throughout late proliferation to senescence phases (3). Addition of recombinant sCSF-1R to PKM cultures resulted in a dose-dependent decrease in macrophage proliferation at nanomolar concentrations, suggesting a role in regulation of proliferation in these cultures. Based on its structure and function, it is predicted

that sCSF-1R functions as a negative inhibitor of CSF-1. In fact, it has previously been shown that sCSF-1R binds to homodimeric CSF-1 (132). However, it is not known if sCSF-1R forms dimers with itself to bind CSF-1, or if sCSF-1R forms dimers with membrane-bound CSF-1R to inhibit downstream signalling (Figure 1.1). Nevertheless, while sCSF-1R is capable of binding to CSF-1, it should be incapable of initiating down-stream signalling pathways and potentially result in decreased positive modulation of macrophage survival, proliferation and differentiation to control the population. It remains unclear if this may be limited to specific macrophage subsets or may differentially impact distinct subsets of this myeloid population. It is further hypothesized that micro-environmental conditions, including cell density, cell size and nutrient depravation, contribute to the activation of negative feedback mechanisms including the production of sCSF-1R (3).

3.6. Teleost model systems

3.6.1. Teleost immunity

Teleost fish have recently become an important model organism for many branches of science that include developmental biology, comparative immunology and medicine. Particularly, fish are emerging as a new model organism for studying the immune system as they serve as a bridge between invertebrates that rely mainly on innate immunity and mammals that rely heavily on classical adaptive mechanisms of immunity (133). Fish have been shown to possess many components of immune signalling pathways, though it is not clear if all components present in higher vertebrates are conserved. Fish possess orthologues of immune components including complement, Toll-like receptors (TLRs), tumour necrosis factors (TNFs), interleukins (ILs), interferons (IFNs), and the inflammatory response (134,135). However, while some components are well conserved, such as intracellular signalling adaptors and enzymes, other components such as the class II cytokines and receptors have evolved independently from their mammalian counterparts and so show species-specific expansions and diversifications (134). Fish also possess many of the cells that are

essential to the immune system including monocytes, granulocytes, tissue macrophages, neutrophils, thrombocytes, B- and T-cells, eosinophils and natural killer (NK) cells (133,135).

While fish do have many similarities to mammals, there are some key differences that need to be taken into account for comparative studies. Unlike mammals, fish do not possess red bone marrow or lymph nodes, which are essential for the mammalian production of myeloid cells (myelopoiesis) and all other blood cells (hematopoiesis) respectively (133). Instead, fish rely primarily on the thymus and the kidney for these processes during adulthood. In addition, while fish do have most of the necessary components of the adaptive immune response, it is innate immunity that is more useful for the defence of the organism. This is attributable to the less specific nature of adaptive immune response compared to mammals, as well as the delayed development of essential components until after the embryo has hatched and been exposed to the aquatic environment (133,136). It has been theorized that innate immunity in the fish results in a more rapid and efficient response to protect the organism from foreign invaders. This is based on the presence of various robust components of the innate response including complement, NK cells, TLR receptors, and novel immune-type receptors unique to zebrafish (136). Based on these components, the pressure to fine tune adaptive immunity may not have been present. Each species of fish also has a unique environment in which it lives, and so immune responses differ based on the unique immune challenges presented (137). Fish also have high genetic variability in immune responses, and on average, genes involved in immune function appear to be more divergent than the rest of the genome, especially those genes whose products interact with the pathogen (134,137). Finally, there is a lack of markers for cellular and molecular components (137), resulting in the need for different techniques when using fish as a model organism.

3.6.2. Use of goldfish and zebrafish

Goldfish have become an important model organism for a variety of scientific fields, including pathogenesis, neurobiology, toxicology, metabolism, physiology and immunology. As a member of one of the largest vertebrate families, *Cyprinidae*, they have many functional and anatomical similarities to higher vertebrates. Goldfish are closely related to many species that have important roles as genetic model organisms, as well as ecological and economical roles (138). Goldfish are easily contained in a laboratory environment and are often cheaper than other vertebrate models (139). Due to their size, they are amenable models for experimental manipulations and allow for easy dissection of tissue. The use of goldfish for tissue culture to study the immune system offers a unique advantage – cultured macrophages secrete all necessary growth factors and as such, exhibit spontaneous growth (131). Teleosts provide sufficient numbers of macrophages and their progenitors to examine individual animal primary cultures without the need to pool samples. However, while there is an abundance of functional tools available to study goldfish, several disadvantages are present. While the need to pool tissues is not necessary in goldfish to obtain sufficient numbers of cells, the outbred nature of goldfish prevents any pooling for immunology studies due to immune responses to non-self tissues. Additionally, as they are long lived-organisms, the time it takes for a goldfish to reach sexual maturity is much greater than other teleost fish, such as the zebrafish (139). Finally, goldfish only breed in early spring which makes it unreasonable to use them to study development (131). Despite these disadvantages, goldfish remain a useful model organism.

Zebrafish have recently emerged as one of the major fish models used for comparative studies. Unlike other models, large numbers of zebrafish are easy and inexpensive to keep. In addition, zebrafish breed well in a laboratory environment year round, with one female capable of producing hundreds of progeny per week. These transparent embryos are fertilized external to the mother and as such their rapid development can be observed from the one-cell stage to adulthood. Each embryo has a fully operational macrophage-based

immune system after only 24 hours post-fertilization (hpf) (135), and all organs are formed after 3 days post-fertilization (dpf) (140). Zebrafish also serve as a model for primitive and definitive hematopoiesis, with distinct similarities to mammalian models (141). Zebrafish are closely related to goldfish which allows for comparison between the species. In addition, both zebrafish and goldfish have robust innate immune responses which allows for study of the associated processes easily. Through the use of zebrafish, much is known about events surrounding cell commitment and development during hematopoiesis. However, due to the small size of zebrafish, functional studies examining primary cells are difficult to perform (145). As such, the use of larger teleosts including carp and goldfish allows for characterization of the mechanism and events of hematopoietic development. Though zebrafish are small in size compared to goldfish and lack in functional tools such as tissue culture, ease of manipulation and genetic tools of zebrafish make them an excellent model organism.

3.6.3. Macrophage development in goldfish

The primary site of hematopoiesis in teleost fish is the head kidney, with monocyte/macrophage maturation occurring in the spleen (143). Progenitor cells in the goldfish kidney can form multiple cell types including monocytes, granulocytes, lymphocytes, erythrocytes and thrombocytes (143-148). The differentiation of these progenitor cells involves tight control of lineage-specific gene expression by interactions between transcription factors (149-153), co-regulatory molecules (154-158), and DNA-binding sequences (159,160). As these transcription factors show differential expression during stages of hematopoietic stem cell development and commitment, the assortment of transcription factors expressed by a cell is characteristic of both the hematopoietic lineage and stage of maturation (143).

Functional studies of goldfish hematopoiesis have been simplified by the ease of culturing teleost kidney macrophages *in vitro*. As teleost macrophages secrete endogenous growth factors, they are capable of self-regulating their growth and additionally exhibit spontaneous growth (161,162). One such growth

factor present in cell-conditioned media (CCM) is macrophage growth factor (MGF) which selectively induces the proliferation of macrophage-like cells from goldfish kidney cultures (131,148). *In vitro* cultures of kidney cells exposed to high levels of MGF possess characteristics of proliferative cultures as discussed below. Through knowledge of MGF and use of primary kidney macrophage (PKM) cultures, the processes surrounding hematopoiesis have been clarified.

PKM cultures show three distinct developmental phases: a lag phase, a proliferative phase and a senescence phase (131). Post-isolation, cultures experience a lag phase which typically lasts 3-5 days. Lag phase is characterized by a sharp decrease in cell numbers and debris formation as cells that are not part of the macrophage lineage die off (131). Some proliferation and differentiation of progenitor cells is also observed. During late lag phase/early proliferative phase, there is a decrease in cellular debris and vacuolisation and an increase in numbers of monocytes. Proliferative phase is characterized by extensive proliferation and differentiation, typically encompassing 6 to 8 generations (131). During this phase, cellular debris and cell clumps are absent, while established populations of adherent and non-adherent cells are observed. Furthermore, three distinct sub-populations are observed, termed R1, R2 and R3 populations (148,163). R1 cells are small (6-10 μm) with a high nucleus to cytoplasm ratio and closely resemble progenitor cells (163). These early progenitors proliferate and differentiate from 4 to 10 day during culturing in response to CCM (158). R2 cells are large (12-20 μm) and irregularly shaped with a low nucleus to cytoplasm ratio and resemble mature macrophages (163). These mature macrophages proliferate in the presence of CCM and are stimulated to produce nitric oxide species in the presence of MAF+LPS (148). R3 cells are large (12-15 μm) and round with a low nucleus to cytoplasm ratio and resemble mammalian monocytes (163). These monocytes were seen to differentiate into mature macrophages in the presence of CCM or MAF+LPS (148). Finally, senescence phase is characterized by a variety of events including cellular clumping, increased debris and cell death via apoptosis, increased cell vacuolisation and cessation of proliferation (131).

The current view for vertebrate hematopoiesis is based on the mononuclear phagocyte system (MPS) present in mammals, whereby macrophages are derived from circulating monocytes in the blood that arise from progenitor stem cells in the bone marrow (163). While MPS-dependent macrophages are conserved in teleosts, macrophage heterogeneity is maintained through MPS-independent macrophages, termed alternative pathway macrophages (AP-macrophages) (163). AP-macrophages are believed to arise directly from early progenitors and thus bypass the monocytic stage of development. It is hypothesized that AP-macrophages are conserved evolutionarily as well as developmentally from primitive hematopoiesis in vertebrate embryos to adult hematopoiesis (163). MPS-independent populations of macrophages have been observed in fetal lungs of mice that arise prior to development of bone marrow and circulating monocytes (51). In addition, microglia in the brain are believed to arise from an MPS-independent pathway and have been shown to be derived from progenitor cells that arose in the yolk sac during development (41). Furthermore, AP-macrophages share characteristics with embryonic macrophages, including self-renewal capabilities, low expression of transcription factor PU.1 and localized myeloperoxidase staining (34,163). The development of embryonic macrophages in zebrafish is discussed below.

3.6.4. Macrophage development in zebrafish

In zebrafish, the head kidney of the adult zebrafish contains myeloid cells of all developmental stages, with macrophages predominately located in the head kidney and spleen (141). However, they do not originate here. Much is known about where development and maturation of macrophages begin in the developing zebrafish. Primitive hematopoiesis begins as early as 12 hpf, when macrophage precursors emerge from the rostral region near the cardiac field of the embryo (135,143,144). Macrophages are present in the hundreds after 22 hpf in two types: half-spread wandering cells near the hatching gland (pre-macrophages), and unspread round cells with little cytoplasm that frequently divide and wander around the embryo (immature macrophages) (144). The onset of blood circulation

(24-26 hpf) takes most of the macrophages away to other parts of the embryo, initially seeding them in the cephalic mesenchyme and brain. The remainder of the macrophages remain near the yolk, and take on an amoeboid shape with a bean-shaped nucleus and possess phagocytic vacuoles (early macrophages).

These early macrophages are unique in that they follow a non-classical-rapid differentiation pathway that bypasses the normal monocyte series typical of macrophage differentiation (144). Definitive hematopoiesis begins shortly afterwards, resulting in stimulation of the caudal hematopoietic tissue, and differentiation of hematopoietic stem cells (141,143). At about 2 dpf, the embryo hatches and becomes exposed to the environment. As lymphocytes have yet to be developed (the thymus is seeded 3 dpf with lymphoblasts), the embryo relies solely on these macrophages, and thus innate immunity, to protect it (141). The early macrophages possess all of the essential abilities needed, including engulfment and digestion of pathogens and apoptotic bodies, the ability to migrate to infected tissue and to remodel the embryo (141,144). These macrophages are also capable of producing cytokines, resulting in activation of the total population of macrophages in the embryo (144). After 4 days, the kidney is seeded with hematopoietic stem cells (141). After the initial developmental stages, all macrophages differentiate using the classical, monocyte-based pathway.

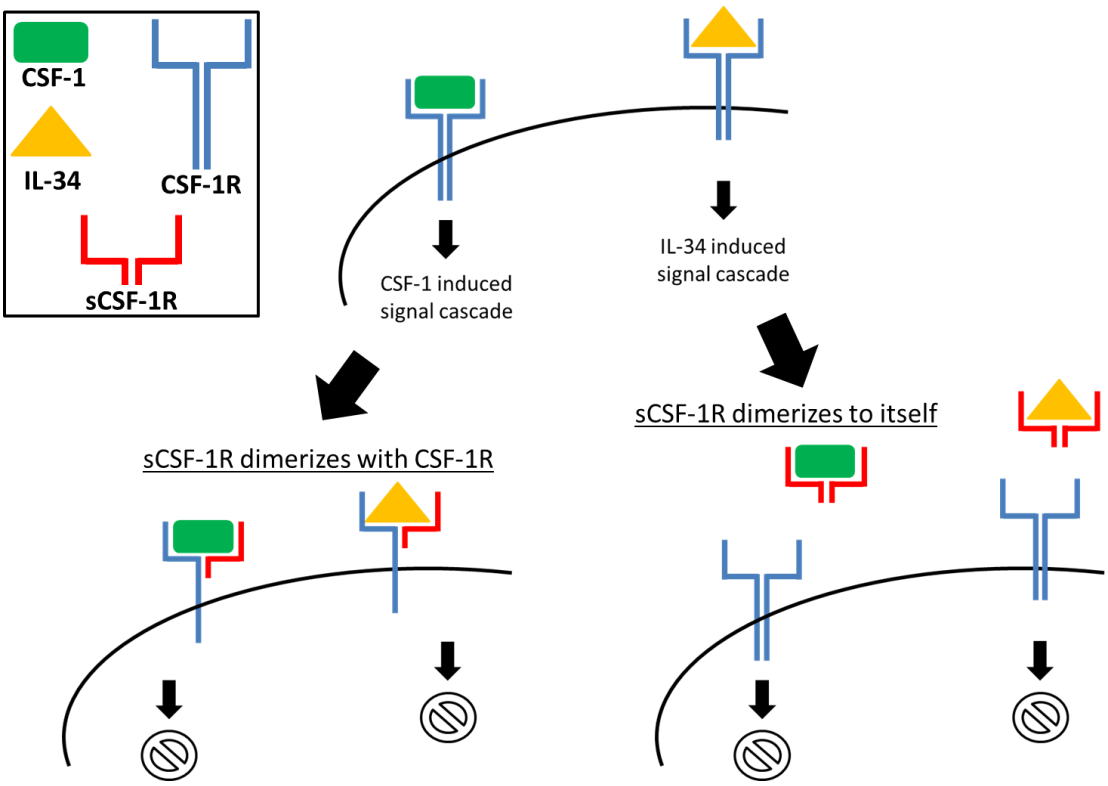


Figure 1.1. Potential mechanisms for regulation of CSF-1R and IL-34 signalling by sCSF-1R. CSF-1R is capable of binding to both CSF-1 and IL-34 to initiate downstream signaling cascades. Upon generation of sCSF-1R, two possible events can occur: i) sCSF-1R dimerizes with CSF-1R, leading to binding of the ligands but no downstream signaling; or ii) sCSF-1R dimerizes to itself, which prevents ligands from binding to CSF-1R and inhibits signaling cascades.

4. REFERENCES

1. Stanley ER and Heard PM. 1977. Factors regulating macrophage production and growth: purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J Biol Chem* **252**: 4305–4312.
2. Barreda DR, Hanington PC and Belosevic M. 2004. Regulation of myeloid development and function by colony stimulating factors. *Dev Comp Immunol* **28**: 509-554.
3. Barreda DR, Hanington PC, Stafford JL and Belosevic M. 2005. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). *Dev Comp Immunol* **29**: 879-894.
4. Mosser DM and Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8(12)**: 958-969.
5. Gordon S. 1998. The role of the macrophage in immune regulation. *Res Immunol* **149**: 685-688.
6. Gordon S. 2007. The macrophage: Past, present and future. *Eur J Immunol* **37**: S9-S17.
7. Gordon S and Taylor PR. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**: 953-964.
8. Mackaness GB. 1977. Cellular immunity and the parasite. *Adv Exp Med Biol* **93**: 65-73.
9. O'Shea JJ and Murray PJ. 2008. Cytokine signaling modules in inflammatory responses. *Immunity* **28**: 477-487.
10. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK and Suttles J. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* **175**: 342-349.
11. Forlenza M, Fink IR, Raes G and Wiegertjes GF. 2011. Heterogeneity of macrophage activation in fish. *Dev Comp Immunol* **35**: 1246-1255.

12. Murray PJ and Wynn TA. 2011. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* **11**: 723-737.
13. Liddiard K, Rosas M, Davies LC, Jones SA and Taylor PR. 2011. Macrophage heterogeneity and acute inflammation. *Eur J Immunol* **41**: 2470-2525.
14. Kreider T, Anthony RM, Urban JF Jr. and Gause WC. 2007. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol* **19**:448-453.
15. Robinson WA, Stanley ER and Metcalf D. 1969. Stimulation of bone marrow colony growth in vitro by human urine. *Blood* **33(3)**:396–399.
16. Callard RE, editor. The cytokine FactsBook. San Diego:Academic Press; 1994. p. 265.
17. Moore MAS. Macrophage colony-stimulating factor. In: Garland JM, Quesenberry PJ, Hilton DJ, editors. Colony stimulating factors: molecular and cellular biology, 2nd ed. New York: Marcell Dekker; 1997. p. 255–289.
18. Stanley ER. Colony stimulating factor-1 (macrophage colony stimulating factor). In: Thompson A, editor. The cytokine handbook, 2nd ed. New York: Academic Press; 1994. p.387–418.
19. Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y and Yeung YG. 1997. Biology and action of colony-stimulating factor-1. *Molec Reprod Dev* **46(1)**: 4–10.
20. Fixe P and Praloran V. 1997. Macrophage colony-stimulating-factor (M-CSF or CSF-1) and its receptor: structure–function relationships. *Eur Cytokine Netw* **8(2)**:125–136.
21. Hamilton JA. 1997. CSF-1 signal transduction. *J Leukoc Biol* **62**:145-155.
22. Wang T, Hanington PC, Belosevic M and Secombes CJ. 2008. Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. *J Immunol* **181**: 3310-3322.

23. Kawasaki ES, Ladner MB. 1990. Molecular biology of macrophage colony-stimulating factor. *Immunol Ser* **49**:155–176.
24. Ladner MB, Martin GA, Noble JA, Nikoloff DM, Tal R, Kawasaki ES and White TJ. 1987. Human CSF-1: gene structure and alternative splicing of mRNA precursors. *Embo J* **6(9)**:2693–2698.
25. Kawasaki ES, Ladner MB, Wang AM, Van Arsdell J, Warren MK, Coyne MY, Schweickart VL, Lee MT, Wilson KJ and Boosman A. 1985. Molecular cloning of a complementary DNA encoding human macrophage-specific colony-stimulating factor (CSF-1). *Science* **230(4723)**: 291–296.
26. Chambers SK, Kacinski BM. 1994. Messenger RNA decay of macrophage colony-stimulating factor in human ovarian carcinomas in vitro. *J Soc Gynecol Investig* **1(4)**: 310–316.
27. Bakheet T, Frevel M, Williams BR, Greer W and Khabar KS. 2001. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res* **29(1)**: 246–254.
28. Rettenmier CW and Roussel MF. 1988. Differential processing of colony-stimulating factor 1 precursors encoded by two human cDNAs. *Mol Cell Biol* **8(11)**: 5026–5034.
29. Manos MM. 1988. Expression and processing of a recombinant human macrophage colony-stimulating factor in mouse cells. *Mol Cell Biol* **8(11)**: 5035–5039.
30. Horiguchi J, Warren MK and Kufe D. 1987. Expression of the macrophage-specific colony-stimulating factor in human monocytes treated with granulocyte-macrophage colony stimulating factor. *Blood* **69(4)**: 1259–1261.
31. Harrington M, Konicek BW, Xia XL and Song A. 1997. Transcriptional regulation of the mouse CSF-1 gene. *Mol Reprod Dev* **46(1)**: 39–44. Discussion-5.

32. Gruber MF, Webb DSA and Gerrard TL. 1992. Stimulation of human monocytes via Cd45, Cd44, and Lfa-3 triggers macrophage colony-stimulating factor production-synergism with lipopolysaccharide and Il-1-Beta. *J Immunol* **148(4)**: 1113–1118.
33. Falkenburg JHF, Harrington MA, Walsh WK, Daub R and Broxmeyer HE. 1990. Gene expression and release of macrophage-colony stimulating factor in quiescent and proliferating fibroblasts. Effects of serum, Fibroblast growth-promoting factors, and Il-1. *J Immunol* **144(12)**: 4657-5662.
34. Oster W, Lindemann A, Horn S, Mertelsmann R and Herrmann F. 1987. Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. *Blood* **70(5)**: 1700–1703.
35. Horiguchi J, Warren MK, Ralph P and Kufe D. 1986. Expression of the macrophage specific colony-stimulating factor (CSF-1) during human monocytic differentiation. *Biochem Biophys Res Commun* **141(3)**: 924–930.
36. Oster W, Lindemann A, Mertelsmann R and Herrmann F. 1988. Regulation of gene expression of M-, G-, GM-, and multi-CSF in normal and malignant hematopoietic cells. *Blood Cells* **14(2–3)**: 443–462.
37. Hofstetter W, Wetterwald A, Cecchini MC, Felix R, Fleisch H and Mueller C. 1992. Detection of transcripts for the receptor for macrophage colonystimulating factor, c-fms, in murine osteoclasts. *Proc Natl Acad Sci USA* **89(20)**: 9637–9641
38. Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB and Stanley ER. 1987. Apparent role of the macrophage growth factor, CSF-1, in placental development. *Nature* **330(6147)**: 484–486.
39. Sapi E, Flick MB, Rodov S, Carter D and Kacinski BM. 1998. Expression of CSF-I and CSF-I receptor by normal lactating mammary epithelial cells. *J Soc Gynecol Investig* **5(2)**: 94–101.

40. Wang Y, Berezovska O and Fedoroff S. 1999. Expression of colony stimulating factor-1 receptor (CSF-1R) by CNS neurons in mice. *J Neurosci Res* **57(5)**: 616–632.
41. Kacinski BM. 1997. CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. *Mol Reprod Dev* **46(1)**: 71–74.
42. Ashmun RA, Look AT, Roberts WM, Roussel MF, Seremetis S, Ohtsuka M and Sherr CJ. 1989. Monoclonal antibodies to the human CSF-1 receptor (c-fms protooncogene product) detect epitopes on normal mononuclear phagocytes and on human myeloid leukemic blast cells. *Blood* **73(3)**: 827–837.
43. Guilbert LJ and Stanley ER. 1980. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. *J Cell Biol* **85(1)**: 153–159.
44. Wang ZE, Myles GM, Brandt CS, Lioubin MN and Rohrschneider L. 1993. Identification of the ligand-binding regions in the macrophage colony-stimulating factor receptor extracellular domain. *Mol Cell Biol* **13(9)**: 5348–5359.
45. Sherr CJ. 1991. Mitogenic response to colony-stimulating factor 1. *Trends Genet* 7**(11–12)**: 398–402.
46. Reilly JT. 2002. Class III receptor tyrosine kinases: role in leukaemogenesis. *Br J Haematol* **116(4)**: 744–757.
47. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U and Ullrich A. 1987. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *Embo J* **6(11)**: 3341–3351.
48. Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, Birg F and Birnbaum D. 1993. Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. *Blood* **82(4)**: 1110–1119.
49. Rosnet O, Marchetto S, deLapeyriere O and Birnbaum D. 1991. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. *Oncogene* **6(9)**: 1641–1650.

50. Claesson-Welsh L, Eriksson A, Westermark B and Heldin CH. 1989. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci USA* **86(13)**: 4917–4921.
51. Yarden Y, Escobedo JA, Kuang WJ, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Francke U, Fried VA, Ullrich A and Williams LT. 1986. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* **323(6085)**: 226–232.
52. Hampe A, Shamoon BM, Gobet M, Sherr CJ and Galibert F. 1989. Nucleotide sequence and structural organization of the human FMS proto-oncogene. *Oncogene Res* **4(1)**: 9–17.
53. Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT and Stanley ER. 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* **41(3)**: 665–676.
54. Visvader J and Verma IM. 1989. Differential transcription of exon 1 of the human c-fms gene in placental trophoblasts and monocytes. *Mol Cell Biol* **9(3)**: 1336–1341.
55. Roberts WM, Shapiro LH, Ashmun RA and Look AT. 1992. Transcription of the human colony-stimulating factor-1 receptor gene is regulated by separate tissue-specific promoters. *Blood* **79(3)**: 586–593.
56. Lee AW and States DJ. Guide to CD115 (CSF-1R). In: Stanley ER, editor. Protein reviews on the web. 2000. p. 13–21.
57. Hamilton JA. 1997. CSF-I signal transduction: what is of functional significance? *Immunol Today* **18(7)**: 313–317
58. Weiss A and Schlessinger J. 1998. Switching signals on or off by receptor dimerization. *Cell* **94(3)**: 277–280.
59. Munn DH and Cheung NK. 1990. Phagocytosis of tumor cells by human monocytes cultured in recombinant macrophage colony-stimulating factor. *J Exp Med* **172(1)**: 231–237.

60. Munn DH and Cheung NK. 1995. Antibody-independent phagocytosis of tumor cells by human monocyte-derived macrophages cultured in recombinant macrophage colony-stimulating factor. *Cancer Immunol Immunother* **41(1)**: 46–52.
61. Bober LA, Grace MJ, Pugliese-Sivo C, Rojas-Triana A, Sullivan LM and Narula SK. 1995. The effects of colony stimulating factors on human monocyte cell function. *Int J Immunopharmacol* **17(5)**: 385–392.
62. Karbassi A, Becker JM, Foster JS and Moore RN. 1987. Enhanced killing of Candida albicans by murine macrophages treated with macrophage colony-stimulating factor: evidence for augmented expression of mannose receptors. *J Immunol* **139(2)**: 417–421.
63. Ralph P and Nakoinz I. 1987. Stimulation of macrophage tumoricidal activity by the growth and differentiation factor CSF-1. *Cell Immunol* **105(2)**: 270–279.
64. Wing EJ, Waheed A, Shadduck RK, Nagle LS and Stephenson K. 1982. Effect of colony stimulating factor on murine macrophages. Induction of antitumor activity. *J Clin Invest* **69(2)**: 270–276.
65. VandePol CJ and Garnick MB. 1991. Clinical applications of recombinant macrophage-colony stimulating factor (rhM-CSF). *Biotechnol Ther* **2(3–4)**: 231–239.
66. Metcalf D and Nicola NA. 1985. Synthesis by mouse peritoneal cells of G-CSF, the differentiation inducer for myeloid leukemia cells: stimulation by endotoxin, M-CSF and multi-CSF. *Leuk Res* **9(1)**: 35–50.
67. Motoyoshi K, Suda T, Kusumoto K, Takaku F and Miura Y. 1982. Granulocyte macrophage colony-stimulating and binding activities of purified human urinary colony-stimulating factor to murine and human bone marrow cells. *Blood* **60(6)**: 1378–1386.
68. Oster W, Brach MA, Gruss HJ, Mertelsmann R and Hermann F. 1992. Interleukin-1 beta (IL-1 beta) expression in human blood mononuclear phagocytes is differentially regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, and IL-3. *Blood* **79(5)**: 1260–1265.

69. Moore RN, Oppenheim JJ, Farrar JJ, Carter CS Jr., Waheed A and Shadduck RK. 1980. Production of lymphocyte-activating factor (Interleukin 1) by macrophages activated with colony-stimulating factors. *J Immunol* **125(3)**: 1302–1305.

70. Warren MK and Ralph P. 1986. Macrophage growth factor CSF-1 stimulates human monocyte production of interferon, tumor necrosis factor, and colony stimulating activity. *J Immunol* **137(7)**: 2281–2285.

71. James SL, Cook KW, Lazdins JK. 1990. Activation of human monocyte-derived macrophages to kill schistosomula of Schistosoma mansoni in vitro. *J Immunol* **145(8)**: 2686–2690.

72. Curley SA, Roh MS, Kleinerman E and Klostergaard J. 1990. Human recombinant macrophage colony stimulating factor activates murine Kupffer cells to a cytotoxic state. *Lymphokine Res* **9(3)**: 355–363.

73. Moore RN, Larsen HS, Horohov DW and Rouse BT. 1984. Endogenous regulation of macrophage proliferative expansion by colony-stimulating factor-induced interferon. *Science* **223(4632)**: 178–181.

74. Wing EJ, Ampel NM, Waheed A and Shadduck RK. 1985. Macrophage colony-stimulating factor (M-CSF) enhances the capacity of murine macrophages to secrete oxygen reduction products. *J Immunol* **135(3)**: 2052–2056.

75. Desai G, Nassar F, Brummer E and Stevens DA. Killing of Histoplasma capsulatum by macrophage colony stimulating factor-treated human monocyte-derived macrophages: role for reactive oxygen intermediates. *J Med Microbiol* **43(3)**: 224–229.

76. Garcia Lloret MI, Rocha Ramirez LM and Ramirez A. 1992. Macrophage colony-stimulating factor enhances the respiratory burst of human monocytes in response to Entamoeba histolytica. *Arch Med Res* **23(2)**: 139–141.

77. Meager A. Cytokines. Englewood Cliffs, NJ: Prentice Hall; 1991.

78. Sherr CJ. 1990. Regulation of mononuclear phagocyte proliferation by colony-stimulating factor-1. *Int J Cell Cloning* **8(Suppl 1)**: 46–60. Discussion-2.

79. Wiktor-Jedrzejczak W, Gordon S. 1996. Cytokine regulation of the macrophage system using the colony stimulating factor-1 deficient op/op mouse. *Physiol Rev* **76**: 927–947.

80. Yong K, Salooja N, Donahue RE, Hegde U and Linch DC. 1992. Human macrophage colony-stimulating factor levels are elevated in pregnancy and in immune thrombocytopenia. *Blood* **80(11)**: 2897–2902.

81. Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O, Russell RG, Pollard JW and Stanley ER. 2001. Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse (Csf1(op)/Csf1(op)) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* **98(1)**: 74-84.

82. Roca FJ, Sepulcre MP, López-Castejón G, Meseguer J and Mulero V. 2006. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molec Immunol* **43**: 1418-1423.

83. Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, Nakanishi T, Yokomizo Y, Takahashi Y, Inagawa H and Soma G-I. 2005. Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. *Molec Immunol* **42**: 1-8.

84. Parichy DM, Ransom DG, Paw B, Zon LI and Johnson SL. 2000. An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in zebrafish *Danio rerio*. *Development* **127**: 3031-3044.

85. Herbomel P, Thisse B and Thisse C. 2001. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol* **238**: 274-288.

86. Garceau V, Smith J, Paton IR, Davey M, Fares MA, Sester DP, Burt DW and Hume DA. 2010. Pivotal advance: Avian colony-stimulating factor 1 (CSF-1), interleukin-34 (IL-34), and CSF-1 receptor genes and gene products. *J Leuko Biol* **87**: 753-764.

87. Wei S, Nandi S, Chitu V, Yeung Y-G, Yu W, Huang M, Williams LT, Lin H and Stanley ER. 2010. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leuko Biol* **88**: 495-505.

88. Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, Halenbeck R, Wu G, Zhou A, Behrens D, Hollenbaugh D, Linnemann T, Qin M, Wong J, Chu K, Doberstein SK and Williams LT. 2008. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science* **320**: 807-811.

89. Mouchemore KA and Pixley FJ. 2012. CSF-1 signaling in macrophages: pleiotrophy through phosphotyrosine-based signaling pathways. *Crit Rev Clin Lab Sci* **49(2)**: 49-61.

90. Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, Kimura F and Okada S. 2010. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell Death Diff* **17**: 1917-1927.

91. Wang Y, Szretter KJ, Vermi W, Gilfillan S, Rossini C, Cella M, Barrow AD, Diamond MS and Colonna M. 2012. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nat Immunol* [Epub ahead of print].

92. Nandi S, Gokhan S, Dai X-M, Wei S, Enikolopov G, Lin H, Mehler MF, Stanley ER. 2012. The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct developmental brain expression patterns and regulate progenitor cell maintenance and maturation. *Dev Biol* **367**: 100-113.

93. Bartocci A, Mastrogiannis DS, Migliorati G, Stockert RJ, Wolkoff AW and Stanley ER. 1987. Macrophages specifically regulate the

concentration of their own growth factor in the circulation. *Proc Natl Acad Sci USA* **84(17)**: 6179–6183.

94. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR and Stanley ER. 1982. Survival of mononuclear phagocytes depends of a lineage specific growth factor that the differentiated cells selectively destroy. *Cell* **28**: 71–81.

95. Guilbert LJ and Stanley ER. 1986. The interaction of 125I-colonystimulating factor-1 with bone marrow-derived macrophages. *J Biol Chem* **261(9)**: 4024–4032.

96. Mancini A, Koch A, Wilms R and Tamura T. 2002. c-Cbl associates directly with the C-terminal tail of the receptor for the macrophage colony-stimulating factor, c-Fms, and down-modulates this receptor but not the viral oncogene v-Fms. *J Biol Chem* **277(17)**: 14635–14640.

97. Li W and Stanley ER. 1991. Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. *Embo J* **10(2)**: 277–288.

98. Zhang DE, Hetherington CJ, Meyers S, Rhoades KL, Larson CJ, Chen HM, Hiebert SW and Tenen DG. 1996. CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol Cell Biol* **16(3)**: 1231–1240.

99. Barreda DR and Belosevic M. 2001. Transcriptional regulation of hemopoiesis. *Dev Comp Immunol* **25(8–9)**: 763–789.

100. Hume DA, Yue X, Ross IL, Favot P, Lichanska A ad Ostrowski MC. 1997. Regulation of CSF-1 receptor expression. *Mol Reprod Dev* **46(1)**: 46–52. Discussion-3.

101. Xie Y, Chen C and Hume DA. 2001. Transcriptional regulation of cfms gene expression. *Cell Biochem Biophys* **34(1)**: 1–16.

102. Zhang DE, Hetherington CJ, Chen HM and Tenen DG. 1994. The macrophage transcription factor PU.1 directs tissue-specific expression of

the macrophage colony-stimulating factor receptor. *Mol Cell Biol* **14(1)**: 373–381.

103. Zhang DE, Hohaus S, Voso MT, Chen HM, Smith LT, Hetherington CJ and Tenen DG. 1996. Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. *Curr Top Microbiol Immunol* **211(3)**: 137–147.

104. Inaba T, Gotoda T, Ishibashi S, Harada K, Ohsuga JI, Ohashi K, Yazaki Y and Yamada N. 1996. Transcription factor PU.1 mediates induction of c-fms in vascular smooth muscle cells: a mechanism for phenotypic change to phagocytic cells. *Mol Cell Biol* **16(5)**: 2264–2273.

105. Reddy MA, Yang BS, Yue X, Barnett CJ, Ross IL, Sweet MJ, Hume DA and Ostrowski MC. 1994. Opposing actions of c-ets/PU.1 and c-myb protooncogene products in regulating the macrophage-specific promoters of the human and mouse colony-stimulating factor-1 receptor (c-fms) genes. *J Exp Med* **180(6)**: 2309–2319.

106. Rabault B, Roussel MF, Quang CT and Ghysdael J. 1996. Phosphorylation of Ets1 regulates the complementation of a CSF-1 receptor impaired in mitogenesis. *Oncogene* **13(4)**: 877–881.

107. Ross IL, Yue X, Ostrowski MC and Hume DA. 1998. Interaction between PU.1 and another Ets family transcription factor promotes macrophage-specific basal transcription initiation. *J Biol Chem* **273(12)**: 6662–6669.

108. Rhoades KL, Hetherington CJ, Rowley JD, Hiebert SW, Nucifora G, Tenen DG and Zhang DE. 1996. Synergistic up-regulation of the myeloid-specific promoter for the macrophage colony-stimulating factor receptor by AML1 and the t(8;21) fusion protein may contribute to leukemogenesis. *Proc Natl Acad Sci USA* **93(21)**: 11895–11900.

109. Zhang DE, Fujioka K, Hetherington CJ, Shapiro LH, Chen HM, Look AT and Tenen DG. 1994. Identification of a region which directs the monocytic activity of the colony-stimulating factor 1 (macrophage colony-stimulating factor) receptor promoter and binds PEBP2/CBF (AML1). *Mol Cell Biol* **14(12)**: 8085–8095.

110. Kee BL and Murre C. 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix–loop–helix transcription factor E12. *J Exp Med* **188(4)**: 699–713.

111. Tamir I, Dal Port JM and Cambier JC. 2000. Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. *Curr Opin Immunol* **12(3)**: 307–315.

112. Helgason CD, Damen JE, Rosten P, Grewal R, Sorenson P, Chappel SM, Borowski A, Jirik F, Krystal G and Humphries RK. 1998. Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev* **12(11)**: 1610–1620.

113. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A and Rohrschneider LR. 1996. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* **10(9)**: 1084–1095.

114. Rovida E, Rigacci S, Paccagnini A, Dello Sbarba P and Berti A. 1998. The low-molecular weight phosphotyrosine protein phosphatase, when overexpressed, reduces the mitogenic response to macrophage colony-stimulating factor and tyrosine phosphorylation of its receptor. *Biochem Biophys Res Commun* **253(2)**: 300–304.

115. Takeshita S, Namba N, Zhao JJ, Jiang Y, Genant HK, Silva MJ, Brodt MD, Helgason CD, Kalensnikoff J, Rauh MJ, Humphries RK, Krystal G, Teitelbaum SL and Ross FP. 2000. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. *Nat Med* **8(9)**: 943–949.

116. Rohrschneider LR, Fuller JF, Wolf I, Liu Y and Lucas DM. 2000. Structure, function, and biology of SHIP proteins. *Genes Dev* **14(5)**: 505–520.

117. Nicola NA, Nicholson SE, Metcalf D, Zhang JG, Baca M, Farley A, Willson TA, Starr R, Alexander W and Hilton DJ. 1999. Negative regulation of cytokine signaling by the SOCS proteins. *Cold Spring Harb Symp Quant Biol* **64**: 397–404.

118. Alexander WS, Starr R, Metcalf D, Nicholson SE, Farley A, Elefanty AG, Brysha M, Kile BT, Richardson R, Baca M, Zhang JG, Willson TA, Vine EM, Sprigg NS, Rakar S, Corbin J, Mifsud S, DiRago L, Cary D, Nicola NA, Hilton DJ. 1999. Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. *J Leukoc Biol* **66(4)**: 588–592.

119. Suzu S and Motoyoshi K. 2002. Signal transduction in macrophages: negative regulation for macrophage colony stimulating factor receptor signaling. *Int J Hematol* **76(1)**: 1–5.

120. Suzu S, Tanaka-Douzono M, Nomaguchi K, Yamada M, Hayasawa H, Kimura F and Motoyoshi K. 2000. p56(dok-2) as a cytokine-inducible inhibitor of cell proliferation and signal transduction. *Embo J* **19(19)**: 5114–5122.

121. Carter DB, Deibel Jr. MR, Dunn CJ, Tomich CS, Laborde AL, Slightom JL, Berger AE, Bienkowski MJ, Sun FF, McEwan RN, Harris PKW, Yem AW, Waszak GA, Chosay JG, Sieu LC, Hardee MM, Zurcher-Neely HA, Reardon IM, Heinrikson RL, Truesdell SE, Shelly JA, Eessalu TE, Taylor BM and Tracey DE. 1990. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature* **344(6267)**: 633–638.

122. Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP and Thompson RC. 1990. Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* **343(6256)**: 336–340.

123. Baynes RD, Reddy GK, Shih YJ, Skikne BS and Cook JD. 1993. Serum form of the erythropoietin receptor identified by a sequence-specific peptide antibody. *Blood* **82(7)**: 2088–2095.

124. Layton MJ, Cross BA, Metcalf D, Ward LD, Simpson RJ and Nicola NA. 1992. A major binding protein for leukemia inhibitory factor in normal mouse serum: identification as a soluble form of the cellular receptor. *Proc Natl Acad Sci USA* **89(18)**: 8616–8620.

125. Nakamura Y, Komatsu N and Nakauchi H. 1992. A truncated erythropoietin receptor that fails to prevent programmed cell death of erythroid cells. *Science* **257(5073)**: 1138–1141.
126. Baumann G, Stolar MW, Amburn K, Barsano CP and DeVries BC. 1986. A specific growth hormone-binding protein in human plasma: initial characterization. *J Clin Endocrinol Metab* **62(1)**: 134–141.
127. Iwasaki H, Shimoda K, Okamura S, Otsuka T, Nagafuji K, Harada N, Ohno Y, Miyamoto T, Akashi K, Harada M and Niho Y. 1999. Production of soluble granulocyte colony-stimulating factor receptors from myelomonocytic cells. *J Immunol* **163(12)**: 6907–6911.
128. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE and Park LS . 1990. Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell* **60(6)**: 941–951.
129. Fernandez-Botran R, Chilton PM and Ma Y. 1996. Soluble cytokine receptors: their roles in immunoregulation, disease, and therapy. *Adv Immunol* **63**: 269–336.
130. Leonard WJ. Type I cytokines and interferons and their receptors. In: Paul WE, editor. Fundamental immunology, 4th ed. Philadelphia: Lippincott-Raven; 1999. p. 741–744.
131. Barreda DR and Belosevic M. 2001. Characterisation of growth enhancing factor production in different phases of in vitro fish macrophage development. *Fish Shellfish Immunol* **11**: 169–186.
132. Hanington PC, Wang T, Secombes CJ and Belosevic M. 2007. Growth factors of lower vertebrates: characterization of goldfish (Carassius auratus L.) macrophage colony-stimulating factor-1. *J Biol Chem* **282(4)**: 31865-31872.
133. Workenhe ST, Rise ML, Kibenge MJT and Kibenge FSB. 2010. The fight between the teleost fish immune response and aquatic viruses. *Molec Immunol* **47**: 2525-2536.

134. Stein C, Caccamo M, Laird G and Leptin M. 2007. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. *Genome Biol* **8**: R251-251.23.

135. Traver D, Herbomel P, Patton EE, Murphey RD, Yoder JA, Litman GW, Catic A, Amemiya CT, Zon LI and Trede NS. 2003. The zebrafish as a model organism to study development of the immune system. *Adv Immunol* **81**: 253-330.

136. Trede NS, Langenau DM, Traver D, Look AT and Zon LI. 2004. The use of zebrafish to understand immunity. *Immunity* **20**: 367-379.

137. Randelli E, Buonocore F and Scapigliati G. 2008. Cell markers and determinants in fish immunology. *Fish Shell Immunol* **25**: 326-340.

138. Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR and Trudeau VL. 2008. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Molec Cell Endocrinol* **293**: 43-56.

139. Powers DA. 1989. Fish as Model Systems. *Science* **246(4928)**: 352-358.

140. Van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CMJE and Bitter W. 2004. A star with stripes: zebrafish as an infection model. *TRENDS Microbiol* **12**: 451-457.

141. Ellett F and Lieschke GJ. 2010. Zebrafish as a model for vertebrate hematopoiesis. *Curr Opin Pharmacol* **10**: 1-8.

142. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC and Layton JE. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**: 3087-3096.

143. Hanington PC, Tam J, Katzenback BA, Hitchen SJ, Barreda DR and Belosevic M. 2009. Development of macrophages of cyprinid fish. *Dev Comp Immunol* **33**: 411-429.

144. Herbomel P, Thisse B and Thisse C. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**: 3735-3745.

145. Kobayashi I, Moritomo T, Ototake M and Nakanishi T. 2007. Isolation of side population cells from ginbuna carp (Carassius auratus langsdorfii) kidney hematopoietic tissues. *Dev Comp Immunol* **31**: 696–707.

146. Kobayashi I, Sekiya M, Moritomo T, Ototake M and Nakanishi T. 2006. Demonstration of hematopoietic stem cells in ginbuna carp (Carassius auratus langsdorfii)kidney. *Dev Comp Immunol* **30**: 1034–1046.

147. Moritomo T, Asakura N, Sekiya M, Ototake M, Inoue Y and Nakanishi T. 2004. Cell culture of clonal ginbuna crucian carp hematopoietic cells: differentiation of cultured cells into erythrocytes in vivo. Dev Comp Immunol **28**: 863–869.

148. Neumann NF, Barreda DR and Belosevic M. 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. *Fish Shell Immunol* **10**: 1–20.

149. Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, Narravula S, Torbett BE, Orkin SH and Tenen DG. 2000. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood* **96**: 2641–2648.

150. Sieweke MH and Graf T. 1998. A transcription factor party during blood cell differentiation. *Curr Opin Genet Dev* **8**: 545–551.

151. Goetz TL, Gu TL, Speck NA and Graves BJ. 2000. Auto-inhibition of Ets-1 is counteracted by DNA binding cooperativity with core-binding factor alpha 2. *Molec Cell Biol* **20**: 81-90.

152. Gu TL, Goetz TL, Graves BJ and Speck NA. 2000. Auto-inhibition and partner proteins, core-binding factor beta (CBFbeta) and Ets-1, modulate DNA binding by CBFalpha2 (AML1). *Molec Cell Biol* **20**: 91-103.

153. Woo AJ, Moran TB, Schindler Y, Choe SK, Langer NB, Sullivan MR, Fujiwara Y, Paw BH and Cantor AB. 2008. Identification of ZBP-89 as a novel GATA-1 associated transcription factor involved in megakaryocytic and erythroid development. *Molec Cell Biol* **28**: 2675–2689.

154. Thomas T, Corcoran LM, Gugasyan R, Dixon MP, Brodnicki T, Nutt SL, Metcalf D and Voss AK. 2006. Monocytic leukemia zinc finger protein is essential for the development of long-term reconstituting hematopoietic stem cells. *Genes Dev* **20**: 1175–1186.

155. Levanon D, Goldstein RE, Bernstein Y, Tang H, Goldenberg D, Stifani S, Paroush Z and Groner Y. 1998. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/ Groucho corepressors. *Proc Natl Acad Sci USA* **95**: 11590-11595.

156. Holmes M, Turner J, Fox A, Chisholm O, Crossley M and Chong B. 1999. hFOG-2, a novel zinc finger protein, binds the co-repressor mCtBP2 and modulates gata-mediated activation. *J Biol Chem* **274**: 23491–23498.

157. Xu L, Glass CK and Rosenfeld MG. 1999. Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* **9**:140–147.

158. Burke L and Baniahmad A. 2000. Co-repressors 2000. FASEB J **14**: 1876-1888.

159. Tenen DG, Hromas R, Licht JD and Zhang DE. 1997. Transcription factors, normal myeloid development, and leukemia. *Blood* **90**: 489–519.

160. Lenny N, Westendorf JJ and Hiebert SW. 1997. Transcriptional regulation during myelopoiesis. *Mol Biol Rep* **24**: 157–168.

161. Hanington PC, Hitchen SJ, Beamish LA and Belosevic M. 2009. Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages. *Fish Shell Immunol* **26**: 1-9.

162. Hanington PC, Barreda DR and Belosevic M. 2006. A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages. *J Biol Chem* **281**: 9963–9970.

163. Belosevic M, Hanington PC and Barreda DR. 2006. Development of goldfish macrophages in vitro. *Fish Shell Immunol* **20**: 152-171.

CHAPTER II: GENERAL MATERIALS AND METHODS

1. MOLECULAR ANALYSIS

1.1 Fish

1.1.1 Goldfish

Goldfish (*Carassius auratus* L.) 10-15 cm in length were purchased from Mount Parnell (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were held at 20°C in a flow-through water system on a simulated natural photoperiod. The fish were fed to satiation daily with trout pellets.

1.1.2 Zebrafish

Adult fish were maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta according to standard protocols (Westerfield, 2000) in accordance with the Canadian Council for Animal Care (CCAC) guidelines. To obtain embryos, crossings were made between two male and 3 female fish. Embryos were grown in the dark at 28°C in embryo media until use. The AB strain of wild-type fish was used for all experiments unless otherwise noted.

1.2 Tissue isolation

Goldfish were anesthetized in a solution of tricane methanesulfonate (Aqualife) and sacrificed via cervical severing. Tissues of interest (eye, brain, heart, gill, liver, spleen, kidney, muscle and gonad) were removed fish using forceps, transferred to individual 50 mL conical tubes and immediately frozen on dry ice. Samples were stored at -80°C until use.

Adult zebrafish were anesthetized in a solution of tricane methanesulfonate (Aqualife). Tissues of interest (head kidney, body kidney and spleen) were removed from approximately 50 adult AB zebrafish and pooled based on tissue

and strain. Additional tissues (eye, brain, heart, gill, liver, spleen, head kidney, body kidney, muscle, female gonads and male gonads) were removed from approximately 30 adult AB zebrafish and pooled based on tissue. All samples were frozen on dry ice and stored at -80°C until use. Embryos (n=50) were collected at 0, 12, 18, 24, 48, 72, 96 and 120 hours post fertilization (hpf) into 15 mL conical tubes. Embryos were stored in RNAlater (Ambion) at 4°C until use.

1.3 RNA isolation and cDNA synthesis

RNA was isolated using TRIzol (Invitrogen) as per manufacturers' directions and quantified using a NanoDrop 2000C (ThermoScientific). Double-stranded cDNA was synthesized from 4 µg (goldfish) or 400 ng (zebrafish) RNA using SmartScribe reverse-transcriptase (Clontech) as per manufacturer's directions with the exception of the use of two reverse primers (0.5 µL each of 20 µM solution, IDT). Primers used for first strand amplification are as follows:

5'oligo (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3');

3'CDS (5'-AAGCAGTGGTATCAACGCAGAGTACTTT$_{(27)}$-3').

The primer used for second strand amplification is as follows:

5'PCR (5'-AAGCAGTGGTATCAACGCAGAGT-3').

In addition, gene-specific cDNA synthesis was performed using a reverse primer specific to CSF-1R or sCSF-1R. Gene-specific synthesis was performed as per manufacturer's directions with the following changes: 3 µl of RNA template was added to 1.5 µl mQH$_2$O and reverse primer (0.5 µL of 20 µM solution, IDT). Primers are as follows:

goldfish CSF-1R (5'-GAARATCTCCCASAGSADGA-3');

goldfish sCSF-1R (5'-CCTTCAGCAAAGTAATGAACT-3');

zebrafish CSF-1R (5'-TCGGGATGTTCTTGTACTC-3');

zebrafish sCSF-1R (5'-AGCACTGTAAATGAAACTC-3').

1.4 Polymerase chain reaction amplification

Alignment of goldfish CSF-1R (AY536523) to zebrafish CSF-1R (AF240639) allowed for prediction of the site of sCSF-1R generation by

comparison of Exon4/Intron4 boundaries. From this, a predicted sequence for zebrafish sCSF-1R was generated via comparison to zebrafish genomic sequence. Primers were designed to amplify the predicted sCSF-1R. Primers for goldfish CSF-1R and sCSF-1R (AY536524) and zebrafish CSF-1R were designed from existing sequences.

PCR amplification for each gene was performed as follows: 2 µL of cDNA template was added to a mastermix containing 40.3 µL mQH_2O, 10x PCR buffer (5 µL of 200 mM Tris-HCl pH8.3, 500 mM KCl, 50 mM $MgCl_2$, 0.1% (w/v) gelatin), dNTPs (0.8 µL of 25 mM solution containing dATP, dCTP, dGTP and dTTP, Invitrogen), forward and reverse primers (1.2 µL of each 20 µM solution, IDT) and Taq DNA polymerase (0.5 µl of 5U/µL solution). PCR amplification was performed in a Mastercycler Gradient (Eppendorf). The amplification consisted of a hot-start at 95°C, followed by 25-40 cycles of 95°C for 20 sec, 55°C for 20 sec, 72°C for 2 min, 30 sec, and a final elongation step at 72°C for 5 min. PCR products were electrophoresed in a 1% agarose gel, stained in ethidium bromide and visualized on an AlphaImager 2200 (Alpha Innotech). β-actin PCR was performed for 25-27 cycles, while CSF-1R and sCSF-1R were performed for 40 cycles. Bands of interest were removed from the agarose gel using a scalpel and extracted using QIAquick Gel Extraction Kit (QIAGEN) as per manufacturer's directions. Gel extracts were stored at -20°C until use.

The primers used for this study can be found in Table 2.1.

1.5 Densitometry analysis

Following gel electrophoresis, gel pictures were analysed using Adobe Photoshop in order to determine relative levels of gene expression. The number of pixels for each band was determined and compared to the background intensity for each sample. This ratio was then compared to β-actin for the corresponding sample and graphed using Microsoft Excel.

1.6 TOPO-TA cloning and screening

Following gel extraction, PCR products were used for TOPO TA cloning (Invitrogen) as per manufacturer's directions. Following cloning, the vector was transformed into chemically-competent DH5α-T1^{R} *E. coli* cells and plated onto LB agar plates containing 100 µg/mL ampicillin and 40 mg/mL X-gal in dimethylformamide (Invitrogen) (for blue-white selection of positive clones). Plates were incubated at 37°C for 16-18 hours.

To identify colonies containing the PCR product, colony PCR was performed as follows: 0.2 mL PCR tubes were prepared with 41.3 µL mQH_2O, 10x PCR buffer (5 µL of 200 mM Tris-HCl pH8.3, 500 mM KCl, 50 mM $MgCl_2$, 0.1% (w/v) gelatin), dNTPs (0.8 µL of 25 mM solution containing dATP, dCTP, dGTP and dTTP), M13 forward and reverse primers (1.2 µL of each 20 µM solution, IDT; forward 5'-gtaaaacgacggccag-3' and reverse 5'-caggaaacagctatgac-3') and Taq DNA polymerase (0.5 µl of 5U/µL solution). An autoclaved toothpick was used to select white colonies off of LB agar plates and to swirl into the PCR tube. The toothpick was then used to inoculate liquid cultures containing 2 mL LB broth and 100 µg/mL ampicillin. The PCR amplification consisted of a 10-min 94°C hot-start, followed by 30 cycles of 94° for 30 sec, 52.5°C for 30 sec, 72°C for 1 min, 30 sec, and a final elongation step of 72°C for 10 min. PCR products were electrophoresed in a 1% agarose gel, stained in ethidium bromide and visualized as above.

Clones containing an insert of the desired size were used to inoculate liquid cultures, which were incubated in a shaking incubator at 37°C for 16-18 hours. Plasmids were then isolated using a QIAprep Spin Miniprep Kit (QIAGEN) following manufacturer's directions. Plasmids were stored at -20°C until use.

1.7 Sequencing and analysis

Following cloning, selected plasmids were used for sequencing. Plasmids were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed at the Molecular Biology Service Unit (MBSU; Department of Biological Sciences, University of Alberta) using a PE Applied

Biosystems 377 automated sequencer. Obtained sequences were analysed to confirm identity using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/). A consensus sequence for predicted zebrafish sCSF-1R was derived through use of the Geneious software program (http://www.geneious.com/).

2. FUNCTIONAL ANALYSIS

2.1 Fish

2.1.1 Zebrafish CSF-1R^{j4e1} mutant fish

Two male and two female CSF-1R^{j4e1} mutant zebrafish were ordered from the Zebrafish International Resource Center (ZIRC, line #ZL74). CSF-1R^{j4e1} zebrafish possess a valine to methionine substitution mutation of residue 614, resulting in a recessive loss-of-function mutation in CSF-1R. Crossings were made in order to obtain a F_1 generation which were raised and cared for as mentioned above.

2.2 Homeostasis and inflammation in goldfish

2.2.1 Peritoneal injections of zymosan and apoptotic bodies

These experiments were done in collaboration with Aja Rieger from the lab of Dr. Daniel Barreda (University of Alberta). Goldfish (10-15 cm) were acclimatized at 20°C for one week in a flow-through system quarantine tank. Goldfish were anesthetized in a solution of tricane methanesulfonate (Aqualife). Zymosan (2.5 mg; 100 µL) or apoptotic bodies ($5x10^6$) were injected into the peritoneal cavity of goldfish either individually, or with apoptotic bodies injected 4 hours prior to zymosan. The fish were placed into the quarantine tank to recover.

Goldfish were anesthetized in a solution of tricane methanesulfonate (Aqualife) and sacrificed by severing the spinal cord. The lavage was performed by passing 10 mL Trypsin-EDTA PBS-/- through the peritoneal cavity, which was then collected into individual 50 mL conical tubes and placed on ice. Cells were enumerated using a haemocytometer and the total cells/mL was calculated for each lavage. Lavages were combined into a 50 mL conical tube and centrifuged

at 1100 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet was flash frozen and stored at -80°C until use for RNA isolation.

2.2.2 PKM culturing and exposure of zymosan and apoptotic bodies

These experiments were done in collaboration with Aja Rieger from the lab of Dr. Daniel Barreda (University of Alberta). In short, primary kidney macrophages (PKM) were cultured by seeding leukocytes and cultured in 15 mL of complete MGFL-15 medium (100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 10% newborn calf serum (Gibco) and 5% carp serum) and 5 mL of cell-conditioned medium from previous cultures. Cultures were incubated for 7 days at 18°C. $2x10^6$ cells were then exposed to zymosan and/or apoptotic bodies at a ratio of 5:1 (particle:cell) for 4 or 24 hours. Cells were collected, flash frozen, and stored at -80°C until use for RNA isolation.

2.2.3 Molecular analysis

Frozen cell pellets from above (n=5 per condition for lavages; n=3 for PKMs) were used to isolate RNA using TRIzol (Invitrogen) as above for each time point. Double-stranded cDNA was synthesized from 300 ng RNA using SmartScribe reverse-transcriptase (Clontech) as above.

PCR amplification was performed as above to determine changes in expression levels of CSF-1R and sCSF-1R. PCR products were electrophoresed in a 1% agarose gel, stained in ethidium bromide and visualized as above. Bands of interest were gel extracted as above and used for cloning and sequencing.

Densitometry analysis was carried out for all gels as described above. A two-tailed unpaired T-test was performed to determine significance using Microsoft Excel.

2.3 Peritonitis model in goldfish peritoneal cavity

2.3.1 Peritoneal injections of zymosan

Thirty-two goldfish (10-15 cm) were acclimatized at 20°C for one week in a flow-through system quarantine tank. Goldfish were anesthetized in a solution of

tricane methanesulfonate (Aqualife). Zymosan (2.5 mg; 100 µL) was injected into the peritoneal cavity of twenty-four goldfish which were then placed back into the quarantine tank to recover. The remaining eight fish were used as a 0 hour uninjected control. Eight fish were used for each time point of 24, 48 and 72 hours post injection.

2.3.2 Peritoneal lavages

Peritoneal lavages were performed on eight fish at each time point. Goldfish were anesthetized in a solution of tricane methanesulfonate (Aqualife) and sacrificed by severing the spinal cord. The lavage was performed by passing 10 mL PBS-/- through the peritoneal cavity, which was then collected into individual 50 mL conical tubes and placed on ice. Cells were enumerated using a haemocytometer and the total cells/mL was calculated for each lavage. Five lavages for each time point were combined into a 50 mL conical tube and centrifuged at 1100 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet was flash frozen and stored at -80°C until use for RNA isolation. The remaining three lavages were processed for flow cytometric characterization.

2.3.3 Flow cytometric characterization

Three lavages from each time point were centrifuged individually at 1100 rpm for 10 min at 4°C. The supernatant from each tube was discarded and the remaining liquid was used to resuspend the cell pellet. 100 µL was transferred to a 5 ml round bottom polystyrene tube containing 100 µl of 2% formaldehyde. Samples were placed on ice for 10 min before analysis. Data was acquired on an ImageStream multi-spectral flow cytometer and analyzed using IDEAS software (Amnis).

2.3.4 Molecular analysis

Frozen cell pellets for the lavages were used to isolate RNA using TRIzol (Invitrogen) as above for each time point. Double-stranded cDNA was

synthesized from 1.5 µg RNA using SmartScribe reverse-transcriptase (Clontech) as above. In addition, gene-specific cDNA was synthesized as above.

PCR amplification was performed as above to determine changes in expression levels of CSF-1R and sCSF-1R. PCR products were electrophoresed in a 1% agarose gel, stained in ethidium bromide and visualized as above. Bands of interest were gel extracted as above and used for cloning and sequencing. Densitometry analysis was carried out for all gels as described above. A two-tailed unpaired T-test was performed to determine significance using Microsoft Excel.

2.4 Pigmentation analysis in zebrafish

2.4.1 Analysis of CSF-1R^{j4e1} mutant fish

CSF-1R^{j4e1} mutant embryos were obtained by crossings between two genetically identical male and three genetically identical female fish. As the pigmentation phenotype has previously been described, embryos were analysed to ensure detection of the phenotype. In short, embryos were incubated at 29°C in E3 media and analysed every 24 hours until the pigmentation phenotype could be detected. Differences between AB and CSF-1R^{j4e1} mutants were documented using an Olympus DP72 microscope digital camera attached to a Leica MZ16F fluorescence stereomicroscope.

2.4.2 Morpholino design and injection

Morpholinos were designed and synthesized using Gene Tools. Two morpholinos were designed for this project: i) a splice-block designed to the exon 4-intron 4 boundary designed to knock-down expression of CSF-1R only (CCAGCACTGTAAATGAAACTCACTT); and ii) a translation-block designed to the ATG start site designed to knock-down expression of both CSF-1R and sCSF-1R (CTCAGAGCTGCTCCTGCTTAACCAT). Morpholinos were injected into 0 hpf embryos at a concentration of 2.5 ng/µL using a glass microcapillary needle. Embryos were incubated at 29°C in E3 media and analysed every 24

hours until a phenotype could be detected. Phenotypes were documented as mentioned previously.

2.5 Analysis of macrophage numbers in developing zebrafish

In order to determine if CSF-1R plays a role in macrophage development in embryonic zebrafish, *in situ* hybridizations were performed using a probe for L-plastin. L-plastin is an actin bundling protein which is expressed in leukocytes in mice and has been used in zebrafish to examine early macrophage precursors. To generate the probe, linearized plasmid containing the L-plastin gene was donated by Laura Pillay from the lab of Dr. Andrew Waskiewicz (Department of Biological Sciences, University of Alberta). A DIG-labelled probe was synthesized using DIG DNA Labeling Kit (Roche) as per manufacturer's directions at 37°C for two hours. To determine the purity of the probe, 2 µL of the probe was electrophoresed on a 1% agarose gel with a RNA ladder standard, stained in ethidium bromide and visualized as above.

Embryos (AB wild type, CSF-1R^{j4e1} mutants or splice-block injected embryos) for *in situ* hybridizations were collected at 30 hpf. Embryos were dechorionated using pronase and were washed for 5 minutes each in the following solutions: 25% MeOH/75% PBST; 50% MeOH/50% PBST; 75% MeOH/25% PBST; and 100% MeOH. Embryos were then stored at -20°C until use. Prior to use, embryos were rehydrated with washes for 5 minutes each in the following solutions: 75% MeOH/25% PBST; 50% MeOH/50% PBST; 25% MeOH/75% PBST; and 100% PBST. Embryos were permeabilized with 10 µg/mL in Proteinase K for 7 minutes and fixed in 4% paraformaldehyde/10x PBS for 20 minutes.

Pre-hybridization, hybridization and wash steps were carried out in a 65°C rotating incubator. The wash steps were performed as follows: 5 minutes each with three solutions: 66% hybridization mix/33% 2x SSC; 33% hybridization mix/33% 2x SSC; and 2x SSC. High stringency washes were as follows: 20 minutes each with two solutions: 0.2x SSC/0.1% Tween-20; and 0.1x SSC/0.1% Tween-20. Following this, wash steps at room temperature were performed as

follows: 5 minutes each with three solutions: 66% 0.2x SSC/33% PBST; 33% 0.2x SSC/66% PBST; and 100% PBST. Embryos were incubated for 1.5 hours in blocking solution and then incubated overnight at 4°C in a 1:5000 dilution of anti-DIG-AP FAB fragments in blocking solution (Roche). Embryos were then washed 5 times of 15 minutes each in PBST at room temperature. Embryos were incubated in Genius 3 buffer (0.1% Tween-20, 1x 1M TrisHCl + 1 M NaCl, pH 2.5, and 1x $MgCl_2$) for 15 minutes. The colouration reaction was performed using NBT/BCIP dissolved in Genius 3 buffer for 2.5 hours. After colouration, embryos were washed twice for 5 minutes each in 100% MeOH + 0.1% Tween-20, following by 2 washes of 15 minutes each. Embryos were stored in this solution at 4°C until use.

Table 2.1. List of primers used for semi-quantitative reverse transcriptase PCR.

Organism	Gene	Orientation	Primer	Sequence	Use
Goldfish	CSF-1R	Forward	Exon 2 (11-34)	5'-TCCTGTTCGTCTGTGGGATCCTTT-3'	RT-PCR
Goldfish	CSF-1R	Forward	Exon 12 (14-33)	5'-AGRTSCGCTGGAARATCATC-3'	RT-PCR
Goldfish	CSF-1R	Reverse	Exon 18 (85-105)	5'-GTTGGAGTCRTTGATGATGTC-3'	RT-PCR
Goldfish	CSF-1R	Reverse	Exon 19 (86-105)	5'-GAARATCTCCCASAGSADGA-3'	gene-specific cDNA
Goldfish	sCSF-1R	Forward	Exon 2 (92-116)	5'-CGGTCGGTACGGATGTGATTC-3'	RT-PCR
Goldfish	sCSF-1R	Reverse	Intron 4 (19-45)	5'-TTTGATTCGTGGGAAAGCACT-3'	RT-PCR, gene-specific cDNA
Goldfish	sCSF-1R	Reverse	Intron 4 (155-181)	5'-CCTTCAGCAAAGTAATGAACT-3'	RT-PCR, gene-specific cDNA
Zebrafish	CSF-1R	Forward	Exon 11 (1767-1782)	5'-GGGCAAAGAGGACAACATCA-3'	RT-PCR
Zebrafish	CSF-1R	Reverse	Exon 14/15 (2166-2180)	5'-CTCCGACGAAGAATCCAGA-3'	RT-PCR
Zebrafish	CSF-1R	Reverse	Exon 19 (2731-2749)	5'-TCGGGATGTTCTTGTACTC-3'	gene-specific cDNA
Zebrafish	sCSF-1R	Forward	5' UTR ([-21]-[-44])	5'-AGACGCTGAAGACTGCTGATGGT-3'	RT-PCR
Zebrafish	sCSF-1R	Forward	Exon 2 (19-42)	5'-TTCCTCATTGGGATCCTGCTTGGT-3'	RT-PCR
Zebrafish	sCSF-1R	Reverse	Intron 4 (625-643)	5'-AGCACTGTAAATGAAACTC-3'	RT-PCR, gene-specific cDNA
Both	β-actin	Forward	Exon 1 (40-60)	5'-TCGCTGCCCTGGTCGTTGATA-3'	RT-PCR
Both	β-actin	Reverse	Exon 3 (702-719)	5'-GGCGGCGGTTCCCATCTC-3'	RT-PCR

CHAPTER III: CHARACTERIZATION OF CSF-1R AND A NOVEL SOLUBLE CSF-1R IN GOLDFISH

1. INTRODUCTION

The CSF-1 system of macrophage control is well conserved in vertebrate species including fish, reptiles, birds, mammals and humans. CSF-1 acts as a hematopoietic growth factor and has been shown to be expressed in a range of cell types (1-4). In general, the CSF-1 system controls the survival, proliferation, differentiation and function of macrophages and their progenitors and, among others, helps maintain cell populations involved in the inflammatory response (5). CSF-1 also contributes to other processes that include development, reproduction and homeostasis, emphasizing the need to tightly regulate its actions.

CSF-1 exerts its activity through its cognate receptor, CSF-1R. This receptor is expressed mainly on cells of the macrophage lineage and promotes survival, proliferation, differentiation and function of macrophages (1). CSF-1R is also found to be expressed on other cell types that include placental trophoblasts (2), osteoclasts (3), mammary epithelial cells (4), and pigment cells in zebrafish (6). Similar to CSF-1, the CSF-1R is highly conserved amongst vertebrates and has most recently been identified in a number fish species. Gilthead seabream show CSF-1R expression in cells of the monocyte/macrophage lineage in tissues that are involved the immune response including thymus, spleen, head kidney, liver, blood and gills (7). Rainbow trout show high expression of CSF-1R in the head kidney, kidney, intestine, spleen, ovary and blood with minimal expression in other tissues including liver (8).

Recently, a novel soluble form of CSF-1R (sCSF-1R) was identified by our group in goldfish primary kidney macrophages (PKM) (9). This soluble CSF-1R appeared to be generated via alternative splicing from the full-length CSF-1R, resulting in a truncated protein possessing only the ligand binding domain of CSF-1R. Soluble CSF-1R appears to function as a competitive inhibitor capable of competing with membrane-bound CSF-1R for binding CSF-1, thus inhibiting

downstream signalling pathways (9). Consistent with this hypothesis, functional analyses revealed that introduction of this soluble receptor to actively growing PKM resulted in significant down-regulation of cellular proliferation (9). Subsequent analysis confirmed that this inhibition was mediated through interaction between CSF-1 and the soluble CSF-1R (10). Interestingly, sCSF-1R does not appear to be broadly expressed in goldfish PKM; instead, it is preferentially expressed in self-renewing macrophages which also actively produce endogenous growth factors (10-14). As such, it provides an opportunity for the establishment of a control loop for the regulation of macrophage numbers in teleost fish. Based on the pleiotropic role of CSF-1, it remains unclear if this sCSF-1R may also contribute to other aspects of macrophage biology such as the control of antimicrobial inflammatory responses.

In this chapter, I take important next steps in the characterization of CSF-1R and sCSF-1R in goldfish. Specifically, I examined the tissue specificity of CSF-1R and sCSF-1R in tissues of adult goldfish. One goal was to assess whether the sCSF-1R was preferentially expressed in the spleen and kidney tissues, which would be expected of a cytokine receptor with a central role in hematopoiesis. Expression in other tissues would suggest potential broader impact of sCSF-1R beyond hematopoiesis. I also examined the *in vivo* contributions of CSF-1R and sCSF-1R to inflammatory or homeostatic responses in peritoneal lavage cells at 24 hours as a first step to characterize the role of these molecules. Finally, a self-resolving *in vivo* peritonitis model of adult goldfish was used to assess the contributions of this novel sCSF-1R to antimicrobial inflammatory responses and their resolution over a 72 hour period in teleost fish.

2. RESULTS

2.1. Tissue expression of CSF-1R and sCSF-1R in goldfish

Previous studies in rainbow trout and gilthead seabream have shown that CSF-1R is primarily expressed in thymus, spleen, kidneys, and blood, with varying levels in the liver and gills (7,8). My first goal was to assess the expression of membrane CSF-1R in the goldfish and then compare its expression

pattern with that of the novel goldfish sCSF-1R. Previous experiments pointed to a role of goldfish sCSF-1R in the control of hematopoiesis, but it remained unclear if additional contributions (e.g. control of inflammation) may result from expression of this soluble receptor in other tissues. Primers were designed and confirmed for specificity to CSF-1R and sCSF-1R. In short, areas specific to the CSF-1R resided downstream of Exon 4 and thus did not overlap with sCSF-1R sequences. In contrast, areas specific for the sCSF-1R took advantage of its unique 3' region. All primer sequences are outlined in Chapter II of this thesis.

Expression of CSF-1R and sCSF-1R was analysed in goldfish using semi-quantitative reverse-transcriptase PCR (Figure 3.1A). In addition, gene-specific cDNAs for CSF-1R and sCSF-1R were used to confirm the presence or absence of CSF-1R and sCSF-1R in each of the tissues examined (Figure 3.1B). Bands for CSF-1R (kidney and liver) and for sCSF-1R (eye and brain) were identified, gel extracted, cloned into TOPO-TA vector and sequenced with BigDye (Applied Biosystems) to confirm identity of CSF-1R and sCSF-1R. Bands of expected size corresponded to the published sequence for goldfish CSF-1R and sCSF-1R (Figure 3.1C). As such, CSF-1R and sCSF-1R are broadly expressed in adult goldfish. These transcripts do not appear to be limited to the hematopoietic compartment (spleen, kidney) as they are also expressed in other tissues.

Densitometry assessment of band intensity provided preliminary insights into the relative abundance of CSF-1R and sCSF-1R transcripts in these tissues. Band intensity for CSF-1R and sCSF-1R are presented relative to β-actin for each tissue examined (Figure 3.2). Results for CSF-1R show high variation between the two fish, which is expected in outbred individuals. Expression of sCSF-1R is also seen to vary greatly, but with increased expression in the gill. This suggests variation in expression of these transcripts within individuals of an outbred population of fish, even when kept in the same conditions.

2.2. Differential contributions of CSF-1R and sCSF-1R to inflammation and homeostasis

The process of inflammation is tightly regulated due to the complexity of responses to pathogens and the potential for tissue damage (15-17). Teleost phagocytes are capable of inducing divergent responses to both homeostatic and pro-inflammatory stimuli (18). Previous studies that have examined the effects of apoptotic cells on phagocytes have observed marked decreases in pro-inflammatory antimicrobial killing mechanisms and production of pro-inflammatory cytokines, as well as increases in anti-inflammatory immune modulators (19-29). Studies from our group have observed an increase in CSF-1R expression upon exposure to apoptotic bodies (18). As such, my goal was to assess the potential contributions of CSF-1R and sCSF-1R to inflammation and homeostasis. To do this, I examined the expression of these genes following *in vivo* induction of inflammation using a model of self-renewing peritonitis.

Goldfish were injected with PBS, zymosan, apoptotic bodies or both (-4hr; apoptotic bodies injected 4 hours prior to zymosan) into the peritoneal cavity. Cells were collected via peritoneal lavage and used to isolate RNA and synthesize cDNA. Semi-quantitative RT-PCR was performed to determine expression of CSF-1R and sCSF-1R under each condition (Figure 3.3A). Densitometry assessment of band intensity provided qualitative insights into the abundance of CSF-1R and sCSF-1R (Figure 3.3B). Band intensity for CSF-1R and sCSF-1R are presented relative to β-actin for each condition, which is normalized to the PBS injection and averaged among individuals (n=5). Expression of both CSF-1R and sCSF-1R was observed to be low in PBS-injected goldfish, with a trend upwards after injection of apoptotic bodies. This was also observed after injection with zymosan, with a significant ($p=0.02$) increase for sCSF-1R expression. Finally, pre-injection of apoptotic bodies followed by zymosan injection 4 hours later resulted in no change from PBS injected individuals, though this response was greatly variable between individuals. This suggests that sCSF-1R expression may be affected more by inflammation than homeostasis and that homeostasis in under inflammatory conditions reduces this effect.

Primary kidney macrophages (PKM) were untreated or exposed to apoptotic bodies or zymosan for 4 or 24 hours. Cells were collected and used to isolate RNA and synthesize cDNA. Semi-quantitative RT-PCR was performed to determine expression of CSF-1R and sCSF-1R under each condition (Figure 3.4A). Densitometry assessment of band intensity provided qualitative insights into the abundance of CSF-1R and sCSF-1R (Figure 3.4B). Band intensity for CSF-1R and sCSF-1R are presented relative to β-actin for each condition and are averaged between each individual. Expression of both CSF-1R and sCSF-1R was observed to be unchanged under each condition at both time points. This suggests that the changes in sCSF-1R expression observed upon zymosan injection *in vivo* are unlikely to be due to a direct effect of apoptotic bodies and zymosan on macrophages.

2.3. Contributions of CSF-1R and sCSF-1R to peritonitis in goldfish

Numerous studies have characterized cellular events during of zymosan-induced peritonitis in the mouse, frog, rat, goldfish and carp (30-33). This model has been well studied in mice (30) and shows changes in both tissue resident macrophages as well as inflammatory macrophages during the course of peritonitis. As CSF-1R and sCSF-1R expression in goldfish was not confined to the hematopoietic organs and showed broad expression, and CSF-1 is known to contribute to macrophage antimicrobial responses (34), the contributions of CSF-1R and sCSF-1R may extend to the regulation of inflammation. My goal was to assess the contributions of CSF-1R and sCSF-1R to inflammation through use of a zymosan-induced peritonitis model in goldfish.

After zymosan injection, cells were obtained through peritoneal lavage from 8 goldfish per time point. Cells were then counted using a haemocytometer. Total cell counts indicated very few cells ($2.91x10^5$ cells) were present in the peritoneal cavity in goldfish at 0 hours (Figure 3.5A). After 18 and 24 hours, a significant increase in cell numbers was observed ($2.68x10^6$ and $4.12x10^6$ cells respectively). Cell numbers were seen to decline 48 hours post injection ($1.48x10^6$ cells) and return to basal levels after 72 hours ($4.54x10^6$ cells). Three

fish from each time point were used to determine cell populations via use of an imaging flow cytometer (ImageStream; Amnis) (Figure 3.5B,C). Lymphocyte/early progenitors appeared to be the prominent cell type at the 0 hour time point. After 18 hours, all cell types were seen to increase with more lymphocyte/early progenitors compared to other cell types. The numbers of lymphocyte/early progenitors and monocyte/neutrophils reached approximately equal levels after 24 hours. After 48 hours, monocyte/neutrophils showed a rapid decline in cell numbers, with lymphocyte/early progenitors again becoming the prominent cell type. After 72 hours, cell populations returned to approximately basal levels. Cells at each time point displayed normal cellular morphology (Figure 3.5C).

In order to determine if CSF-1R and sCSF-1R expression levels are altered during peritonitis, cells from five fish per time point were pooled and used to isolate RNA and synthesize cDNA. Semi-quantitative RT-PCR was performed using total cDNA (Figure 3.6A). Densitometry assessment of band intensity provided qualitative insights into the abundance of CSF-1R and sCSF-1R during zymosan-induced peritonitis (Figure 3.6B). Band intensity for CSF-1R and sCSF-1R are presented relative to β-actin for each time point, which is normalized to 0 hours post injection. Levels of CSF-1R expression trend upwards from 0 to 24 hours post injection. The 48 hour time point showed variability in levels of CSF-1R expression, while CSF-1R expression at 72 hours was consistent with 24 hours. This was mirrored by a similar upwards trend in levels of sCSF-1R between 0 and 24 hours, with a decreasing trend for 48 and 72 hours post injection.

3. DISCUSSION

As CSF-1R plays an important role in hematopoiesis control of macrophage function, it was hypothesized that tissues involved in these processes would show abundant CSF-1R expression (spleen, kidney). This has indeed been observed in several other fish species, including gilthead seabream and rainbow trout, where there is abundant expression of CSF-1R in the spleen, kidney and blood (7,8).

However, these fish also show CSF-1R expression in other tissues, indicating that teleost CSF-1R may not be confined to hematopoiesis. As sCSF-1R had only been identified in primary kidney macrophages and appears to have a role in control of macrophage populations, it was hypothesized that sCSF-1R would be expressed in areas where CSF-1R is found.

Based on tissue expression results from goldfish, it is obvious that CSF-1R and sCSF-1R are not confined to hematopoietic organs. We do see substantial expression of CSF-1R in the spleen and gill and sCSF-1R in the gill, however there is expression in tissues not known for abundant macrophage populations or hematopoiesis, including the heart, muscle and gonads. Upon further research, the expression pattern of CSF-1R and sCSF-1R may not be entirely unexpected. Previous studies with rainbow trout and gilthead seabream have shown expression of CSF-1R in all tissues examined, with increased expression in tissues directly related to aspects of immunity (7,8). In fact, all the tissues examined in goldfish appear to have a population of macrophages in mammals with varying roles including repair processes, protective roles, support during development, fertility, and hematopoiesis (35-42). In addition, mice express CSF-1R in a large range of tissues, from the kidney and spleen to the lymph nodes and cerebrum, indicating a larger role for this transcript (43,44).

As discussed in more detail in Chapter I, primitive macrophages in teleost zebrafish originate near the cardiac region. These macrophages have typical protective and homeostatic roles. However, macrophages have also been shown to be essential for heart development in *Xenopus* (39) and the development and growth of myocytes (36). In addition, cardiac macrophages are involved in the repair process after myocardial infarctions (35), a role which is mirrored in the repair of skeletal muscle (37). Macrophages also have an essential role in male and female gonads. In the testes of mice, 25% of the interstitial cells are macrophages, which are responsible for steroid hormone production and support of Leydig cells (36,40,42). Male mice lacking in CSF-1 itself have a low sperm count and libido, highlighting the importance of the CSF-1 system in fertility. In female mice, ovarian macrophages are also present in the interstitial tissue and

have shown to be needed for steroid hormone production and ovulation (8,36). Females with mutations in CSF-1 or CSF-1R also display reduced fertility. As CSF-1R and sCSF-1R are expressed in these tissues, as well as in other tissues that have support and repair roles, they may contribute to numerous processes in a range of different tissues that contain a macrophage population. While the role that macrophages play in these tissues is not always a typical immune response, the presence of CSF-1R and sCSF-1R in these tissues may point to contributions of theses transcripts for function. Of course, in order to assess this, gene expression would have to be performed in these populations of macrophages. The considerable variation observed in expression of these transcripts in these tissues between fish is an interesting observation. The closely related zebrafish shows significant genetic variability in individuals within highly inbred strains, highlighting that individuals may show differential expression of certain genes (45). While the goldfish used for this thesis are raised in the same conditions, they represent outbred populations. As such, the differential expression of CSF-1R and sCSF-1R between individuals may be attributable to heterogeneity within outbred populations of fish either at the level of gene expression or macrophage quantities.

As the contributions of CSF-1R and sCSF-1R were not confined to hematopoiesis, I examined the potential contributions of CSF-1R and sCSF-1R to homeostatic responses compared to an inflammatory event. Due to the nature of inflammation, a balance between pathogen and homeostatic phagocytosis is essential to allow for pathogen clearance with minimal tissue damage (46,47). As such, teleost phagocytes have evolved to produce differential responses to pathogens and tissue damage (18). These phagocytes are essential to remove apoptotic cells that are generated through homeostatic responses. However, phagocytosis of these apoptotic cells leads to decreased pro-inflammatory antimicrobial killing mechanisms and production of pro-inflammatory cytokines, and increased production of anti-inflammatory immune modulators (19-28). As such, it was of interest to examine the contributions of CSF-1R and sCSF-1R to these two essential processes. CSF-1R expression appears to trend upwards upon

injection of apoptotic body and zymosan into the peritoneal cavity of goldfish, consistent with what has previously been observed in our group (18). However, this increase, along with the variability observed in the -4hr treatment, was not significant, suggesting heterogeneity between individuals or inefficiency with the chosen detection method. In fact, the only significant increase in gene expression was observed for sCSF-1R following zymosan injection. This suggests that sCSF-1R may be more involved in regulating inflammatory responses than homeostatic responses, as we see an increase in expression following zymosan injection. Alternatively, this increase could also be attributed to more sCSF-1R-expressing cells present following zymosan injection. To further understand this response, *in vitro* PKM cultures were examined after exposure to apoptotic bodies and zymosan. Gene expression appeared unchanged between all conditions and both time points. This suggests that the effects of zymosan and apoptotic bodies on sCSF-1R expression *in vivo* are not likely to be a direct effect on the macrophages themselves, or that the changes in infiltrating cells are masking gene expression changes. Previous studies examining murine macrophage cell lines have noted changes in CSF-1R expression upon activation of macrophages, most notably with LPS. When examining levels of transcripts, CSF-1R mRNA expression increases in bone marrow macrophages after just 1 hour of LPS activation (48). On the other hand, membrane-bound CSF-1R protein shows a decrease in expression following LPS activation of macrophage cell lines, resulting from CSF-1R release from the membrane (49). Clearly the changes in gene and protein expression need to be examined in more detail in the goldfish system in order to elucidate the mechanisms under which CSF-1R and sCSF-1R are regulated and function. To more definitively characterize these changes and assess the contributions of these transcripts, a more sensitive-approach such as real-time PCR should be utilized to allow for a more in-depth analysis.

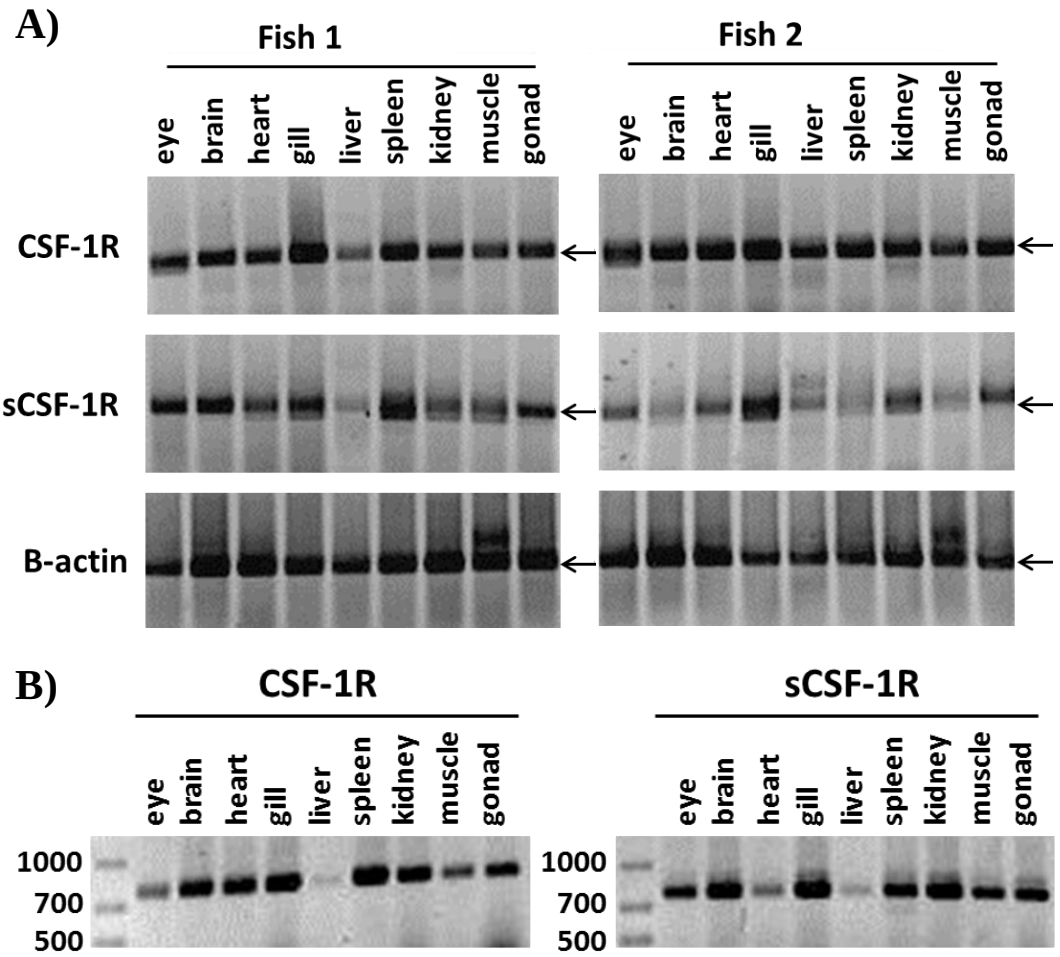
Finally, I examined CSF-1R and sCSF-1R in the context of acute inflammation in the peritoneal cavity of goldfish to assess contributions of these transcripts in the progression and resolution of peritonitis. Previous studies have examined the role of resident macrophages versus infiltrating macrophages in

mice during zymosan-induced peritonitis (30). They observed that after 24 hours, there was a burst in numbers of cells present in the peritoneal cavity. This burst consisted of primarily neutrophils, which were cleared 48 hours after injection, as well as inflammatory monocyte/macrophages that entered the site. By 72 hours, the levels of inflammatory macrophages had declined to basal levels, with resident macrophages recolonizing the site. Other studies from our group have examined the differences in peritonitis responses in goldfish and mice (18). Goldfish were seen to respond quickly to zymosan, with robust recruitment of several cell types including lymphoid and myeloid. On the other hand, mice responded more subtly to zymosan, with increases in neutrophils specifically. My data closely matches this goldfish data, showing an initial burst of lymphocyte/early progenitor cells after 18 hours with a smaller increase in monocyte/neutrophils. After 24 hours, the numbers of monocyte/neutrophils and lymphocyte/early progenitors are observed to be approximately equal. These cells are then seen to decline 48 hours post injection and return to basal levels 72 hours post injection.

CSF-1R and sCSF-1R appear to be associated with the cellular events during zymosan-induced peritonitis. CSF-1R expression is seen to increase 18 and 24 hours post injection, a time when the number of infiltrating monocytes and macrophages is also seen to increase. After 48 hours, these infiltrating cells greatly decline as the inflammatory response is resolved and resident cells have yet to recolonize the site. After 72 hours, CSF-1R-expressing resident macrophages are again found in the peritoneal cavity. During the 18-24 hour time period, sCSF-1R expression is also seen to increase. As the inflammatory response needs to be tightly regulated to prevent excessive tissue injury, the relative abundance of sCSF-1R may suggest that this molecule contributes to the control of this response. After 48 hours when the numbers of infiltrating monocyte/macrophages are declining, sCSF-1R would be essential to remove circulating CSF-1 to prevent further differentiation or proliferation of these cell types. After 72 hours, resident macrophages will have recolonized the site, resulting in reduced need for sCSF-1R control. Further characterization of

changes in gene expression of CSF-1R and sCSF-1R during zymosan-induced peritonitis through real-time PCR would allow for more detailed analysis of the contributions of these transcripts.

These data suggest a role for CSF-1R and sCSF-1R outside of classical hematopoiesis. More specifically, they may have a role in regulating populations of macrophages throughout the organism. These transcripts are expressed in a broad pattern throughout the adult goldfish. In many of these tissues, macrophages have been observed to possess protective or supportive roles that are not always immune-based. As such, CSF-1R and sCSF-1R are essential to many processes in goldfish. These transcripts appear to play a role in both homeostatic and inflammatory processes. As such, CSF-1R and sCSF-1R likely play an essential role to goldfish and possibly other teleosts as well.



C)

Gene	Tissue	BLAST	Accession	% similarity	% identity
CSF-1R	kidney	CSF-1R	AY536523.1	98%	98%
	liver	CSF-1R	AY536523.1	99%	98%
sCSF-1R	eye	sCSF-1R	AY536524.1	99%	99%
	brain	sCSF-1R	AY536524.1	99%	99%

Figure 3.1. Expression of CSF-1R and sCSF-1R in all tissues of two goldfish. Tissues of interest were isolated from two goldfish and used to isolate RNA and synthesize cDNA. **A**) Semi-quantitative reverse transcriptase PCR was performed using primers designed to Exon12-Exon18 (CSF-1R; 789 bp) and Exon2-Intron4 (sCSF-1R; 650 bp) and β-actin (786 bp). **B**) Gene-specific cDNAs for CSF-1R and sCSF-1R were used to confirm presence or absence of transcripts. **C**) Bands of interest were gel extracted, cloned into a TOPO-TA vector and sequenced. Sequences were analysed using BLAST (NCBI) for identity.

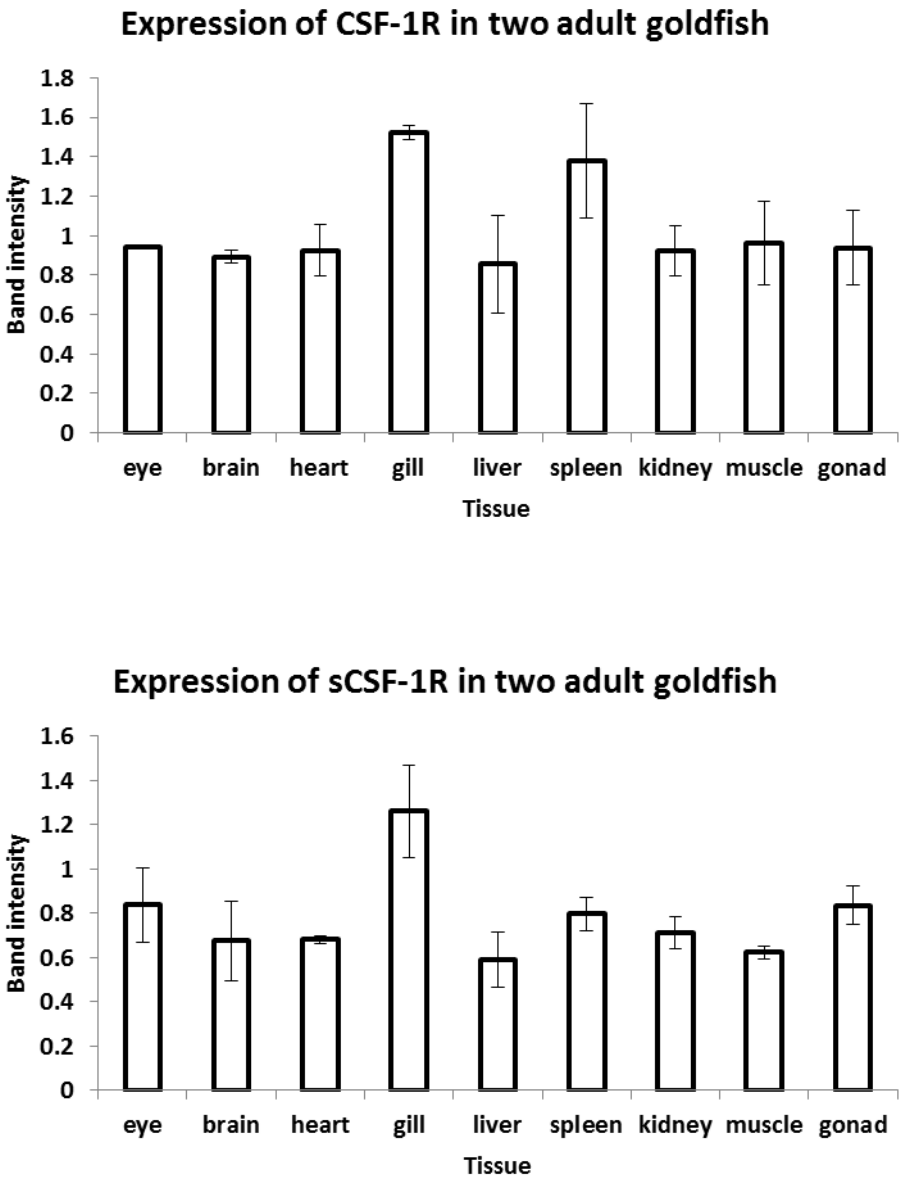


Figure 3.2. Levels of CSF-1R and sCSF-1R expression in tissues of two goldfish. Images of agarose gels were analysed using Adobe Photoshop software to determine band intensity in tissues of interest. Band intensity for each tissue was normalized to corresponding B-actin band and graphed using Excel software. Error bars represent standard error between RT-PCR results of the two goldfish.

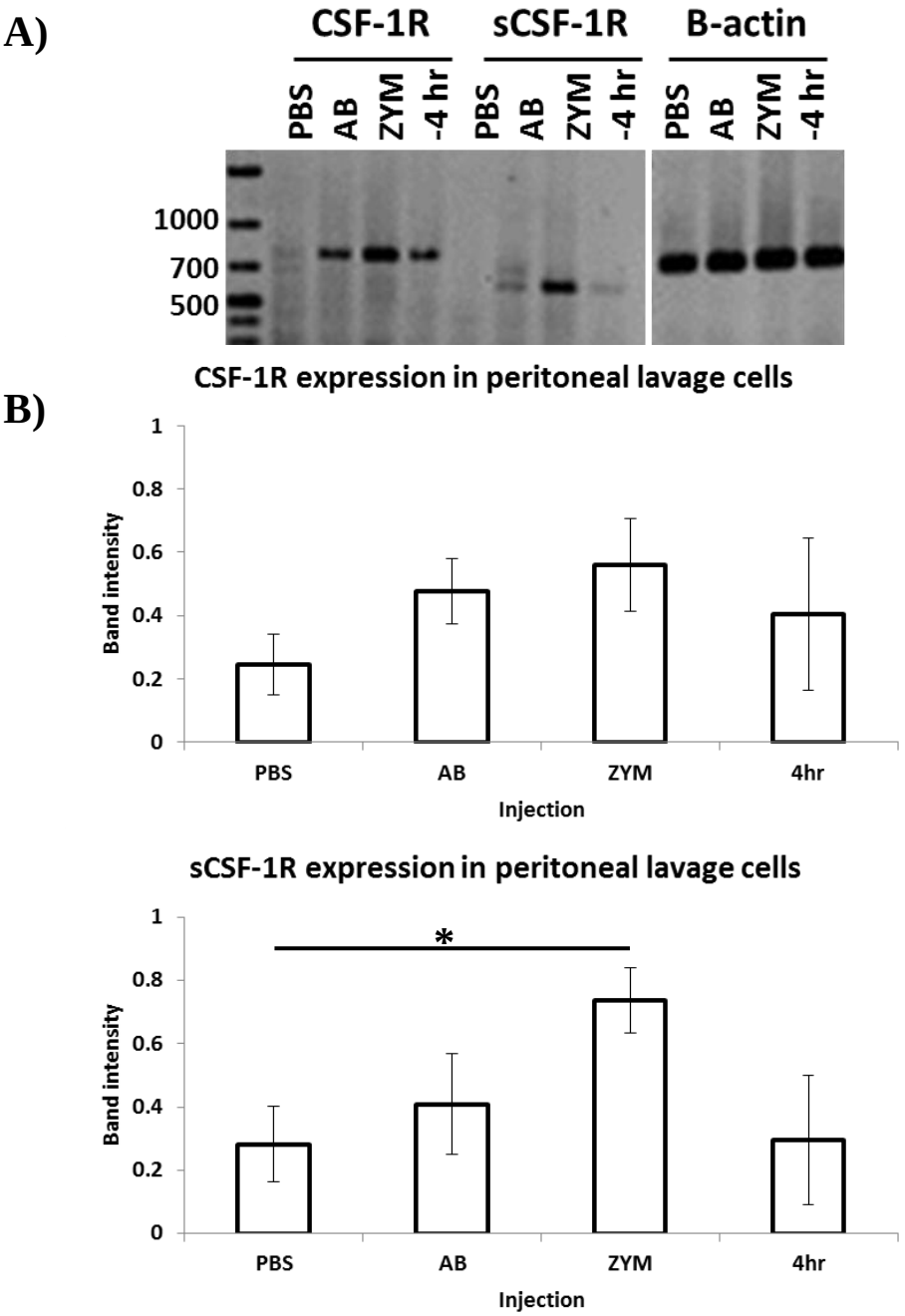


Figure 3.3. Expression of CSF-1R and sCSF-1R in peritoneal lavage cells following PBS, zymosan, apoptotic bodies or -4h injections. Zymosan (2.5 mg; 100 µL) or apoptotic bodies ($5x10^6$) were injected into the peritoneal cavity of goldfish for 24 hours prior to analysis. Apoptotic bodies were additionally injected into the peritoneal cavity 4 hours prior to zymosan (-4hr). Cells were isolated by peritoneal lavage using 10 mL Trypsin-EDTA PBS -/- and counted using a haemocytometer. Cells from lavages were pooled and used for RNA isolation and cDNA synthesis. **A**) Semi-quantitative reverse-transcriptase PCR was performed to detect CSF-1R (789 bp) and sCSF-1R (650 bp) expression. **B**) Densitometry analysis was performed to determine relative abundance of CSF-1R and sCSF-1R transcripts. * denotes significance ($p<0.05$) compared to PBS.

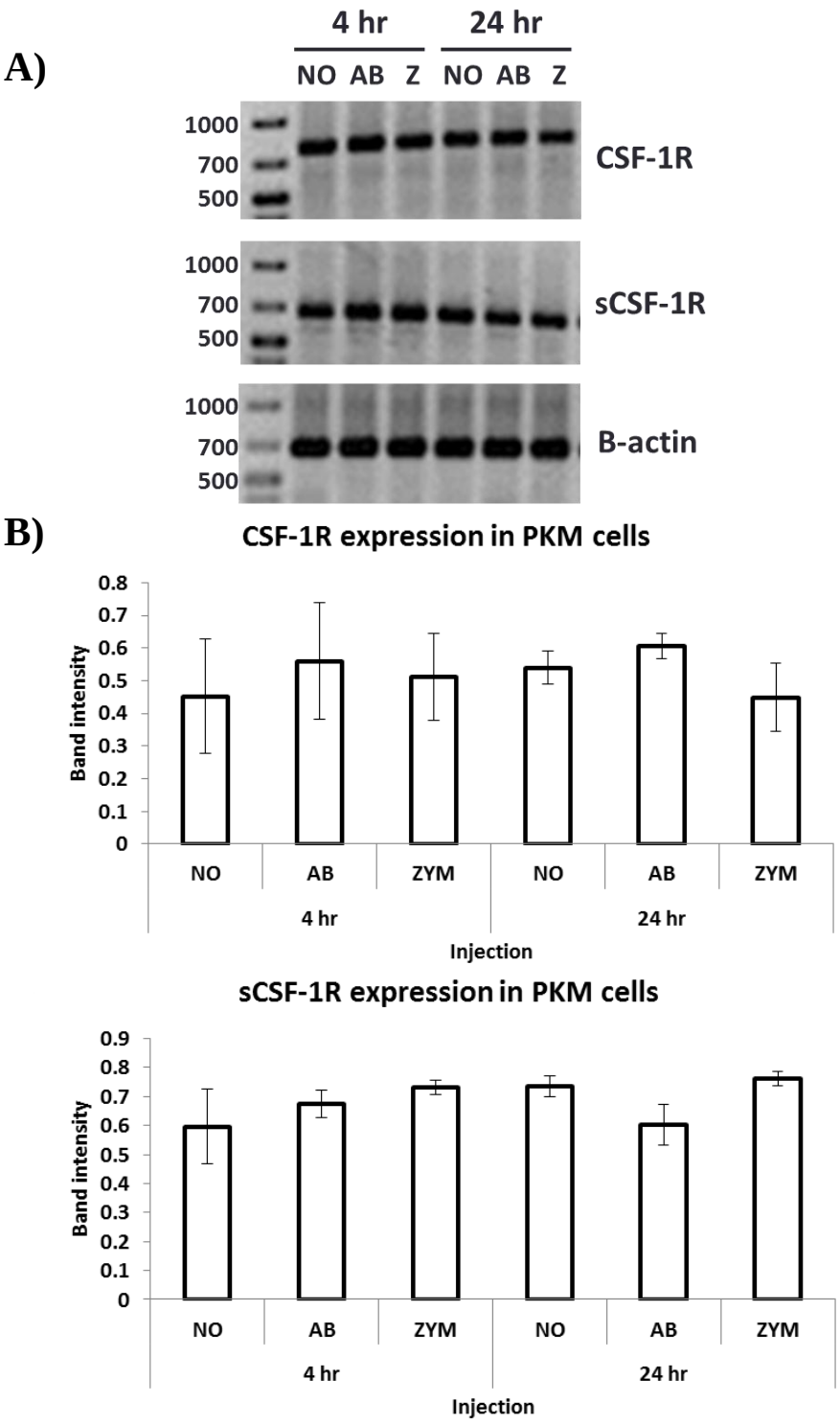
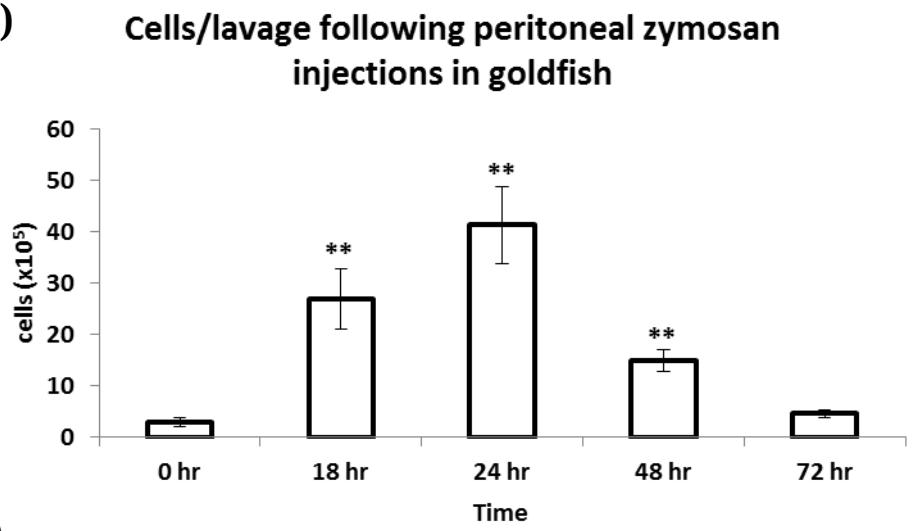
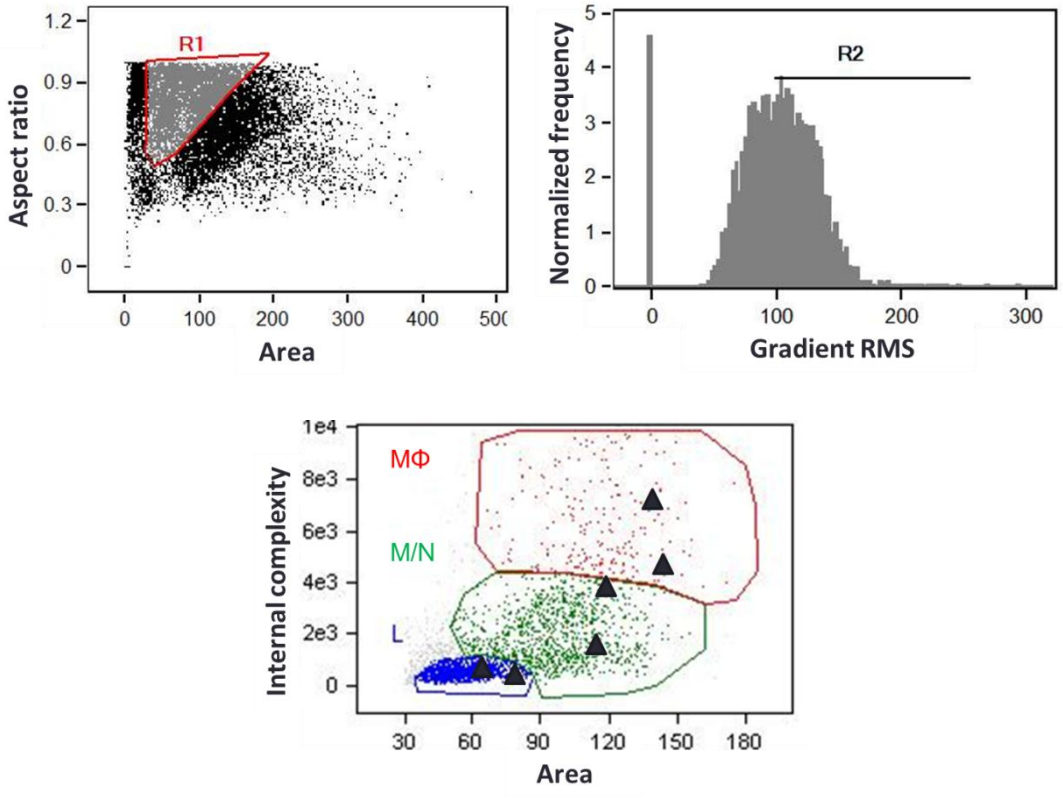


Figure 3.4. Expression of CSF-1R and sCSF-1R in PKMs following zymosan or apoptotic body or exposure. Zymosan or apoptotic bodies (5:1 ratio) were added to primary kidney macrophage (PKM) cultures for 4 or 24 hours. Cells were harvested and used for RNA isolation and cDNA synthesis. **A**) Semi-quantitative reverse-transcriptase PCR was performed to detect CSF-1R (789 bp) and sCSF-1R (650 bp) expression. **B**) Densitometry analysis was performed to determine relative abundance of CSF-1R and sCSF-1R transcripts.

A)


Cells/lavage following peritoneal zymosan injections in goldfish
cells (x10⁵)
60
50
40
30
20
10
0
**
**
**
0 hr
18 hr
24 hr
48 hr
72 hr
Time

B)


Aspect ratio
1.2
0.9
0.6
0.3
0
R1
0
100
200
300
400
500
Area
Normalized frequency
5
4
3
2
1
0
R2
0
100
200
300
Gradient RMS
Internal complexity
1e4
8e3
6e3
4e3
2e3
0
MΦ
M/N
L
30
60
90
120
150
180
Area

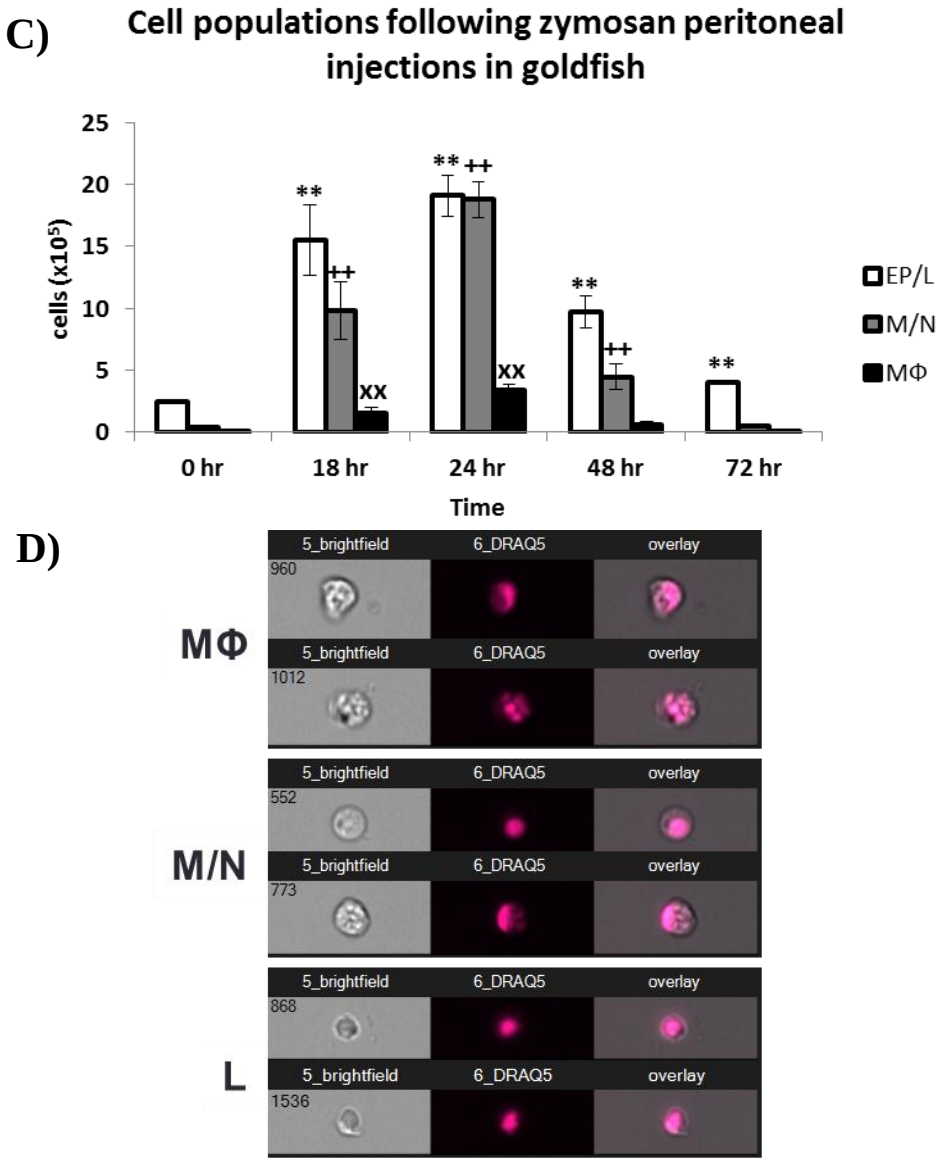


Figure 3.5. Changes in cell populations following zymosan-induced inflammation. Zymosan (2.5 mg; 100 µL) was injected into the peritoneal cavity of 24 goldfish, with 8 un-injected fish serving as a 0 hour control. Cells from animals were isolated by peritoneal lavage using 10 mL PBS -/- and counted using a haemocytometer. **A**) Cell counts for individual lavages at 0, 18, 24, 48 and 74 hours post injection (n=16, except n=8 for 18 hours). * denotes significance ($p<0.05$) to 0 hours. **B)** Three lavages from each time point were used for ImageStream analysis to determine cell populations. Single cells were gated for R1, focused cells were gated for R2 and cell populations were determined based on size and internal complexity. **C**) Cell populations are graphed based on number of cells from A) and proportion of cells from B). ** denotes EP/L (early progenitor/lymphocyte) significance ($p<0.01$); ++ denotes M/N (monocyte/neutrophil) significance ($p<0.01$); and xx denotes MΦ (macrophage) significance ($p<0.01$). All statistics are compared to 0 hours. **D)** Sample cell images from ImageStream.

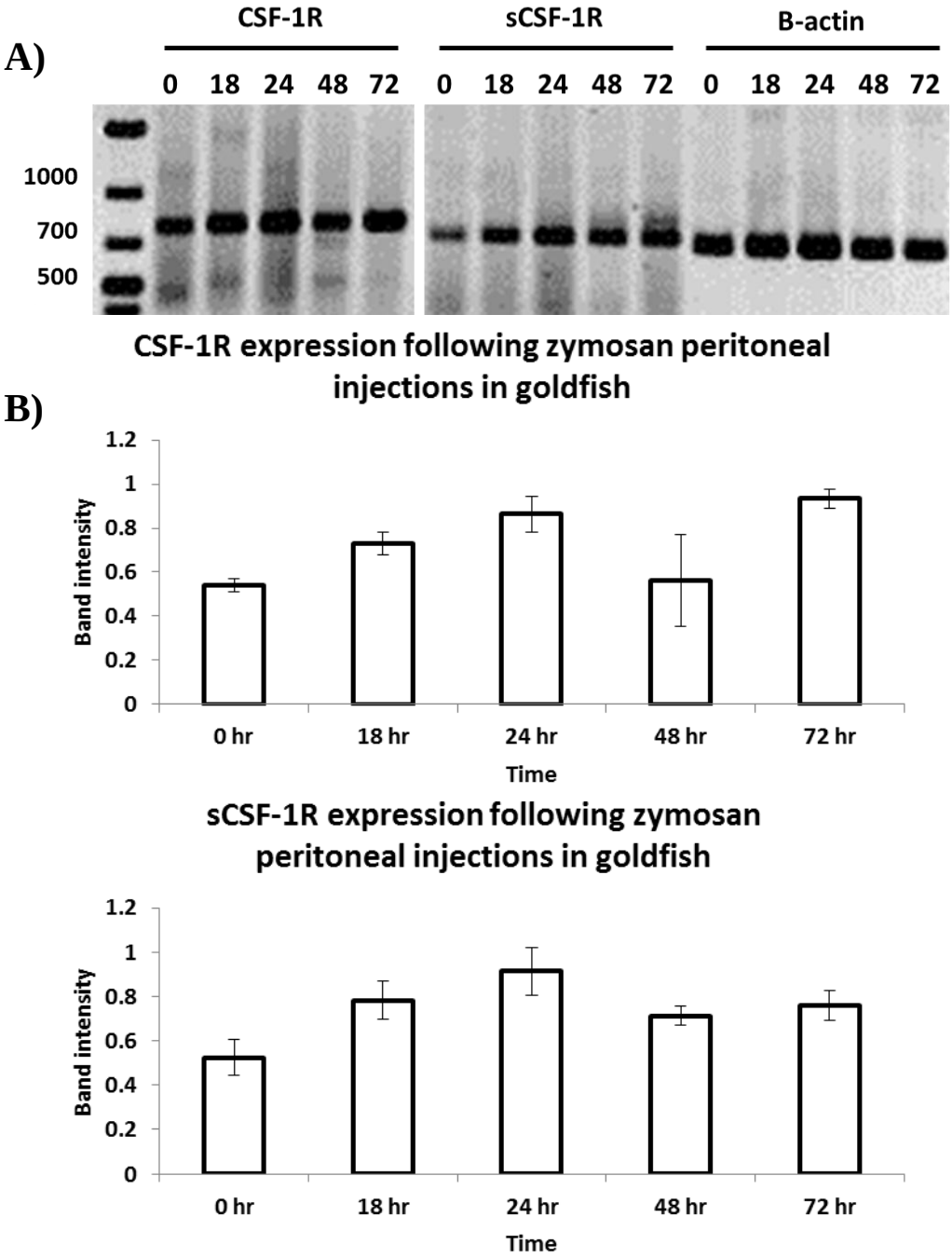


Figure 3.6. Changes in CSF-1R and sCSF-1R expression upon zymosan-induced inflammation. Zymosan (2.5 mg; 100 µL) was injected into the peritoneal cavity of 24 goldfish, with 8 un-injected fish serving as a 0 hour control. Cells from animals were isolated by peritoneal lavage using 10 mL PBS -/- and counted using a haemocytometer. Five lavages from each time point were pooled and used for RNA isolation and cDNA synthesis. **A**) Semi-quantitative reverse-transcriptase PCR was performed to detect CSF-1R (789 bp) and sCSF-1R (650 bp) expression. **B**) Densitometry analysis was performed to determine relative abundance of CSF-1R and sCSF-1R transcripts. Error bars represent standard error between two rounds of RT-PCR.

4. REFERENCES

1. Hanington PC, Tam J, Katzenback BA, Hitchen SJ, Barreda DR and Belosevic M. 2009. Development of macrophages of cyprinid fish. *Dev Comp Immunol* **33**: 411-29.
2. Randelli E, Buonocore F and Scapigliati G. 2008. Cell markers and determinants in fish immunology. *Fish Shell Immunol* **25**:326-40.
3. Pollard JW, Bartocci A, Arceci R, Orlofsky A, Ladner MB and Stanley ER. 1987. Apparent role of the macrophage growth factor, CSF-1, in placental development. *Nature* **330**:484-6.
4. Hofstetter W, Wetterwald A, Cecchini MC, Felix R, Fleisch H and Mueller C. 1992. Detection of transcripts for the receptor of macrophage colony-stimulating factor, c-fms, in murine osteoclasts. *Proc Natl Acad Sci USA* **89**:9637-41.
5. Sapi E, Flick MB, Rodov S, Carter D and Kacinski BM. 1998. Expression of CSF-1 and CSF-1 receptor by normal lactating mammary epithelial cells. *J Soc Gynecol Investig* **5**:94-101.
6. Parichy DM, Ransom DG, Paw B, Zon LI and Johnson SL. 2000. An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in zebrafish *Danio rerio*. *Development* **127**:3031-44.
7. Roca FJ, Sepulcre MP, López-Castejón G, Meseguer J and Mulero V. 2006. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molec Immunol* **43**:1418-23.
8. Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, Nakanishi T, Yokomizo Y, Takahashi Y, Inagawa H and Soma G-I. 2005. Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. *Molec Immunol* **42**:1-8.
9. Barreda DR, Hanington PC, Stafford JL and Belosevic M. 2005. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing

macrophages of goldfish (*Carassius auratus* L.). *Dev Comp Immunol* **29**:879-94.

10. Hanington PC, Wang T, Secombes CJ and Belosevic M. 2007. Growth factors of lower vertebrates: characterization of goldfish (Carassius auratus L.) macrophage colony-stimulating factor-1. *J Biol Chem* **282**: 31865-31872.
11. Neumann NF, Barreda D and Belosevic M. 1998. Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. *Dev Comp Immunol* **22**: 417-432.
12. Barreda DR and Belosevic M. 2001. Characterisation of growth enhancing factor production in different phases of in vitro fish macrophage development. *Fish Shell Immunol* **11**: 169-185.
13. Hanington PC, Barreda DR and Belosevic M. 2006. A novel hematopoietic granulin induces proliferation of goldfish (Carassius auratus L.) macrophages. *J Biol Chem* **281**: 9963-9970.
14. Belosevic M, Hanington PC and Barreda DR. 2006. Development of goldfish macrophages in vitro. *Fish Shell Immunol* **20**:152-171.
15. Poon IK, Hulett MD and Parish CR. 2010. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ* **17**:381-397
16. Gregory CD and Devitt A. 2004. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically?. *Immunology* **113**:1-14.
17. Duffield JS. 2003. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)* **104**:27-38.
18. Rieger AM, Konowalchuk JD, Grayfer L, Katzenback BA, Belosevic M and Barreda DR. *Manuscript in progress*.
19. Zamboni DS and Rabinovitch M. 2003. Nitric oxide partially controls *Coxiella burnetii* phase II infection in mouse primary macrophages. *Infect Immun* **71**: 1225-1233.

20. Johann AM, von Knethen A, Lindemann D, and Brune B. 2006. Recognition of apoptotic cells by macrophages activates the peroxisome of proliferator-activated receptor-gamma and attenuates the oxidative burst. *Cell Death Differ* **13**:1533-1540.
21. Reiter I, Krammer B, and Schwamberger G. 1999. Cutting edge: differential effect of apoptotic versus necrotic tumor cells on macrophage antitumor activities. *J Immunol* **163**: 1730-1732.
22. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY and Henson PM. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit inflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2 and PAF. *J Clin Invest* **101**: 890-898.
23. Maderna P and Godson C. 2003. Phagocytosis of apoptotic cells and the resolution of inflammation. *Biochim Biophys Acta* **1639**: 141-151.
24. Weigert A, Jennewein C and Brune B. 2009. The liason between apoptotic cells and macrophages – the end programs the beginning. *Biol Chem* **390**: 379-390.
25. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR and Girkontaite I. 1997. Immunosuppressive effects of apoptotic cells. *Nature* **390**: 350-351.
26. Bellingan GJ, Caldwell H, Howie SE, Dransfield I and Haslett C. 1996. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol* **157**: 2577-2585.
27. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS and Ley K. 2005. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17 . *Immunity* **22**: 285-294.
28. Tassiulas I, Park-Min KH, Hu Y, Kellerman L, Mevorach D and Ivashkiv LB. 2007. Apoptotic cells inhibit LPS-induced cytokine and chemokine production and IFN responses in macrophages. *Hum Immunol* **68**:156-164.

29. Kim S, Elkon KB and Ma X. 2004. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity* **21**: 643-653.

30. Davies LC, Rosas M, Smith PJ, Fraser DJ, Jones SA and Taylor PR. 2011. A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *EurJ Immunol* **41**: 2155-2164.

31. Chadzinska M, Kolaczkowska E, Scislowska-Czarnecka A, van Rooijen N and Plytycz B. 2004. Effects of macrophage depletion on peritoneal inflammation in swiss mice, edible frogs and goldfish. *Folia Biologica* **52**:225-231.

32. Cash JL, White GE and Greaves DR. 2009. Zymosan-induced peritonitis as a simple experimental system for the study of inflammation. *Methods Enzymol* **461**:379-396. Chapter Seventeen.

33. Chadzinska M, Leon-Kloosterziel KM, Plytycz B and Verburg-van Kemenade BML. 2008. *In vivo* kinetics of cytokine expression during peritonitis in carp: Evidence for innate and alternative macrophage polarization. *Dev Comp Immunol* **32**:509-518.

34. Grayfer L, Hanington PC and Belosevic M. 2009. Macrophage colony-stimulating factor (CSF-1) induces pro-inflammatory gene expression and enhances antimicrobial responses of goldfish (*Carassius auratus* L.) macrophages. *Fish Shell Immunol* **26**: 406-413.

35. Herbomel P, Thisse B and Thisse C. 2001. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol* **238**:274-288.

36. Van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CMJE and Bitter W. 2004. A star with stripes: zebrafish as an infection model. *Trends Microbiol* **12**:451-457.

37. Ellett F and Lieschke GJ. 2010. Zebrafish as a model for vertebrate hematopoiesis. *Curr Opin Pharmacol* **10**:1-8.

38. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC and Layton JE. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**:3087-3096.
39. Herbomel P, Thisse B and Thisse C. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**:3735-3745.
40. Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR and Trudeau VL. 2008. The goldfish (*Carassius auratus*) as a model for neuroendocrine signaling. *Molec Cell Endocrinol* **293**: 43-56.
41. Powers DA. 1989. Fish as Model Systems. *Science* **246(4928)**: 352-358.
42. Hutson JC. 1992. Development of cytoplasmic digitations between Leydig cells and testicular macrophages of the rat. *Cell Tissue Res* **267**:385-389.
43. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, Williams LT, Lin H and Stanley ER. 2010. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leukoc Biol* **88(3)**: 495-505.
44. Byrne PV, Guilbert LJ and Stanley ER. 1981. Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues. *J Cell Biol* **91**: 848-853.
45. Guryev V, Koudijs MJ, Berezikov E, Johnson SL, Plasterk RHA, van Eeden FJM and Cuppen E. Genetic variation in the zebrafish. *Genome Research* **16**: 491-497.
46. Fadok VA, Bratton DL and Henson PM. 2001. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest* **108**: 957-962.
47. Medzhitov R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* **449**: 819-826.
48. Myers MJ, Ghildyal N and Schook LB. 1995. Endotoxin and interferon-γ differentially regulate the transcriptional levels of proto-oncogenes and cytokine genes during the differentiation of colony-stimulating factor type-1-derived macrophages. *Immunology* **85**: 318-324.

49. Sester DP, Trieu A, Brion K, Schroder K, Ravasi T, Robinson JA, McDonald RC, Ripoll V, Wells CA, Suzuki H, Hayashizaki Y, Stacey KJ, Hume DA and Sweet MJ. 2005. *Immunobiology* **210**: 97-107.

CHAPTER IV: IDENTIFICATION OF sCSF-1R AND CHARACTERIZATION OF CSF-1R AND sCSF-1R IN ZEBRAFISH

1. INTRODUCTION

The CSF-1 system of macrophage control is broadly conserved amongst vertebrates, including those of the teleost linage. While CSF-1 itself has been identified in few teleost fish (rainbow trout, goldfish and zebrafish), the orthologues of the receptor CSF-1R have been identified in greater number in teleosts. These include rainbow trout, gilthead seabream, East African cichlids, pufferfish, goldfish, and zebrafish (1-7). The CSF-1R orthologue in gilthead seabream shows expression exclusively in cells of the monocyte/macrophage lineage in immunologically-relevant tissues including the spleen, thymus, head kidney, liver, blood and gills (2). This is similar to that of rainbow trout, with high expression of CSF-1R in the head kidney, kidney, intestine, spleen, ovary and blood with minimal expression in other tissues (1). As seen in Chapter III of this thesis, goldfish show broad expression of CSF-1R, including in the hematopoietic organs and other tissues, and varies considerably between individuals. However, higher expression levels were seen in the spleen and gill. As such, it is probable that CSF-1R has a variety of roles outside of immune function in teleost fish.

Generally, CSF-1R expression in zebrafish is similar to the fish above, including a presence in macrophage lineage cells as well as in osteoclasts (7). CSF-1R has been shown to be essential during macrophage development and differentiation in the embryo. CSF-1R^{j4blue} mutants possess a deletion in the second kinase domain of CSF-1R resulting in a frameshift mutation (7,8). This results in a pronounced defect in proper macrophage migration, leading to decreased numbers of macrophages in sites distant to the yolk sac including the eye, brain and caudal regions. These mutants also showcase a unique role of zebrafish CSF-1R during pigmentation patterning. CSF-1R^{j4blue} mutants show a

decreased number of xanthophores and melanocytes during development, resulting in an absence of normal yellow and black pigment respectively that is retained throughout adulthood (5). This role is also observed in East African cichlids, where an orthologue of CSF-1R is expressed in egg-dummies (consisting of xanthophores) which play important functions during mating rituals (3). This role for CSF-1R in pigmentation is believed to be linked to the evolution of coloration in vertebrates and specifically teleost fish (4).

As discussed in Chapter III, the alternative splice form of CSF-1R, sCSF-1R, has to date only been identified in goldfish in senescent primary kidney macrophage cultures (6). Based on its structure, goldfish sCSF-1R is believed to function as a negative inhibitor of CSF-1. While sCSF-1R is capable of binding to CSF-1, it is predicted to be incapable of initiating down-stream signalling pathways and potentially result in decreased positive modulation of macrophage survival, proliferation and differentiation to control the population (6). Based on my data from Chapter III, sCSF-1R appears to be expressed in all tissues examined in goldfish and similar to CSF-1R, sCSF-1R expression varies between individuals. As sCSF-1R has only to date been identified in goldfish, not much is known outside of this model. Zebrafish, a close relative of goldfish, will be used to determine if production of sCSF-1R is conserved in other cyprinid species.

Zebrafish have emerged as one of the major fish models used for comparative studies due to their numerous advantages over other fish or mammalian models as discussed in Chapter I. Specifically for this study, zebrafish reproduce rapidly and in high numbers and develop externally to the mother. These embryos develop fully operational macrophage-based immunity 24 hours post fertilization (hpf) and possess fully formed organs 3 days post fertilization (dpf) (9,10). Additionally, adult zebrafish rely on their robust innate immune response for lifelong protection from pathogens and for homeostatic control.

In this chapter, I describe steps in the identification of the novel sCSF-1R in adult and embryonic zebrafish. Additionally, I characterized CSF-1R during development of the organism and immune system using discrete embryo stages.

Zebrafish CSF-1R was characterized in its role in pigmentation and macrophage development in zebrafish and shows expression in both embryos and adults. In goldfish, I have shown that CSF-1R and sCSF-1R are expressed in all tissues examined (Chapter III). My next step was to determine the homogeneity of this expression pattern in the closely related zebrafish and examine the relevance of these transcripts during development. In particular, I was interested on the temporal regulation of sCSF-1R compared to CSF-1R and their relationship to previously delineated primitive and definitive waves of hematopoiesis. It was hypothesized that CSF-1R and sCSF1R would have roles in macrophage control and hematopoiesis and as such should be confined to the teleost hematopoietic organs (spleen, head kidney and body kidney) or areas with abundant macrophage populations.

2. RESULTS

2.1. Identification of sCSF-1R in hematopoietic organs

As sCSF-1R had, to date, only been identified in goldfish primary kidney macrophages, it was unknown if sCSF-1R was a unique regulator confined to goldfish immune function control. As such, my first goal was to identify sCSF-1R in zebrafish, a closely related species to goldfish. A predicted sequence for zebrafish sCSF-1R was determined from comparison to goldfish CSF-1R and sCSF-1R. From this, primers to detect zebrafish sCSF-1R were designed and confirmed for specificity.

Zebrafish sCSF-1R was identified from the hematopoietic organs of zebrafish and was shown to correspond to the predicted sequence based on comparison to goldfish (Figure 4.1A). Sequences for all CSF-1R transcripts that have been annotated from various species (in order: dog, cat, cow, horse, human, gorilla, mouse, chicken, turkey, anole lizard, tropical frog, coelacanth, tilapia, goldfish, zebrafish and medaka) were used to generate a cladogram to determine protein relationships between CSF-1R and sCSF-1R (Figure 4.1B). Both goldfish and zebrafish sCSF-1R were more similar to their respective CSF-1R transcripts than to other species as expected.

Goldfish possess a divergence from the splice site consensus sequence (AG|GT(A/G)AGT) at Exon4/Intron4 boundary, which is predicted to allow for the generation of sCSF-1R (6). Zebrafish appear to also possess divergences in this sequence (Figure 4.2A). To elucidate if other species may generate sCSF-1R, sequences 45 base pairs upstream and 10 base pairs downstream of the Exon4/Intron4 boundary were aligned and examined from gorilla, human, cow, horse, dog, mouse, chicken, goldfish, zebrafish and medaka (Figure 4.2B). All species utilized show some degree of divergence in the consensus region, though this varies between species. Sequences were further analyzed to detect in-frame stop codons as well as hydrophilic tails which indicated secreted proteins (Figure 4.3A). All species appear to possess an in-frame stop codon in Intron 4. Of all species examined, four did not possess a hydrophilic tail, including cow, dog, coelacanth and lamprey. Hydrophilic plots were generated using ExPASy ProtScale (Figure 4.3B). As seen, cow, dog and lamprey do not possess hydrophilic tails, while mouse, goldfish and zebrafish do. This sequence analysis leads to the hypothesis that species other than goldfish and zebrafish may be capable of generating a soluble CSF-1R through alternative splicing.

Expression of sCSF-1R was analysed in the hematopoietic organs of zebrafish, namely the spleen, head kidney and body kidney. cDNA was amplified for an additional 24 cycles before being used as a template for reverse-transcriptase PCR. After amplification and agarose electrophoresis, a band of the predicted size for sCSF-1R was detected in the three tissues examined (Figure 4.4).

2.2. Tissue expression pattern of CSF-1R and sCSF-1R in adults

While it was predicted CSF-1R and sCSF-1R would be mainly confined to the hematopoietic organs, it was of interest to determine if they additionally play a role elsewhere in the organism. This was especially of interest based on observations of CSF-1R and sCSF-1R tissue expression in goldfish. It has previously been shown that CSF-1R plays a role in pigmentation patterning in zebrafish, showing expression in both melanocytes and xanthophores (7). As

such, a determination of the expression of CSF-1R and sCSF-1R throughout the entire zebrafish was undertaken in order to elucidate the possible roles of CSF-1R and sCSF-1R based on tissue specificity.

Expression was analysed from pools of tissues from AB adult fish, comprising the eye, brain, heart, gill, liver, spleen, head kidney, body kidney, muscle, female gonads and male gonads. Total cDNA was utilized as a semi-quantitative measure of CSF-1R and sCSF-1R expression levels (Figure 4.5A). Expression of CSF-1R was detected in all tissues examined, indicating that zebrafish CSF-1R is not confined to the teleost hematopoietic organs. Zebrafish sCSF-1R was also detected in all tissues examined. Bands of interest were sequenced and shown to correspond to the published sequence of CSF-1R and the consensus sequence of sCSF-1R from generated sequencing results (Figure 4.5B). Densitometry assessment of band intensity was performed to provide insights into the relative abundance of these transcripts. Band intensity for CSF-1R and sCSF-1R are compared relative to β-actin for each respective tissue (Figure 4.6). Gene-specific cDNA was utilized as a template to confirm the presence or absence of CSF-1R and sCSF-1R in each of the tissues examined (Figure 4.7). These results correlate expression seen with total cDNA.

2.3. Expression of CSF-1R and sCSF-1R in embryo stages

Previous studies have examined expression of CSF-1R during development using *in situ* hybridizations. These studies found CSF-1R detected as early as 5.25 hpf during formation of the gastrula, with expression in early macrophages at approximately 19 hpf (11,12). However, these results have not been confirmed using reverse-transcriptase PCR analysis during development. In addition, as sCSF-1R had previously not been identified in zebrafish, it is unknown if or when it is expressed during development. It was also of interest to determine if expression of CSF-1R and sCSF-1R was involved in primitive (0 to 30 hpf), definitive (30 hpf to 5 dpf) or both waves of hematopoiesis. As such, I examined the expression of CSF-1R and sCSF-1R in 0, 12, 18, 24, 48, 72, 96 and 120 hpf embryos.

Total cDNA (Figure 4.8) was synthesized from pools of n=50 embryos and used for reverse-transcriptase PCR. Expression of CSF-1R in a total cDNA pool is detected as early as 24 hpf, with a distinct increase at 48 hpf. Expression of sCSF-1R in a total cDNA pool is detected as early as 12 hpf and retains fairly equal levels of expression until 120 hpf. Densitometry assessment of band intensity was performed to provide insights into the relative abundance of these transcripts during development (Figure 4.9). Band intensity for CSF-1R and sCSF-1R are compared relative to β-actin for each respective stage. Gene-specific cDNA was utilized as a template to confirm the presence or absence of CSF-1R and sCSF-1R in each of the stages examined (Figure 4.10). With use of gene-specific cDNA, we see that the presence of CSF-1R can be detected as early as 0 hpf, with a distinct increase at 24 hpf. With use of gene-specific cDNA, presence of sCSF-1R can be detected as early as 0 hpf. As such, it is hypothesized that during development, sCSF-1R may play a role earlier in the developmental processes of embryonic fish than CSF-1R.

2.4. Mutant and morpholino validation

Several mutant alleles of CSF-1R exist, including CSF-1R^{j4e1} which possesses a substitution (V614M) in the first kinase domain resulting in a recessive loss-of-function mutation (7). This phenotype presents as reduced xanthophores and melanocytes (responsible for yellow and black pigmentation, respectively) in both adults and embryos. Fish possessing this mutation as well as ABxWik wild-type fish were used for the remainder of this chapter.

Additionally, two morpholinos were designed for this project (Figure 4.11A). A splice-block morpholino was designed to the boundary of Exon 4/Intron 4 of CSF-1R. By this design, two outcomes are possible: 1) Exon 5 is skipped, resulting in a product lacking this exon; or 2) Intron 4 is retained, resulting in generation of a product that resembles sCSF-1R. Either outcome likely results in a knock-down of CSF-1R. A translation block morpholino was designed to the ATG start site of the CSF-1R transcript. As such, this would result in a knock-down of both CSF-1R and sCSF-1R.

Injections of 2.5 ng of splice-block morpholino resulted in a phenotype similar to that of CSF-1R^{j4e1} mutants (Figure 4.11B). Mutants examined at 4 days post fertilization showed decreased xanthophore pigmentation in the head and along the spinal cord, as well as disrupted melanocyte pigmentation in stripes along the spinal cord. This was mirrored in morpholino-injected fish at 3 days post fertilization. Injections of translation-block morpholino (1 ng to 2.5 ng) resulted in no visible phenotype. Further optimization of this morpholino will need to be carried out to determine effect of knock-down of CSF-1R and sCSF-1R.

2.5. Role of CSF-1R during myeloid development

Previous studies have examined the role CSF-1R plays in early macrophage migration throughout the embryo. It has been found that in CSF-1R^{j4blue} mutants have macrophage phenotypes consisting of normal development, but a complete lack of migration to colonize sites in the embryo (8). This phenotype was examined through use of *in situ* hybridizations using a probe for L-plastin. In mice, L-plastin is an actin-bundling protein that is expressed specifically in leukocytes and plays a role in their adhesion and activation (7). Recent studies have shown that L-plastin expression is conserved in zebrafish early macrophages and is retained in mature macrophages (12). As such, a probe for L-plastin was utilized to quantify and locate macrophages in wild-type, CSF-1R^{j4e1} mutant and splice-block injected embryos.

Distinct and punctate labelling of myeloid cells was observed in the head, yolk sac and caudal region of 30 hpf embryos (Figure 4.12). When quantified, there was no observable difference in total number macrophages or spatial organization of macrophages. This is in contrast to what was previously observed in CSF-1R^{j4blue} mutants, which showed normal numbers of macrophages in the yolk sac but a drastic decrease in macrophages in the head region throughout early development of the embryo (8). I interpret this to mean that the CSF-1R^{j4e1} allele used here is less severe than the CSF-1R^{j4blue} allele, resulting in a milder macrophage phenotype.

3. DISCUSSION

In zebrafish, sCSF-1R was initially identified in the spleen, head kidney and body kidney, which correspond to the sites of hematopoiesis in the adult zebrafish. This was expected as in goldfish sCSF-1R likely plays a role in controlling alternative pathway macrophages and is believed to function as a negative regulator of macrophage proliferation, differentiation, survival and function (6). Zebrafish sCSF-1R was shown to correspond to the predicted sequence based on comparison between goldfish and zebrafish CSF-1R and goldfish sCSF-1R. Goldfish CSF-1R possesses a divergence from the typical splice site consensus sequence (AG|GT(A/G)AGT) at the Exon4/Intron4 which is hypothesized to be the major contributor to generation of sCSF-1R (6). Zebrafish also appear to possess this divergence, further supporting the generation of sCSF-1R in this closely related species.

The possibility of another species possessing this alternative splice form of the full-length CSF-1R is not surprising. CSF-1 itself has splice variants, as well as closely related CSF molecule receptors, including granulocyte colony-stimulating factor receptor (G-CSFR) and granulocyte macrophage colony-stimulating factor receptor (GM-CSFR) (14). As seen previously in this chapter, many species from mammals to birds to fish have some level of divergence from the splice site consensus sequence. In addition, intronic polyadenylation signals have been found downstream of some intron-exon boundaries, suggesting that other alternative splice forms of CSF-1R may exist. It has also previously been noted that human and puffer fish also possess consensus sequence divergences in the ligand binding domains which may lead to inefficient splicing of introns (15). Furthermore, as these divergences are found in the ligand binding domain, alternatively-spliced transcripts may have differential affinities for the CSF-1 ligand, resulting in a variety of contributions from these molecules (15). Previous studies have also identified the presence of two CSF-1 molecules in various teleost species, including rainbow trout, zebrafish and goldfish (16). In rainbow trout, these two CSF-1 molecules are differentially expressed *in vivo* with MCSF1

more highly expressed in the spleen, brain and intestine and MCSF2 more highly expressed in the head kidney, gills and muscles (16). Thus, one can expect that with time and resources, sCSF-1R could be identified in a variety of organisms throughout different branches of evolution.

I previously described tissue expression of CSF-1R and sCSF-1R in goldfish in Chapter III. It was observed that CSF-1R and sCSF-1R did not appear to be confined to any particular location in adult goldfish, though the levels of expression varied between individuals. This observation pointed towards heterogeneity between individuals of an outbred population raised in similar conditions. Further, this suggests that CSF-1R and sCSF-1R are involved in roles outside of hematopoiesis or what would be expected for these genes based on macrophage quantities. This observation is mirrored in zebrafish adult tissues. Expression of CSF-1R and sCSF-1R was detected in all tissues examined. Additionally, as there did not appear to be increased expression in the hematopoietic compartment, it does not appear that the roles of CSF-1R and sCSF-1R are confined to hematopoiesis. This was already expected due to the role of zebrafish CSF-1R in pigmentation, an observation which has not to date been noted in other species besides East African cichlids (3-4). As noted in goldfish in Chapter III, there are populations of macrophages or macrophage-like cells that may rely on CSF-1R present in all tissues examined in various vertebrate species. These macrophages have numerous roles that vary based on tissue examined, consisting of repair processes, protective roles, support during development, fertility, and of course hematopoiesis (17-24). The presence of CSF-1R and sCSF-1R in all tissues examined of zebrafish point to the role of the CSF-1 system in various processes in teleost fish.

The role of CSF-1R was examined in more detail during development. Like previously reported, CSF-1R was essential for proper pigmentation patterning, as both mutants and splice-block injected fish possess abnormal xanthophore and melanocyte pigmentation at 4 and 3 dpf, respectively. However, unlike previous studies, CSF-1R did not appear to play a role in the total number or spatial distributions of macrophages in embryos at 30 hpf. This may have a simple

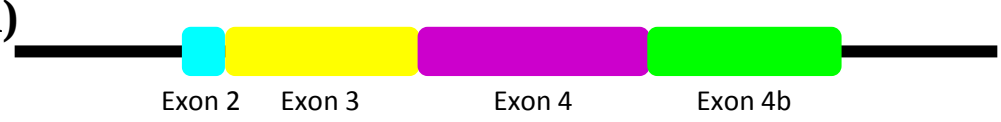
explanation based on the materials used. The mutant utilized was CSF-1R^{j4e1} which has been described as a recessive loss-of-function mutation. These mutants possess a substitution (V614M) in the first tyrosine kinase domain that is hypothesized to prevent proper signalling of CSF-1R. Previous studies have used CSF-1R^{j4blue} mutants, which possess a frameshift mutation (L847Δ) in the second tyrosine kinase domain resulting in a truncated protein (7). As the mutation is less severe in CSF-1R^{j4e1} mutants, it is possible that it is not sufficient to cause the abnormal macrophage development that is observed in CSF-1R^{j4blue} mutants.

As CSF-1R plays an essential role in the regulation of macrophages in the adult and has been shown to be required for proper macrophage tissue colonization, the role CSF-1R plays in the activity of developing macrophages will need to be examined in the future. I developed a combined FITC-zymosan/neutral red approach in order to assess this. FITC-zymosan can be used as an assay for macrophage activity as it would be phagocytosed by early macrophages. Neutral red is a vital stain that has previously been shown to be readily taken up by phagocytic cells through fluid-phase endocytosis and accumulate in lysosomes of macrophages in zebrafish embryos (8). As such, it can be used as a marker for macrophages that have engulfed FITC-zymosan. Preliminary experiments utilizing this technique have not yielded quantifiable results due to the lack of co-localization between the FITC-zymosan and neutral red. This could potentially be explained by a few theories: i) zymosan is clumping outside of phagocytic cells; ii) zymosan is being internalized by phagocytes that are not being labelled by neutral red; or iii) neutral red is staining cell types that are not phagocytic. As such, further characterization of a possible macrophage phenotype would involve optimizing the combined FITC-zymosan and neutral red approach to allow for quantification of the phagocytic capabilities of macrophages possessing a mutation in CSF-1R.

Based on the very broad expression of CSF-1R and sCSF-1R and the likely diverse role they play in the zebrafish, one aspect that will be examined at a later date is that of the role of CSF-1R and sCSF-1R in a peritonitis model in zebrafish. The close relationship between goldfish and zebrafish will allow for comparison

of the responses between the fish and aid in determining if sCSF-1R plays a conserved role in the immune response between vertebrates. Similar to that in goldfish (examined in detail in Chapter III), it is expected that the cellular response in the zebrafish during peritonitis will be mirrored by changes in the expression of CSF-1R and sCSF-1R. With this experiment, the contributions of CSF-1R and sCSF-1R to an immune function unrelated to hematopoiesis can be examined. Of course, until a mutant of sCSF-1R can be generated specifically, the role of sCSF-1R in zebrafish will be hard to assess.

A)



ATGTTCTTCATGCTCTTATTCCTCATTGGGATCCTGCTTGGTCAAGTTCA
GGGTTGGTCTGAACCGCGGATCAGACTGAGCTCTGGTGCTCTGGCCGGCA
CAGATGTGATCCTGGATTCCGGATCTCCTCTTCAGCTGGTCTGTGAGGGC
GACGGTCCAGTAACGTTTCTTCCCCGTCTGGCCAAACACAAGCGCTACAT
TTCCAAAGAAGTGGGGAAAATACGCAGTTTCCTCGTGGAAAAGGCAACGG
TGGACTTCACAGGAACATACAAGTGCGTCTATATGAATGGAAATGATTCA
AACTTGTCGTCTTCGGTTCATGTGTTTGTCCGGGACTCCCGAGTTCTCTT
TGTTTCTCCGAGTACGTCTCTGCGTTATGTGCGGAAAGAAGGTGAAGATC
TGTTGCTTCCGTGTCTTCTGACCGACCCGGAGGCCACAGACTTCACATTT
CGCATGGACAACGTTTCAGCAGCGCCCTACGGCATGAACATCACCTACGA
CCCCAGAAAGGGTGTCCTGATCCGCAAAGCTCATCCTGGATTTAATGGTG
AATACATCTGCAGCGCTAGAATCAGAGGAATTGAGAAAGTGTCAAAGATA
TTCTCGAATAACATCATTCAAAGTGAGTTTCATTTACAGTGCTGGGTGTT
TCCATACAAATCAGCTGACAGAAGCTCCACATTTTAA

B)

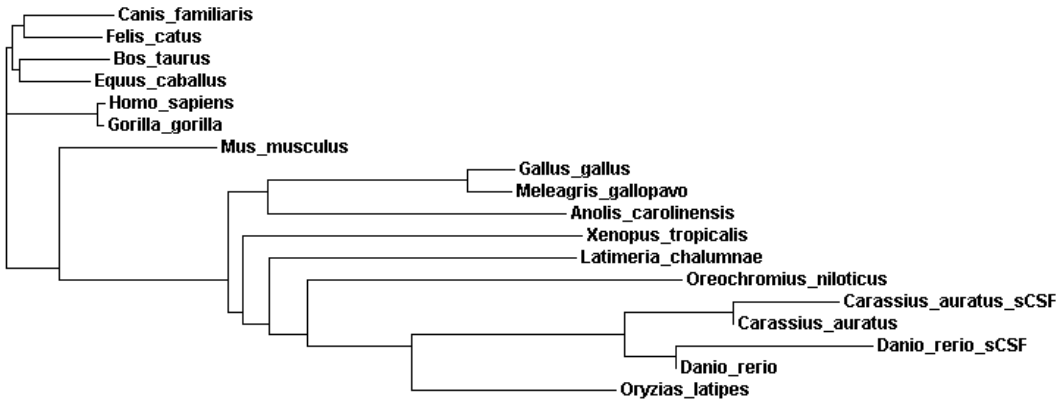


Figure 4.1. Identification and alignment of zebrafish soluble CSF-1R. Reverse-transcriptase PCR was performed using primers designed to Exon 2 and Exon 4b of sCSF-1R. Bands of interest were gel extracted, cloned into TOPO-TA vector and sequenced using BigDye. **A**) A consensus sequence was determined using Geneious software. Sequencing data correlates with the predicted zebrafish sequence of sCSF-1R based on comparison to goldfish. The red boxes correspond to the ATG start site and the TGA stop codon. **B**) Cladogram of CSF-1R transcripts from various species compared to goldfish and zebrafish sCSF-1R. Sequences for CSF-1R from species were obtained through Ensembl Genome Browser or NCBI and analysed using ClustalW.

A)

zebrafish

```
                                    ↓
CSF-1R   TTCTCGATAAACATCATTCAAAGGTTACGTTTTCCACCGTATGTGTACCTGAAGAGGAACGAGTATGT
sCSF-1R  TTCTCGAATAACATCATTCAAAGTGAGTTTCATTTACAGTGCTGGGTGTTTCCATACAAATCAGCTGA
         *******::****************.:.  * :*  **.**.   * :: * ...:.:**. .. .**:
```

goldfish

```
                                   ↓
CSF-1R   TTCTCGATAAACGTCATTCAGCGATTACATTTTCCA
sCSF-1R  TTCTCGATAAACGTCATTCAGCGTGAGTTTCATTGA
         ************************: :. :* :* :*
```

B)

```
CONSENSUS                                                    AGGTRAGT
Gorilla gorilla       GCAGGAAGGTGATGTCCATCAGCATCCGGCTGAAAGTGCAGAAAGGTGCGTGGGG
Homo sapiens          GCAGGAAGGTGATGTCCATCAGCATCCGGCTGAAAGTGCAGAAAGGTGCGTGGGG
Bos taurus            GCAGGGTGGTGAAGTCCCTCAGCATCCGGCTTAAAGTTCAGAAAGGTGCGTGGAC
Equus caballus        GCAGGACGGCGACGTCGATCAGCATCCGGCTTAAAGTGCAGAAAGGTGCGTGGCG
Canis familiaris      GCAGGACGGTAACGTCGATGGGCATCTGGCTTAAAGTGCAGAAAGGTGTGTGGGG
Mus musculus          GTAGGGAATCCACCTCCACTGGCATCTGGCTTAAGGTGAATCGAGGTATGTAGGA
Gallus gallus         GAAAAATAGAGAAGTCATCAAGAATAAGACTGATTGTGGTTGAAGGTAAAGGATT
Carassius auratus     GGGTTTGGAAAGTGTCGAAGATATTCTCGATAAACGTCATTCAGCGTGAGTTTCA
Danio rerio           GAATTGAGAAAGTGTCAAAGATATTCTCGAATAACATCATTCAAAGTGAGTTTCA
Oryzias latipes       GAGTGGAGAAGACCTCGAAGGCCTTTTCCATCAATGTTATTCAGAGTGAGACCTT
                      * .    .   . :**    . .:*  : .: *: .*  : ... **. .
```

Figure 4.2. Alignment of CSF-1Rs from various species. Sequences for CSF-1R from species were obtained through Ensembl Genome Browser or NCBI and analysed using ClustalW. **A)** Alignment of goldfish CSF-1R/sCSF-1R and zebrafish CSF-1R/sCSF-1R. The arrow corresponds to the Exon4/Intron4 boundary. **B)** Alignment of Exon4/Intron4 boundary of CSF-1R transcripts. Underlined nucleotides correspond to the consensus splice site recognition sequence. Bold nucleotides indicate divergences from the consensus sequence. R corresponds to an A or G nucleotide.

A)

Species	Splice site sequence	In-frame STOP?	Hydrophilic tail
Homo sapiens	AG|GTG**C**GT	yes	yes
Gorilla gorilla	AG|GTG**C**GT	yes	yes
Bos taurus	AG|GTG**C**GT	yes	no
Equus caballus	AG|GTG**C**GT	yes	yes
Canis familiaris	AG|GTG**T**GT	yes	no
Mus musculus	AG|GTA**T**GT	yes	yes
Gallus gallus	AG|GTAA**AG**	yes	yes
Xenopus tropicalis	AG|GT**TT**GT	yes	yes
Oryzias latipes	**GA**|GTGAG**A**	yes	yes
Danio rerio	A**A**|GTGAGT	yes	yes
Carassius auratus	**GC**|GTGAGT	yes	yes
Latimeria chalumnae	**T**G|GTAA**AC**	yes	no
Petromyzon marinus	A**T**|GTGAG**C**	yes	no

B)

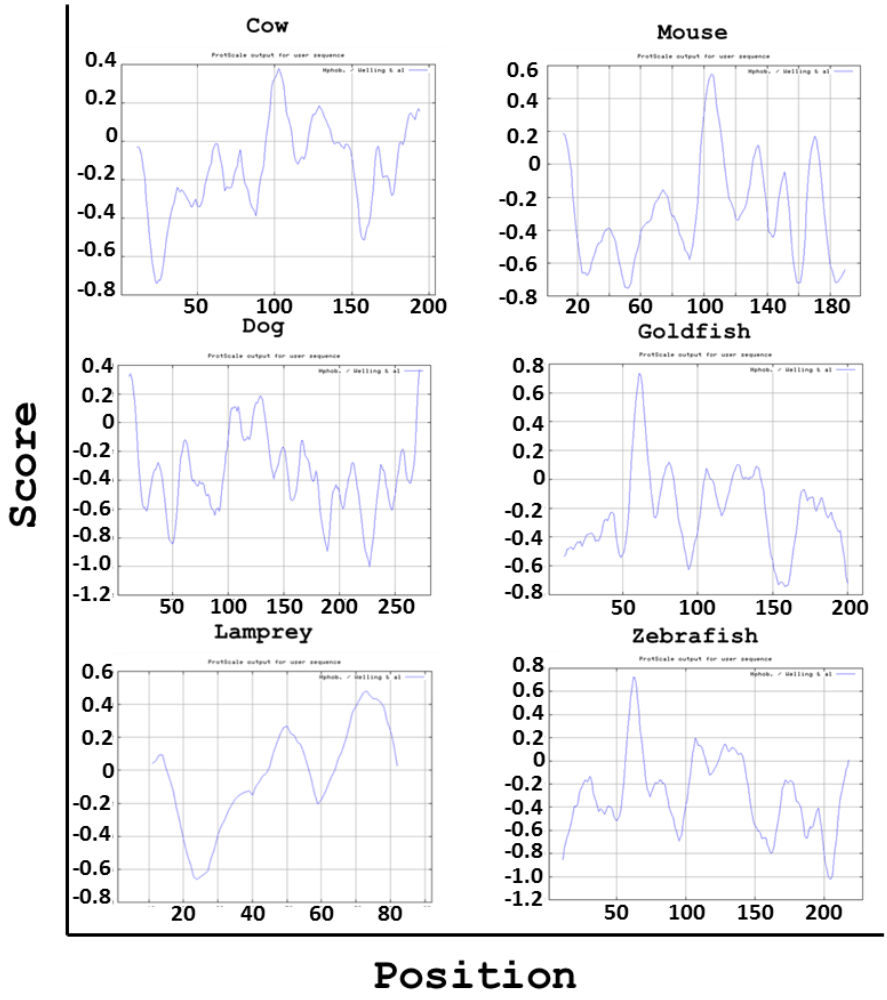


Figure 4.3. sCSF-1R prediction from various species. Sequences for CSF-1R from species were obtained through Ensembl Genome Browser or NCBI and analysed at Exon4/Intron4 boundary. **A)** Analysis of splice site sequence, stop codons and hydrophilic tail. **B)** Hydrophilic tail plots were obtained from http://web.expasy.org/protscale/ using "Hphob./Welling & al" scale. Positive scores correspond to hydrophobic residues while negative scores correspond to hydrophilic residues.

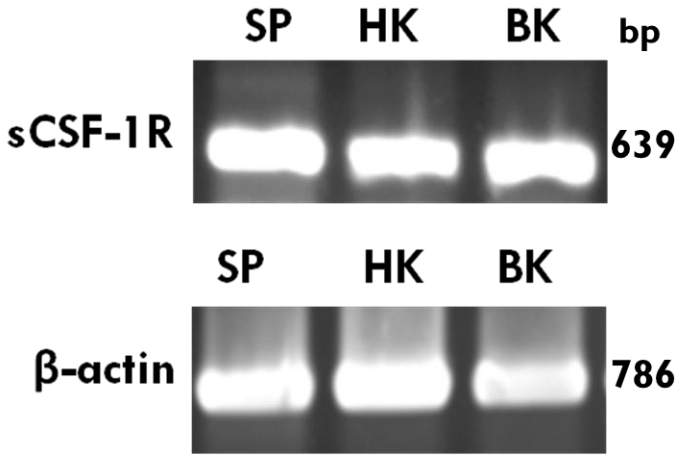
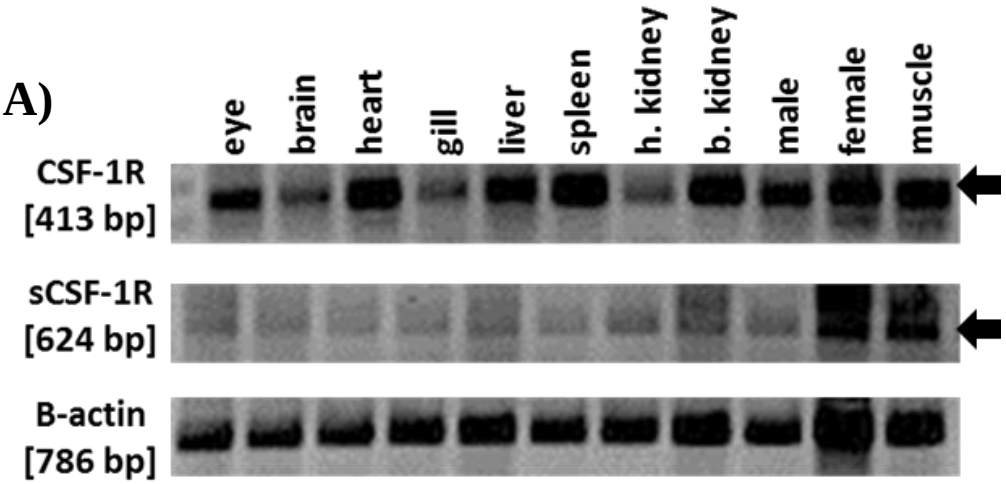


Figure 4.4. Expression of sCSF-1R in hematopoietic organs of zebrafish. Spleen, head kidney and body kidney were collected from n=30 adult zebrafish (AB) and used to isolate RNA and synthesize cDNA. Reverse-transcriptase PCR was performed using primers designed to Exon 2 and Exon4b of sCSF-1R.

A)



B)

Gene	Tissue	BLAST	Accession	% identity
CSF-1R	liver	CSF-1R	BC162803.1	98%
	spleen	CSF-1R	BC162803.1	99%
	body kidney	CSF-1R	BC162803.1	99%
sCSF-1R	liver	Compared to zebrafish CSF-1R and sCSF-1R consensus sequence		90%
	spleen			95%
	head kidney			95%
	body kidney			95%
	female gonad			99%

Figure 4.5. Expression of CSF-1R and sCSF-1R in tissues of adult zebrafish. Tissues of interest were isolated from n=30 AB zebrafish and used to isolate RNA and synthesize cDNA. **A**) Semi-quantitative reverse transcriptase PCR was performed using primers designed to Exon11-Exon14/15 (CSF-1R) and Exon2-Exon4b (sCSF-1R). **B**) Bands of interest were gel extracted, cloned into a TOPO-TA vector and used for sequencing. Sequences were analysed using BLAST (NCBI) for identity.

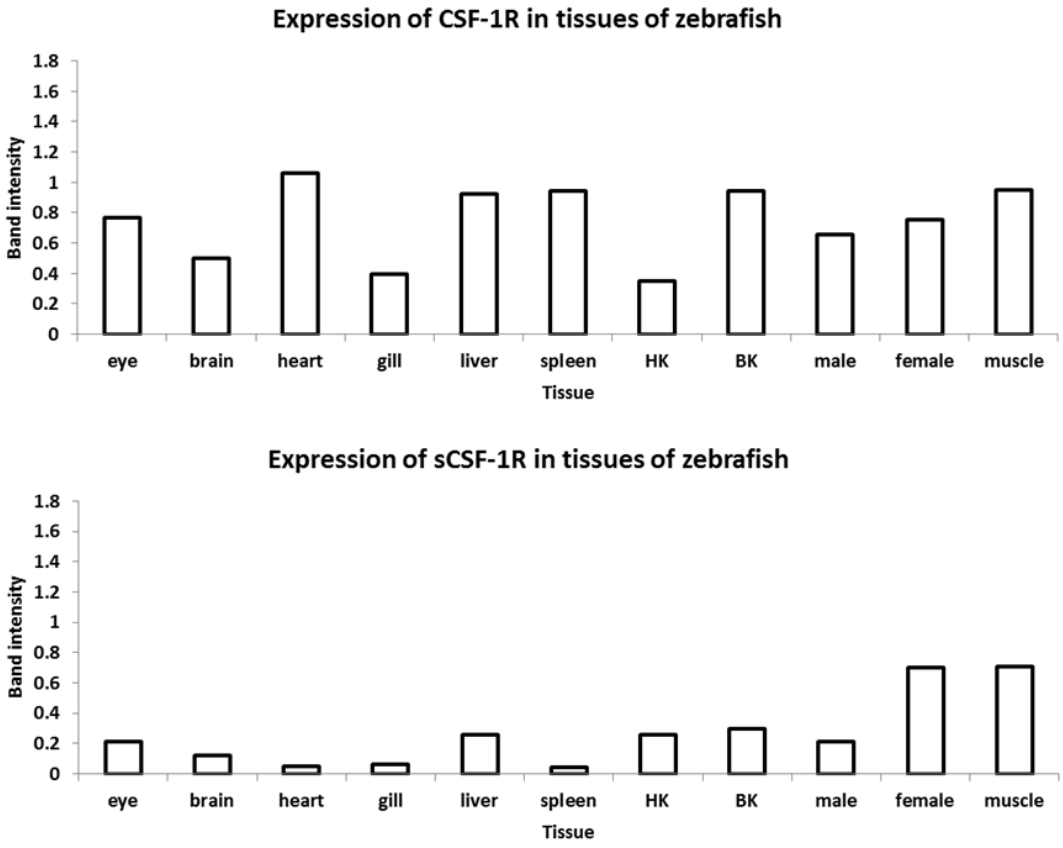


Figure 4.6. Levels of CSF-1R and sCSF-1R expression in tissues of adult zebrafish. Images of agarose gels were analysed using Adobe Photoshop software to determine band intensity in tissues of interest. Band intensity for each tissue was normalized to corresponding B-actin band and graphed using Excel software.

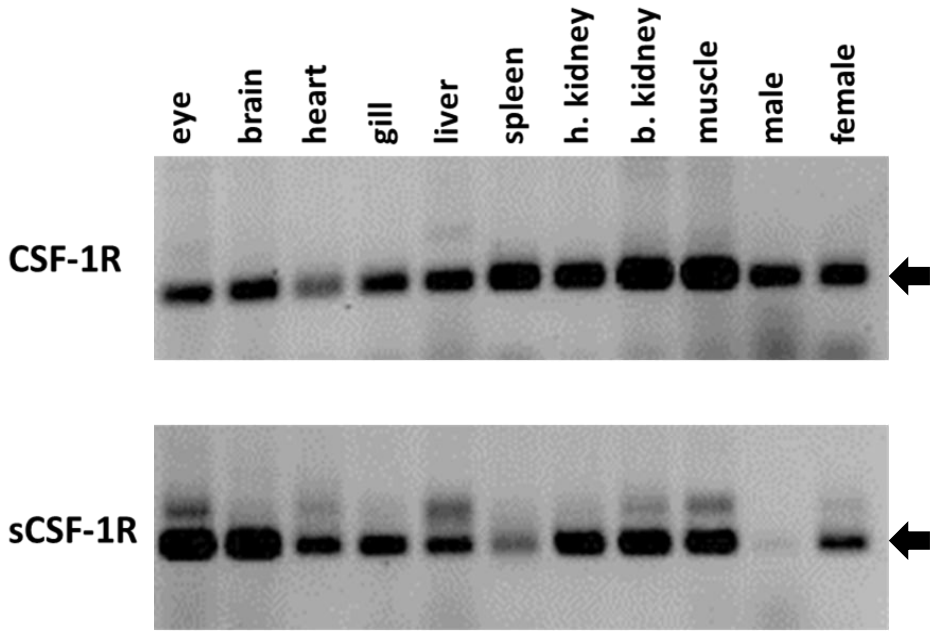


Figure 4.7. Presence or absence of CSF-1R and sCSF-1R in adult zebrafish tissues. Tissues of interest were collected from n=30 adult zebrafish and used to isolate RNA and synthesize gene-specific cDNA. Semi-quantitative RT-PCR was performed with zebrafish gene-specific cDNA and primers designed for Exon 11 to Exon 14/15 of CSF-1R and Exon 2 to Exon 4b of sCSF-1R.

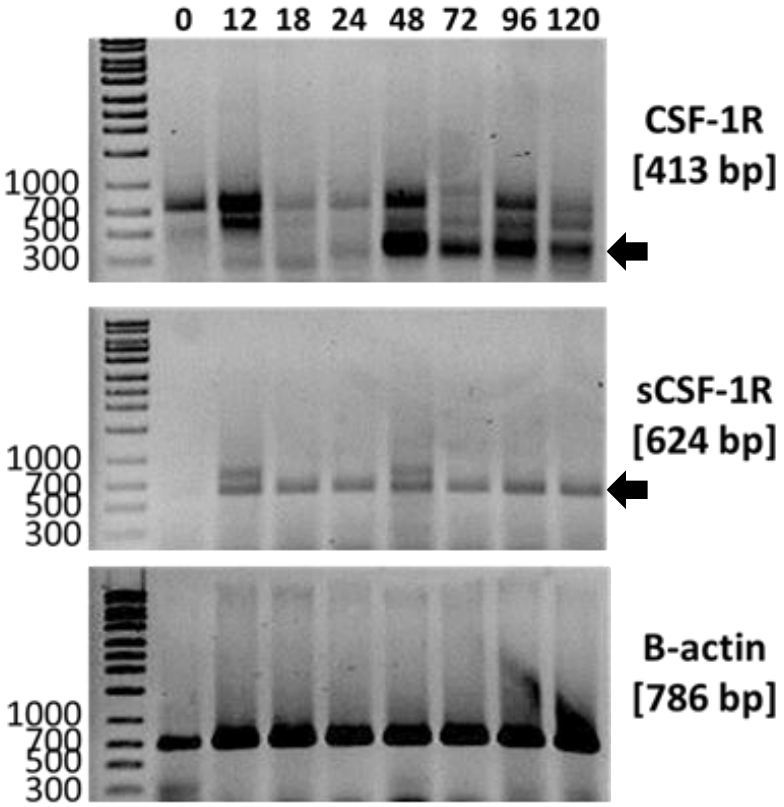


Figure 4.8. Expression of CSF-1R and sCSF-1R in embryo stages. Approximately 50 embryos of each stage were collected from crossing of ABxWik adults and used to isolate RNA and synthesize cDNA. Semi-quantitative RT-PCR was performed using primers for CSF-1R spanning from Exon 11 to Exon 14/15 and primers for sCSF-1R spanning from Exon 2 to Exon 4b. Results show CSF-1R expression beginning between 24 and 48 hpf and sCSF-1R expression beginning between 0 and 12 hpf.

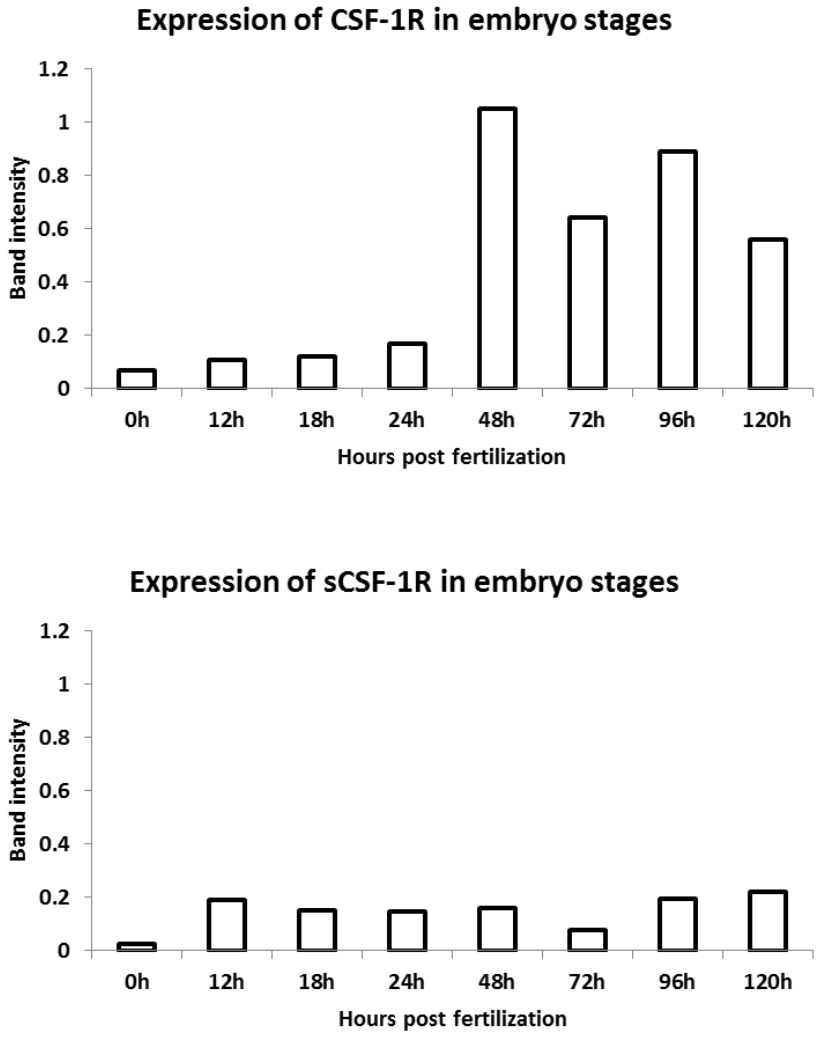


Figure 4.9. Levels of CSF-1R and sCSF-1R expression in stages of zebrafish embryos. Images of agarose gels were analysed using Adobe Photoshop software to determine band intensity in tissues of interest. Band intensity for each tissue was normalized to corresponding B-actin band and graphed using Excel software.

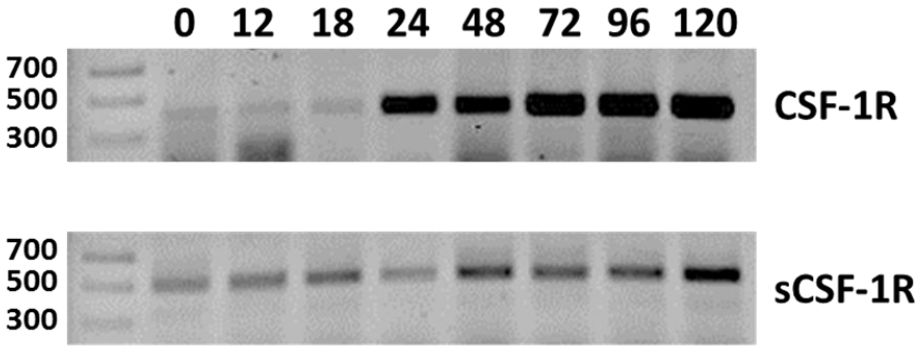


Figure 4.10. Presence or absence of CSF-1R and sCSF-1R in embryo stages. Approximately 50 embryos of each stage were collected from crossings of ABxWik adults and used to isolate RNA and synthesize gene-specific cDNA. Semi-quantitative RT-PCR was performed with primers designed for Exon 11 to Exon 14/15 of CSF-1R and Exon 2 to Exon 4b of sCSF-1R. Results show CSF-1R and sCSF-1R detected between 0 and 12 hpf.

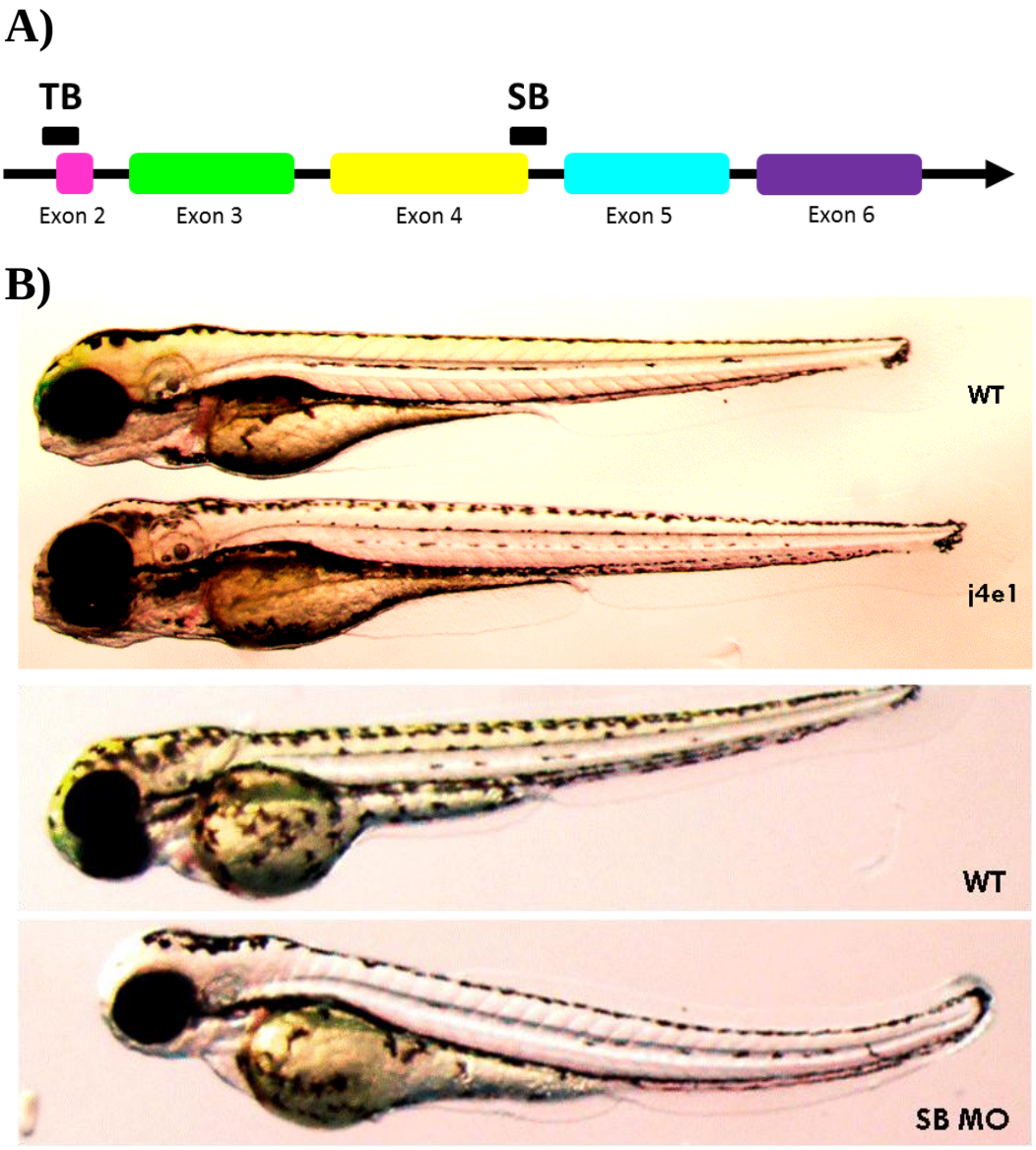


Figure 4.11. Pigment development in wild-type AB embryos compared to mutant CSF-1R^{j4e1} and splice-block morpholino injected embryos. A) Diagram of translation block (TB) and splice block (SB) morpholinos. **B**) Crossings were made between groups of AB fish or groups of CSF-1R^{j4e1} mutant fish. Embryos were incubated at 28°C for 4 days before being photographed. Crossings were made between groups of adult AB zebrafish. Half of the collected embryos were incubated at 28°C for 3 days. The other half were injected with 2.5 ng of the CSF-1R splice-block morpholino and then incubated at 28°C for 3 days.

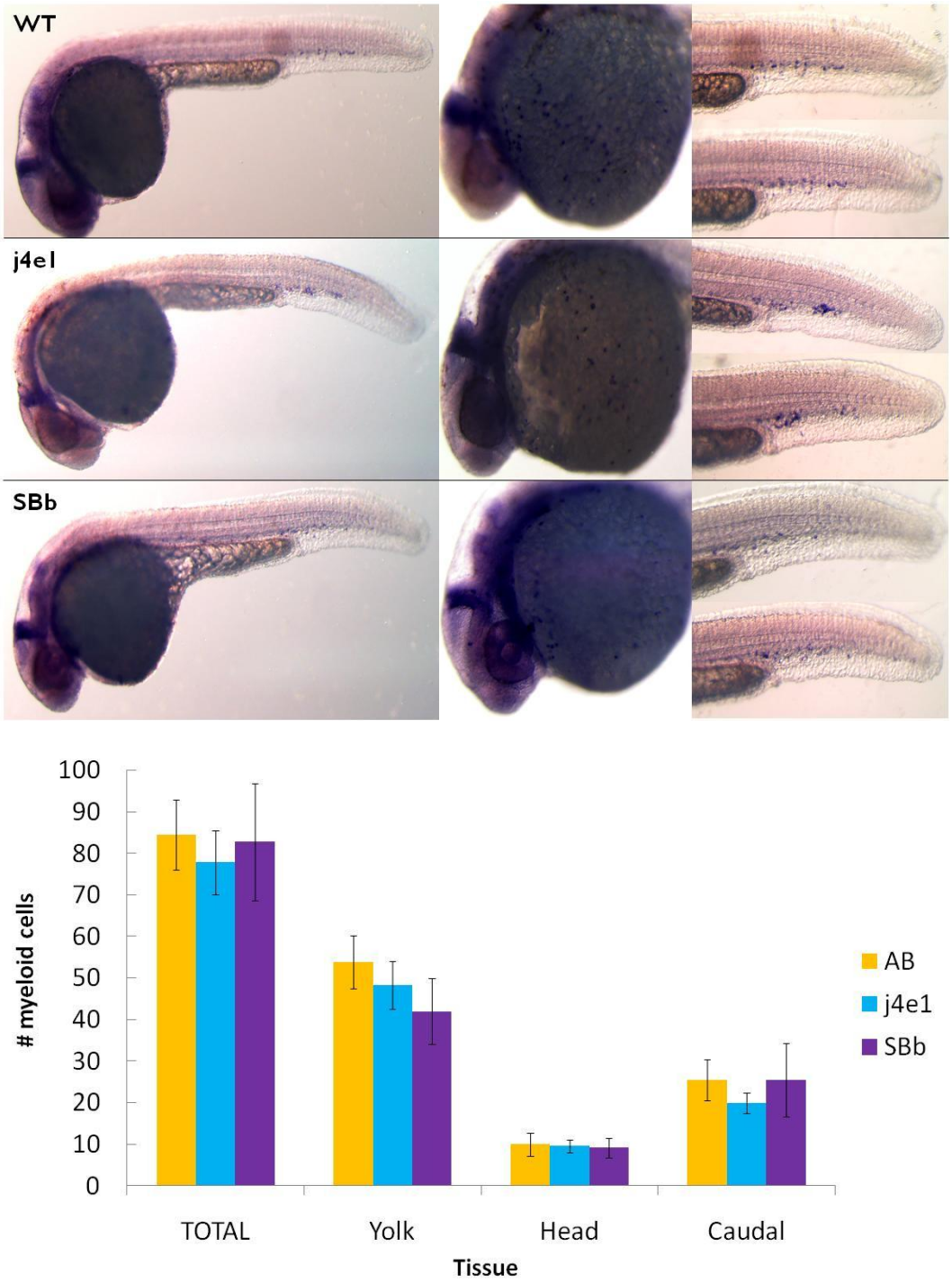


Figure 4.12. *in situ* hybridization data for wild-type, CSF-1R^{j4e1} and splice-block morpholino injected embryos. Embryos were collected and incubated at 28°C for 30 hours. Embryos were dechorionated and fixed in solution of 4% paraformaldehyde/PBS overnight at 4°C. *in situs* were performed using probes for L-plastin, eng2 and krox20. Fish were photographed using a light microscope with mounted camera. ImageJ software was using to quantify number of visible macrophages. Results were then graphed and analyzed in Excel.

4. REFERENCES

1. Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, Nakanishi T, Yokomizo Y, Takahashi Y, Inagawa H and Soma G-I. 2005. Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. *Molec Immunol* **42**:1-8.
2. Roca FJ, Sepulcre MP, López-Castejón G, Meseguer J and Mulero V. 2006. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molec Immunol* **43**:1418-23.
3. Salzburger W, Braasch I and Meyer A. 2007. Adaptive sequence evolution in a color gene involved in the formation of the characteristic egg-dummies of male haplochromine cichlid fishes. *BMC Biol* **5**:51.
4. Braasch I, Salzburger W and Meyer A. 2006. Asymmetric evolution in two fish-specifically duplicated receptor tyrosine kinase paralogons involved in teleost coloration. *Mol Biol Evol* **23**:1192-1202.
5. Williams H, Brenner S and Venkatesh B. 2002. Identification and analysis of additional copies of the platelet-derived growth factor receptor and colony stimulating factor 1 receptor genes in fugu. *Gene* **295**:255-264.
6. Barreda DR, Hanington PC, Stafford JL and Belosevic M. 2005. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). *Dev Comp Immunol* **29**:879-94.
7. Parichy DM, Ransom DG, Paw B, Zon LI and Johnson SL. 2000. An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in zebrafish *Danio rerio*. *Development* **127**:3031-44.
8. Herbomel P, Thisse B and Thisse C. 2001. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol* **238**:274-88.

9. Traver D, Herbomel P, Patton EE, Murphey RD, Yoder JA, Litman GW, Catic A, Amemiya CT, Zon LI and Trede NS. 2003. The zebrafish as a model organism to study development of the immune system. *Adv Immunol* **81**: 253-330.
10. Ellet F and Lieschke GJ. 2010. Zebrafish as a model for vertebrate hematopoiesis. *Curr Opin Pharmacol* **10**: 1-8.
11. Thisse B, Wright GJ and Thisse C. 2008. Embryonic and larval expression patterns from a large scale screening for novel low affinity extracellular protein interactions. ZFIN Direct Data submission (http://zfin.org).
12. Herbomel P, Thisse B and Thisse C. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**:3735-3745.
13. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC and Layton JE. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**: 3087-3096.
14. Barreda DR, Hanington PC and Belosevic M. 2004. Regulation of myeloid development and function by colony stimulating factors. *Dev Comp Immunol* **28**:509-554.
15. Belosevic M, Hanington PC and Barreda DR. 2006. Development of goldfish macrophages in vitro. *Fish Shell Immunol* **20**:152-171.
16. Wang T, Hanington PC, Belosevic M and Secombes CJ. 2008. Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. *J Immunol* **181**: 3310-3322
17. Frangogiannis NG, Mendoza LH, Ren G, Akrivakis S, Jackson PL, Michael LH, Smith CW and Entman ML. 2003. MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype. *Am J Physiol Heart Circ Physiol* **285**: H483-H492.
18. Pollard JW. 2009. Trophic macrophages in development and disease. *Nat Rev Immunol* **9**: 259-270.

19. Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, Gherardi RK and Chazaud B. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis. *J Exp Med* **204(5)**: 1057-1069.
20. Wilson HM, Walbaum D and Rees AJ. 2004. Macrophages and the kidney. *Curr Opin Nephrol and Hypertens* **13**: 285-290.
21. Smith SJ and Mohun TJ. 2011. Early cardiac morphogenesis defects caused by loss of embryonic macrophage function in *Xenopus*. *Mech Dev* **128***:* 303-315.
22. Cohen PE, Zhu L and Pollard JW. 1997. Absence of colony stimulating factor-1 in osteopetrotic ($csfm^{op}/csfm^{op}$) mice disrupts estrous cycles and ovulation. *Biol Reprod* **56**: 110-118.
23. Niemi M, Sharpe RM and Brown WRA. 1986. Macrophages in the interstitial tissue of the rat testis. *Cell Tissue Res* **243**:337-344.
24. Ovchinnikov DA. 2008. Macrophages in the embryo and beyond: much more than just giant phagocytes. *Genesis* **46**: 447-462.

CHAPTER V: GENERAL DISCUSSION

1. OVERVIEW OF FINDINGS

Teleost fish rely strongly on the innate immune response as a first line of defense against pathogens. One of the key cells of the innate immune response is the macrophage, which acts as a central effector of innate defence mechanisms and as a regulator of innate and adaptive responses. Due to their importance in the body, macrophage function and development needs to be tightly regulated. The primary regulation of macrophage survival, proliferation, differentiation and function occurs through the CSF-1 system. The CSF-1 system and its regulation are poorly understood in teleost fish. In 2005, a soluble CSF-1R was identified from goldfish primary kidney macrophages as a novel method of CSF-1 regulation (1). The presence of sCSF-1R in other vertebrates has not been determined to date.

The main focus of my M.Sc. program was to expand our understanding of the role sCSF-1R in the regulation of the CSF-1 system. In particular, I focused on expanding upon current knowledge of goldfish sCSF-1R and identifying sCSF-1R in zebrafish to assess its contributions to aspects of development and immune function. Examination of tissue expression patterns of both CSF-1R and sCSF-1R lead to the conclusion that these genes were not specific to hematopoietic organs as hypothesized (Chapter III). Rather, CSF-1R and sCSF-1R appear to be expressed in all tissues examined. CSF-1R and sCSF-1R showed differentially expression between tissues and between fish, suggesting that these two transcripts may be differentially expressed in outbred goldfish.

In order to assess the contributions of CSF-1R and sCSF-1R to an inflammatory process, I examined the changes in expression of these genes during zymosan-induced peritonitis in goldfish. Peritonitis was characterized both *in vitro* and *in vivo* by examining changes in total cell number, cell populations, and gene expression of CSF-1R and sCSF-1R (Chapter III). At basal conditions, very few cells were localized to the peritoneal cavity. Total cell counts were seen to

rapidly increase 18 and 24 hours after injection. After 48 hours, cell counts decreased with a large drop in all populations. Finally at 72 hours, cell counts and populations returned to approximately basal conditions. Expression of both CSF-1R and sCSF-1R were altered during the time course of this experiment. Both transcripts showed a trend upwards from 0 to 24 hours, with lower expression after 48 hours. Levels of CSF-1R and sCSF-1R remained stable after 72 hours.

I successfully identified sCSF-1R from zebrafish hematopoietic tissues (Chapter IV). Zebrafish sCSF-1R showed high identity (82%) to that of goldfish sCSF-1R, additionally possessing splice site consensus sequence divergences at the Exon4/Intron4 boundary. I further examined the expression of CSF-1R and sCSF-1R in tissues isolated from zebrafish. Both genes showed expression in all tissues examined, a result that was mirrored in goldfish.

I was also interested in determining the temporal expression of CSF-1R and sCSF-1R during development in zebrafish (Chapter IV). CSF-1R was readily detectable as early as 24-48 hours post fertilization (hpf) through RT-PCR. Through use of gene-specific cDNA, the transcript for CSF-1R was identified shortly after fertilization, with a rapid increase at 24 hpf. sCSF-1R was detectable 0-12 hpf through RT-PCR, which was similar to the use of gene-specific cDNA. Thus, it appears that sCSF-1R may play a role earlier in development than CSF-1R.

Finally, I examined the contributions of CSF-1R and sCSF-1R to zebrafish development and to the development and activity of primitive macrophages (Chapter IV). I observed that CSF-1R is indeed essential for proper pigmentation patterning during zebrafish development, a result which was expected based off of previous studies. I was unable to observe any changes in spatial location or numbers of developing macrophages at 30 hpf in CSF-1R^{j4e1} mutants or splice-block morpholino injected embryos. To assess the contributions of CSF-1R and sCSF-1R to primitive macrophage activity, I suggest further fine-tuning a technique I developed utilizing FITC-zymosan and neutral red would yield data as to the contributions of CSF-1R and sCSF-1R to primitive macrophage activity.

2. ASSESSMENT OF THE CONTRIBUTIONS OF CSF-1R AND sCSF-1R TO TELEOSTS: ROLE IN DEVELOPMENT AND INFLAMMATION

While previous work in our group and others has shown that CSF-1 and CSF-1R are conserved in teleost fish, little is known about their function (1,2). A recent study examining CSF-1 in teleost fish identified two copies of CSF-1 in zebrafish, rainbow trout and salmon, suggesting that two CSF-1 genes may be present in many fish species (3). Further examination of rainbow trout CSF-1 molecules showed differential expression based on tissues, with MCSF1 more highly expressed in the spleen, brain and intestine and MCSF2 more highly expressed in the head kidney, gills and muscles (3).

Several CSF-1R orthologues have also been examined in teleost fish, including rainbow trout, gilthead seabream, East African cichlids, pufferfish, goldfish and zebrafish (1,4-9). Orthologues in gilthead seabream and rainbow trout show expression in cells of the monocyte/macrophage lineage in immunologically-relevant tissues (4,5). On the other hand, orthologues in zebrafish and East African cichlids play a unique role in pigmentation patterning of melanocytes (zebrafish) and xanthophores (both) (6,7,9). To date sCSF-1R has only been identified in goldfish in senescent primary kidney macrophage cultures (1). sCSF-1R is believed to function as a negative inhibitor of CSF-1 based on its structure. Cultures exposed to increasing levels of recombinant sCSF-1R show dose-dependent decreases in proliferation, further supporting this hypothesis (1).

My studies have shown that sCSF-1R is conserved in zebrafish with high identity to that of goldfish sCSF-1R. Similar to that of CSF-1R, sCSF-1R showed expression in all tissues examined in both goldfish and zebrafish, indicating roles outside of typical hematopoiesis. CSF-1R and sCSF-1R appear to contribute to the cellular events of the inflammatory process, as expression was observed to change as cell populations were altering. In the following sections I will discuss possible contributions of CSF-1R and sCSF-1R to development of the immune system and organism as well as during inflammation in the peritoneal cavity. Overall, I believe that these transcripts are critical to both development and function of an adult organism. As they are essential to proper macrophage

regulation, CSF-1R and sCSF-1R should play a role in the development and function of primitive macrophages. Further, it is predicted CSF-1R and sCSF-1R will play a role in the inflammatory response of the peritoneal cavity based the role macrophages play during this response.

2.1. Contributions during development

The development and maturation of macrophages has been well studied in zebrafish. Primitive hematopoiesis can be observed as early as 12 hpf as macrophage precursors emerge near the cardiac field of the embryo (10-12). These macrophage precursors are the first producers of CSF-1R in the organism (9). After 22 hpf, macrophages can be observed in the hundreds after 22 hpf as pre-macrophages or immature macrophages (12). Upon the onset of blood circulation between 24 and 26 hpf, the majority of the macrophages are seeded in the cephalic mesenchyme and brain. This process has been shown to be CSF-1R-dependent, as mutant embryos do not display this characteristic migration (9). The remainder of the macrophages stay in the yolk sac and mature into early macrophages. Shortly after this migration, definitive hematopoiesis begins in the caudal hematopoietic tissue and in the aorta-gonad-mesonephros (AGM) region (13).

Early macrophages are unique in their development. They appear to bypass the monocytic development stage and instead follow a non-classical-rapid differentiation pathway similar to that of goldfish alternative-pathway (AP) macrophages (12). Both early macrophages and AP-macrophages are capable of self-renewal, a trait that is not observed in typical MPS-monocyte derived macrophages. In addition, both early macrophages and AP-macrophages display similar myeloperoxidase staining and transcription factor PU.1 expression (14-16). As such, it is believed that early macrophages and/or their precursors are retained in the adult fish and function as adult AP-macrophages (17). As expression of sCSF-1R is found in AP-macrophages in goldfish, it is hardly surprising that sCSF-1R expression is detectable in zebrafish early macrophages as well (Chapter IV). Further, expression of sCSF-1R appears to precede

expression of CSF-1R during development, suggesting an important role for sCSF-1R in control of the development of early macrophages.

The full spectrum of functions of these early macrophages has not been fully characterized. These macrophages are capable of phagocytic activity, can effectively clear bacterial challenges, and can remove apoptotic bodies (12,19). However, the killing mechanisms of these macrophages are poorly understood. Zebrafish macrophages deficient in CSF-1R still display normal functions, but it is unknown if the killing mechanisms are still intact. As the CSF-1 system controls the function of macrophages (amongst other roles), it likely controls the function of these early macrophages as well. The expression of CSF-1R and sCSF-1R during a time when early macrophages are present may indicate that these transcripts contribute to the regulation of early macrophage function.

CSF-1R has previously been shown to be involved in pigmentation patterning in zebrafish as well as in East African cichlids (6,7,9). Zebrafish with a CSF-1R^{j4blue} mutant display severely decreased numbers of xanthophores and melanocytes during development and adulthood (9). This has been attributed to a decrease migration of cells of the xanthophore lineage, as well as increased death of melanocytes along the zebrafish body. This CSF-1R^{j4blue} mutation results from a frameshift in the second kinase domain leading to a non-functional truncated protein (9). As such, it can be expected that levels of sCSF-1R likely remain unchanged in these fish. Thus, this effect on pigmentation may be due to the hypothesized negative regulator role of sCSF-1R as sCSF-1R would still be capable of proper function in this situation. This suggests that sCSF-1R may contribute to processes during development and not confined to typical immune protection in adulthood.

2.2. Contributions during inflammation

The cellular events during zymosan-induced peritonitis have been studied in numerous organisms including mouse, frog, rat, goldfish and carp (19-22). It has been observed that course of peritonitis can be modulated by a variety of endogenous or exogenous factors including season, ambient temperature, stress or

pharmacological factors including opioids (20). Nevertheless, in all organisms examined a rapid increase in infiltrating cells is observed 24 hours post injection, typically comprised of neutrophils. In carp, respiratory burst activity of these infiltrating inflammatory neutrophils is greatly increased compared to resting neutrophils (23). In addition, maximum expression of IL-1β, TNF-α, CXCa and CXCR1 is observed after 24 hours (24). In mice, levels of tissue resident macrophages rapidly drop during the first hours of inflammation as levels of inflammatory macrophages increase to a maximum at 24 hours (19). This decrease has been attributed to three possible factors: i) increased tissue adherence; ii) emigration to draining lymph nodes; and/or iii) cell death (19). After two days, inflammatory macrophages decline while tissue resident macrophages begin to repopulate the peritoneal cavity. The majority of neutrophils are cleared by 48 hours post injection.

Zymosan-induced peritonitis has additionally been studied in SWISS mice, frogs and goldfish following ablation of macrophages by clodronate (CL) treatment (20). In SWISS mice, an increased influx of polymorphonuclear leukocytes (PMNs) is observed with prolonged accumulation in the peritoneal cavity when macrophages are ablated from the mouse. This leads to the hypothesis that macrophages are essential for limiting the numbers of inflowing PMNs during peritonitis. Interestingly, frogs and goldfish that have been treated with CL do not display a typical response to peritonitis (20). This may be explained by the observation that CL-treatment itself leads to PMN influx in the peritoneal cavity, which may obscure the typical response.

As macrophages are seen to be altered during the peritonitis response, it is expected that the gene expression of these macrophages is also altered. In SWISS mice, IL-10 has been implicated in the PMN-limiting abilities of macrophages (20). Furthermore, it is hypothesized that based on the function of CSF-1R and sCSF-1R, these transcripts should be actively involved in and/or contributing to this response. It has been noted in mice that CSF-1 promotes the persistency of tissue resident macrophages (25). As tissue resident macrophages decline and inflammatory macrophages increase, differential changes in CSF-1R and sCSF-

1R expression are expected. This is indeed what is observed in goldfish. Between 0 and 24 hours post zymosan injection, the levels of expression of sCSF-1R and CSF-1R are seen to steadily increase. As macrophages and monocytes are entering the inflammatory site during this period, the increasing expression of CSF-1R is hardly surprising. These infiltrating myeloid cells would rely directly upon CSF-1R for proper function. As discussed above, macrophages in mice have been hypothesized to regulate the infiltration of PMNs during peritonitis (20). Thus, increasing sCSF-1R expression may point to regulation of this response to prevent unnecessary tissue damage that stems from an excessive inflammatory response. In fact, sCSF-1R may be essential to remove circulating CSF-1 to prevent further differentiation or proliferation of cells that are no longer needed in the peritoneal cavity. While it is clear that CSF-1R and sCSF-1R are involved in the inflammatory process, further characterization as to the contributions of these transcripts will need to be performed.

3. FUTURE DIRECTIONS

The results presented in this thesis indicate goldfish sCSF-1R and CSF-1R show diverse expression in all tissues examined of goldfish, indicating a role for these genes outside of typical hematopoiesis. The contributions of these transcripts to inflammation in the peritoneal cavity were assessed at different time points and were shown to play a role in this response. Zebrafish also possess sCSF-1R with high identity to goldfish sCSF-1R. Similar to goldfish, zebrafish sCSF-1R and CSF-1R show expression in all tissues examined. These genes are also expressed early in development, indicating possible contributions to early hematopoiesis.

While sCSF-1R and CSF-1R have been identified and further characterized through my studies, detailed tissue expression in the tissues of goldfish and zebrafish should be performed. Through use of a more sensitive technique, such as real-time PCR, expression of sCSF-1R and CSF-1R can be accurately determined in tissues examined. This would yield data to allow for a more detailed analysis of the potential roles of these genes to teleost immune function.

The contributions of sCSF-1R and CSF-1R to development of macrophages in embryonic zebrafish need to be further examined. I anticipate that fully optimizing a developed FITC-zymosan/neutral red technique would allow for generation of quantifiable data to assess the roles of sCSF-1R and CSF-1R during development. I expect that macrophages deficient in the CSF-1 system of control would display comprised activity resulting in a poorly defended embryo that would be sensitive to pathogens. Furthermore, with recent developments in using zinc finger nucleases in zebrafish to knock-out specific genes, a sCSF-1R and/or CSF-1R knock-out mutant can be generated to allow for examination of macrophage function throughout development and adulthood of a zebrafish.

As stated above, while the contributions of sCSF-1R and CSF-1R to inflammation in the peritoneal cavity have been assessed, real-time PCR would allow for a more sensitive assessment of changes in expression of these genes. In addition, it may be possible to examine individual fish without the need to pool in order to determine if sCSF-1R and CSF-1R expression levels vary between fish during inflammation. Zymosan injections into the peritoneal cavity of zebrafish should be performed to determine if sCSF-1R and CSF-1R between goldfish and zebrafish provide the same contributions during inflammation. Taking advantage of the already generated mutants, including CSF-1R^{j4e1} and CSF-1R^{j4blue}, would allow for assessment of acute inflammation in teleosts that do not have a fully functioning CSF-1 system.

The contributions of sCSF-1R to inflammation in goldfish are currently being examined by our lab. The differential expression of sCSF-1R following exposure of zymosan and apoptotic bodies to PKM cultures is being examined at the 48 hour time point. In addition, the expression of sCSF-1R in sorted cell populations from peritoneal lavages will be examined to determine which cell type is the major contributor of sCSF-1R production. Finally, through generation of recombinant sCSF-1R we will be able to assess the contributions of sCSF-1R to a variety of factors *in vitro* and *in vivo*.

The main objectives of my thesis were to further characterize goldfish sCSF-1R and CSF-1R, to identify sCSF-1R in zebrafish, and to assess the

contributions of these genes during development and inflammation. My research has provided information that sCSF-1R is not unique to goldfish, and is in fact expressed in zebrafish, a close relative. This suggests that other vertebrate species may also express a sCSF-1R transcript. Identifying sCSF-1R in other vertebrates and the role(s) it plays in the innate immune response will open new doors for the characterization of CSF-1 biology.

4. REFERENCES

1. Barreda DR, Hanington PC, Stafford JL and Belosevic M. 2005. A novel soluble form of the CSF-1 receptor inhibits proliferation of self-renewing macrophages of goldfish (*Carassius auratus* L.). *Dev Comp Immunol* **29**:879-94.
2. Barreda DR, Hanington PC and Belosevic M. 2004. Regulation of myeloid development and function by colony stimulating factors. *Dev Comp Immunol* **28**: 509-554.
3. Wang T, Hanington PC, Belosevic M and Secombes CJ. 2008. Two macrophage colony-stimulating factor genes exist in fish that differ in gene organization and are differentially expressed. *J Immunol* **181**:3310-3322.
4. Honda T, Nishizawa T, Uenobe M, Kohchi C, Kuroda A, Ototake M, Nakanishi T, Yokomizo Y, Takahashi Y, Inagawa H and Soma G-I. 2005. Molecular cloning and expression analysis of a macrophage-colony stimulating factor receptor-like gene from rainbow trout, *Oncorhynchus mykiss*. *Molec Immunol* **42**:1-8.
5. Roca FJ, Sepulcre MP, López-Castejón G, Meseguer J and Mulero V. 2006. The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Molec Immunol* **43**:1418-23.
6. Salzburger W, Braasch I and Meyer A. 2007. Adaptive sequence evolution in a color gene involved in the formation of the characteristic egg-dummies of male haplochromine cichlid fishes. *BMC Biol* **5**:51.

7. Braasch I, Salzburger W and Meyer A. 2006. Asymmetric evolution in two fish-specifically duplicated receptor tyrosine kinase paralogons involved in teleost coloration. *Mol Biol Evol* **23**:1192-1202.
8. Williams H, Brenner S and Venkatesh B. 2002. Identification and analysis of additional copies of the platelet-derived growth factor receptor and colony stimulating factor 1 receptor genes in fugu. *Gene* **295**:255-264.
9. Parichy DM, Ransom DG, Paw B, Zon LI and Johnson SL. 2000. An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in zebrafish *Danio rerio*. *Development* **127**:3031-44.
10. Traver D, Herbomel P, Patton EE, Murphey RD, Yoder JA, Litman GW, Catic A, Amemiya CT, Zon LI and Trede NS. 2003. The zebrafish as a model organism to study development of the immune system. *Adv Immunol* **81**:253-330.
11. Hanington PC, Tam J, Katzenback BA, Hitchen SJ, Barreda DR and Belosevic M. 2009. Development of macrophages of cyprinid fish. *Dev Comp Immunol* **33**: 411-29.
12. Herbomel P, Thisse B and Thisse C. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* **126**:3735-45.
13. Dzierzak E. 2002. Hematopoietic stem cells and their precursors: developmental diversity and lineage relationships. *Immunol Rev* **187**: 126-38.
14. Shepard JL and Zon LI. 2000. Developmental derivation of embryonic and adult macrophages. *Curr Opin Hematol* 7**(1)**: 3-8.
15. Takahashi K, Naito M and Takeya M. 1996. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. *Pathology International* **46**: 473-85.
16. Takahashi K, Yamamura F and Naito M. 1989. Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J Leukoc Biol* **45(2)**: 87-96.

17. Belosevic M, Hanington PC and Barreda DR. 2006. Development of goldfish macrophages in vitro. *Fish and Shellfish Immunology* **20**: 152-171.
18. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC and Layton JE. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**:3087-3096.
19. Davies LC, Rosas M, Smith PJ, Fraser DJ, Jones SA and Taylor PR. 2011. A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation. *EurJ Immunol* **41**: 2155-2164.
20. Chadzinska M, Kolaczkowska E, Scislowska-Czarnecka A, van Rooijen N and Plytycz B. 2004. Effects of macrophage depletion on peritoneal inflammation in swiss mice, edible frogs and goldfish. *Folia Biologica* **52**:225-231.
21. Cash JL, White GE and Greaves DR. 2009. Zymosan-induced peritonitis as a simple experimental system for the study of inflammation. *Methods Enzymol* **461**:379-396. Chapter Seventeen.
22. Chadzinska M, Leon-Kloosterziel KM, Plytycz B and Verburg-van Kemenade BML. 2008. *In vivo* kinetics of cytokine expression during peritonitis in carp: Evidence for innate and alternative macrophage polarization. *Dev Comp Immunol* **32**:509-518.
23. Serada K, Moritomo T, Teshirogi K, Itou T, Shibashi T, Inoue Y and Nakanishi T. 2005. Comparison of respiratory burst activity of inflammatory neutrophils in ayu (*Plecoglossus altivelis*) and carp (*Cyprinus carpio*). *Fish Shell Immunol* **19**:363-373.
24. Chadzinska M, Leon-Kloosterziel KM, Plytycz B and Verburg-van Kemenade BML. 2008. *In vivo* kinetics of cytokine expression during peritonitis in carp: Evidence for innate and alternative macrophage polarization. *Dev Comp Immunol* **32**:509-518.

25. Hume DA and MacDonald KPA. 2012. Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. *Blood* **119**:1810-1820.