University of Alberta

The analysis of cytokine regulation of macrophage antimicrobial responses of the goldfish (*Carassius auratus* L.)

by

Leon Grayfer

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

Doctor of Philosophy

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences

©Leon Grayfer

Fall 2011
Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

In loving memory of

FAINA BORISOVNA RUMANOVSKI

ABSTRACT

Inflammation is a highly regulated immune response to tissue damage, infiltrating pathogens or both. Cells of the myeloid lineage such as macrophages are indispensable for the initiation, progression and resolution of inflammation. Macrophages possess an armamentarium of antimicrobial responses that are under cytokine regulation. The key inflammatory cytokines have been extensively characterized in mammals and their homologues have been identified across bony fish (teleosts). However, the specific functional roles of these teleost inflammatory cytokines have not been extensively studied.

My doctoral research focused on characterizing the roles of key goldfish (*Carassius auratus* L.) inflammatory cytokines in the regulation of macrophage antimicrobial processes and in host defense against the fish pathogen, *Mycobacterium marinum*. I examined at the molecular and functional levels major goldfish pro-inflammatory cytokines TNFα, IFNγ, IFNγrel, their cognate receptors as well as the anti-inflammatory cytokine, IL-10. I also assessed the inflammatory mechanisms governing the antithesis of antimicrobial defenses and pathogen evasion during *M. marinum* infections of goldfish monocytes and macrophages.

Unlike mammals, goldfish possess two Type II interferons, IFNγ and IFNγ related protein (IFNγrel). I established that zebrafish and goldfish also possess two interferon gamma receptor 1 chains (IFNGR1-1 and IFNGR1-2), where the goldfish rgIFNγ strictly bound rgIFNGR1-2 while rgIFNγrel exclusively engaged rgIFNGR1-1. Furthermore, rgIFNγ and rgIFNγrel elicited distinct antimicrobial

responses and immune gene expression patterns in goldfish monocytes and macrophages. While the recombinant goldfish tumor necrosis factor alpha (rgTNFα) exhibited pro-inflammatory roles attributed to its mammalian counterpart, goldfish TNFα ligands (rgTNFα1 and rgTNFα2) and receptors (rgTNF-R1 and rgTNF-R2) formed dimers and not trimers, as in mammals. In the first functional analysis of a teleost interleukin-10, I demonstrated that rgIL-10 conferred anti-inflammatory properties towards goldfish monocytes. *M. marinum* infections of goldfish monocytes and macrophages ablated their cytokine-induced antimicrobial responses through several mechanisms. Intermittently, infected monocytes and macrophages increased their expression of pro-inflammatory genes while cells activated with rgTNFα2, rgIFNγ, or rgIFNγrel exhibit decreased viability of infecting *M. marinum*. Together, these findings provide an *in vitro* model system for investigating cytokine regulation of monocyte/macrophage antimicrobial responses and mycobacterium-host cell immune interactions of a lower vertebrate species.

ACKNOWLEDGEMENTS

Dr. Midrag Belosevic, I am forever grateful for the supervision and support that you have given me over these years. You are truly one of a kind and I feel incredibly fortunate to have worked under your supervision. You have been a great inspiration and a motivational force during my studies. Thank you for igniting the passion of science in me. I feel that all I am as a scientist, I owe to you. You have always been there to guide me and provide the best advise. You have taught me a great deal about research, community and about myself. Thank you for always giving me the creative freedom to pursue areas that interested me and renewing my enthusiasm towards my own work. I will always value the experiences that I have gained working with you.

To my mother, Rita Grayfer and father, Dmitri Beliavski, thank you for all of your support and encouragement. You have always believed in me an encouraged me to follow my passions in life. I see more and more of you in myself everyday. Thank you for your unwavering support.

To my wife Candace, you have always been extremely understanding and supportive of me. You have been by my side since the beginning of my career in science and you have always been a voice of encouragement in difficult times. I feel that I owe you a great deal and would not have accomplished what I have, without you with me.

To my dear friend Andrew Keith, though you are gone, you are always with me. I am forever grateful for the friendship that we had and I will always hold the memories of you closest to me. Thank you for always being a true friend.

To my PhD committee, candidacy and defense members: Dr. Greg Goss, Dr. Larry Guilbert, Dr. Catherine Field, Dr. Reuben Kaufman, Dr. Declan Ali and Dr. Brian Dixon, thank you for being a part of my doctoral experience and making inspiring in me the sense of scientific community.

To my dear friends and colleagues, Ayoola Oladiran, Barbara Katzenback, Benjamin Montgomery, Caleb Grey, Cheryl Miller, Dr. Daniel Barreda, Jacqueline Mewes-Ares, Dr. James Stafford, Jaswinder Toor, Jennifer Kerr, Jill Metcalf, Dr. John Walsh, Jonathan Pun, Jordan Hodgkinson, Johnathan Ussher, Karlijn Verheijen, Kristina Petkau, Dr. Patrick Hanington, Matt Polack, Mariel Hagen, Scott Mitchell, Stanislav Baichev, Steven Hitchen and Travis Franko, thank you for your continued friendship and support.

To Alex Royblat, thank you for your support and encouragement over the years.

To the past and present Belosevic lab members, I feel that I have learned and benefited from each and every one of you. You have all been inspiring colleagues, great friends and a source of encouragement.

To Dr. Allen Shostak, thank you for your support and constructive advise throughout my time in the lab. You have always been a source of encouragement and advise.

To collaborators, Dr. John Chang, Caleb Grey, Dr. Andrew Waskiewicz, Laura Pillay, Dr. Daniel Barreda, Aja Rieger, Dr. Martin Olivier, Issa Abu-Dayyeh, Dr. Teruyuki Nakanishi, Yasuhiro Shibasaki, I am truly thankful for having had the opportunity to work with and learn a great deal from you.

To everyone in the Biological Sciences Department, I feel very fortunate to have done my doctoral work here. I take away with me many fond memories, amazing experiences and great friends.

I would also like to acknowledge the financial support provided by Natural Sciences and Engineering Research Council of Canada (NSERC; PGSD Scholarship), the Department of Biological Sciences (Teaching Assistantships and Dr Richard E (Dick) Peter Memorial Graduate Scholarship) and the Faculty of Graduate Studies (Andrew Stuart Memorial Graduate Scholarship).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α:	alpha/anti
Ab:	antibody
ACAMP	apoptotic cell associated molecular pattern(s)
AIM:	absence in melanoma
ALR	absence in melanoma (AIM)-like receptor
APP:	acute phase protein
BMDM:	bone marrow-derived macrophage
CCM:	cell conditioned medium
cfu:	colony forming units
ConA:	concanavalin A
CRP:	complement-reactive protein
DAMP:	damage associated molecular pattern(s)
DHR:	dihydrorhodamine
DMSO:	dimethyl sulfoxide
ELR:	glutamic acid-leucine-arginine (Glu-Leu-Arg)
EST:	expression sequence tag
FACS:	fluorescence activated cell sorter
FBS:	fetal bovine serum
FPLC:	fast performance liquid chromatography
GAS:	gamma-IFN activated sequences
G-CSF:	granulocyte-colony stimulating factor
GPI:	glycophosphatidylinositol
HKC:	head kidney cells
HSP:	heat shock protein
ICAM:	Intracellular adhesion molecule
ICE:	Interleukin-1β-converting enzyme (caspace-1)
IFN:	interferon
IFNAR:	interferon alpha receptor
IFNGR:	interferon gamma receptor
IL:	interleukin
iNOS:	inducible nitric oxide synthase
IPTG:	isopropyl β-D-thiogalactoside
IRAK:	IL-1R associated kinase
IRF:	interferon regulatory factor
ISGF:	interferon stimulated gene factor
IVDKM:	in vitro derived kidney macrophages
Jak	janus activated kinase
kDa:	kilodultans
LB:	Luria-Bertani
LPS:	lipopolysacharide
LRR:	Leucine-rich repeats
MAC:	membrane attack complex
MAF:	macrophage activating factors
MASP:	mannose-associated serine protease

MBP:	mannose binding protein
MCP:	monocyte chemotactic protein
M-CSF:	macrophage-colony stimulating factor
MDF:	macrophage deactivating factor
MIP:	macrophage inflammatory protein
MLR:	mixed leukocyte reaction
MMP:	matrix metalloproteinase
NBT	nitro blue tetrazolium
NET:	neutrophil extracellular traps
NFkB:	nuclear factor kappa B
NiNTA:	nickel-nitrilotriacetic acid
NLR:	nucleotide-binding domain-leucine-rich repeat containing receptors
NLS:	nuclear localization signal
NO:	nitric oxide
NOD:	nucleotide oligomerization domain
NRAMP:	natural resistance associated macrophage protein
O.D.:	optical density
PAMP:	pathogen associated molecular pattern(s)
PBLs:	peripheral blood leukocytes
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEG:	polyethelyne glycol
PGN:	peptidoglycan
PHA:	phytohaemagglutinin
PKM:	primary kidney macrophages
PMA:	phorbol myristate acetate
poly(I:C):	polyinosinic:polycytidylic acid
PRR:	pattern recognition receptor
Q-PCR:	quantitative-polymerase chain reaction
Ra:	receptor antagonist
RACE:	rapid amplification of cDNA ends
RCA:	regulator of complement activation
rg:	recombinant goldfish
RIG:	retinoic acid inducible gene
RLR:	retinoic acid inducible gene-like receptor
RNI:	reactive nitrogen intermediates
ROI:	reactive oxygen intermediates
RT-PCR	reverse transcriptase polymerase chain reaction
SA:	serum amyloid
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS:	suppressor of cytokine signaling
SR:	scavenger receptor
SSH:	selective subtractive hybridization
Stat:	signal transducer and activator of transcription
TACE	tumor necrosis factor alpha converting enzyme

TGF	transforming growth factor
THD	tumor necrosis factor homology domain
TIR:	toll/interleukin-1 receptor (TIR) domain
TLR:	toll-like receptor
TNF:	tumor necrosis factor
TNF-R:	tumor necrosis factor receptor
VCAM:	vascular cell adhesion molecule

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

The field of comparative immunology largely focuses on research dealing with comparing/contrasting the immune mechanisms of lower vertebrates and invertebrates to those of higher vertebrates. A significant branch of this research area focuses on the immune responses of bony fishes (teleosts). The teleosts represent one of the lowest vertebrate classes to exhibit functional innate and adaptive immune components akin to those of mammals, thus allowing for assessment of the evolution of immune mechanisms of the metazoa. To date, the majority of studies of teleost immune responses have focused on gene identification and relatively little on the mechanisms of immunity in host-pathogen interactions.

The innate immune response is of central importance for host defense in lower vertebrates such as fish. The major component of innate immunity is the process called inflammation. During my thesis work I cloned a number of fish pro-inflammatory cytokines and their receptors. Amongst these are interferon-gamma and interferon-gamma-related (IFNγ and IFNγrel, respectively), IFNγ receptors (IFNGR1-1 and IFNGR1-2), tumor necrosis factor alpha (isoforms TNFα1 and TNFα2) and TNFα receptors (TNF-R1 and TNF-R2). Additionally, I have identified and cloned the anti-inflammatory cytokine, interleukin-10 (IL-10). I have examined the inflammatory roles of recombinant goldfish (rg) IFNγ, rgIFNγrel, rgTNFα and rgIL-10. I have also functionally characterized goldfish

TNF-R1 and TNF-R2 and IFNGR1-1 and IFNGR1-2. The central focus of my thesis research was to elucidate the cytokine regulation of the inflammatory response in the goldfish, with specific focus on macrophage antimicrobial functions.

2. THESIS OBJECTIVES

The main objective of my research was to characterize the key innate immune mediators and examine their role in host defense of goldfish (*Carassius auratus* L.) against *Mycobacterium marinum*. The specific aims of my research were: (1) To characterize at the molecular and functional levels major goldfish pro-inflammatory cytokines TNFα, IFNγ, IFNγrel and their cognate receptors as well as the anti-inflammatory cytokine, IL-10; and (2) to examine the mechanisms of cytokine-induced macrophage antimicrobial responses against *Mycobacterium marinum*.

2.1. Outline of thesis

In the first chapter, I review the current literature pertaining to macrophage-mediated inflammatory responses in mammals and fish. Specifically, this chapter outlines the fundamental steps involved in the inflammatory responses of mammals and expands on the current understanding of these respective mechanisms in fish species. Chapter II represents a detailed description of the Materials and Methods employed throught this thesis. In Chapter III of this thesis, I describe the identification, characterization and

functional analysis of the goldfish pro-inflammatory cytokine, tumor necrosis factor alpha (isoform 2) and in Chapter IV I describe the identification and the characterization of the cognate goldfish TNFα receptors, TNF-R1 and TNF-R2. Chapter V details the identification, characterization and the functional assessment of the goldfish inflammatory mediator, interferon gamma (one of the two type II IFNs of the goldfish); Chapter VI describes the identification and the characterization of two distinct IFNγ receptors isoforms in both zebrafish and the goldfish; while Chapter VII recounts a comprehensive functional comparison of the two type II IFNs of the goldfish, IFNγ and IFNγ related. Chapter VIII details the identification, characterization and functional analysis of the anti-inflammatory goldfish cytokine, IL-10. Chapter IX represents a comprehensive assessement of the respective immune roles of the key goldfish inflammatory mediators (characterized in Chapters III-VIII) in the context of *in vitro* goldfish macrophage infections with the natural fish/amphibian pathogen, *Mycobacterium marinum*. Finally, in Chapter X, I provide an overview of my findings and describe how these findings relate to the current understanding of the inflammatory processes of bony fish; suggest future directions for the described research and propose potential evolutionary origins of the fundamental regulation and effector mechanisms of inflammation. In this chapter I also contend that the goldfish kidney-derived monocyte and macrophage cultures combined with my thesis findings pertaing to the cytokine-regulation of the antimicrobial mechansism of these cells and my observations regading the effects of *Mycobacterial* infections of goldfish monocytes/macrophages on these

antimicrobial pathways, provide an ideal natural host cell modle system for furthering the understanding *M. marinum*-immune cell interface.

3. LITERATURE REVIEW: CYTOKINE REGULATION OF THE INFLAMMATORY RESPONSES OF BONY FISH

3.1. Introduction

The inflammatory response is a highly regulated process initiated by tissue damage, infiltrating pathogens or both. The primary role of inflammation is the resolution of tissue damage, including the elimination of damaged or dead cells and any infiltrating pathogens and restoration of homeostasis. The initial recognition of tissue damage and/or pathogens is mediated by tissue resident macrophages primarily through various sentinel pattern recognition receptors such as toll-like receptors (discussed below). In response to and in accordance with distinct stimuli, macrophages become activated to produce a wide range of bioactive molecules some of which attract other cells to the site of inflammation and others that dictate the course of an inflammatory response and eventual tissue repair. Recent evidence suggests that there are at least two activation states of monocytes/ macrophages (13, 303, 512, 516). The classically activated monocytes/macrophages possess significant antimicrobial armamentarium, secrete a plethora of factors that propagate and enhance the microbicidal activities and in general mediate pathogen clearance. The non-classically or alternatively activated monocytes/macrophages secrete factors that ablate the destructive components of the inflammatory response and promote tissue healing, repair and

angiogenesis. The processes involved in the onset, progression and resolution of inflammation are highly complex and remain to be fully elucidated. However, it is widely believed that this inflammatory process can be subdivided into four phases: neutrophil egress, monocyte recruitment, termination of neutrophil recruitment, and return to homeostasis.

3.2. Phase I of inflammation: resident macrophage recruitment of neutrophilic granulocytes

3.2.1. Recognition of immune insult

During the initial onset of inflammation, resident macrophages at the sites of injury detect tissue damage and/or infiltrating pathogens through sentinel pattern recognition receptors. This process is tightly regulated and the resulting immune response is orchestrated in accordance with the form of perturbation sensed by these cells. The existence of immune pattern recognition receptors (PRRs) was first proposed by Dr. Charles Janeway over 20 years ago (196). Since then a great deal of insight has been gained into immune recognition of pathogen associated molecular patterns (PAMPs) via these PRRs. Currently, the known PRRs can be subdivided based on structure and function into five groups: toll-like receptors (TLRs), C-type lectins, retinoic acid inducible gene 1 (RIG1)-like receptors (RLRs), nucleotide-binding domain-leucine-rich repeat containing receptors (NLRs or NOD-like), and absence in melanoma (AIM)-like receptors (ALRs) [reviewed in reference (166)]. These TLRs, RLRs, NLRs and ALRs are expressed predominantly on immune cells, including neutrophils, monocytes,

macrophages, dendritic cells and also on specific epithelial and endothelial cells (166).

In addition to specializing in the recognition of distinct pathogenic components (LPS, dsRNA, flagelin, GPI anchors etc.), some of these PRRs also detect damage associated molecular patterns (DAMP) associated with tissue damage. The recognition of DAMPs is less understood, but modified-self components such as necrotic cells, disrupted cell membranes and chromatin components are amongst the postulated ligands for these receptors (220, 393, 501).

Presumably in anticipation of the potential source of PAMP/DAMP contact, the cellular localization of these PRRs varies accordingly. Of the 10 human TLRs, TLR 1,2,4,5,6 and 10 are membrane bound while the TLRs 3,7,8 and 9 are located in endosomes. (333). In contrast the NLRs, RLRs and ALRs, which have evolved to respond to viral infections, exhibit exclusively cytosolic localization (333).

3.2.2. Pattern recognition receptors of bony fish

The Toll-like receptor family is the most thoroughly studied PRR family, representing an ancient mechanism of pattern recognition with a common ancestral gene/protein predating the plant and animal split. Members of the TLR family share the intracellular toll-interleukin-1 receptor (TIR) motifs (31). Initially identified in *Drosophila* spp. for anti-fungal properties (244), members of this family are now widely believed to be indispensable immune components in

most metazoans. Humans are currently known to have 10 TLRs (TLR 1-10) while mice possess 12, some of which are shared with man (TLR1-9), while others (TLR 11-13) recognizing unique PAMPs (166). Birds also possess 10 TLRs of which some are counterparts of the mammalian receptors (TLR 3-5, 7 and 2 forms of each TLR 1 and 2) (208), while others (TLR15, 16, 21) are not found in higher vertebrates (458). Amphibian species are predicted to possess around 20 TLRs, with multiple isoforms of TLR2, 6, 8 and 14 (192). Bony fish possess 17 distinct TLRs including gene orthologs of most mammalian, avian and amphibian TLRs as well as TLRs unique to fish (TLR 20-23) (181, 325, 366). Interestingly, while certain fish species possess TLR4 homologues, the mammalian counterpart of which recognizes bacterial lipopolysaccharide (LPS), there is no TLR4 in *fugu*, *tetraodon* or stickleback (166). Furthermore, it has been demonstrated that the zebrafish TLR4 does not recognize LPS and negatively regulates NFkB signaling (405, 441). This implies that the other lower vertebrate orthologs of the mammalian TLRs might not necessarily recognize the same components as their higher vertebrate counterparts. Further research into these sentinel receptors in fish, amphibian and bird species will likely shed new light on the evolutionary history of these important innate immune receptors.

Other PRR families have also been traced down to lower vertebrates. Gene synteny analysis led to the identification of RLRs in avian and teleost species (18, 518). However, investigations failed to identify RLR genes in select fish species, suggesting that these genes are either absent or are otherwise diverged beyond recognition. Functional confirmations of teleost RLRs,

supporting conserved recognition roles have been published (32, 436). Also, many orthologs of mammalian NLRs as well as a unique NLR subfamily of receptors appear to be present in bony fish (235).

3.2.3. Neutrophil mobilization

Upon receiving the "danger" signal through PRRs, resident macrophages initiate chemoattraction of neutrophils to localized inflammatory sites (132). In fact macrophage-produce neutrophil chemoattractants are pivotal during inflammatory neutrophil recruitment (22, 51, 52). At this stage in the inflammatory response, the chemokine profiles are geared strictly towards angiogenesis and neutrophil attraction and as such predominantly bear the CXC and Glu-Leu-Arg (ELR) motifs, characteristic of neutrophil chemoattractants (434). The key mammalian CXC chemokines involved in neutrophil recruitment include CXCL1, CXCL5 and CXCL8 (3, 22, 52, 459).

3.2.4. CXC-motif chemokines of bony fish

Several CXC motif chemokines have been identified and characterized in teleost fish. The trout and carp CXC homologs of the mammalian IFNγ-inducible chemokines CXCL9, 10 and 11 were identified (232, 389). A zebrafish CXCL14-like molecule was also reported (260). Interestingly while the central neutrophil chemoattractive chemokine, CXCL8 (IL-8), has been identified in numerous fish species (78, 183, 237, 239), with the exception of the haddock CXCL8, all of these lack the ELR motif. Despite this, the fish CXCL8 have been demonstrated

to be chemoattractive to fish neutrophils (170, 514). In fact, it would appear that certain fish species such as carp possess two lineages of CXCL8 chemokines (one of which is more closely related to the mammalian CXCL8) that display distinct expression patterns and yet are both chemotactic to fish phagocytes (470). Interestingly, salmonids such as trout appear to possess at least four copies of the IL-8 gene (118)

3.2.5. Production, storage, release and extravasation of mammalian granulocytes

In mammals, there are large storage pools of neutrophils in the bone marrow, retained there by the stromal cell-produced CXC chemokines (35, 122, 275). Shortly after an inflammatory challenge, there is a spike in blood levels of mediators such as leukotriene B4, C5a (complement component) and CXCL8, which lead to rapid mobilization of these neutrophil pools through the sinusoidal endothelium of the bone marrow into the blood (194, 195, 459). Granulocyte-colony stimulating factor (G-CSF) cytokine maintains granulopoiesis and neutrophil steady state mobilization during homeostasis (253, 403), and stimulates neurophil mobilization into the blood during an inflammatory response, while other soluble mediators navigate these cells to desired sites (121, 122).

In response to inflammatory mediators, endothelial cells up-regulate the expression of the adhesion molecules P-selectin (CD62P) and intracellular adhesion molecule (ICAM1 and ICAM2), which are bound by neutrophil surface β-integrins, promoting adhesion and egress [reviewed in (248)]. Presumably in

order to facilitate rapid neutrophil mobilization, the sinusoidal endothelium cells of the bone marrow constitutively express such adhesion molecules, favoring neutrophil adhesion and extravasation (399, 414).

3.2.6. Production, storage, release and extravasation of bony fish granulocytes

The kidney is the major hematopoietic organ of bony fish. Granulopoiesis in fish kidney, including the formation of the major neutrophilic cell differentiation stages and the presence of nucleated, azurophilic and specific granules in mature kidney granulocytes was observed over 20 years ago (288). Since then it has been well established that in all respects, the teleost kidney serves as a reservoir of mature neutrophilic granulocytes akin to the mammalian bone marrow (62, 165, 347). The kidney neutrophil populations are readily mobilized following bacterial challenges (218) or intraperitoneal injections of thioglycolate (33). Furthermore, these cells appear to be fully functional immediately upon harvesting. Kidney neutrophils of fish display reactive oxygen-dependent tumoricidal activities (230), degranulation, myloperoxidase activity and release of neutrophil extracellular traps (NETs) (329). These cells readily upregulate ROI and phagocytosis in response to recombinant cytokine stimulation (149) and are chemoattracted to CXC motif chemokines (170, 183, 514).

The growth factor that is required for granulocyte development is granulocyte-colony stimulating factor (G-CSF). Relatively little is known about

the fish G-CSF. Low identity G-CSF sequences of Japanese flounder, fugu and green-spotted puffer fish have been identified (387). It is believed that this immune gene has been subject to rapid and very selective evolutionary pressures, manifested in low identities of respective G-CSF gene and protein sequences across vertebrate species. Interestingly, duplicated ancestral G-CSF paralogs are present in the genomes of fugu and black rockfish (307, 387), suggesting that possibly distinct evolutionary pressures caused the retention of both gene duplicates. The increased expression of the fish G-CSF in kidneys and PBLs following stimulation with LPS or mitogens (387) suggests retained inflammatory roles of this molecule. Furthermore, in an elegant set of studies it was demonstrated that the zebrafish G-CSF/G-CSF receptors signaling pathway is indispensible in orchestrating the hematopoiesis of several distinct myeloid cell lineages under homeostatic and immune stimulus conditions (255). Additionally, the zebrafish G-CSF signaling pathways appear to orchestrate the early migration of embryonic myloid cells (255). Further research of the teleost G-CSF will establish the exact functions of this primordial growth factor.

3.3. Phase II of inflammation: Recruitment of inflammatory monocytes

3.3.1. Recruitment of monocyte to site of inflammation in mammals

Upon extravasation, neutrophils secrete various factors that attract monocytes to sites of inflammation. Neutrophils release preformed granules and secretory vesicles while passing through the vasculature (43, 107). The content of these include various antimicrobial factors and agents that propagate the

inflammatory response by promoting extravascular migration of other inflammatory cells by causing an increase in vascular permeability and enhancing the immune cell migration to the inflammatory sites (27, 133, 175, 421, 422). For example, neutrophils secrete cationic protein azuricidin, which not only serves as an antimicrobial agent but also induces increased monocyte vascular adhesion and endothelial transmigration to the inflamed sites (240).

The monocyte recruitment is further facilitated by a shift in the profiles of adhesion molecules expressed by endothelial cells, at this point favoring monocyte rather than neutrophil adhesion/extravastaion events. For example, neutrophils secrete bioactive molecules that elicit the expression of E-selectins (CD62E) and vascular cell adhesion molecule 1 (VCAM-1/CD106), which are bound by monocyte β1 integrins, promoting adhesion and extravasation of these cells [reviewed in (248)].

In favor of monocyte/macrophage recruitment, at this point in the inflammatory response, the profiles of produced chemokine shift from CXCL to CCL-types. The plethora of neutrophil-released bioactive molecules activate local endothelium to produce CC-motif chemokines such as CCL2 (372, 448) while systemic azurocidin up-regulates monocyte/macrophage receptors for the CCL chemokines (328). Furthermore, in response to specific inflammatory stimuli such as IFNγ, neutrophils shift from producing CXCL8 to secreting CCL3 and CCL4 (205, 206), while neutrophil-secreted proteolytic enzymes enhance the chemotactic potentials of monocyte-specific chemokines as much as 1000 fold (28).

3.3.2. Recruitment of monocytes to sites of inflammation in bony fish

Teleosts appear to exhibit waves of neutrophil and subsequent monocyte inflammatory recruitment akin to those described for mammals. In a recent study it was elegantly demonstrated that upon injury-induced inflammation, zebrafish neutrophils are quickly deployed to the inflammatory sites, with migration peaking 6 hours after the inflammatory stimulus, while optimal monocyte migration occurs 48 hours after stimulus (146). Furthermore, as in mammals, the zebrafish monocyte recruitment appears to also be facilitated by endothelial contact events, since the enhancement of monocyte/endothelium interactions results in faster monocyte recruitment (146).

3.3.3. CC-motif chemokines

In mammals the CC-motif chemokines can be functionally subdivided into inflammatory and homeostatic groups. The inflammatory CCL chemokines consist of two main clusters, monocyte chemotactic protein (MCP) and macrophage inflammatory protein (MIP), while the homeostatic CCL chemokines group into small clusters or are otherwise phylogenetically unclustered (296). Chemokines of the MCP and MIP groups are thought to have arisen out of tandem duplication events, hence sharing higher identity between chemokines within a given species than between specific chemokines across distinct species. Because of this, phylogenetic studies of these chemokines have been difficult.

It has been suggested that there are at least 26 CC-motif chemokines in teleosts (17), where zebrafish have as many as 40 putative CCL gene loci (338).

Extensive phylogenetic analysis has demonstrated that the fish and other vertebrate CCL chemokines can be subdivided into five distinct groups: MCP group; MIP group; CCL17/22 group; CCL19/21/25 group; a distinct fish CCL group (with no homologs in other vertebrates); a CCL20 group and a CCL27/28 group (338).

3.3.4. Acute phase response

During a rigorous inflammatory response, activated macrophages produce cytokines and oxidative radicals that induce and modulate the production of acute phase proteins (APPs) by hepatic cells [reviewed in reference (160)]. These acute phase reactants facilitate several immune functions including pathogen opsonization, trapping of invading microorganisms or their products, activation of complement, neutralization of enzymes, and scavenging of free hemoglobin and radicals, and modulating the host inflammatory response. The exact constituents of the acute phase response vary across mammalian species, but generally they can be categorized into positive and negative acute phase reactants (proteins), depending on whether their relative blood levels are increased or decreased during an inflammatory response.

Upon hepatic cell activation there is a rapid increase of 5 to 1000 fold levels of blood C-reactive protein (CRP), a 50% increase in blood ceruloplasmin and complement C3, 2-3 fold increases in hemoglobins, fibrinogen, alpha-globulin and LPS binding protein. Conversely, hepatic cell activation leads to a general decrease in serum levels of zinc, iron, albumin, transferrin, cortisol-

bindind globulin, retinol (vitamin A)-binding protein and transthyretin (187). Because the majority of these proteins serve as hormone binding molecules, the decrease in their levels causes increased availability of free hormones in the blood (187).

While viral infections induce modest acute phase responses (213), bacterial infections elicit potent production of these soluble mediators (160, 171, 172, 472). Upon recognition of bacterial components such as LPS, monocytes and macrophages also produce gratuitous amounts of TNFα, IL-6 and IL-1β, which exacerbate this response (59, 171, 172, 238, 402). Feedback mechanisms governing the termination of APP production are also largely dependent on cytokines produced by macrophages. For example, when Kupffer cells (liver macrophages) are stimulated with IL-6 during the onset of the acute phase response, they down-regulate their production of TNFα and IL-1β, thus decreasing the autocrine hepatic cell stimulation by these cytokines (395). Furtermore, the Kupffer cells begin to produce IL-10, which in turn suppresses the local production of IL-6, further ablating hepatic cell stimulation and APP production (216).

3.3.5. Acute phase reactants of bony fish

Bony fish species appear to possess a fully functional repertoire of acute phase reactants, including many of the mammalian APPs as well as additional reactants unique to fish species. The serum-CRP levels of salmonid fish have been used as a measure of fish stress in response to water pollutants and

chemicals (137, 256, 493). It has also been demonstrated that the trout CRP is capable of activating complement (306). A serum amyloid-A (SAA) and a serum amyloid P-CRP like pentraxin proteins have also been identified in salmonids (199). *Aeromonas salmonicida* infections of salmon caused increased levels of SAA protein (199). The salmon hepatocyte expression of SAA also increases after stimulation of these cells with supernatants from LPS-activated macrophages, or human recombinant TNFα, IL-1β or IL-6, which suggests conserved cytokine-mediated APP regulation mechanisms in this species (200). Interestingly, while LPS stimulations increased the expression of the SAP-like fish pentraxin, *Aeromonas salmonicida* infections causes a decrease in the expression of this gene, suggesting that pentraxin may be a negative APP or conversely exhibiting more ancestral roles outside the acute phase response (199, 268).

A selective subtractive hybridization (SSH) study of hepatic transcripts in unchallenged and bacterially challenged trout confirmed that a fully functional, broad-repertoire acute phase response is in place in this fish species, akin to that present in mammals (20). It has also been documented that following distinct immune stimuli such as bacterial, viral or fungal challenges, trout exhibit overlapping but partially distinct profiles of APP production (136). A well developed acute phase response has also been confirmed in catfish using microarray analysis, where bacterial infections led to greater than 50 fold increases in transcripts of numerous iron homeostatic proteins, coagulation factors, lectins, and most complement components including constituents of the

membrane attack complex as well as certain inhibitors of the complement pathways (337). Furthermore, SSH studies of zebrafish infected with *A. salmonicida* and *Staphylococcus aureus* demonstrated that cyprinids possess overlapping as well as unique APPs to those reported in mammals, and confirmed that the induction and regulation of this fish response is both pattern-recognition and cytokine-dependent (254). Revisiting some of the earlier fish acute phase response work using newer techniques such as recombinant cytokines and morpholino knockdowns will yield a better understanding of this primordial immune mechanism.

3.3.6. Complement

As described above, during the inflammatory response hepatocyte activation by pro-inflammatory cytokines leads to systemic up-regulation of APPs, amongst them complement components [reviewed in references (274, 282)]. Together these factors implement immune recognition and response to exogenous as well as endogenous inflammatory stimuli such as bacterial infiltrations or autoimmunity, respectively. In brief, the complement cascades are triggered by one of three pathways utilizing antibody, or pattern-recognition of inflammatory antigens. The three pathways of complement include: the classical antibody-mediated pathway, where complement components bind to and activate in response to Ab-antigen complexes; the mannose binding-lectin pathway, during which complement activation is propagated following mannose-binding lectins (MBL, structurally related to C1q) association with pathogen surface

carbohydrates; and the alternative complement cascade, which occurs from spontaneous hydrolysis events. Cleaved complement components (C3a, C4a and C5a) are referred to as anaphylatoxins and mediate numerous inflammatory roles including increased mast cell degranulation and histamine release, vascular permeability, leukocyte chemotaxis and extravasation. Additionally complement activation on pathogenic surfaces leads to the formation of the membrane attack complex (MAC) pores that causes lysis of the pathogen.

3.3.7. Complement of bony fish

Most of the mammalian complement components are present in bony fish [reviewed in reference (317)]. In fact in comparison to mammals, birds, amphibians, and teleosts possess a full set of complement genes with the exception of Factor D, some regulators of complement activation (RCAs) and the absence of teleost MASP-1 and MASP-2 (317). Despite this, bony fish express multiple forms of several key complement components including C3 and C5 proteins (207, 376, 443-445).

A number of key fish complement components have been demonstrated to exhibit conserved inflammatory roles akin to mammals. The teleost C5a possesses chemotactic properties (207, 445) while the trout C3a isoforms elicit fish leukocyte ROI and phagocytosis (376, 443). Furthermore, an activated trout serum fraction containing C3a, C4a, and C5a has been shown to have chemoattractive activity for both head kidney leukocytes and PBLs, but only induces phagocytic activity in kidney leukocytes (249). Presumably, both cell

populations possess the receptors for these components, while the PBL cell populations might have lower phagocytic potentials. Alternatively, kidney leukocytes and PBLs may have receptors for the complement chemotactic component(s) while PBLs may lack the receptor(s) required for complement-mediated phagocytosis. The teleost complement biology has been fully addressed in a recent review (442).

3.3.8. Phagocyte antimicrobial functions

The process of cellular endocytosis of large particles (microorganisms, intact cells, macro-molecules or cellular debris) also known as phagocytosis is the primordial defense mechanism of all metazoan organisms. During the inflammatory response phagocytes such as neutrophils, monocytes and macrophages undergo phagocytosis either through the engagement of a plethora of phagocytic receptors or by means of hydrophobic interactions of the phagocyte membrane and the target particles. In addition to orchestrating the release of numerous inflammatory components, phagocytes are equipped with an armamentarium of antimicrobial responses deployed during an inflammatory response. Upon engulfment of foreign organisms, phagocytes release a slate of potent pre-formed antimicrobial molecules into the pathogen-enclosed phagolysosomes. Among these numerous compounds are degradative enzymes (proteases, nucleases, phosphatases, esterases, and lipases) and antimicrobial peptides (neutrophilic peptides and basic proteins), which aid in the destruction of the phagocytosed pathogens (4, 5, 42, 127, 204, 424).

Through numerous mechanisms including cytokine stimulation, pathogen pattern recognition as well as after particle uptake, phagocytes are activated to produce several cytotoxic antimicrobial components, amongst them reactive oxygen (ROI) and reactive nitrogen (RNI/NO) intermediates.

Monocytes, macrophages and neutrophils are capable of undergoing respiratory bursts or the production of ROIs. The multi-component enzyme responsible for ROI production and referred to as NADPH oxidase assembles on inner surfaces of phagocyte plasma membranes in response to immune stimuli (45). This complex comprises of the perpetually membrane bound $gp91^{phox}$ and $p22^{phox}$ subunits (334-336) as well as the cytosolic $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits (245, 464, 465, 478), which are recruited to the cell membrane following immune stimuli (such as cytokine priming, Fig. 1.1). The activation of the NADPH oxidase complex leads to the formation of the superoxide anion (O_2^-), which via spontaneous or enzyme-catalyzed pathways is converted into an array of reactive oxygen species including hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), hypochlorous acid (OCl^-) and peroxynitrite ($ONOO^-$) (43, 190, 407). Neutrophils and monocytes are the most potent producers of ROI, although as monocytes differentiate into mature macrophages, they gradually lose the capacity to undergo this response (57). For a more detailed review of NADPH oxidase and its products please refer to the following excellent contributions (293, 368, 369, 407).

3.3.9. Antimicrobial responses of bony fish phagocytes

It has been well established that fish phagocytes possess the oxidative burst response, comparable to that of mammals. Due to the absence of readily available fish cytokines, early research into the production of reactive radicals relied on cell stimulation with pathogen products and/or crude supernatants from activated cells, presumed to contain hallmark inflammatory agents. This work set up the foundation for current studies of fish phagocyte-mediated inflammatory processes and has been comprehensively reviewed (315). Since then the specific genes encoding the components of the fish NADPH oxidase complex have been cloned in various fish species (41, 189, 285) and their expression has been correlated with reactive oxygen radical production (41, 150, 322). The priming of the fish phagocyte ROI response by recombinant fish cytokines such as TNFα (153, 309, 521), IFNγ (149, 150, 517) and IL-1β (124, 221, 339) has also been reported. Similar to the mammalian monocyte/macrophage paradigm (57), fish monocytes have a greater capacity to produce ROIs following a short stimulation with activating agents (153, 313, 364), whereas mature fish macrophages require relatively prolonged immune stimulations to achieve comparable magnitude of this response (143, 144, 313, 401).

As described above, in addition reactive oxygen production, phagocytes (primarily mature macrophages) respond to appropriate immune stimuli by producing microbicidal/tumoricidal reactive nitrogen intermediates. The enzyme that catalyzes the conversion of arginine to citruline is called the inducible nitric oxide synthase (iNOS, Fig. 1.2), resulting in the production of nitric oxide (NO)

and other products including nitrite, nitrate, and nitrosamines (193, 310, 435). The NADPH oxidase produced superoxide anion can also react with nitric oxide to form the peroxynitrite intermediate [$ONOO^-$] that also has potent microbiacidal activity (86, 377, 461, 515). The biochemistry and biology of the iNOS enzyme and its product have been reviewed in references (8, 265, 295).

The capability of fish macrophages to produce NO as a microbicidal response has been well established [reviewed in reference (315)]. The iNOS gene transcript has been cloned in several fish species (233, 234, 385, 485) and fish macrophages have been demonstrated to up-regulate the expression of iNOS and produce copious amounts of NO in response to a plethora of immune stimuli (149-153, 191, 202, 321, 380, 428, 510). The inflammatory cytokine regulation of fish iNOS and NO production is described below.

3.4. Phase III of inflammation: Termination of neutrophil-mediated response

3.4.1. Monocyte abrogation of nuetrophil inflammatory responses of mammals

During the inflammatory response there is cooperation between neutrophils and monocytes (413, 421, 423), however as the inflammatory response progresses, various mediators secreted by activated monocytes, macrophages, neutrophils and endothelial cells actively terminate the neutrophil recruitment/persistence (120, 247, 406). A large proportion of these are lipid mediators. For example secretion of prostaglandin E2 and prostaglandin D2

promotes the synthesis of anti-inflammatory mediators and resolution of inflammation (120). Monocyte-produced lipoxins hasten neutrophil apoptosis, decrease their oxidative burst capabilities, NFkB activation and ablate the production of chemokines (e.g. CXCL8) and other cytokines by these cells (71, 117, 201). Additionally, the release of apoptotic cell products such as Annexin1, alters activation states of local monocytes/macrophages and promotes their clearance of apoptotic neutrophils (394), in turn leading to further decreases of neutrophil accumulation (344, 345, 394).

At present, the monocyte and macrophage regulation of neutrophils during inflammation is poorly understood, and active research in this area suggests that this is a highly complex process. For example, in addition to monocyte/macrophage products that clearly antagonize neutrophil recruitment and activity, pro-inflammatory cytokines such as the soluble form of TNFα may prevent neutrophil apoptosis at lower concentrations while greater amounts of the soluble cytokine as well as neutrophil engagement by the membrane bound TNFα actually induce neutrophil programmed cell death (469).

3.4.2. Monocyte abrogation of neutrophil inflammatory responses in teleosts

It is not clear weather similar monocyte-mediated neutrophil modulation occurs during the inflammatory responses of lower vertebrates such as bony fish. As described above, live imaging studies of zebrafish demonstrated temporally distinct recruitment of respective immune population to the inflammatory site (146). However, in a parallel study of mice, frogs and goldfish suffering from

induced peritoneal inflammation, when animals were depleted of resident macrophage populations mice had increased influx and prolonged accumulation of neutrophils, while frogs and goldfish did not exhibit this phenotype (64). This suggests that the regulation of amphibian and teleost inflammatory processes might be distinct from those reported for mammals. Further work will reveal whether the above observations were variable-specific or globally representative of the teleost management of their inflammatory processes.

3.5. Phase IV of inflammation: Return to homeostasis

3.5.1. Clearance of apoptotic cells and debris

Resident macrophages and their secreted products are pivotal to orchestrating the resolution phase of the inflammatory response. The uptake of apoptotic cells by monocytes/macrophages leads to their alternative activation and the production of immunosuppressive mediators and growth factors. For example LPS-stimulated monocytes exposed to apoptotic bodies reduce their TNFα production and in turn secrete the anti-inflammatory mediators IL-10 and TGFβ (104, 105, 477). In fact macrophages express specific phagocytic receptors that specialize in the clearance of apoptotic cells [reviewed in (158)]. Receptors that fall into this category include the proposed receptor(s) for inner membrane component phosphatidylserine, scavenger receptors, integrins and complement CR1 and CR3 receptors (251, 290). Appropriately, the ligands for such receptors have been coined apoptotic cell associated molecular patterns (ACAMPs).

3.5.2. Wound healing

During wound healing, dermal cells secrete proteases and collagenases that degrade the extracellular matrix and facilitate keratinocyte migration from the wound's edge onto the center of the wound (348-351). Activated fibroblasts migrate into the wound and in cooperation with resident macrophages form granulated tissue around the wound area (417, 418). These fibroblasts then differentiate into contractile myofibroblasts that bridge the gap between the wound-edge while growth factors produced by the newly formed granulating tissues promote the proliferation and differentiation of epithelial cells, reforming the epithelial barrier (123-126). Finally, myofibrobalsts, endothelial cells and macrophages that participated in the formation of the granulating tissue apoptose, while the type III collagen at the site is remodeled by metalloproteinases produced by fibroblasts, macrophages, epidermal and endothelial cells and replaced with type I collagen (123-126). Together it would appear that aside form ablating the inflammatory components, the main roles of macrophages in wound repair are to facilitate infiltration of fibroblast into the wound and the induction of fibrosis (241-243).

The processes of tissue repair and wound healing in bony fish are currently not well understood and have not been extensively studied. The specifics of what is currently known on this topic can be accessed in the following reviews (355, 453).

3.6. Cytokine regulation of the inflammatory responses

At distinct times during the onset, progression and resolution of the inflammatory response, different cells at the sites of inflammation secrete a number of immunoregulatory mediators (cytokines, monokines, chemokines) that orchestrate inflammation. Pro-inflammatory cytokines such as TNFα, IFNγ, and IL-1β have the capacity to induce the antimicrobial functions of immune cells and facilitate the pathogen clearance by enhancing various antimicrobial mechanisms. Conversely, cytokines that have been labeled as "anti-inflammatory" such as TGFβ and IL-10 down-regulate the inflammatory response and gear cell functions towards tissue repair mechanisms. It is important to note that in addition to the cytokines indicated above other cytokines, such as chemokines and growth factors, also participate in the regulation of an inflammatory response.

In the last decade, significant steps have been made in enhancing our understanding of inflammatory responses in lower vertebrates, such as bony fish. The genes encoding various teleost cytokines have been cloned and characterized in a number of fish species. Furthermore, the structure of the fish genes encoding these cytokines and often the chromosomal organization of the neighboring genes are in general comparable to the counterpart genes of higher vertebrates although multiple isoforms of a specific cytokine may be present in different fish species. This review will primarily focus on the comparative biology of pro- and anti-inflammatory cytokines (TNFα, IFNγ, IL-1β, TGFβ and IL-10) of bony fish and mammals.

3.6.1. Tumor necrosis factor alpha (TNFα)

3.6.1.1. Mammalian TNFα and TNFα receptors

Tumor necrosis factor alpha is a highly pleiotropic cytokine, and one of the central mediators of the inflammatory processes in vertebrates. This cytokine was initially identified as the serum component that caused "hemorrhagic necrosis" of certain tumors present in bacillus Calmette-Guerin (BCG) primed, endotoxin-treated mice (55). Since its discovery, TNFα has been linked to a plethora of immune-related responses and produced by several cell types including cells of the monocyte/macrophage lineage, polymorphonuclear granulocytes, mast cells, as well as smooth muscle cells (98, 488, 506). The repertoire of functions attributed to this cytokine increases every year and spans a wide range of physiological mechanisms. In the context of the inflammatory response, TNFα has been demonstrated to promote the chemotaxis of neutrophils and monocytes/macrophages (292, 504), enhance phagocytic capacity of myeloid cells (215, 258, 473), prime immune cells for the production of reactive oxygen and reactive nitrogen species (93, 312), as well as serving as a chemoattractant to fibroblasts (396) and promoting the release of platelet activating factor (53, 163, 250).

The mammaian TNFα mediates its biological effects both as a 26 kDa type II trans-membrane protein as well as a 17 kDa soluble moiety, following enzymatic cleavage and release by the metalloproteinase, TNFα cleaving enzyme (TACE) (225, 298, 343). The majority of the biological effects are induced following the engagement of a homotrimerized TNFα (soluble or membrane

bound) to one of two cognate receptors, TNF-R1 or TNF-R2, that in turn trimerize around the ligand (16, 99). Currently, there is no consensus as to the respective contribution of these two receptors to the biological effects caused by TNFα. Some lines of evidence suggest that TNF-R1 propagates the signal from the soluble TNFα, while the membrane-bound TNFα mediates its effects exclusively through TNF-R2 (156). Some reports suggest that the primary outcome of the ligation of TNF-R1 is apoptosis while ligation of TNF-R2 results in proliferation and cell survival (300), while other reports suggest that cooperation between the two receptors is required for specific biological outcomes (490). The prevailing theory is that TNF-R1 is the central receptor involved in propagation the signaling while TNF-R2 plays a minor role in binding and redistribution of the TNFα ligand to TNF-R1 in a process coined "ligand passing" (68, 97, 480). Despite this, accumulating current literature suggests that TNF-R2 is directly involved in many inflammatory processes including the activation of T lymphocytes (211, 212); stimulation of myofibroblasts (460); as well as tumor suppression (513). The TNF-R1 and TNF-R2 utilize extremely complex and largely non-overlapping signaling mechanisms that rely on the recruitment of a plethora of downstream signaling molecules, the relative abundance of which ultimately dictate signaling outcomes [for a current review of TNFα signal transduction please refer to the following (330)]. The relative utilization of respective TNF receptors in signal transduction and the biological outcomes of TNFα stimulation appear to be dependent on cell type and the physiological state of the target cells.

3.6.1.2. Identification of TNFα in teleosts

The presence of an endogenous tumor necrosis factor system in bony fish was first proposed in the early 1990s when it was observed that the human recombinant TNFα induced the production of reactive oxygen intermediates in trout leukocytes that was ablated following the administration of monoclonal anti-TNF-R1 antibody (167, 198). These findings were confirmed by Hirono *et al.* (176) who identified and characterized the first transcript encoding a Japanese flounder TNFα. The flounder TNFα held only 20-30% amino acid sequence identity with the mammalian TNFs, and had very similar intron/exon organization compared to the mammalian TNFs. The expression of this flounder TNFα in fish PBLs was upregulated following LPS, ConA or PMA stimulation, suggesting a conserved role for this cytokine in fish inflammatory responses. Shortly after this discovery, the rainbow trout TNFα cDNA was identified, demonstrated to be constitutively expressed in the gill and kidney tissues and was shown to be upregulated in LPS-stimulated or IL-1β-treated kidney leukocytes and the trout macrophage cell line, RTS11 (236). In the catfish, TNFα was constitutively expressed across tissues of healthy fish and was also expressed in a number of catfish immune cell lines including macrophage (42TA) and T cell lines (G14D, TS32.17) but not in B cell (1G8) or fibroblast (3B11) cell lines (522). The catfish TNFα mRNA levels were upregulated in PBLs, G14D cells, 42TA cells but not in GB11 cells following stimulation with PMA and calcium ionophore. Notably, all fish TNFα proteins had the TNF family signature, [LV]-x-[LIVM]-x_3-G-

[LIVMF]-Y-[LIMVMFY]$_2$-x$_2$-[QEKHL] (236), further underlining the evolutionary conservation of this cytokine.

3.6.1.3. Multiple TNFα isoforms are present in bony fish

The presence of an additional trout TNF isoform was reported (523) and the trout TNFα mRNA transcript stability was shown to be prolonged in head kidney-derived monocytes and macrophages following stimulation with large doses of LPS (272), which is similar to what has been observed for mammalian TNFα. The presence of multiple TNFα isoforms in bony fish was confirmed in common carp, in which first two isoforms were reported (384), followed shortly by a discovery of a third carp TNFα isoform (392). The increased mRNA expression of carp TNF isoforms was demonstrated in head kidney phagocytes stimulated with LPS and after infection of carp with the protozoan blood parasite, *Trypanoplasma borreli* (384). The treatment of carp kidney leukocytes with pentoxyfilline, a selective pharmacological inhibitor of TNFα, inhibited the LPS-induced production of reactive nitrogen intermediates and proliferation of these cells, suggesting a role of the carp TNFα in the induction of these biological functions (384). Additionally, the polymorphism in the gene encoding the carp TNFα2 isoform was linked with fish resistance to *T. borreli* infections in individual carp.

3.6.1.4. Inflammatory roles of the teleost TNFα

The first functional characterization of a fish TNFα was reported in 2003, when Zou *et al.* (521) demonstrated that recombinant trout TNFα1 and TNFα2 (isoforms) both induced the expression of several trout immune genes including IL1β, TNFα1, TNFα2, IL-8 and COX-2 in primary kidney leukocytes and in the RTS11 trout macrophage cell line. This report also demonstrated that both recombinant TNFα isoforms induced dose-dependent chemotaxis of trout kidney leukocytes and enhanced the ability of the cells to phagocytose yeast particles. Additionally, SDS-PAGE analysis of the recombinant TNFα1 and TNFα2 suggested that both of the recombinant molecules existed in monomeric, dimeric as well as trimeric states. Together, these findings indicated highly conserved pro-inflammatory role for teleost TNFα.

The putative mature (cleaved) form of the TNFα of gilthead sea bream was expressed as a recombinant molecule in a prokaryotic expression system and functionally assessed (131). Intraperitoneal administration of this TNFα to fish resulted in rapid recruitment of phagocytic granulocytes to the sites of injection, granulopoeisis and the priming for enhanced ROI by cells from peritoneal cavity and primary kidney leukocytes. Also, size exclusion chromatography of the recombinant sea bream TNFα indicated that this protein existed primarily in a dimeric state (131) in contrast to trimeric state adopted by the mammalian TNFα.

Surprisingly, the same research group subsequently reported that the pro-inflammatory effects of the sea bream and zebrafish TNFα proteins were not direct but resulted from the stimulation of endothelial cells (371). When sea

bream peritoneal exudate cells and head kidney leukocytes were primed for ROI production with recombinant TNFα (100 ng/mL) or bacterial genomic DNA (*Vibrio anguillarum*, 50 μg/mL), a 16 hour treatment with TNFα elicited a significant response, that was substantially more modest in comparison to the one induced by *V. anguillarum* DNA, leading the authors to conclude that the sea bream TNFα was not an efficient mediator of this particular response. Intraperitoneal injections of the recombinant TNFα resulted in upregulated expression of a panel of pro-inflammatory genes in sea bream peritoneal exudate cells, while *in vitro* TNFα treatment of the sea bream endocardium endothelial cells (EECs) also increased their immune gene expression. Interestingly, the *in vitro* TNFα stimulation of sea bream EECs and macrophages resulted in increased expression of a number of pro-inflammatory genes, with significantly more robust mRNA levels exhibited by the macrophage population. Also, the stimulation of EECs with TNFα (when EECs were subsequently stimulated with LPS/ VaDNA) enhanced lymphocyte and granulocyte adhesion to these cells. While TNFα failed to chemo-attract leukocytes, conditioned medium from TNFα-stimulated EECs and supernatants from TNFα-injected peritoneal exudate cells elicited potent chemo-attraction of sea bream leukocytes. In the same report, the author also demonstrated that the zebrafish TNFα induced neutrophil recruitment while paradoxically at the same time caused an increase in fish susceptibility to bacterial and viral infections. Accordingly, the authors of this work suggested that although the fish TNFα is an inflammatory mediator, its mechanisms of function are to a large extent non-overlapping with the mammalian TNFs.

It is of interest that carp TNFα1 and TNFα2, as well as zebrafish, trout and sea bream TNFα (recombinant) proteins were all shown to confer trypanolytic activity of *Trypanoplasma borreli*, akin to the trypanolytic capacity of mammalian TNFs (116). These authors also elegantly demonstrated that *in vivo*, this lytic activity was attributed to membrane bound rather than the soluble form of TNFα. The supernatants from cardiac endothelial cells stimulated with recombinant TNFα proteins conferred the ability to prime kidney-derived phagocytes for enhanced ROI while neither recombinant carp TNFα1 nor TNFα2 (similar to the sea bream TNFα) were capable of directly enhancing phagocytosis or the production of reactive oxygen and nitrogen intermediates by carp phagocytes. Based on these observations and by drawing parallels to the sea bream TNFα work described above (371), Florenza *et al.* (2009) suggested that while the trypanolyitic roles of TNFα are evolutionarily conserved, the pro-inflammatory mechanisms elicited by this molecule are not.

Contrary to the sea bream and carp work, numerous other reports suggest that like the mammalian cytokine, teleost TNFα directly elicits pro-inflammatory cellular functions. The tilapia non-specific cytotoxic cells (NCCs) that constitutively express membrane bound as well as soluble forms of TNFα are thought to utilize this molecule (in addition to granzymes and Fas ligand) to facilitate cytotoxicity of target cells and when stimulated with recombinant tilapia TNFα, become protected from activation-induced apoptosis (356). While the turbot recombinant TNFα failed to enhance macrophage reactive oxygen production, it effectively elicited *in vitro* NO production and *in vivo* inflammatory

cell recruitment and activation (324). The Ayu fish recombinant TNFα was also shown to induced ROI production by kidney cells (467) and the blue fin tuna recombinant TNFα, isoforms 1 and 2, enhanced the phagocytic response of the tuna PBLs (203).

Zebrafish TNFα-induced signaling was elegantly demonstrated to confer increased resistance to *Mycobacterium marinum* (75). Furthermore, the knockdown of the TNF-R1 in zebrafish led to enhanced mycobacterial disease progression, increased fish mortality, accelerated bacterial growth, granuloma breakdown and necrotic macrophage cell death (75). Thus, it appears that the zebrafish TNFα is pivotal to the maintenance of encapsulated *M. marinum* granulomas and the restriction of the growth of this pathogen. My recent work (Chapter IX) supports these findings, where the pre-treatment of goldfish macrophages with recombinant goldfish TNFα2 ablated the *M. marinum*-mediated down-regulation of NO production by these cells and reduces intracellular bacterial survival.

The TNFα2 is one of two isoforms recently identified and characterized in the goldfish (153). The functional biology of the recombinant TNFα2 was assessed using a primary kidney-derived goldfish macrophage culture system (PKMs, Chapter III) (153). The recombinant TNFα2 induced a dose-dependent chemotaxis of goldfish macrophages and enhanced their ability to phagocytose particles and produce NO. Using a flow cytometery-based ROI assay I demonstrated that the recombinant TNFα2 effectively primed the ROI responses

of goldfish PKMs and that the majority of this ROI production was due to the monocyte rather then mature macrophage subpopulations.

The extent of the conservation of the biological functions of the teleost TNFα will become more evident with increased availability of tools, reagents and cell culture systems that at present are lacking. While some reports suggest a lack of conservation in pro-inflammatory roles of teleost TNFα, others strongly implicate recombinant forms of this molecule in enhancing antimicrobial functions of fish inflammatory cells. It seems unlikely that the latter reports are artifacts since the prokaryotic-expressed recombinant TNFα proteins used in these studies are generally purified to a high degree and are assessed for possible contamination by SDS-PAGE and western blotting. Furthermore, LPS contamination is not a probable source of confoundment. It has been well established that fish are extremely insensitive to endotoxin and studies that use LPS as a stimulus resort to high (microgram concentrations) to elicit immune responses in fish cells. It should be noted that the majority of the studies that suggest that fish recombinant TNFα has pro-inflammatory role have used nanogram concentrations of respective recombinants. Possibly, the discrepancies in these findings lie not in the recombinant proteins but rather the cell types used to examine the TNFα functional biology. For example, both the sea bream and carp studies that failed to observe direct effects of fish TNFα on antimicrobial functions such as ROI response, utilized freshly isolated adherent kidney phagocyte populations (116, 371). It has been well established that isolated mammalian myeloid populations are highly dynamic in their respective

capabilities to undergo distinct antimicrobial processes (277). Similarly, in the context of the goldfish kidney derive macrophage system, we have observed that with culture time and the development and maturation of distinct subpopulations, there is a concomitant gain and loss of capabilities of distinct subpopulations to undergo stimulus-induced antimicrobial functions (153, 313). In particular, the production of reactive oxygen intermediates by goldfish PKM cultures appears to be primarily mediated by monocytes rather than mature macrophages, while RNI is predominantly produced by mature macrophages (153, 313).

3.6.1.5. TNFα receptors of bony fish

Tumor necrosis factor alpha has now been identified in a number of fish species including flounder (176), trout (236, 272), catfish (522), carp (384, 392), sea bream (50, 131), tilapia (356), turbot (324), ayu (467), fugu (390), zebrafish (390), sea bass (309), goldfish (153) and tuna (203). There is also a report indicating the existence of an additional novel TNF molecule with unique intron/exon organization in zebrafish and flounder (390). Despite this, information regarding the cognate receptors for the teleost TNF proteins is relatively limited.

A death domain containing TNF receptor has been identified in zebrafish ovarian tissues and has accordingly been coined the ovarian TNF receptor (OTR) (38). Predicted zebrafish TNF-R1 and TNF-R2 sequences are in the NCBI database, with zebrafish TNF-R1 showing extremely high sequence homology to the OTR. The goldfish TNF-R1 and TNF-R2 cDNAs (Chapter IV) were

identified based on these zebrafish sequences (148). The predicted protein sequences of the goldfish TNF-R1 and TNF-R2 are conserved compared to the mammalian TNF receptors. The conserved regions included cysteine residues as well as predicted docking sites for downstream signaling. In addition, goldfish TNF-R1 possesses a conserved death domain including the highly conserved motif (W/E)-X_{31}-L-X_2-W-X_{12}-L-X_3-L (with R in the W/E position) and six conserved or semi-conserved residues that are crucial for the TNF-R1 mediated cytotoxicity (457). Interestingly, while the mammalian TNF-R1 and TNF-R2 as well as the fish TNF-R2 each contain four TNF homology domains (THD, as defined be placement of specific cysteine residues), the known fish TNF-R1 proteins possess only 3 full THDs (148). The expression of both of these receptors in goldfish macrophages changed after treatment *in vitro* with recombinant TNFα2, IFNγ, or TGFβ.

The recombinant forms of the extracellular domains of both goldfish TNF-R1 and TNF-R2 bound to either goldfish recombinant TNFα1 or TNFα2 in *in vitro* binding assays (Chapter IV). As reported for the sea bream TNFα (131), the goldfish recombinant TNFα ligands and receptors 1 and 2 all adopted dimeric conformations and interacted as dimers rather then trimers *in vitro*. Weather this observation is an artifact of the recombinant technology or a true representation of the teleost TNF system remains to be established. However it is worth mentioning that several reports indicated dimerized forms of the mammalian TNF-R1 (304, 305, 342). Furthermore, the mammalian p75/NTR neurotrophin receptor, a member of the TNF superfamily of proteins with many structural similarities to

teleost and mammalian TNF-R1, binds to its cognate dimerized NTR ligand as a dimer (66, 140).

By examining the TNF system in lower vertebrate such as teleost fish, we can gain greater insight into the evolutionary origins of our own immune systems as well as the selective pressures that have molded the higher vertebrate immune processes. As in mammals, TNFα appears to be of central importance to the regulation and maintenance of inflammatory responses of bony fish. Further examination of biological effects of this molecule using different lineages of fish immune cells will yield a more concrete understanding of this system in teleosts.

3.6.2. Interferon-gamma (IFNγ)

3.6.2.1. Mammalian IFNγ and IFNγ receptors

Interferon gamma is an extremely pleiotropic, pro-inflammatory and anti-viral cytokine, initially identified in the supernatants of PHA-activated lymphocyes based on its unique anti-viral properties (491). IFNγ is produced primarily by activated Th1 phenotype CD4+ cells (297), CD8+ cells (383) and natural killer (NK) cells (346). Aside from exhibiting modest antiviral properties IFNγ is a central cytokine that mediates host defense against a plethora of obligate and facultative intracellular pathogen (26, 209, 226, 427, 432). For example, IFNγ gene knock-out mice are incapable of controlling infections with *Leishmania major* (487), *Listeria monocytogenes* (182), and *Mycobacterium* (77), underlining the importance of this cytokine in the regulation of antimicrobial responses (26, 30, 56, 111, 276).

The biological effects exhibited by the mammalian IFNγ are a direct result of its ligation of the interferon gamma receptor 1 (IFNGR1), which subsequently associates with IFNGR2, forming a signaling complex. This complex assembly results in the activation of Janus kinases (Jak) 1 and 2, associated with the receptor chains 1 and 2, respectively (186). The activated Jak1 and Jak2 are then phosphorylated and activate the IFNGR1-associated signal transducer of activation-1 (Stat1) transcription factor (81). The IFNGR ligation may also activate and utilize Stat2 (450) in the downstream signal propagation, albeit to a much lesser extent than Stat1. A subsequent transcriptional regulation of several other genes then ensues through homodimeric Stat1, heterodimeric Stat1:Stat2 as well as through the transcription factor complexes ISGF3 and Stat1-p48, composed of Stat1:Stat2:IRF-9 and Stat1:Stat1:IRF-9, respectively (37, 284, 450, 451). These transcription factors mediate gene regulation by recognition of IFNγ-activated sequences (GAS) in the promoter regions of target genes (451). Within 30 minutes of IFNγ receptor ligation, there is a concomitant increases in the transcript levels of several interferon regulatory factors (IRFs), which then modulate subsequent waves of gene expression in the IFNγ signaling cascade (505).

3.6.2.2. Identification of IFNγ in bony fish

The existence of fish IFNγ was first proposed when supernatants form trout mitogen-simulated leukocytes were demonstrated to possess macrophage activating factor (MAF) activities analogous to the mammalian IFNγ (143, 144).

Based on the fact that type I fish IFNs were known to exist, coupled with the biochemical and functional analyses of these crude MAFs, it was suggested that it was likely that a homolog of the mammalian IFNγ was present in these MAF supernatants. It was additionally established that the downstream signaling factors employed by the mammalian IFNγ (Stats), were also present in fish (382). Specifically, using a monoclonal anti-mammalian Stat 6, a homologous protein was identified in catfish cell lysates and nuclear isolates (382). When purified, this factor showed the capability of binding to the mammalian IFNγ promoter activation site, that when coupled with its nuclear localization, strongly implicated this protein as a Stat transcription factor (382).

The identification of the first fish IFNγ homolog was done by examination of the fugu gene scaffolds (524). Zou *et al.* (2004) reported that the homologs of the mammalian genes that display chromosomal synteny to the IFNγ gene were also present on the same fugu gene scaffold. The fugu IFNγ homolog displayed the 4 exon/3 intron organization similar to its mammalian counterpart and shared 32.3-32.7 % identity with the bird and 34.9-43.3% identity with the mammalian IFNγ sequences, respectively. The trout IFNγ protein sequence shared a low sequence similarity with other vertebrate IFNγ proteins, but possessed the conserved signature motif ([IV]-Q-X-[KQ]-A-X_2-E-[LF]-X_2-[IV]) as well as C-terminal nuclear localization signal (NLS) characteristic of the mammalian IFNγ proteins (517). The expression of the trout IFNγ was inducible in trout head kidney leukocytes following PHA and poly(I:C) stimulations and in the kidney

and spleen tissues of fish injected with poly(I:C), suggesting conserved roles of the fish cytokine in immune response.

3.6.2.3. Inflammatory roles of the teleost IFNγ

The stimulation of the trout monocyte-macrophage cell line, RTS-11, with a recombinant trout IFNγ (rtIFNγ) caused increased expression of the immune genes γIP-10, MHC class II β-chain, and Stat1 (517). The rtIFNγ-induced expression of γIP-10 could be pharmacologically abrogated with ERK (transcription factor) or protein kinase C (PKC) inhibitors as well as by deleting the C-terminal NLS on the rtIFNγ, while rtIFNγ treatment of trout kidney leukocyte significantly enhanced their ROI production. Together, this report strongly suggested that biological mechanisms of the mammalian IFNγ were conserved in fish (517).

The proteomic analysis of the Atlantic salmon head-kidney derived cell line SHK-11 stimulated with rtIFNγ, indicated changes in the panel of proteins including the increased production of the antiviral Mx protein, complement C5-2 component and a proteosome subunit (278). The treatment of the rainbow trout macrophage cell line RTS-11 with rtIFNγ resulted in increased mRNA levels of several immune genes including C-type lectin, IL-1β, IFNγ, TAP-1, tapasin, IRF-1, IkB and JunB. It has also been demonstrated that the recombinant trout IL-15 and rtIFNγ reciprocally induce each other's expression in trout kidney leukocytes (484). Together it would appear that the salmonid IFNγ possesses inflammatory capabilities similar to the mammalian cytokine.

Microinjections of recombinant IFNγ into adult zebrafish failed to induce pro-inflammatory and antiviral gene expression or to protect fish against infections with *Streptococcus iniae* and spring viremia of carp virus (SVCV) (264). This observed lack of stimulation or protection following these IFNγ microinjections could stem from the recombinant IFNγ being bound up by cells expressing only the IFNGR1 without eliciting a detectable level of response from the relatively few cells that would have been expressing the IFNGR2. Thus the results of this study may underline the localized nature of the zebrafish IFNγ function (based on the receptor presence) rather then the efficacy of the induction of antimicrobial functions by this cytokine.

I (149) as well as others (9) have reported on the functional characterization of the cyprinid IFNγ, in the goldfish and common carp species, respectively. I initially identified the cDNA sequence of the goldfish IFNγ (Chapter V) and found that this gene was predominantly expressed in the spleen tissues, isolated splenocytes and PBLs (149). Kidney leukocytes activated by poly(I:C), PHA, or through a mixed leukocyte reaction, up-regulated the expression of the goldfish IFNγ, as did kidney leukocytes, PBLs and splenocytes, following stimulation with recombinant goldfish TNFα2.

A recombinant form of the goldfish IFNγ (rgIFNγ, Chapter V) rgIFNγ primed goldfish monocytes for an enhanced ROI response in a concentration dependent manner (149). In congruence with the mammalian IFNγ (161, 378), I observed that at lower concentrations, the rgIFNγ and rgTNFα2 elicited additive effects on the priming of the ROI responses of goldfish monocytes. Additionally,

I demonstrated that the rgIFNγ effectively enhanced the expression of the ROI enzyme NADPH oxidase catalytic subunits, $p67^{phox}$ and $gp91^{phox}$ (Chapter VII). Similarly, the recombinant carp IFNγ (rcIFNγ) also primed carp kidney phagocytes for enhanced ROI production (9).

The rgIFNγ also induced modest but significant increases in the phagocytic ability of goldfish monocytes and NO production by goldfish mature macrophages while lower doses of rgIFNγ and rgTNFα2 again elicited additive effects with respect to both of these antimicrobial responses (149). In addition, rgIFNγ increased the expression of iNOS gene isoforms A and B in mature macrophage in a time-dependent manner, with iNOS A exhibiting greatest expression following a 12 hour stimulation while iNOS B reaching significant levels of expression after 24 hours of treatment. Conversely, carp kidney phagocytes only displayed significant iNOS gene expression and NO production when treated with a combination of carp recombinant IFNγ and 30 μg/mL LPS, but not following rcIFNγ treatments alone (9). Surprisingly, it was determined that the carp phagocytes expressing the greatest magnitude of iNOS and primarily responsible for the production of reactive nitrogen intermediates were granulocytes (9).

There are several differences between the experimental designs of the above two studies that might account for the differences in the function of IFNγ proteins of these closely related species. First, the description of method for the isolated carp kidney phagocytes describes the presence of 2 fractions: one consisting of 65% macrophages, 10% granulocytes and 25% small macrophages

and lymphocytes and the other fraction consisting of 85% granulocytes and 15% macrophages (9). Thus a mixed population of cells (granulocytes and macrophages) was used to determine the NO response of carp phagocytes. In the goldfish, kidney leukocytes cultured with conditioned medium gain the ability to produce reactive nitrogen species with the development and maturation of macrophage populations, which occurs six to eight days following initial culture of these cells. This is akin to bone-marrow-derived macrophages, which acquire antimicrobial capabilities with culture time. Hence, the ability of the goldfish IFNγ to induce iNOS gene expression and NO production was assessed in six-eight day cultures that were confirmed by flow-cytometery to comprise of primarily mature macrophages (313, 314). Second, pooled carp phagocytes, or individual carp leukocyte fractions were stimulated for 4 or 5 hours (respectively) with the rcIFNγ prior to measuring iNOS expression and for 0, 4, 19, or 25 hours prior to assessment of NO production (9). In the goldfish system, rgIFNγ was shown to induce optimal expression of iNOS A and iNOS B after 12 and 24 , respectively, and the NO response was determined 72 hour after treatment of macrophages with IFNγ (149). In general, it is accepted that while the production of ROIs can be seen as soon as 1 hour following immune stimulation, the production RNIs is first detected approximately 24 hours post stimulation (398). For this reason, most Griess reaction-based NO assays (employed in both of the above studies) usually utilize 48-72 hour incubation times before measuring nitrite levels (an indirect determination of NO production).

The treatment of mature goldfish macrophages with rgIFNγ resulted in increased expression of TNFα1 and TNFα2; IL-1β1 and IL-1β2; IL-12 subunits p35 and p40; IFNγ; IL-8 (CXCL-8); CCL-1; and viperin (an anti-viral molecule) (149). The treatment of carp phagocytes with a combination of carp IFNγ and LPS resulted in increased expression of TNFα; IL-1β; IL-12 subunits p35 and isoforms of IL-12 p40 subunit (9). The carp IFNγ also induced the expression of the CXCL-10 like chemokine, CXCLb, while inhibiting the LPS-induced expression of CXCL-8 isoforms, CXCL-8_L1 and CXCL-8_L2 (9). The latter observations are reminiscent of the relationships of mammalian IFNγ and CXCL-8. While shorter treatments of mammalian granulocytes with IFNγ results in a decreased production of CXCL-8 (58, 205, 287), prolonged treatment with IFNγ increases the CXCL-8 mRNA and CXCL-8 protein production (205). Additionally, mammalian blood monocytes and macrophage cell lines stimulated with IFNγ have been shown to up-regulate the mRNA and protein levels of CXCL-8 (44, 87) which is due to post-transcriptional stabilization of the CXCL-8 mRNAs rather than increases in gene expression (44). Together, these findings suggest that the pro-inflammatory roles of IFNγ, including synergism with LPS, are conserved in cyprinid fish, while it seems probable that the discrepancies in the immune gene expression profiles are a result of employing distinct immune cell model systems (primary kidney phagocytes versus cultured mature macrophages).

3.6.2.4. The two type II IFNs of bony fish

Using gene synteny analysis, Igawa *et al*. (185) discovered two tandem genes encoding IFNγ isoforms, residing next to the fish IL-22 and IL-26 genes. These two IFNγ sequences, coined IFNγ1 and IFNγ2, shared only 17 % amino acid identity with each other, both exhibited exon/intron organization of the mammalian and fugu IFNγ genes. Furthermore, the putative residue sequences of both of these type II IFNs possessed the IFNγ signature motif ([IV]-Q-X-[KQ]-A-X_2-E-[LF]-X_2-[IV]), while only IFNγ but not IFNγrel had a C-terminal nuclear localization signal (NLS). These authors suggested that prior to the teleost split, a duplication event occurred that resulted in the formation of these two IFNγ isoforms.

The existence of two IFNγ isoforms was soon confirmed in siluriformes and other cypriniformes when IFNγ1 and IFNγ2 were identified in the catfish (291) and the common carp (433), respectively. Catfish and carp were both reported to possess two distinct, alternatively spliced transcripts of IFNγ2 as well as a single transcript of IFNγ1 (291, 433). At the protein level, catfish and carp IFNγ1 and IFNγ2 possessed an IFNγ signature sequence, 6 putative helical regions and mRNA instability motifs in their UTRs. Like the zebrafish and fugu IFNγ isoforms, only the IFNγ2 of the latter fish species had the C-terminal NLS squence. The mRNA levels of the catfish IFNγ1 and IFNγ2 genes differed across tissues of healthy fish, but had similar expression pattern in various cell lines and immune cells, with high expression in macrophage, T cell and NK cell lines as well as in mixed leukocyte reaction cultures (291). Additionally, low transcript

levels of IFNγ1 but not IFNγ2 were also reported in a catfish B-cell line, 1G8. Expression of the carp IFNγ2 was shown to increase in carp T lymphocytes following stimulation with PHA, while the mRNA levels of carp IFNγ1 increased in IgM+, B cell enriched immune cell fractions, stimulated with large quantities of LPS (433). Furthermore, increased transcript levels of the carp IFNγ2, but not IFNγ1, were observed in the head kidneys of fish infected with *Trypanoplasma borreli*. Interestingly, the goldfish IFNγ1 and IFNγ2 displayed similar mRNA levels across tissues and immune cell type (150).

In an assessment of the evolutionary history of IFNγ across vertebrates, it was suggested that because IFNγ2 displayed all of the characteristics of the mammalian IFNγ, it should be denoted as simply IFNγ (391). Conversely, because IFNγ1 appears to be structurally related to the classical IFNγ, while lacking some of its characteristics (notably NLS), it should be named IFNγ related, or IFNγrel. Hence there is somewhat of a consensus in the field to now refer to fish IFNγ1 as IFNγrel and to IFNγ2 as IFNγ. For that reason this nomenclature will be adopted for remainder of this article.

Grass carp infected with Reo virus exhibited a long lasting (7 days) up-regulation of mRNA levels of IFNγrel in the spleen (70). The IFNγrel expression was elevated in the blood, head and trunk kidney and spleen following administration of peptidoglycan (PGN), poly(I:C) or LPS, and this expression was observed to parallel that of the PGN receptor, nucleotide oligomerization domain 2 (NOD2), suggesting a possible NOD2-dependent activation mechanism for the carp IFNγrel gene.

3.6.2.5. Functional roles of teleost IFNγrel

While the functional properties of IFNγrel have to date not been examined in siluriformes, there has been some published work addressing this topic in cyprinid fish. The expression of IFNγrel was seen in zebrafish eggs immediately after they were laid, suggesting that this mRNA is maternally supplied because at this stage of development, the zygotic transcription has not yet began (410). The elevated expression of IFNγrel continued into the embryonic development, while the expression of IFNγ was not detected until much later stages of development. Embryos injected with *in vitro* transcribed mRNAs for IFNγ or IFNγrel displayed similar up-regulation in the expression of immune genes, known to be transcriptionally regulated by the mammalian IFNγ. However when both IFNγ and IFNγrel mRNAs were injected, further increases in certain genes were observed, suggesting non-overlapping roles for the respective IFNγ proteins.

The morpholino knock-downs of either IFNγ or IFNγrel alone had negligible effects on embryo survival following *Escherichia coli* infections, the knock-down of both IFNγs resulted in a substantial drop in embryo survival following infection (410). Interestingly, individual morpholino knock-downs of IFNγ or IFNγrel caused a decline in survival rates of embryos infected with *Yersinia ruckeri,* while double knock-downs further decreased embryo survival following infection with this bacterium. Presumably IFNγ and IFNγrel may induce some redundant as well as distinct antimicrobial mechanisms that are comparable in efficacy. Thus, the presence of one of these cytokines may be sufficient in eliciting ample microbicidal functions in dealing with certain

pathogens. It is also noteworthy that while the *E. coli* (strain DH5α) is not a natural fish pathogen, the *Y. ruckeri* has evolved as such and has been demonstrated to persist in fish macrophage phagocytic compartments and prevent phago-lysosomal fusion (381).

I performed a comprehensive functional characterization and direct comparison of the fish type II IFNs, IFNγ and IFNγrel (150). I observed that the recombinant goldfish (rg) IFNγrel and rgIFNγ possessed distinct capacities to mediate specific pro-inflammatory responses of goldfish myeloid cells (Chapter VII). While monocytes stimulated with rgIFNγ exhibited long-lasting ROI priming effects, the rgIFNγrel elicited relatively short-lived priming effects that caused subsequent unresponsiveness to the priming for ROI production by other recombinant cytokines (rgIFNγ or rgTNFα2). As observed previously, rgIFNγ elicited relatively modest phagocytosis and nitric oxide responses in goldfish monocytes and macrophages, respectively (149, 150). In contrast, rgIFNγrel induced significantly greater phagocytosis, higher iNOSA and iNOSB gene expression and nitric oxide production compared to rgIFNγ. Furthermore, rgIFNγ and rgIFNγrel induced different gene expression profiles in goldfish monocytes. Significantly more robust induction of TNFα2, CXCL8 and ceruloplasmin expressions were observed after activation of monocytes with rgIFNγrel as compared to rgIFNγ. The rgIFNγrel incubated with monocytes was more abundant in whole cell lysates compared to rgIFNγ, suggesting a stronger receptor-ligand interaction of the IFNγrel. Interestingly, while both cytokines induced the phosphorylation of Stat1, the nuclear translocation of this

transcription factor was only observed following treatment of monocytes with rgIFNγ (but not with rgIFNγrel). Together, my findings suggested the presence of functional segregation of the goldfish type II interferons at least with respect to relative capabilities of these molecules to elicit antimicrobial functions.

3.6.2.6. Teleost IFNγ receptors

Homologues of the IFNγ have now been identified in a number of bony fish species including zebrafish (185), Japanese pufferfish (524), trout (517), Atlantic salmon (367), catfish (291), common carp (433) and goldfish (149). Despite the growing breadth of work being published regarding the teleost type II IFNs, the subject of the receptor system by which these cytokines signal has largely not been addressed until recently.

As described above, the mammalian IFNγ dimer ligates two chains of the IFNγ receptor 1 (IFNGR1) and this complex further associates with two chains of IFNγ receptor (IFNGR2) to mediate signal transduction primarily through Stat1 but also, to a lesser extent other Stats and interferon regulatory factors (IRFs). The IFNGR1 and IFNGR2 chains were recently identified in the rainbow trout (130). The mammalian chromosomal gene synteny around these receptors was conserved in this fish species and the expression of the IFNGR1 was generally greater then that of IFNGR2, with highest expression observed in the brain of trout and the macrophage cell line RTS-11. A decrease of IFNGR1 expression was seen in the trout fibroblast cell line RTG-2 following stimulation with either rIFNγ or rIL1β while the same treatments increased the expression of the

IFNGR2 in these cells. Furthermore RTG-2 cells transfected with an IFNGR1 construct or CHO (Chinese hamster ovary cell line) cells transfected with constructs expressing IFNGR1 and IFNGR2, both displayed binding of the rtIFNγ. This report also demonstrated that, as in mammals, the expression of the IFNGR2 chain was essential for the IFNγ-induced activity, measured by the ability of rtIFNγ to up-regulate the expression of the γIP antiviral gene. It should be noted that a reliable trout IFNγ reporter cell line has been established and effectively demonstrated to specifically increase luciferase reporter expression following IFNγ stimulation but not in response to a range of other stimuli (60, 61). Therefore the system is in now in place to elucidate the distinct mechanisms and the downstream signaling molecules involved in the salmonid IFNγ signaling and biological processes. Development of tools such as the one described above will be invaluable in conducting true comparative studies of pro-inflammatory mediators of lower vertebrates.

Because the IFNγ and IFNγrel fish molecules possess unique protein sequences (notably the presence of NLS on the former but not latter), distinct expression profiles following immune stimulation and at least partially non-overlapping functions, it was hypothesized that these cytokines might each have distinct cognate receptor(s). When examining chromosomal locations and organization of zebrafish genes that are known to be syntenic to the mammalian IFNγ receptor, I observed that while some of these genes were localized to the chromosome bearing the known zebrafish IFNGR1 gene, others were present on a distinct chromosome (Chapter VI). Through further analysis of the chromosomal

region flanked by these genes, I discovered a second gene, encoding a distinct IFNGR1 protein (147). I denoted these as IFNGR1-1 and IFNGR1-2 and subsequently identified the corresponding goldfish cDNA transcripts of these zebrafish receptor genes. All of the known fish IFNGR1 protein sequences displayed putative Janus kinase (Jak1) and Stat1 binding sites, that are pivotal for the biological functions of the mammalian IFNγ (106, 154, 155). While the zebrafish IFNGR1 isoforms displayed similar tissue expression patterns, the goldfish IFNGR1-1 exhibited substantially greater mRNA levels than the IFNGR1-2 in all tissues and immune cell types examined. Interestingly, relative goldfish tissue expression of the IFNGR1-1 was greatest in the spleen while IFNGR1-2, which is phylogenetically closer to the trout IFNGR1, displayed the greatest expression in the brain, as is the case with the trout IFNGR1 (130). Modest increases in the expression of the goldfish macrophage IFNGR1-1 and IFNGR1-2 were also observed following stimulation with rgTNFα2 and rgIFNγ.

In order to elucidate possible binding partners for the goldfish IFNGR1-1 and IFNGR1-2, recombinant forms of the extracellular domains of these respective molecules were produced and *in vitro* binding assays were performed. My results showed that while IFNGR1-1 bound exclusively to IFNγrel (IFNγ1), IFNGR1-2 bound strictly to IFNγ (IFNγ2, receptors were named after the fact). It has recently been reported that morpholino knockdowns of IFNGR1-1, IFNGR1-2 or a putative IFNGR2 abolished zebrafish IFNγ-induced gene expression (2). In contrast, only the knock-down of IFNGR1-1, but not the knock-down of IFNGR1-2 or IFNGR2, abrogated gene expression induced by IFNγrel. The

authors of this article suggested that IFNγ might signal through a heterodimer of IFNGR1-1 and IFNGR1-2 and a homodimer of IFNGR2 while the IFNγrel would ligate with a homodimeric IFNGR1-1 and as of yet unidentified receptor 2 chains.

During my *in vitro* binding studies of the goldfish recombinant type II IFNs and the IFNGR1 chains, I did not detect an interaction between the rgIFNγ and the rgIFNGR1-1. There are a number of possible explanations for this discrepancy. It is possible that IFNGR1-1 has a much lower affinity for IFNγ and/or requires additional components in order to facilitate the interaction, either of which would manifest as a lack of visible *in vitro* interaction. Alternatively, since the IFNγrel transcript is present in zebrafish embryos immediately after they are laid, one could argue that this cytokine likely has important roles in the development of zebrafish (and presumably of other cyprinids). It is then possible that IFNGR1-1 interacts strictly with IFNγrel and that it has a pivotal in zebrafish immune development, for example in inducing the expression of certain IFNγ signaling components. Under such biological circumstances, a morpholino knock-down of IFNGR1-1 would manifest itself in an abrogation of IFNγ functions.

It would appear that in certain teleost species the receptor signaling systems are in place to facilitate distinct biological outcomes induced by IFNγ and IFNγrel. Furthermore, the presence of the putative Stat1 binding site on the IFNGR1-1 partially explains our observation that goldfish Stat1 is phosphorylated following IFNγrel stimulation, but does not account for its nuclear absence in this response. Possibly, the IFNGR1-1 gene arose as a duplication of an ancestral

IFNGR1 and has subsequently evolved to rely on other signaling mechanisms for function, where the Stat1 phosphorylation is an artifact, remnant of the ancestral gene. Indeed, the importance of the NLS on the C-terminus of the fish (and mammalian) IFNγ has been demonstrated (521) while the lack of this NLS on IFNγrel proteins is the key distinguishing feature of the latter cytokine. The leading model for mammalian IFNγ signaling (439) suggests that following the ligation of IFNγ to its receptor complex, Stat1 is delivered into the nucleus via the IFNγ NLS in a complex consisting of Stat1:IFNGR1:IFNγ. Therefore, one can speculate that due to the lack of this NLS, IFNγrel might have evolved to resort to distinct signaling mechanisms alternative to Stat1 nuclear translocation. The available evidence suggests that certain teleost species (cyprinids) have distinct type II IFN responses. The development of tools such as a cyprinid IFNγ specific reporter cell lines similar to the one described for trout and the elucidation of the involvement of the IFNGR2 chain(s) in cyprinids will be invaluable for elucidating distinct mechanisms by which these respective receptors and ligands operate in teleosts.

3.6.3. Interleukin-1 beta (IL-1β)

3.6.3.1. Interleukin-1 cytokines family

The interleukin-1 family of cytokines is highly complex with new family members being discovered and/or assigned to this family. In addition to the well characterized IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18 the new members of the family now include the IL-1F5-10, and IL-1F11 (85, 462).

Accordingly, it has been proposed that IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) should be renamed IL-1F1, IL-1F2, IL-1F3 and IL-1F4, respectively (416). The exact biological roles of these new IL-1 cytokines (IL-1F5-11) have not been fully elucidated, and it was reported that concentrations of 100-1000 fold greater than those of IL-1β are required to them to induce biological effects (416, 419). These new family members will not be discussed further, instead the remainder of this section will deal with the classical IL-1 cytokines and primarily IL-1β.

3.6.3.2. Mammalian IL-1α, IL-1β and IL-1 receptors

The IL-1α (259) and IL-1β (15) were initially identified as transcripts from distinct monocyte genes. They have only 23% amino acid identitiy, but are structurally similar. Both of these cytokines are produced as leaderless 31 kDa pro-peptides that undergo cleavage events to generate mature 17 kDa cytokines that mediate their biological effects by binding to the IL-1RI (88, 89, 91). The synthesis of IL-1 has been reported in several cell types, including keratinocytes, Langerhan's cells, synovial fibroblasts, mesangial cells, astrocytes, microglia, corneal cells, gingival cells, thymic epithelial cells, in addition to myeloid and some lymphoid cell types, reviewed by Dinarello (89, 91). Although IL-1α and IL-1β signal through the same receptor, they induced different biological functions. It has been suggested that IL-1α is produced primarily by epithelial cells and keratinocytes (294) and is involved in mediating local inflammatory processes and intracellular events. In contrast, IL-1β is synthesized by cells such as monocytes, macrophages, Langerhan's cells and dendritic cells (89), and

mediates systemic inflammatory processes (88). This is corroborated by the fact that while IL-1α may act as a membrane-bound pro-IL-1 through myristoylation of the protein (231, 431), IL-1β requires intracellular processing for activation.

As mentioned, IL-1α and IL-1β are both produced as pro-peptides and while IL-1α can mediate biological effects as pro-IL-1α or as a mature IL-1α following enzymatic cleavage (e.g. with calpain) (217), only the proteolytically cleaved, mature IL-1β, but not the pro-IL-1β can induce biological effects. In fact, the release of IL-1β appears to be a two-step process whereby the first stimulus such myeloid cell encounter with a pathogen results in increased synthesis and cytosolic accumulation of pro-IL-1β, while a second (as of yet poorly defined) stimulus induces the proteolytic processing of pro-IL-1β by caspase-1/IL-1β-converting enzyme (ICE) and the subsequent release of the biologically active mature IL-1β (90). This processing event is further enhanced by the presence of extra-cellular ATP, that binds to the ATP receptor $P2X_7R$, causing an efflux of K^+ and a concomitant activation of ICE (110). A number of mechanisms have been suggested for the release of the mature IL-1β into the extracellular milieu. These include: exocytosis of IL-1β containing lysosomes, through microvesicular budding of the plasma membrane, release in exosomes by fusion of multivesicular bodies with the plasma membrane, export through specific membrane transporters, and cell-lysis-mediated release [reviewed in reference (100)]. Possibly, these mechanisms are mutually exclusive and/or cell type dependent, and further research in multiple model organism and cell types is needed before specific mechanism(s) can be defined.

Both IL-1α and IL-1β bind to IL-1RI, the ligation of which results in recruitment of the IL-1R associated protein (IL-1RAcP), that amplifies the signal transduction (415). The IL-1RI is structurally related to the toll-like pattern recognition receptors and the signal propagation through the IL-1RI utilizes the same downstream signaling components (MyD88, IL-1R associated kinase (IRAK), and NFkB) as those employed in TLR signaling (320). IL-1α and IL-1β also bind to the IL-1RII, but because this receptor lacks the intracellular signaling components of IL-1RI, it functionally serves as a "decoy" receptor by dampening the IL-1α/β signal transduction (76). Additionally, the IL-1 receptor antagonist (IL-1Ra) was reported to also exhibits competitive inhibition of the IL-1α/β signaling by interacting with the IL-1RI without eliciting the downstream signaling events (76).

Unlike most other cyokines and growth factors IL-1α and IL-1β (primarily IL-1β) target nearly every cell type and induces an overwhelming range of biological processes, reviewed by Dinarello (88, 89, 92). The known pro-inflammatory properties of IL-1β include increase of collagen and procollagenase synthesis, increase chondrocyte protease and proteoglycan release, increase in osteoclast-activating factor release and hence bone resorption, induction of the synthesis of lipid mediators such as PGE_2, and enhancement of the proliferation of fibroblasts, keratinocytes, mesangial, glial cells and smooth muscle cells. The IL-1β also induces chemotaxis of T and B lymphocytes, the synthesis of thromboxane by neutrophils and monocytes, basophil histamine release and eosinophil degranulation. Additionally IL-1β induces synthesis of

type I IFNs, endothelial plasminogen activator inhibitors and expression of leukocyte adherence receptors on endothelial cell surfaces. Based on the above it is not surprising that IL-1β has been implicated in numerous disease conditions including cardiac disease (46), rheumatoid arthritis (210), and neurodegenerative diseases (157).

3.6.3.3. Identification of IL-1β in teleosts

Fish were first suspected of possessing an IL-1 homolog when it was observed that the mammalian PBL-derived IL-1 enhanced the proliferation of catfish T-lymphocytes in response ConA (164), and observations that carp epithelial cells (411), carp macrophages and granulocytes (475) and catfish monocytes (102), produced factor(s) with properties similar to the mammalian IL-1. Gel filtration analysis of catfish monocyte revealed two distinct bands of approximately 70 kDa and 15 kDa, recognized by antiserum raised against mammalian IL-1α and IL-1β. The catfish 70 kDa molecule activated catfish (but not mouse) T cells, while the 15 kDa molecule activated mouse (but not catfish) T cells (102).

The first fish IL-1β sequence was identified in trout that had 49-56% amino acid identity to the mammalian IL-1β (400). Interestingly, the trout IL-1β did not possess a putative ICE cleavage site required for the maturation-cleavage of the mammalian IL-1β, while the expression of this trout cytokine could be induced in trout tissues and head kidney leukocytes following LPS stimulation (400, 520).

3.6.3.4. Inflammatory roles of the teleost IL-1β

Using the selective subtractive hybridization technique, a carp IL-1β was identified (119) and the C-terminus of the cytokine produced as a recombinant protein (503). The recombinant carp IL-1β was capable of dimerization and shown to enhance carp antibody response to *Aeromonas hydrophila*, where sera from carp co-injected with the IL-1β and *A. hydrophila* had higher agglutination capacity than the respective controls.

A number of reports have since utilized recombinant technology to investigate the functions of the fish IL-1β. A recombinant form of the mature trout IL-1β (rtIL-1β) was produced by Hong and co-workers (180) and shown to enhance the expression of the MHCII β chain, IL-1β and COX-2 genes in trout head kidney leukocytes and the macrophage cell line, RTS-11. Functionally, rtIL-1β induced the proliferation of head kidney cells and that of a murine cell line, D10.G4.1 that specifically proliferate when treated with mammalian IL-1β (180). Also rtIL-1β enhanced the phagocytosis of yeast particles by trout HKCs (180). Peritoneal injection of trout with rtIL-1β induced migration of trout leukocytes to the site of injection, enhanced the phagocytic potentials of peritoneal cells and increased the systemic expression of IL-1β, COX-2 and lysozyme II (177). The injections of fish with rtIL-1β also enhanced trout resistance to infection by the pathogen *Aeromonas salmonicida*. Additionally, the rtIL-1β and the recombinant sea bass IL-1β were demonstrated to induce Ca^{2+} mediated downstream signaling events that could be abrogated by trypsin-treaments of leukocytes, indicating a requirement for receptor engagement (27). Interestingly, these authors reported

that IL-1β of trout, sea bass and humans were very highly species specific. This observation is in contradiction of early fish IL-1β reports using carp and catfish, where cross-reactivity was observed (27, 102, 164, 411, 475).

The distinct biological roles of individual sub-domains of the trout IL-1β were also examined by generating appropriate peptides (178, 339, 340). While a control scrambled peptide (P2) had no effect, the peptide corresponding to the putative trout IL-1β receptor binding region (P1) had little effect on its own, while a peptide (P3) corresponding to an alternative receptor binding area of the mammalian IL-1β (14, 476), exhibited chemotactic properties towards HKCs alone or (at suboptimal concentrations) in combination with the P1 peptide (340). Intra-peritoneal injections of the rtIL-1β P3 peptide induced peritoneal leukocyte migration and enhanced phagocytosis and reactive oxygen production by these cells. Additionally, the rtIL-1β P3 conferred fish resistance to viral hemorrhagic septicemia virus (VHCV) early after injection. Surprisingly, injection of rtIL-1β P1 caused an increase in *in vivo* expression of the antiviral gene Mx, inhibition of trout TNFα expression and no change in the expression of a panel of other pro-inflammatory genes (178). The injection of the rtIL-1β P3 peptide resulted in a more robust, widespread inflammatory response with increased expression in trout rtIL-1β, IL-8 and lysozyme. This work suggests the fish IL-1β utilizes a very complex receptor-ligand interaction system, perhaps more complicated than that of the mammalian IL-1β.

The functional roles of the trout IL-1β have been corroborated in others fish species. For example, injection of carp with the a plasmid encoding the carp

IL-1β gene caused enhancement of the PHA-induced proliferation of carp lymphocytes, increased carp macrophage reactive oxygen production, enhancement of phagocytosis and resulted in protection against *A. hydophila* challenge (221). A sea bass recombinant IL-1β displayed immuno-adjuvant properties when combined with rsIL-1β in immunization trials against the pathogen, *Vibrio anguillarum*. The sea bass IL-1β also induced the proliferation of the murine IL-1β reporter cell line (D10.G4.1) and sea bass thymocytes, enhanced kidney leukocyte phagocytosis and activated peritoneal macrophages when administered i.p. (47, 48, 72). The orange spotted grouper rIL-1β stimulated the proliferation of grouper head kidney leukocytes, increased the gene expression of IL-1β and COX-2 and was shown to utilize p38 MAPK and Jnk signaling pathways to mediate the changes in gene expression (266). Together, these above findings suggest that the function of IL-1β has been evolutionarily conserved in vertebrates.

3.6.3.5. Multiple IL-1β isoforms are present in teleosts

Because many fish species are tetraploid and have undergone additional genome duplication events, it is not surprising that additional isforms of IL-1β exist in certain fish species. An identified second trout IL-1β, denoted IL-1β2, also has the 6 exon/5 intron organization, 82% amino acid homology to the trout IL-1β1 and no putative ICE cleavage site (354). In the catfish, two IL-1β genes have been described and shown to undergo distinct expression patterns following challenge with *Edwardsiella ictaluri* (486). Transcripts for additional carp IL-1β,

IL-1β2 were identified and found to have 74% identity with the carp IL-1β1 and 95-99% identity between individual IL-1β2 transcripts (103). Since several distinct IL-1β2 sequences were identified in a homozygous individual, it was suggested that there might be multiple IL-1β2 genes. The expression of the carp IL-1β1 and IL-1β2 differed following immune stimuli and the expression of IL-1β1 was found to be at least ten fold greater on average than that of IL-1β2. It was also reported that transcripts of IL-1β2 had a high substitution numbers in the coding regions, including those predicted to be involved in receptor binding. The authors of this report speculated that the IL-1β2 may be a pseudogene, still under transcriptional regulation since a pharmacological NFkB inhibitor abrogated the carp IL-1β2 mRNA expression while stimuli such as LPS could still enhance the transcription of this gene (103). In support of this theory, we have also noted that in the goldfish, a close carp relative, the predicted IL-1β2 protein is truncated compared to the IL-1β1, and while the expression of both IL-1β isoforms may be altered following immune stimuli, certain treatments elicited greater and more robust expression increase of IL-1β1 compared to IL-1β2 (Grayfer *et al.*, unpublished observations)

3.6.3.6. Maturation cleavage of the teleost IL-1β

Despite the fact that the fish IL-1β molecules are functionally similar to their mammalian counterpart, all of the IL-1β fish proteins identified to date lack the ICE cleavage site required for the functional maturation of the mammalian IL-1β1. Despite this, there are some lines of evidence indicating that fish IL-1β is

cleaved. Early fish IL-1 studies using anti-mammalian IL-1α/β serum to surveys supernatants of activated fish cells demonstrated the recognition of distinct, multiple IL-1 protein species in these supernatants. While the catfish monocyte supernatants were demonstrated to contain a 70 kDa and a 15 kDa molecules with IL-1-like activity (102), carp macrophages secreted a 22 kDa and a 15 kDa IL-1-like factors that were recognized by mammalian anti-IL-1 antibodies (475). The immunoprecipitation experiments using the anti-mammalian IL-1 serum recognized the 15 kDa factor from the macrophage supernatants, suggesting possible maturation events of the carp IL-1 (475). In an independent survey of PHA-stimulated carp leukocytes using a monoclonal anti-carp IL-1β antibody, the 15 kDa protein was confirmed to be a mature carp IL-1β, with the putative carp IL-1β cleavage site situated approximately 15 amino acids downstream of the mammalian ICE site (283). Analysis of the supernatants of the trout macrophage cell line, RTS-11, using an anti-trout IL-1β polyclonal antibody detected putative native as well as potentially cleaved trout IL-1β proteins of 29 kDa and 24 kDa, respectively (179). When the RTS-11 cells were transfected with a plasmid encoding an C-terminally His^6 tagged trout IL-1β and the RTS-11 supernatants assayed using an Ni-NTA-column specific for the His^6 tagged IL-1β, the authors detected primarily a mature, 24 kDa IL-1β protein, confirming that maturation cleavage of IL-1β occurs in the trout.

In an elegant set of studies it was demonstrated that combined immune stimuli of sea bream head kidney leukocytes resulted in an accumulation of a 30 kDa, pro-IL-1β protein, which, unlike the mammalian counterpart (271), did not

exhibit a maturation cleavage or secretion following stimulation of the cells with extra-cellular ATP (341). Further, it was reported that sea bream acidophilic granulocyte populations in peritoneal exudates and peripheral blood leukocytes accumulated the 30 kDa form of IL-1β following challenge with *Vibrio anguillarum* (67). In contrast, the sea bream SAF-1 fibroblast cell line was shown to shed a mature, 22 kDa IL-1β protein through microvesicular plasma membrane budding within 30 minutes of treatment with extracellular ATP (271). Interestingly this IL-1β maturation/shedding process could be ablated with a pharmacological inhibitor of the mammalian ICE, suggesting a presence of a homologous sea bream enzyme in this process.

The primary receptor responsible for the recognition of extra-cellular ATP and the concomitant release of mature IL-1β is $P2X_7R$ (110). The mammalian cell line, HEK 293 cells expressing rat $P2X_7R$, human ICE and sea bream IL-1β, upon activation with ATP secreted an non cleaved (30 kDa) sea bream IL-1β (263). Interestingly, neither sea bream nor zebrafish $P2X_7R$ expression in ATP-stimulated HEK 293 cells resulted in sea bream or mammalian IL-1β secretion. In contrast, the expression of a chimeric $P2X_7R$ with the sea bream ATP-binding and rat intracellular domains led to maturation/secretion of the mammalian IL-1β, while the possible effects of these conditions on the sea bream IL-1β, were not described (263). The authors of this work suggest that the mechanisms involved in IL-1β secretion are conserved across vertebrates while the distinct stimuli that elicit the maturation events are not. An alternative hypothesis is that the functional specificity stems from the $P2X_7R$ intracellular signaling, activation of

species specific ICE (or alternative maturation mechanisms), and the substrate specificity of the fish Caspase1/ICE. It is noteworthy that the above group has also cloned and characterized the sea bream Caspase 1 (261), demonstrating that the recombinant form of this enzyme can cleave a commercial substrate known to be cleaved by the mammalian counterpart. Additional studies are needed to establish the association between sea breem Caspase I/ICE and $P2X_7R$ and IL-1β.

3.6.3.7. Novel teleost nIL-1 family member

Although there have been no reports of a fish IL-1α, recently a novel IL-1 family member, nIL-1F has been identified in trout and tetraodon and has been reported to have IL-1 family signature motif as well as a putative ICE cleavage site (483). The expression of nIL-1F increased after activation of macrophages with LPS or recombinant IL-1β. Furthermore, a recombinant form of the C-terminal nIL-1F abrogated the rIL-1β-induced immune gene expression in RTS-11 macrophage-like cells, suggesting a possible competitive inhibition through the trout IL-1RI. This mechanism would be analogous to the mammalian IL-1Ra, which also competes with IL-1β for IL-1RI binding, without inducing downstream events.

3.6.3.8. Teleost IL-1 receptors

All of the IL-1 receptor components known to be involved in IL-1β signaling have been described in fish. A fish IL-1RI was first described in Atlantic salmon with 43-44% similarity to chicken and 31% similarity to the

human IL-1RI, respectively (440). The expression of this gene was increased in fish tissues following LPS injection. The identification of a carp IL-1R1 was also described and the receptor characterized in the context of acute stress conditions (289).

The trout IL-1RII was identified through a selective subtractive hybridization of genes up-regulated following immune stimulation (386). This receptor shared low homology with the mammalian IL-1RII, but exhibited very similar overall organization including a very short intracellular domain. The sea bream IL-1RII was identified and found to be up-regulated following macrophage stimulation to levels 15 times greater than concomitant IL-1β expression increases under the same conditions (262). This suggests that the role of the fish IL-1RII as a "decoy" receptor may be conserved in the sea bream. Furthermore, HEK 293 cells expressing the sea bream IL-1RII were demonstrated to bind the recombinant IL-1β, confirming the specific receptor-ligand interaction.

A better understanding of the evolutionary mechanisms that have been involved in shaping the mammalian IL-1 biological pathways will be achieved through studies that examine the IL-1 receptor-ligand relationships and biological outcomes of these interactions in lower vertebrates such as teleosts.

3.6.4. Transforming growth factor beta (TGFβ)

3.6.4.1. Mammalian TGFβ and TGFβ receptors

There are currently 5 known transforming growth factor-beta (TGFβ) isoforms sharing 64-82% homology, of which TGFβ1-3 are found in mammals,

that are members of a cytokine/growth factor super-family comprised of more then 40 factors. It has been proposed that these TGF family members arose from a common ancestral gene through duplication events (214). The TGFβ1-3 are thought to have partially overlapping roles, although they are produced exclusively by distinct tissues and cell types (142). Of these, TGFβ1 is a key immunomodulatory molecule, while the functional roles of the other TGFβ isoforms in immune processes and regulation are not well established.

The TGFβ protein is synthesized and cleaved intracellularly, producing a mature 24 kDa homodimer which is non-covalently associated with the 80 kDa latency-associated peptide (LAP) in a complex referred to as the "small latency complex" (142). The LAP functionally serves a secretion mechanism for TGFβ, prevents the engagement of TGFβ to the ubiquitously expressed cognate receptors and maintains availability of large extracellular reservoirs of this cytokine, readily accessible for activation. Furthermore, this small latency complex is further bound by large glycoproteins, latent TGFβ binding proteins (LTBPs), forming a "large latency complex" and serving as further sequestering mechanisms onto extracellular matrices and regulation of TGFβ activation (142). It is believed that LTBPs are essential for maintaining the proper folding of TGFβ and targeting the complexes to extracellular structures. The mechanisms involved in the dissociation of the latency complexes and concomitant activation of the TGFβ homodimers are at present not completely known, but is believed that these events may occur through proteolytic and non-proteolytic events and involve various cellular interactions (139, 301, 319).

The immunoregulatory roles of TGFβ1 are complex and dependent on not only the concentration of the cytokine, but also on the presence of other cytokines/growth factors and the target cell types and differentiation states. TGFβ1 knockout mice display an excessive inflammatory response, cardiopulmonary complications, extensive multiple organ infiltration by macrophages and lymphocytes and typically succumb to these condition (228, 229, 408). Presumably, this is due to the loss of anti-inflammatory and anti-proliferative effects controlled by TGFβ1. The TGFβ knockout animals also develop severe autoimmune diseases (73, 502), suggesting an *in vivo* role for TGFβ1 in regulation of tolerance and adaptive immune responses. In contrast, TGFβ2 or TGFβ3 mouse knockouts have developmental defects resulting in death of the animals before the full development of the immune system is even formed in these animals (141).

There is a substantial amount of contradicting literature pertaining to the effects of *in vitro* administration of TGFβ1 on lymphocyte functions. Overall, TGFβ1 appears to inhibit the proliferation of both T and B lymphocytes and increases the apoptotic death of these cells (142). It has been proposed that TGFβ1 may have a role in controlling T cell responses by inducing programmed cell death in post-activated T cells in a process coined “death by neglect” (412). The apoptotic death of the T cell in turn result in the release of additional TGFβ1 (69), thus propagating this ablation of T cell mediated immune functions. Some reports suggest that TGFβ1 may actually propagate the Th1 response by stimulating CD4+ cells to produce T cell growth factor IL-2 and the Th1

cytokine/central mediator of inflammation, IFNγ (63). Arguably, these effects may be indirect, resulting from the presence of unaccounted factor(s) that contributing to these phenotypes (142). Of note is the fact that in addition to down-regulating the killer receptors on natural killer cells (374), TGFβ1 also decreases IFNγ production by this cell type (25). In fact, TGFβ1 appears to inhibit both Th1 and Th2 responses of naïve T lymphocytes while inhibiting the functions of only Th1 (but not Th2) memory T cells (267). An additional proposed mechanism by which TGFβ1 may down-regulate a Th1 response is by inducing an increases in the numbers of CD4+Foxp3+ T regulatory cells (409). Interestingly, TGFβ1 also induces B cell immunoglobulin class switch and the maturation of the B-lymphocytes, suggesting a possible role for this cytokine in regulating Th2-mediated responses. (246).

TGFβ1 also confers a number of regulatory mechanisms on various lineages of phagocytes. For example, TGFβ1 affects the differentiation and antigen presenting ability of dendritic cells, inhibits MHC class II expression and ablates IL-1β- and TNFα- induced IL-12 production by this cell type (135). The stimulation of monocytes with TGFβ1 has been suggested to be one of the mechanisms by which myeloid derived suppressor cells (alternatively activated macrophages, M2) arise (279). Following stimulation with TGFβ1 these cells up-regulate the IL-4 receptor, secrete Th2-biased cytokines, reduce their killing capabilities and exhibit tissue remodeling/repair properties. It has been documented that TGFβ1 prevents the LPS/TLR4-induced release of pro-inflammatory cytokines such as IL-1β and TNFα by macrophages (39).

One of the mechanisms by which TGFβ1 subverts the macrophage pro-inflammatory properties is by down-regulating the expression of receptors for central inflammatory cytokines of macrophages. For example, treatment of murine peritoneal macrophages resulted in a 40-50% loss in TNFα binding, accompanied by a nearly 100% abolished capability of these cells to produce ROI or NOI, after treatment with TNFα (93, 94). Similarly, bone marrow derived murine macrophages exposed to TGFβ1, exhibited a 30-35% reduction in the IFNγ receptor expression, accompanied by 70% reduction in IFNγ-induced macrophage antimicrobial responses (352). Keeping in mind that many pro-inflammatory cytokines tend to exhibit synergistic effects and result in reciprocal cytokine production, it is not surprising the magnitudes of TGFβ1-mediated down-regulation of macrophage inflammatory functions far exceeds the magnitude of the down-regulation of the individual cytokine-specific receptors.

Interestingly, low concentrations of TGFβ1 appear to be chemotactic to human blood monocytes (361, 479, 494) and neutrophils (331), which may explain the loss of adherence and adoption of a rounded state by otherwise adherent macrophages treated with TGFβ1 (466). It has been observed that while some antimicrobial macrophage responses, such as reactive oxygen production are ablated by TGFβ1 stimulation, others such as phagocytosis remain intact (466). In fact, macrophages treated with picomolar TGFβ1 levels have been reported to up-regulate the expression of the phagocytic receptor FcγRIII (CD16), which also enhances ROI production (479).

Despite the many anti-inflammatory effects attributed to TGFβ1 there are also reports suggesting pro-inflammatory roles for this cytokine, underlining the complexity of its biological outcomes and the cell state and cell type dependency of its functions. For example, resting human monocytes and mixed lineages of mononuclear cells treated with TGFβ1 up-regulate the mRNA levels of IL-1α, IL-1β, TNFα, and IL-6 (286, 479, 494). In contrast, when the same cells are also treated with LPS (subsequent to the TGFβ1 treatment), the release of IL-1β, TNFα, and IL-6 cytokines is diminished compared to untreated, LPS stimulated controls (39, 65). Taking into account that more recent reports implicate TGFβ1 in eliciting, rather then dampening antimicrobial mechanisms such as ROI production (34, 252, 302, 308) suggests that the full understanding of the immunoregulatory spectrum of TGFβ remains to be determined.

TGFβ1-3 signal through the same heteromeric complex composed of multiple TGFβ serine/threonine kinase receptor chains, reviewed by Massague and colleagues (280, 281). Upon ligation of TGFβ receptor II, the TGFβ/RII complex associates with and results in the phosphorylation of TGFβ receptor I (495, 496), which in turn leads to the phosphorylation of Smad anchor for receptor activation, SARA (173), a protein associated with the RI chain (463). SARA then propagates the TGFβ signal through downstream signaling Smad2 and Smad3 proteins (173), that are transiently associated with TGFβ receptor (10, 11, 173). The phosphorylated Smad2 and Smad3 complex with Smad4, forming a transcription factor group that translocates into the nucleus and results in transcriptional regulation changes in the TGFβ stimulated cells (511). A third

receptor chain, TGFβ receptor III also aids in propagating the TGFβ signal, where increasing the RIII expression appears to be sufficient to initiate signal transduction, suggesting potentially non-overlapping roles for this receptor chain (36). It is not surprising that with such a complex receptor-signaling system in place, (likely the case across distinct cell types and cellular activation states), that TGFβ controls a spectrum of biological processes.

3.6.4.2. Identification of TGFβ in bony fish

In comparison to mammals, relatively little is known about TGFβ in teleosts. The initial suggestion that TGFβ homologs might exist in fish came from a Russian group that performed acid-ethanol extractions and gel filtration of factors from blastoderm yolk of a cyprinid fish, with one of these extracted fractions exhibiting TGFβ-like properties (108). Treatment of MAF or TNFα activated trout head kidney macrophages with a bovine TGFβ1 resulted in decreased ROI production (197), while incubation of MAF secreting cells with anti-TGFβ1 serum resulted in the generation of more potent MAF activity (198), suggesting the presence of both the receptor and ligand TGFβ1 components, respectively.

The first identified TGFβ1 homolog was reported in trout where a cDNA transript with 68% homology to the human TGFβ1 was isolated from macrophage-enriched head kidney cell cultures (168). This transcript encoded a putative mature TGFβ protein with a TGFβ motif, 3-cysteine pairs and had a cysteine knot, characteristic of the TGFβ family (400). The genomic organization

of the trout TGFβ appeared to be similar to that of higher vertebrates, comprised of 7 coding exons as seen in amphibians, bird and mammalian TGFβ, while displaying unique intron organization (80).

Subsequently, TGFβ1 sequences were identified in several other fish species. The sequence of the carp TGFβ1 was isolated form head kidney leukocytes stimulated with mitogen ConA and it was demonstrated that ConA increased the otherwise low expression of carp TGFβ (508). A sea bream TGFβ1 was identified in fish that had previously been infected with nodavirus (449). The cDNA sequences of striped bass and tilapia TGFβ1 were identified in respective fish species subsequent to infections of those fish with *Mycobacterium marinum* (169). While the *M. marinum* infections decreased the expression of TGFβ1 in the bass splenic mononuclear cells, the overall expression of the tilapia TGFβ1 was unaffected. Interestingly, 3 out of the 10 fish that had the most notable inflammatory response also had the lowest expression levels of TGFβ1.

3.6.4.3. Biological roles of the teleost TGFβ

Despite the fact that TGFβ1 of a number of fish species has been cloned, majority of studies on the functional aspects of TGFβ1 in fish have employed mammalian recombinant TGFβ1. We produced a recombinant form of the goldfish TGFβ1 (rgTGFβ1) and examined its effects on goldfish immune cells (162). The rgTGFβ1 formed a functional homodimer and elicited the proliferation of a goldfish fibroblast cell line, CCL-71 in a dose dependent

manner. This recombinant cytokine also effectively decreased the nitric oxide responses of goldfish macrophages stimulated with rgTNFα (162).

Recently a recombinant form of the mature red sea bream TGFβ1 was demonstrated to induce a dose-dependent migration of peripheral blood leukocytes, while inhibiting this migration response when the cells were first activated with LPS (49). A polyclonal antibody specific for a sea bream chemokine partially abolished the migration activity induced by this sea bream TGFβ1, suggesting that this response may be indirectly mediated by TGFβ1-induced secretion of other factors. These observations are reminiscent of the effects of the mammalian TGFβ1 on murine monocytes, suggesting a potential conservation in the role of this cytokine in vertebrates.

3.6.4.4. Teleost TGFβ receptors

The first identified fish TGFβ receptor was TGFβ Receptor II, found in zebrafish ovarian tissues (219). This transcript exhibited a 70-85% identity with the RII sequences of other vertebrate species, with the greatest conservation seen in the putative kinase domain region. Also present in the NCBI database are sequences for zebrafish TGFβ receptor Ia and Ib, predicted zebrafish RIII as well as salmon and sea bream RII. To date, there have been no other reports of TGFβ receptors or studies addressing TGFβ receptor binding in teleosts.

There have been no reports of a latency associated peptide in fish, however a trout LTBP-3 homolog was identified and was shown to exhibit a great degree of homology to the mammalian LTBP-3 (7). This suggests that the

mammalian TGFβ secretion/regulation system may be conserved in lower vertebrates. Notably, the availability of reagents and powerful techniques in the field of zebrafish embryonic development has allowed for the study of TGFβ family members during development. In general, the findings suggest that the fish TGF family members have conserved developmental roles and utilize similar biological mechanisms to their mammalian counterparts. As research tools for the study of fish immunology become more readily available, it will be interesting to determine which biological processes controlled by TGFβ1 have truly been conserved and whether any of the TGFβ1-elicited immunoregulatory functions are novel in teleosts.

3.6.5. Interleukin-10 (IL-10)

3.6.5.1. Mammalian IL-10 and IL-10 receptors

Interleukin-10 is a central anti-inflammatory cytokine that regulates inflammatory as well as other immune processes. The importance of this immune mediator in the maintenance of homeostasis and immune regulation is evident from studies employing the IL-10 knockout mice (227, 362, 363). These IL-10 -/- mice appear to possess no developmental problems as neonates, however, despite being raised in pathogen free environments, 2-3 months into their lives these animals develop severe enterocolitis in response to their natural enteric flora. The IL-10 -/- mice exhibit highly polarized Th1 biases, with excessive production of pro-inflammatory mediators such as IFNγ. Furthermore, administration of anti-IFNγ or anti-TNFα antibodies ablates these symptoms, emphasizing the

involvement of IL-10 in regulation the production of these powerful pro-inflammatory cytokines.

Despite its anti-inflammatory function, IL-10 is also indispensable in control of immune responses to viral, bacterial, fungal, protozoan and helminth infections. IL-10 is crucial to ameliorating the inflammation-related pathology caused by IFNγ and/or TNFα during a wide range of pathogenic infections including *Mycobacterium spp.* (23); *Plasmodium spp.* (145, 174, 379, 497); *Toxoplasma gondii* (134, 371, 492); *Trypanosma spp.* (138, 184, 273); as well as in viral infections (446). In fact, the absence of IL-10 has been well documented to result in increased severity of immunopathology in response to a spectrum of pathogenic infections (134, 184, 492). Furthermore, IL-10 has been suggested to regulate the Th2 response, preventing severe fibrosis induced by IL-4, IL-5, and IL-13, observed during *Mycobacterium* infections (174), viral infections (311) and infections with *Schistosoma mansoni* (498, 499).

Interestingly, the immune evasion strategies of certain pathogens rely on intentionally inducing the production of IL-10 by the host, which functionally ablates the inflammatory response and promotes the survival of these pathogens in the host. The specific examples of this evasion strategy have been documented for *Leishmania spp.* (6, 24), *Mycobacterium spp.* (375), *Plasmodium spp.* (323, 497), and *Trypanosoma spp.* (360). Induced over-expression of IL-10 in phagocytic cells invariably leads to increased accumulation (decreased killing) and growth of infiltrating pathogens such as *Leishmania*, *Listeria* and *Mycobacterium* (109, 159, 360). At present, it is difficult to ascertain whether the

production of IL-10 is the result of the inflammatory/infectious process progression, or specifically induced by the pathogen itself.

Interleukin-10 was initially identified as a secreted product of Con-A-stimulated T cells that inhibited the synthesis of pro-inflammatory cytokines (112). In addition to T cells, IL-10 is also produced by B cells, eosinophils, epithelial cells, keratinocytes, mesangial cells, monocytes/macrophages, NK cells and tumor cells (96). Although initially assumed to inhibit T and NK cell functions, IL-10 has been shown to act primarily on cells of the monocyte/macrophage lineage, while the IL-10-mediated inhibition of lymphocyte function is believed to be a result of an indirect bystander effect (40, 95, 113, 114). Aside from abrogating the monocyte/macrophage pro-inflammatory cytokine expression and secretion, IL-10 has also been shown to down-regulate the production of reactive oxygen and to a much lower extent, reactive nitrogen intermediates (40, 326). This is though to occur primarily as a result of IL-10-mediated inhibition of TNFα synthesis (326).

The interleukin-10 ligand-receptor interactions are analogous to those of seen for IFNγ. IL-10 forms a functional homodimer (447, 481) that is structurally similar to IFNγ (482, 507), and induces its biological effects by ligating a receptor complex composed of ligand binding (257, 454, 455), and accessory (223, 425) receptor subunits (IL10R1 and IL10R2, respectively). The IL-10 primarily utilizes the a Jak-Stat signaling pathway, with the transcription factor, signal transducer of activation 3 (Stat3), recruited to the IL-10-bound receptor complex and activated through phosphorylation (489). The activation of Stat3 is indispensable to the

anti-inflammatory properties conferred by IL-10 (365, 452) while the phosphorylation of Stat3 leads to a rapid transcriptional up-regulation of suppressor of cytokine signaling-3 (SOCS-3) (12), believed to be one of the central mechanisms controlling the IL-10 induced anti-inflammatory effects (29, 357).

3.6.5.2. Identification of IL-10 in bony fish

Interleukin-10 has been identified in several bony fish species including puffer fish (519), carp (388), trout (189) zebrafish (509), sea bass (353) cod (404) and goldfish (152). It has been established that in contrast to the pro-inflammatory fish cytokines that are present in multiple isoforms, a single copy of the IL-10 gene exists in teleosts.

The initial fish IL-10 sequence identification was reported in the puffer fish using computational analysis of the puffer fish genome database (524). This homolog shared 44-50% homology with the mammalian IL-10 and exhibited the 5 exon/4 intron organization of the mammalian counterparts, but with much shorter introns. Southern blot analysis demonstrated the presence of a single IL-10 gene in the pufferfish while expression analysis showed low constitutive expression in some but not all puffer fish tissues.

The carp IL-10 sequence derived form head kidney cells stimulated with ConA and LPS had the IL-10 signature motif ([KQS]-x(4)-C-[QYC]-x(4)-[LIVM](2)-x-[FL]-[LMV]-x-[DERT]-[IV]-[LMF], with an N in position [KQS]) and the characteristic 5 exon/4 intron organization (388). Interestingly, while the

puffer fish and carp IL-10 genes are substantially smaller then the mammalian counterpart, the IL-10 gene identified in trout (189) was similar in size to the human IL-10 gene, possessing longer intron regions.

While the puffer fish tissue IL-10 mRNA levels were relatively low (519), The IL-10 genes of carp, trout and zebrafish exhibited the greatest tissue expression in the head kidney, spleen and gill tissues and were subject to up-regulation following administration of LPS (189, 388, 509). Cod injected with formaline-killed *Vibrio anguillarum* and polyI:C also demonstrated an up-regulation of spleen IL-10 expression (404) while sea bass challenged with UV-killed *Photobacterium damselae ssp. piscicida* displayed had elevated mRNA levels of IL-10 in the spleen and kidney (353). The expression of the goldfish IL-10 mRNA was found to be the highest in the spleen tissues, peripheral blood leukocyte (PBL) and granulocyte immune populations (152). Interestingly, goldfish monocytes and granulocytes stimulated with rgTNFα2 exhibited significantly down-regulated IL-10 message, underlining the possible dichotomy between these pro- and anti-inflammatory mediators of the goldfish. In Atlantic salmon infected with pancreatic necrosis virus exhibited elevated mRNA levels of IL-10 (188), while carp with soybean meal-induced enteritis displayed fluctuations in their expression of IL-10 (468).

3.6.5.3. Anti-inflammatory roles of IL-10 in teleosts

Despite numerous findings suggesting immunoregulatory role for the fish IL-10, relatively little functional analyses have been performed. I have recently

described the first comprehensive functional characterization of a bony fish interleukin-10 (Chapter VIII) (152). Subsequent to my identification of the goldfish IL-10 cDNA transcript, I produced a recombinant form of this cytokine (rgIL-10) using a prokaryotic expression system. This rgIL-10 substantially reduced the expression of TNFα1, TNFα2, IL-1β1, IL-10, CXCL-8, and NADPH oxidase component, p47phox in monocytes activated with heat-killed *A. salmonicida.* The rgIL-10 also down-regulated the IFNγ expression in *A. salmonicida*-activated splenocytes. Pre-treatment of monocytes with rgIL-10 resulted in reduced ability of these cells to generate reactive oxygen intermediates in response to *A. salmonicida* or rgIFNγ. Western blot analysis suggested that rgIL-10 adopted multimerized states, conferred cellular interactions and induced the phosphorylation and nuclear translocation of the goldfish Stat3. Furthermore, rgIL-10 elicited rapid and robust mRNA expression increase of the goldfish monocyte suppressor of cytokine signaling-3 gene (SOCS-3), suggesting that, as in mammals, this might be a mechanism by which the goldfish IL-10 down-regulates immune processes.

3.6.5.4. Teleost IL-10 receptors

In mammals, IL-10 signals through a receptor complex composed of two alpha (IL-10R1) and two beta (IL-10R2) chains. The IL-10R2 is located on the human chromosome 21 in an IFN/IL-10 receptor gene cluster linked to the GART gene. This cluster also includes the genes encoding the type I IFN receptors, IFNAR1 and IFNAR2 as well as the beta chain of the IFNγ receptor, IFNGR2. In

the initial attempt to evaluate this gene syntenic organization in lower vertebrates, the chicken cluster composed of GART, IFNAR1, IFNAR2 and IL-10R2 was identified, while the puffer fish counterparts were not found (359). Subsequently, the same research group employed amino acids sequence and gene structure homologies to identify several Class II helical cytokines and cytokine receptors (270). A gene encoding the puffer fish IL-10R2 chain was one of the genes identified using this approach. Other components of the IFN/IL-10 receptor gene cluster were also identified and found to be syntenic, with the exception of the GART gene, explaining the previous failure of this group to locate these genes by looking for gene neighbors to the puffer fish GART.

To my knowledge, with the exception to the above work, there have been no further reports describing IL-10 receptors of teleosts. The GenBank has published salmon and zebrafish IL-10R2 sequences, and further research of the IL-10 ligand-receptor biology may pave the way for therapeutic applications for control of excessive inflammatory responses in fish diseases.

3.7. Pathogen evasion and infiltration of inflammatory phagosytes

3.7.1. Pathogen evasion strategies of phagocye antimicrobial responses

Despite the fact that the inflammatory response is geared to generate battery of antimicrobial mechanisms to eliminate infiltrating pathogens, certain microbes have evolved not only to withstand the numerous components of inflammation, but also to thrive within macrophages, the cellular components of this response. These pathogenic infiltrators utilize host cells for growth,

replication and/or maintenance of their life cycles. Since macrophages are amongst the first cells encountered by infectious organisms, the survival of different pathogens depends on their ability to thwart the macrophage-mediated microbiacidal mechanisms. After circumventing the macrophage microbicidal armamentarium, these pathogens then utilize the macrophage to disseminate throughout the host.

There are numerous examples of viral, bacterial and protozoan pathogens of mammals that have evolved to adopt just such a strategy. These pathogens often utilize distinct evasion strategies to facilitate their survival within the macrophage. They have adopted mechanisms to withstand the low pH of mature phagolysosomes (332, 474), actively degrade antimicrobial proteins released into the phagolysosomes (397), subvert phagocyte oxidative bursts by producing detoxifying enzymes (316, 426), and prevent the assembly of enzymatic components involved in these responses (82, 299). Furthermore, they have evolved to overcome phagocyte iron depletion (269, 358). In some cases, the pathogens have evolved to even escape the phagosomes entirely and thrive in the macrophage cytosol (21, 471).

3.7.2. *Mycobacterium marinum* macrophage evasion strategies

Mycobacterium species are prototypical pathogens that have evolved to circumvent the armamentarium of phagocyte antimicrobial mechanisms, allowing them to thrive within the host cells. In particular, the relatively fast growing fish and amphibian pathogen, *Mycobacterium marinum*, which shares a close genetic

relationship with the *M. tubeculosis* complex, has recently been utilized as a surrogate model system to study infections and evasion mechanisms of *Mycobacterium* spp. Here, I will outline some of the recently identified key macrophage evasion strategies utilized by this fish pathogen as an example of the level of sophistication of this host cell -pathogen relationships.

By infecting zebrafish embryos with *M. marinum*, it was demonstrated that macrophages play a pivotal role in dissemination as well as clearance of this pathogen, where macrophage depletion led to significantly more robust infections and bacterial growth (74). Zebrafish embryo infections (lacking adaptive immune components) result in the formation of macrophage aggregates with hallmark granuloma structures even in the absence of lymphocytes, while the infecting *M. marinum* begin expressing newly identified, granuloma-spesific genes (83). Where as classical granuloma formation has been described as an organization of differentiated macrophages and lymphocytes, these observations suggest that the adaptive component is dispensable in the formation of these structures. Furthermore, where as it has been widely proposed that granuloma formation is the host immune strategy for containing mycobacterial infections, it has now become evident that this structure is actively induced and maintained by the parasite as a dissemination strategy. In the zebrafish embryo infections, upon granuloma formation *M. marinum* utilizes its ESX-1/RD1 virulence locus to recruit macrophages to these sites, facilitating infection of new host cells and rapid movement within the granulomas (84). The recruited phagocytes ingest apoptotic cells containing the pathogen thus enhancing the infection and

facilitating systemic dissemination where the primary granuloma serves to seed secondary granuloma structures (84). In fact it would appear that *M. marinum* actively redirects embryonic macrophages from predetermined migration pathways dictated by development (83).

In the literature, the signaling mechanisms employed by TNFα have been coined as a "double edged sword" and this appears to hold true for the TNFα responses to *M. marinum* infections as well. In context of the zebrafish infections, the loss of TNFα signaling results in increased host mortality, increased bacterial growth and a relatively rapid granuloma formation (75). This suggests that TNFα is essential for the maintenance of granuloma structures and reduced bacterial growth within infected macrophages. Thus although TNFα serves to negate the mycobacterial infection, it would appear that this pathogen has evolved to utilize aspects of this cytokine's effect to its strategy, exemplified by the TNFα-mediated maintenance of granuloma integrity.

Upon infection, *M. marinum* has been demonstrated to subvert phagosome maturation of both mammalian (19) and fish (101) macrophages. It has been shown that the secretion of early secreting antigenic 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP_10) by the *M. marinum* ESX-1 (type VII) secretion system plays a pivotal role in arresting phagosome maturation, where bacterial mutants of this pathway are incapable of preventing phagosome acidification (456, 500).

In addition to active *M. marinum* strategies that ensure its survival within phagosomes, mycobacterial cell wall components also aid in evading degradative

macrophage mechanisms. For example, the *M. marinum* pmiA gene product blocks phagosome maturation by dictating the lipid composition of the bacterial cell envelope (370). The virulence determinant gene Erp facilitates bacterial cell wall integrity and by doing so ensures *M. marinum* survival with macrophages (79). The mycobacterrial genes coined invasion and intracellular survival A and B (iiA and iiB), confer antibiotic resistance and protection from lysozomal enzymes by maintaining bacterial cell wall structural integrity (129), where mutants of these genes were avirulent to zebrafish. Also, the *M. marinum* gene involved in biosynthesis of mycolic acids (cell wall components) is directly involved in the bacterial ability to maintain cell wall integrity and survival within macrophages (128).

The intracellular pathogens that reside in macrophages must not only withstand and/or evade the pH and enzymatic degradation following phagolysosome maturation, but also must survive a battery of antimicrobial responses, amongst them the macrophage oxidative burst. It would seem that *M. marinum* has evolved to deal with phagocyte ROI and NO production through a number of strategies. A mutation in the *M. marinum* mel2 gene renders the pathogen susceptible to the ROI and NO produced by an IFNγ-stimulated macrophage cell line, where the addition of iNOS inhibitors and ROS scavengers revert the growth defects of the bacteria within these cells (437, 438). *M. marinum* as well as most other slow growing mycobacteria (eg. *M. avium*) also express the oxyR gene, a central regulator of the oxidative stress response, while this gene is inactive in *M. tuberculosis*. It would appear that this strategy is

superfluous to other *M. marinum* oxidative burst evasion mechanisms, since its inactivation did not affect infectivity or survival in fish macrophages (327). Furthermore, my studies of *M. marinum* infections of goldfish monocytes and macrophages indicate that this pathogen is able to significantly ablate pro-inflammatory cytokine-induced ROI and NO production by down-regulating the expression of NADPH oxidase components and up-regulating immunosupressive macrophage genes such as IL-10, TGFβ and SOCS-3 (Chapter IX).

It has been generally assumed that mycobacteria reside strictly within phagocytic vesicles, however, recent reports indicate that *M. tuberculosis* and *M. leprae* (471) as well as *M. marinum* (429) are capable of escaping the phagolysosome entirely and reside in macrophage cytosols. The *M. marinum* ESX-1 system facilitates this escape (222, 420) through secretion of ESAT-6 that confers phagosomal membrane pore formation (420). Once in the cytosol, *M. marinum* sequesters the host cell Wiscott-Aldrich syndrome protein (WASP) family members to mobilize actin, forming actin tails and facilitatintg its motility throughout the cell (429, 430).

Not surprisingly, *M. marinum* has also evolved mechanisms to modulate the profiles of the cytokines produced by the infected macrophages. The *M. marinum* cell wall glycolipids have been demonstrated to suppress TNFα in LPS stimulated human macrophages (373). This pathogen employs the ESX-5 secretion system to actively suppress the production of mammalian macrophage IL-12p40, TNFα and IL-6 while inducing the production of IL-1β by these cells (1). Interestingly the *M. marinum* ESX-1 secretion system also actively elicits

macropahge IL-1β and IL-18 (IL-1 family member) production (54, 222), underlying a potential importance of these cytokines in this evasion strategy. The mycobacterium elicited IL-1β production exacerbates inflammation to the detriment of the host with no apparent detrimetal effects on pathogen survival (54). Indeed, TLR signaling such as that utilized by the IL-1 receptor has been implicated as a mycobacterial evasion strategy (318). Further investigation into the respective roles of the inflammatory cytokines induced and/or down-regulated during mycobacterial infections will yield a better understanding of the macrophage-mycobacterial interactions and possibly provide new therapeutic alternatives

Undoubtedly the many pathogen evasion mechanisms described here are probably only a fraction of the strategies that mycobacteria utilize to survive in host cells. For a comprehensive review of immune evasion strategies see these excellent reviews (115, 224).

3.8. Concluding remarks

A successful and efficient inflammatory response is self-limiting with inflammatory agents such as TNFα, IFNγ and IL-1β inducing downstream events that capsize their influence on various biological processes. Some of these downstream inflammatory events include the production of "decoy" as well as soluble receptors, that down-regulate cytokine cellular signaling, and the secretion of anti-inflammatory mediators such as TGFβ1 and IL-10, that not only abrogate

the inflammatory processes that are induced by pro-inflammatory cytokines, but also effectively redirect cell functions towards tissue healing and repair.

The regulation of the vertebrate inflammatory response is an extremely complex process, involving numerous mechanisms, some of which are poorly understood while others likely remain to be identified. This is particularly true for the teleost model systems, where lack of specific reagents for different fish species hampers our ability to examine different aspects of the regulation of inflammation at a mechanistic level. However, there is growing evidence that the key immune components required to mount an effective inflammatory response are present in teleosts. In addition, certain fish species possess additional pathways that regulate inflammatory processes (for example IFNγrel and its receptor IFNGR1-1, novel chemokines and PRRs), distinct from those reported for mammals. The elucidation of the coordination of inflammatory responses by these factors may shed new light on the evolution of innate host defense mechanisms in lower vertebrates.

It would appear that, as in mammals, the monocyte/macrophage cell types are indispensable in orchestrating the inflammatory processes of bony fish. Therefore it comes as no surprise that fish pathogens such as *M. marinum* have adopted the evolutionary strategies of residing within and subverting the phagocyte inflammatory and immune processes. It is interesting that where it would be advantageous to the host to polarize these phagocytes towards the inflammatory antimicrobial responses following infection,

monocyte/macrophage-infiltrating *M. marinum* actively capsized antimicrobial mechanisms and instead promotes alternative-activation like states in these cells.

By examining the mechanisms employed by bony fish to orchestrate inflammatory responses combined with investigations of the strategies utilized by pathogens such as *M. marinum* to evade these responses, we will gain a better understanding the evolution of host defense.

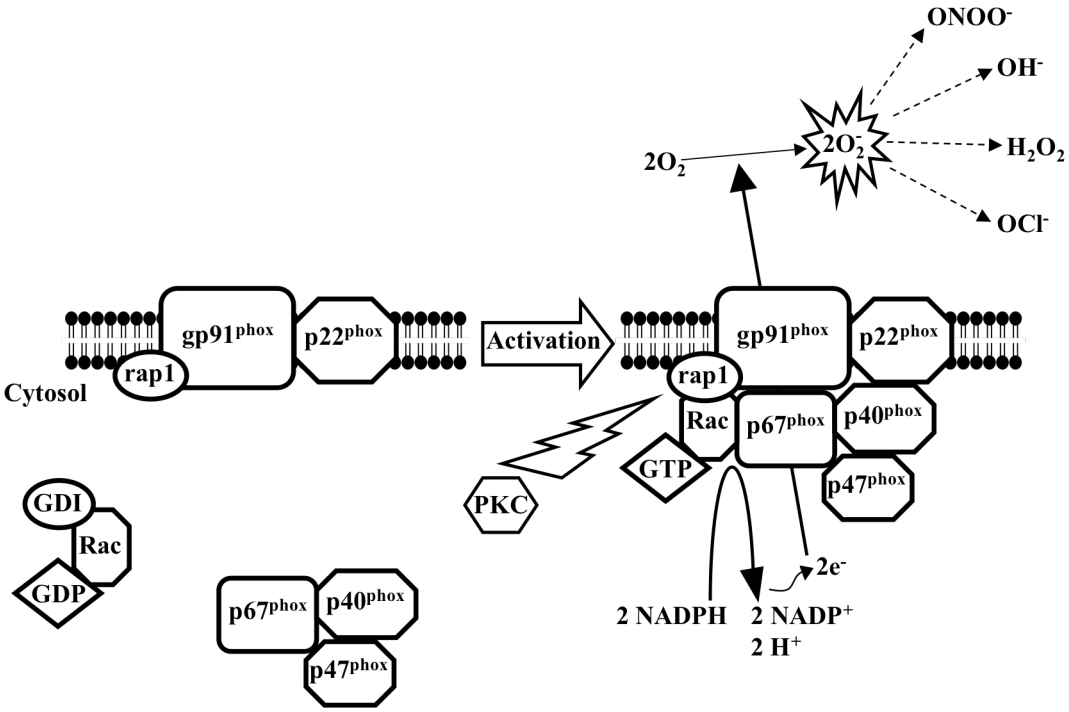

Figure 1.1. Schematic representation of the NADPH oxidase complex mobilization and reactive oxygen production following phagocyte activation. In a resting state, the gp91^{phox} and p22 ^{phox} components are membrane bound while the p40 ^{phox}, p47 ^{phox} and p67 ^{phox} components are located in the cytosol and the small G-protein Rac is GDP bound. Upon cell activation, Rac is rapidly converted from a GDP- to a GTP-bound state and facilitates the translocation and assembly of the cytosolic NADPH oxidase components at the cell membrane. Following PKC activation, Rap1 is thought to serve as the final switch in the activation of the NADPH complex. The activated NADPH oxidase complex accepts the electrons form the reduced NADPH and transfers these to molecular oxygen, forming the superoxide anion (O_2^-). The generated O_2^- can subsequently be converted to other reactive oxygen species. Reviewed in references (293, 368, 369, 407).

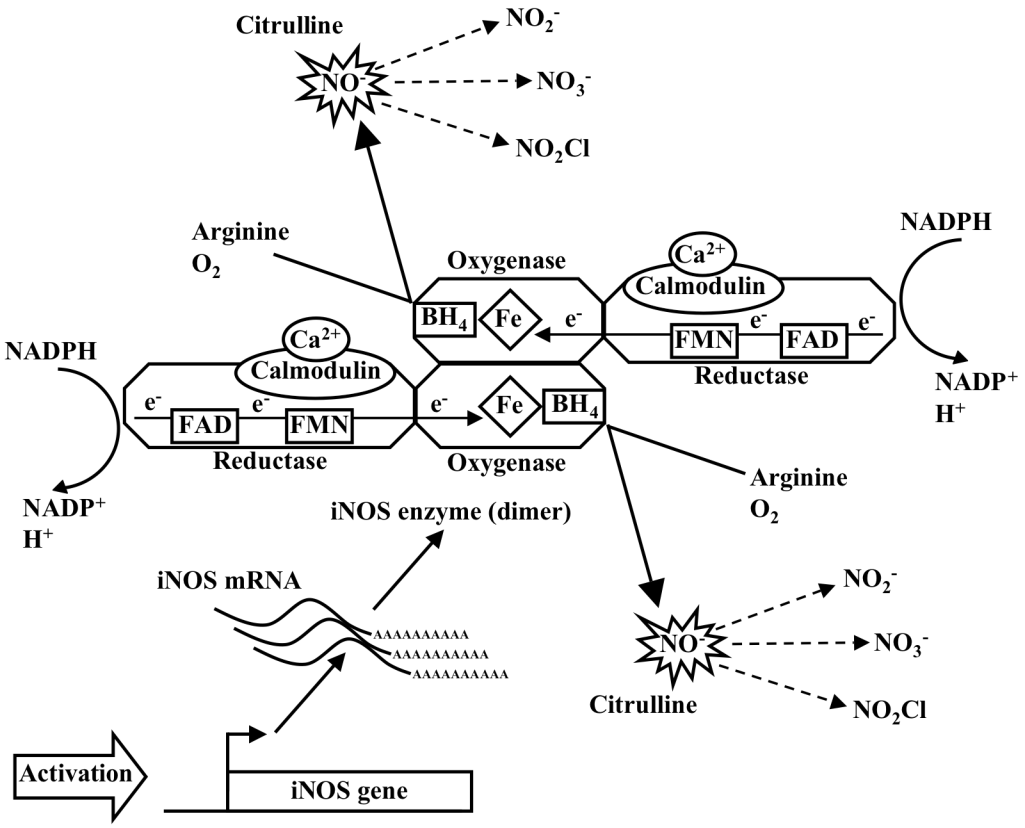

Figure 1.2. Schematic representation of iNOS gene expression, iNOS protein synthesis and enzymatic production of nitric oxide by the iNOS dimer complex. Upon phagocyte activation, there is a substantial increase in the expression from the iNOS gene and de novo synthesis of the iNOS enzyme. The iNOS enzyme forms a dimer and associates with a Ca^{2+} bound calmodulin, stabilizing the structure and facilitating the function of the enzymatic complex. The iNOS dimer catalyzes the transfer of electrons from a reduced NADPH, through a series of cofactors (FAD, FMN, Fe, BH_4) in the oxidation of L-Arginine to L-Citrulline with a concomitant production of nitric oxide ($NO^{\cdot}$). The generated $NO^{\cdot}$ can subsequently be converted to other reactive nitrogen species. Reviewed in references (8, 265, 295).

4. REFERENCES

1. **Abdallah, A. M., N. D. Savage, M. van Zon, L. Wilson, C. M. Vandenbroucke-Grauls, N. N. van der Wel, T. H. Ottenhoff, and W. Bitter.** 2008. The ESX-5 secretion system of Mycobacterium marinum modulates the macrophage response. J Immunol **181:**7166-7175.
2. **Aggad, D., C. Stein, D. Sieger, M. Mazel, P. Boudinot, P. Herbomel, J. P. Levraud, G. Lutfalla, and M. Leptin.** 2010. In vivo analysis of Ifn-gamma1 and Ifn-gamma2 signaling in zebrafish. J Immunol **185:**6774-6782.
3. **Ajuebor, M. N., A. M. Das, L. Virag, R. J. Flower, C. Szabo, and M. Perretti.** 1999. Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10. J Immunol **162:**1685-1691.
4. **Alberdi, F., Jr., M. R. Alderton, P. J. Coloe, and S. C. Smith.** 1995. Characterization of immunorelated peptides to porcidin P1. Immunology and cell biology **73:**505-510.
5. **Alberdi, F., Jr., M. R. Alderton, V. Korolik, P. J. Coloe, and S. C. Smith.** 1995. Antibacterial proteins from porcine polymorphonuclear neutrophils. Immunology and cell biology **73:**38-43.
6. **Anderson, C. F., S. Mendez, and D. L. Sacks.** 2005. Nonhealing infection despite Th1 polarization produced by a strain of Leishmania major in C57BL/6 mice. J Immunol **174:**2934-2941.
7. **Andersson, M. L., and R. I. Eggen.** 2006. Transcription of the fish Latent TGFbeta-binding protein gene is controlled by estrogen receptor alpha. Toxicol In Vitro **20:**417-425.
8. **Andrew, P. J., and B. Mayer.** 1999. Enzymatic function of nitric oxide synthases. Cardiovascular research **43:**521-531.
9. **Arts, J. A., E. J. Tijhaar, M. Chadzinska, H. F. Savelkoul, and B. M. Verburg-van Kemenade.** 2010. Functional analysis of carp interferon-gamma: evolutionary conservation of classical phagocyte activation. Fish & shellfish immunology **29:**793-802.
10. **Attisano, L., and J. L. Wrana.** 1996. Signal transduction by members of the transforming growth factor-beta superfamily. Cytokine & growth factor reviews **7:**327-339.
11. **Attisano, L., and J. L. Wrana.** 2000. Smads as transcriptional co-modulators. Curr Opin Cell Biol **12:**235-243.
12. **Auernhammer, C. J., C. Bousquet, and S. Melmed.** 1999. Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. Proc Natl Acad Sci U S A **96:**6964-6969.
13. **Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann.** 2007.

Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science (New York, N.Y **317:**666-670.

14. **Auron, P. E.** 1998. The interleukin 1 receptor: ligand interactions and signal transduction. Cytokine & growth factor reviews **9:**221-237.
15. **Auron, P. E., A. C. Webb, L. J. Rosenwasser, S. F. Mucci, A. Rich, S. M. Wolff, and C. A. Dinarello.** 1984. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proceedings of the National Academy of Sciences of the United States of America **81:**7907-7911.
16. **Banner, D. W., A. D'Arcy, W. Janes, R. Gentz, H. J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer.** 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. Cell **73:**431-445.
17. **Bao, B., E. Peatman, X. Peng, P. Baoprasertkul, G. Wang, and Z. Liu.** 2006. Characterization of 23 CC chemokine genes and analysis of their expression in channel catfish (Ictalurus punctatus). Developmental and comparative immunology **30:**783-796.
18. **Barber, M. R., J. R. Aldridge, Jr., R. G. Webster, and K. E. Magor.** 2010. Association of RIG-I with innate immunity of ducks to influenza. Proceedings of the National Academy of Sciences of the United States of America **107:**5913-5918.
19. **Barker, L. P., K. M. George, S. Falkow, and P. L. Small.** 1997. Differential trafficking of live and dead Mycobacterium marinum organisms in macrophages. Infection and immunity **65:**1497-1504.
20. **Bayne, C. J., L. Gerwick, K. Fujiki, M. Nakao, and T. Yano.** 2001. Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, Oncorhynchus mykiss, by means of suppression subtractive hybridization. Developmental and comparative immunology **25:**205-217.
21. **Beauregard, K. E., K. D. Lee, R. J. Collier, and J. A. Swanson.** 1997. pH-dependent perforation of macrophage phagosomes by listeriolysin O from Listeria monocytogenes. The Journal of experimental medicine **186:**1159-1163.
22. **Beck-Schimmer, B., R. Schwendener, T. Pasch, L. Reyes, C. Booy, and R. C. Schimmer.** 2005. Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. Respir Res **6:**61.
23. **Bekker, L. G., G. Maartens, L. Steyn, and G. Kaplan.** 1998. Selective increase in plasma tumor necrosis factor-alpha and concomitant clinical deterioration after initiating therapy in patients with severe tuberculosis. J Infect Dis **178:**580-584.
24. **Belkaid, Y., K. F. Hoffmann, S. Mendez, S. Kamhawi, M. C. Udey, T. A. Wynn, and D. L. Sacks.** 2001. The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. The Journal of experimental medicine **194:**1497-1506.
25. **Bellone, G., M. Aste-Amezaga, G. Trinchieri, and U. Rodeck.** 1995. Regulation of NK cell functions by TGF-beta 1. J Immunol **155:**1066-1073.

26. **Belosevic, M., C. E. Davis, M. S. Meltzer, and C. A. Nacy.** 1988. Regulation of activated macrophage antimicrobial activities. Identification of lymphokines that cooperate with IFN-gamma for induction of resistance to infection. J Immunol **141:**890-896.
27. **Benedetti, S., E. Randelli, F. Buonocore, J. Zou, C. J. Secombes, and G. Scapigliati.** 2006. Evolution of cytokine responses: IL-1beta directly affects intracellular Ca2+ concentration of teleost fish leukocytes through a receptor-mediated mechanism. Cytokine **34:**9-16.
28. **Berahovich, R. D., Z. Miao, Y. Wang, B. Premack, M. C. Howard, and T. J. Schall.** 2005. Proteolytic activation of alternative CCR1 ligands in inflammation. J Immunol **174:**7341-7351.
29. **Berlato, C., M. A. Cassatella, I. Kinjyo, L. Gatto, A. Yoshimura, and F. Bazzoni.** 2002. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol **168:**6404-6411.
30. **Berton, G., L. Zeni, M. A. Cassatella, and F. Rossi.** 1986. Gamma interferon is able to enhance the oxidative metabolism of human neutrophils. Biochemical and biophysical research communications **138:**1276-1282.
31. **Beutler, B., and M. Rehli.** 2002. Evolution of the TIR, tolls and TLRs: functional inferences from computational biology. Current topics in microbiology and immunology **270:**1-21.
32. **Biacchesi, S., M. LeBerre, A. Lamoureux, Y. Louise, E. Lauret, P. Boudinot, and M. Bremont.** 2009. Mitochondrial antiviral signaling protein plays a major role in induction of the fish innate immune response against RNA and DNA viruses. Journal of virology **83:**7815-7827.
33. **Bielek, E., J. Bigaj, M. Chadzinska, and B. Plytycz.** 1999. Depletion of head kidney neutrophils and cells with basophilic granules during peritoneal inflammation in the goldfish, Carassius auratus. Folia biologica **47:**33-42.
34. **Black, D., S. Lyman, T. Qian, J. J. Lemasters, R. A. Rippe, T. Nitta, J. S. Kim, and K. E. Behrns.** 2007. Transforming growth factor beta mediates hepatocyte apoptosis through Smad3 generation of reactive oxygen species. Biochimie **89:**1464-1473.
35. **Bleul, C. C., R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti, and T. A. Springer.** 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). The Journal of experimental medicine **184:**1101-1109.
36. **Blobe, G. C., X. Liu, S. J. Fang, T. How, and H. F. Lodish.** 2001. A novel mechanism for regulating transforming growth factor beta (TGF-beta) signaling. Functional modulation of type III TGF-beta receptor expression through interaction with the PDZ domain protein, GIPC. The Journal of biological chemistry **276:**39608-39617.
37. **Bluyssen, H. A., R. Muzaffar, R. J. Vlieststra, A. C. van der Made, S. Leung, G. R. Stark, I. M. Kerr, J. Trapman, and D. E. Levy.** 1995. Combinatorial association and abundance of components of interferon-

stimulated gene factor 3 dictate the selectivity of interferon responses. Proceedings of the National Academy of Sciences of the United States of America **92:**5645-5649.

38. **Bobe, J., and F. W. Goetz.** 2001. Molecular cloning and expression of a TNF receptor and two TNF ligands in the fish ovary. Comp Biochem Physiol B Biochem Mol Biol **129:**475-481.
39. **Bogdan, C., J. Paik, Y. Vodovotz, and C. Nathan.** 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. The Journal of biological chemistry **267:**23301-23308.
40. **Bogdan, C., Y. Vodovotz, and C. Nathan.** 1991. Macrophage deactivation by interleukin 10. The Journal of experimental medicine **174:**1549-1555.
41. **Boltana, S., C. Donate, F. W. Goetz, S. MacKenzie, and J. C. Balasch.** 2009. Characterization and expression of NADPH oxidase in LPS-, poly(I:C)- and zymosan-stimulated trout (Oncorhynchus mykiss W.) macrophages. Fish & shellfish immunology **26:**651-661.
42. **Borelli, V., E. Banfi, M. G. Perrotta, and G. Zabucchi.** 1999. Myeloperoxidase exerts microbicidal activity against Mycobacterium tuberculosis. Infection and immunity **67:**4149-4152.
43. **Borregaard, N., and J. B. Cowland.** 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood **89:**3503-3521.
44. **Bosco, M. C., G. L. Gusella, I. Espinoza-Delgado, D. L. Longo, and L. Varesio.** 1994. Interferon-gamma upregulates interleukin-8 gene expression in human monocytic cells by a posttranscriptional mechanism. Blood **83:**537-542.
45. **Briggs, R. T., D. B. Drath, M. L. Karnovsky, and M. J. Karnovsky.** 1975. Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. The Journal of cell biology **67:**566-586.
46. **Bujak, M., and N. G. Frangogiannis.** 2009. The role of IL-1 in the pathogenesis of heart disease. Arch Immunol Ther Exp (Warsz) **57:**165-176.
47. **Buonocore, F., M. Forlenza, E. Randelli, S. Benedetti, P. Bossu, S. Meloni, C. J. Secombes, M. Mazzini, and G. Scapigliati.** 2005. Biological activity of sea bass (Dicentrarchus labrax L.) recombinant interleukin-1beta. Mar Biotechnol (NY) **7:**609-617.
48. **Buonocore, F., M. Mazzini, M. Forlenza, E. Randelli, C. J. Secombes, J. Zou, and G. Scapigliati.** 2004. Expression in Escherchia coli and purification of sea bass (Dicentrarchus labrax) interleukin 1beta, a possible immunoadjuvant in aquaculture. Mar Biotechnol (NY) **6:**53-59.
49. **Cai, Z., C. Gao, L. Li, and K. Xing.** 2010. Bipolar properties of red seabream (Pagrus major) transforming growth factor-beta in induction of the leucocytes migration. Fish & shellfish immunology **28:**695-700.
50. **Cai, Z. H., L. S. Song, C. P. Gao, L. T. Wu, L. H. Qiu, and J. H. Xiang.** 2003. [Cloning and expression of tumor necrosis factor

(TNFalpha) cDNA from red seabream pagrus major]. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai) **35:**1111-1116.

51. **Cailhier, J. F., M. Partolina, S. Vuthoori, S. Wu, K. Ko, S. Watson, J. Savill, J. Hughes, and R. A. Lang.** 2005. Conditional macrophage ablation demonstrates that resident macrophages initiate acute peritoneal inflammation. J Immunol **174:**2336-2342.
52. **Cailhier, J. F., D. A. Sawatzky, T. Kipari, K. Houlberg, D. Walbaum, S. Watson, R. A. Lang, S. Clay, D. Kluth, J. Savill, and J. Hughes.** 2006. Resident pleural macrophages are key orchestrators of neutrophil recruitment in pleural inflammation. Am J Respir Crit Care Med **173:**540-547.
53. **Camussi, G., F. Bussolino, G. Salvidio, and C. Baglioni.** 1987. Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. The Journal of experimental medicine **166:**1390-1404.
54. **Carlsson, F., J. Kim, C. Dumitru, K. H. Barck, R. A. Carano, M. Sun, L. Diehl, and E. J. Brown.** 2010. Host-detrimental role of Esx-1-mediated inflammasome activation in mycobacterial infection. PLoS pathogens **6:**e1000895.
55. **Carswell, E. A., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences of the United States of America **72:**3666-3670.
56. **Cassatella, M. A., F. Bazzoni, R. M. Flynn, S. Dusi, G. Trinchieri, and F. Rossi.** 1990. Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. The Journal of biological chemistry **265:**20241-20246.
57. **Cassatella, M. A., V. Della Bianca, G. Berton, and F. Rossi.** 1985. Activation by gamma interferon of human macrophage capability to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. Biochemical and biophysical research communications **132:**908-914.
58. **Cassatella, M. A., I. Guasparri, M. Ceska, F. Bazzoni, and F. Rossi.** 1993. Interferon-gamma inhibits interleukin-8 production by human polymorphonuclear leucocytes. Immunology **78:**177-184.
59. **Castell, J. V., M. J. Gomez-Lechon, M. David, T. Andus, T. Geiger, R. Trullenque, R. Fabra, and P. C. Heinrich.** 1989. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. FEBS letters **242:**237-239.
60. **Castro, R., S. A. Martin, S. Bird, J. Lamas, and C. J. Secombes.** 2008. Characterisation of gamma-interferon responsive promoters in fish. Molecular immunology **45:**3454-3462.

61. **Castro, R., S. A. Martin, J. Zou, and C. J. Secombes.** 2010. Establishment of an IFN-gamma specific reporter cell line in fish. Fish & shellfish immunology **28:**312-319.
62. **Castro, R., M. C. Piazzon, M. Noya, J. M. Leiro, and J. Lamas.** 2008. Isolation and molecular cloning of a fish myeloperoxidase. Molecular immunology **45:**428-437.
63. **Cerwenka, A., and S. L. Swain.** 1999. TGF-beta1: immunosuppressant and viability factor for T lymphocytes. Microbes and infection / Institut Pasteur **1:**1291-1296.
64. **Chadzinska, M., E. Kolaczkowska, A. Scislowska-Czarnecka, N. Van Rooijen, and B. Plytycz.** 2004. Effects of macrophage depletion on peritoneal inflammation in swiss mice, edible frogs and goldfish. Folia biologica **52:**225-231.
65. **Chantry, D., M. Turner, E. Abney, and M. Feldmann.** 1989. Modulation of cytokine production by transforming growth factor-beta. J Immunol **142:**4295-4300.
66. **Chapman, B. S., and I. D. Kuntz.** 1995. Modeled structure of the 75-kDa neurotrophin receptor. Protein Sci **4:**1696-1707.
67. **Chaves-Pozo, E., P. Pelegrin, J. Garcia-Castillo, A. Garcia-Ayala, V. Mulero, and J. Meseguer.** 2004. Acidophilic granulocytes of the marine fish gilthead seabream (Sparus aurata L.) produce interleukin-1beta following infection with Vibrio anguillarum. Cell and tissue research **316:**189-195.
68. **Chen, G., and D. V. Goeddel.** 2002. TNF-R1 signaling: a beautiful pathway. Science (New York, N.Y **296:**1634-1635.
69. **Chen, W., M. E. Frank, W. Jin, and S. M. Wahl.** 2001. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. Immunity **14:**715-725.
70. **Chen, W. Q., Q. Q. Xu, M. X. Chang, J. Zou, C. J. Secombes, K. M. Peng, and P. Nie.** 2009. Molecular characterization and expression analysis of the IFN-gamma related gene (IFN-gammarel) in grass carp Ctenopharyngodon idella. Veterinary immunology and immunopathology.
71. **Chiang, N., C. N. Serhan, S. E. Dahlen, J. M. Drazen, D. W. Hay, G. E. Rovati, T. Shimizu, T. Yokomizo, and C. Brink.** 2006. The lipoxin receptor ALX: potent ligand-specific and stereoselective actions in vivo. Pharmacol Rev **58:**463-487.
72. **Chistiakov, D. A., F. V. Kabanov, O. D. Troepolskaya, and M. M. Tischenko.** 2010. A variant of the interleukin-1beta gene in European sea bass, Dicentrarchus labrax L., is associated with increased resistance against Vibrio anguillarum. J Fish Dis **33:**759-767.
73. **Christ, M., N. L. McCartney-Francis, A. B. Kulkarni, J. M. Ward, D. E. Mizel, C. L. Mackall, R. E. Gress, K. L. Hines, H. Tian, S. Karlsson, and et al.** 1994. Immune dysregulation in TGF-beta 1-deficient mice. J Immunol **153:**1936-1946.
74. **Clay, H., J. M. Davis, D. Beery, A. Huttenlocher, S. E. Lyons, and L. Ramakrishnan.** 2007. Dichotomous role of the macrophage in early

Mycobacterium marinum infection of the zebrafish. Cell host & microbe **2:**29-39.

75. **Clay, H., H. E. Volkman, and L. Ramakrishnan.** 2008. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity **29:**283-294.
76. **Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J. G. Giri, S. K. Dower, J. E. Sims, and A. Mantovani.** 1993. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science (New York, N.Y **261:**472-475.
77. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. The Journal of experimental medicine **178:**2243-2247.
78. **Corripio-Miyar, Y., S. Bird, K. Tsamopoulos, and C. J. Secombes.** 2007. Cloning and expression analysis of two pro-inflammatory cytokines, IL-1 beta and IL-8, in haddock (Melanogrammus aeglefinus). Molecular immunology **44:**1361-1373.
79. **Cosma, C. L., K. Klein, R. Kim, D. Beery, and L. Ramakrishnan.** 2006. Mycobacterium marinum Erp is a virulence determinant required for cell wall integrity and intracellular survival. Infection and immunity **74:**3125-3133.
80. **Daniels, G. D., and C. J. Secombes.** 1999. Genomic organisation of rainbow trout, Oncorhynchus mykiss TGF-beta. Developmental and comparative immunology **23:**139-147.
81. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science (New York, N.Y **264:**1415-1421.
82. **Davis, A. S., I. Vergne, S. S. Master, G. B. Kyei, J. Chua, and V. Deretic.** 2007. Mechanism of inducible nitric oxide synthase exclusion from mycobacterial phagosomes. PLoS pathogens **3:**e186.
83. **Davis, J. M., H. Clay, J. L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan.** 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity **17:**693-702.
84. **Davis, J. M., and L. Ramakrishnan.** 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell **136:**37-49.
85. **Debets, R., J. C. Timans, B. Homey, S. Zurawski, T. R. Sana, S. Lo, J. Wagner, G. Edwards, T. Clifford, S. Menon, J. F. Bazan, and R. A. Kastelein.** 2001. Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. J Immunol **167:**1440-1446.
86. **Denicola, A., H. Rubbo, D. Rodriguez, and R. Radi.** 1993. Peroxynitrite-mediated cytotoxicity to Trypanosoma cruzi. Archives of biochemistry and biophysics **304:**279-286.

87. **Dhawan, S., A. Heredia, R. B. Lal, L. M. Wahl, J. S. Epstein, and I. K. Hewlett.** 1994. Interferon-gamma induces resistance in primary monocytes against human immunodeficiency virus type-1 infection. Biochemical and biophysical research communications **201:**756-761.
88. **Dinarello, C. A.** 1996. Biologic basis for interleukin-1 in disease. Blood **87:**2095-2147.
89. **Dinarello, C. A.** 1988. Biology of interleukin 1. Faseb J **2:**108-115.
90. **Dinarello, C. A.** 1998. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. Annals of the New York Academy of Sciences **856:**1-11.
91. **Dinarello, C. A.** 1998. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. Int Rev Immunol **16:**457-499.
92. **Dinarello, C. A.** 1987. The biology of interleukin 1 and comparison to tumor necrosis factor. Immunology letters **16:**227-231.
93. **Ding, A. H., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol **141:**2407-2412.
94. **Ding, A. H., and F. Porteu.** 1992. Regulation of tumor necrosis factor receptors on phagocytes. Proc Soc Exp Biol Med **200:**458-465.
95. **Ding, L., and E. M. Shevach.** 1992. IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. J Immunol **148:**3133-3139.
96. **Ding Y, F. S., Zamarin D, Bromberg JS.** 2003. Interleukin-10. In: Thomson AW, Lotze MT, editors. The cytokine handbook. California: Academic Press 603-625.
97. **Dranoff, G.** 2004. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer **4:**11-22.
98. **Dubravec, D. B., D. R. Spriggs, J. A. Mannick, and M. L. Rodrick.** 1990. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. Proceedings of the National Academy of Sciences of the United States of America **87:**6758-6761.
99. **Eck, M. J., B. Beutler, G. Kuo, J. P. Merryweather, and S. R. Sprang.** 1988. Crystallization of trimeric recombinant human tumor necrosis factor (cachectin). The Journal of biological chemistry **263:**12816-12819.
100. **Eder, C.** 2009. Mechanisms of interleukin-1beta release. Immunobiology **214:**543-553.
101. **El-Etr, S. H., L. Yan, and J. D. Cirillo.** 2001. Fish monocytes as a model for mycobacterial host-pathogen interactions. Infection and immunity **69:**7310-7317.
102. **Ellsaesser, C. F., and L. W. Clem.** 1994. Functionally distinct high and low molecular weight species of channel catfish and mouse IL-1. Cytokine **6:**10-20.
103. **Engelsma, M. Y., R. J. Stet, J. P. Saeij, and B. M. Verburg-van Kemenade.** 2003. Differential expression and haplotypic variation of two

interleukin-1beta genes in the common carp (Cyprinus carpio L.). Cytokine **22:**21-32.

104. **Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson.** 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. The Journal of clinical investigation **101:**890-898.
105. **Fadok, V. A., P. P. McDonald, D. L. Bratton, and P. M. Henson.** 1998. Regulation of macrophage cytokine production by phagocytosis of apoptotic and post-apoptotic cells. Biochem Soc Trans **26:**653-656.
106. **Farrar, M. A., J. Fernandez-Luna, and R. D. Schreiber.** 1991. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. The Journal of biological chemistry **266:**19626-19635.
107. **Faurschou, M., and N. Borregaard.** 2003. Neutrophil granules and secretory vesicles in inflammation. Microbes and infection / Institut Pasteur **5:**1317-1327.
108. **Fedishin, I., R. S. Stoika, and S. I. Kusen.** 1990. [Detection of protease-sensitive factors with properties of beta-type transforming growth factor in early loach embryos]. Ontogenez **21:**63-68.
109. **Feng, C. G., M. C. Kullberg, D. Jankovic, A. W. Cheever, P. Caspar, R. L. Coffman, and A. Sher.** 2002. Transgenic mice expressing human interleukin-10 in the antigen-presenting cell compartment show increased susceptibility to infection with Mycobacterium avium associated with decreased macrophage effector function and apoptosis. Infection and immunity **70:**6672-6679.
110. **Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio.** 2006. The P2X7 receptor: a key player in IL-1 processing and release. J Immunol **176:**3877-3883.
111. **Fertsch, D., and S. N. Vogel.** 1984. Recombinant interferons increase macrophage Fc receptor capacity. J Immunol **132:**2436-2439.
112. **Fiorentino, D. F., M. W. Bond, and T. R. Mosmann.** 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. The Journal of experimental medicine **170:**2081-2095.
113. **Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra.** 1991. IL-10 inhibits cytokine production by activated macrophages. J Immunol **147:**3815-3822.
114. **Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra.** 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol **146:**3444-3451.
115. **Flynn, J. L., and J. Chan.** 2003. Immune evasion by Mycobacterium tuberculosis: living with the enemy. Current opinion in immunology **15:**450-455.

116. **Forlenza, M., S. Magez, J. P. Scharsack, A. Westphal, H. F. Savelkoul, and G. F. Wiegertjes.** 2009. Receptor-mediated and lectin-like activities of carp (Cyprinus carpio) TNF-alpha. J Immunol **183:**5319-5332.
117. **Freire-de-Lima, C. G., D. O. Nascimento, M. B. Soares, P. T. Bozza, H. C. Castro-Faria-Neto, F. G. de Mello, G. A. DosReis, and M. F. Lopes.** 2000. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature **403:**199-203.
118. **Fujiki, K., J. Gauley, N. C. Bols, and B. Dixon.** 2003. Genomic cloning of novel isotypes of the rainbow trout interleukin-8. Immunogenetics **55:**126-131.
119. **Fujiki, K., D. H. Shin, M. Nakao, and T. Yano.** 2000. Molecular cloning and expression analysis of carp (Cyprinus carpio) interleukin-1 beta, high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. Fish & shellfish immunology **10:**229-242.
120. **Funk, C. D.** 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science (New York, N.Y **294:**1871-1875.
121. **Furze, R. C., and S. M. Rankin.** 2008. Neutrophil mobilization and clearance in the bone marrow. Immunology **125:**281-288.
122. **Furze, R. C., and S. M. Rankin.** 2008. The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. Faseb J **22:**3111-3119.
123. **Gabbiani, G.** 1977. Reparative processes in mammalian wound healing: the role of contractile phenomena. International review of cytology **48:**187-219.
124. **Gabbiani, G.** 1979. The role of contractile proteins in wound healing and fibrocontractive diseases. Methods and achievements in experimental pathology **9:**187-206.
125. **Gabbiani, G., and M. C. Badonnel.** 1976. Contractile events during inflammation. Agents and actions **6:**277-280.
126. **Gabbiani, G., C. Chaponnier, and I. Huttner.** 1978. Cytoplasmic filaments and gap junctions in epithelial cells and myofibroblasts during wound healing. The Journal of cell biology **76:**561-568.
127. **Ganz, T., and R. I. Lehrer.** 1997. Antimicrobial peptides of leukocytes. Current opinion in hematology **4:**53-58.
128. **Gao, L. Y., F. Laval, E. H. Lawson, R. K. Groger, A. Woodruff, J. H. Morisaki, J. S. Cox, M. Daffe, and E. J. Brown.** 2003. Requirement for kasB in Mycobacterium mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Molecular microbiology **49:**1547-1563.
129. **Gao, L. Y., M. Pak, R. Kish, K. Kajihara, and E. J. Brown.** 2006. A mycobacterial operon essential for virulence in vivo and invasion and intracellular persistence in macrophages. Infection and immunity **74:**1757-1767.
130. **Gao, Q., P. Nie, K. D. Thompson, A. Adams, T. Wang, C. J. Secombes, and J. Zou.** 2009. The search for the IFN-gamma receptor in fish: Functional and expression analysis of putative binding and signalling

chains in rainbow trout Oncorhynchus mykiss. Developmental and comparative immunology **33:**920-931.

131. **Garcia-Castillo, J., E. Chaves-Pozo, P. Olivares, P. Pelegrin, J. Meseguer, and V. Mulero.** 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci **61:**1331-1340.
132. **Garcia-Ramallo, E., T. Marques, N. Prats, J. Beleta, S. L. Kunkel, and N. Godessart.** 2002. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. J Immunol **169:**6467-6473.
133. **Gautam, N., A. M. Olofsson, H. Herwald, L. F. Iversen, E. Lundgren-Akerlund, P. Hedqvist, K. E. Arfors, H. Flodgaard, and L. Lindbom.** 2001. Heparin-binding protein (HBP/CAP37): a missing link in neutrophil-evoked alteration of vascular permeability. Nature medicine **7:**1123-1127.
134. **Gazzinelli, R. T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher.** 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol **157:**798-805.
135. **Geissmann, F., P. Revy, A. Regnault, Y. Lepelletier, M. Dy, N. Brousse, S. Amigorena, O. Hermine, and A. Durandy.** 1999. TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. J Immunol **162:**4567-4575.
136. **Gerwick, L., R. Steinhauer, S. Lapatra, T. Sandell, J. Ortuno, N. Hajiseyedjavadi, and C. J. Bayne.** 2002. The acute phase response of rainbow trout (Oncorhynchus mykiss) plasma proteins to viral, bacterial and fungal inflammatory agents. Fish & shellfish immunology **12:**229-242.
137. **Ghosh, S., and S. Bhattacharya.** 1992. Elevation of C-reactive protein in serum of Channa punctatus as an indicator of water pollution. Indian journal of experimental biology **30:**736-737.
138. **Gilchrist, M., and A. D. Befus.** 2008. Interferon-gamma regulates chemokine expression and release in the human mast cell line HMC1: role of nitric oxide. Immunology **123:**209-217.
139. **Gleizes, P. E., J. S. Munger, I. Nunes, J. G. Harpel, R. Mazzieri, I. Noguera, and D. B. Rifkin.** 1997. TGF-beta latency: biological significance and mechanisms of activation. Stem Cells **15:**190-197.
140. **Gong, Y., P. Cao, H. J. Yu, and T. Jiang.** 2008. Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. Nature **454:**789-793.
141. **Goumans, M. J., and C. Mummery.** 2000. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. Int J Dev Biol **44:**253-265.

142. **Govinden, R., and K. D. Bhoola.** 2003. Genealogy, expression, and cellular function of transforming growth factor-beta. Pharmacol Ther **98:**257-265.
143. **Graham, S., and C. J. Secombes.** 1990. Do fish lymphocytes secrete interferon-y? J. Fish Biol. **36:**563-573.
144. **Graham, S., and C. J. Secombes.** 1988. The production of a macrophage-activating factor from rainbow trout Salmo gairdneri leucocytes. Immunology **65:**293-297.
145. **Grau, G. E., L. F. Fajardo, P. F. Piguet, B. Allet, P. H. Lambert, and P. Vassalli.** 1987. Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria. Science (New York, N.Y **237:**1210-1212.
146. **Gray, C., C. A. Loynes, M. K. Whyte, D. C. Crossman, S. A. Renshaw, and T. J. Chico.** 2011. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. Thrombosis and haemostasis **105**.
147. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of novel interferon gamma receptor 1 isoforms in zebrafish (Danio rerio) and goldfish (Carassius auratus L.). Molecular immunology **46:**3050-3059.
148. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of tumor necrosis factor receptors 1 and 2 of the goldfish (Carassius auratus L.). Molecular immunology **46:**2190-2199.
149. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization, expression and functional analysis of goldfish (Carassius auratus L.) interferon gamma. Developmental and comparative immunology **33:**235-246.
150. **Grayfer, L., E. G. Garcia, and M. Belosevic.** 2010. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (Carassius auratus L.). The Journal of biological chemistry **285:**23537-23547.
151. **Grayfer, L., P. C. Hanington, and M. Belosevic.** 2009. Macrophage colony-stimulating factor (CSF-1) induces pro-inflammatory gene expression and enhances antimicrobial responses of goldfish (Carassius auratus L.) macrophages. Fish & shellfish immunology **26:**406-413.
152. **Grayfer, L., J. W. Hodgkinson, S. J. Hitchen, and M. Belosevic.** 2010. Characterization and functional analysis of goldfish (Carassius auratus L.) interleukin-10. Molecular immunology **486:**563-571
153. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Developmental and comparative immunology **32:**532-543.
154. **Greenlund, A. C., M. A. Farrar, B. L. Viviano, and R. D. Schreiber.** 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). The EMBO journal **13:**1591-1600.
155. **Greenlund, A. C., M. O. Morales, B. L. Viviano, H. Yan, J. Krolewski, and R. D. Schreiber.** 1995. Stat recruitment by tyrosine-phosphorylated

cytokine receptors: an ordered reversible affinity-driven process. Immunity **2:**677-687.

156. **Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich.** 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell **83:**793-802.
157. **Griffin, W. S., J. G. Sheng, M. C. Royston, S. M. Gentleman, J. E. McKenzie, D. I. Graham, G. W. Roberts, and R. E. Mrak.** 1998. Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol **8:**65-72.
158. **Grimsley, C., and K. S. Ravichandran.** 2003. Cues for apoptotic cell engulfment: eat-me, don't eat-me and come-get-me signals. Trends in cell biology **13:**648-656.
159. **Groux, H., F. Cottrez, M. Rouleau, S. Mauze, S. Antonenko, S. Hurst, T. McNeil, M. Bigler, M. G. Roncarolo, and R. L. Coffman.** 1999. A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. J Immunol **162:**1723-1729.
160. **Gruys, E., M. J. Toussaint, T. A. Niewold, and S. J. Koopmans.** 2005. Acute phase reaction and acute phase proteins. Journal of Zhejiang University. Science **6:**1045-1056.
161. **Gupta, J. W., M. Kubin, L. Hartman, M. Cassatella, and G. Trinchieri.** 1992. Induction of expression of genes encoding components of the respiratory burst oxidase during differentiation of human myeloid cell lines induced by tumor necrosis factor and gamma-interferon. Cancer Res **52:**2530-2537.
162. **Haddad, G., P. C. Hanington, E. C. Wilson, L. Grayfer, and M. Belosevic.** 2008. Molecular and functional characterization of goldfish (Carassius auratus L.) transforming growth factor beta. Developmental and comparative immunology **32:**654-663.
163. **Hajjar, K. A., D. P. Hajjar, R. L. Silverstein, and R. L. Nachman.** 1987. Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells. The Journal of experimental medicine **166:**235-245.
164. **Hamby, B. A., E. M. Huggins, Jr., L. B. Lachman, C. A. Dinarello, and M. M. Sigel.** 1986. Fish lymphocytes respond to human IL-1. Lymphokine research **5:**157-162.
165. **Hamdani, S. H., D. N. McMillan, E. F. Pettersen, H. Wergeland, C. Endresen, A. E. Ellis, and C. J. Secombes.** 1998. Isolation of rainbow trout neutrophils with an anti-granulocyte monoclonal antibody. Veterinary immunology and immunopathology **63:**369-380.
166. **Hansen, J. D., L. N. Vojtech, and K. J. Laing.** 2011. Sensing disease and danger: A survey of vertebrate PRRs and their origins. Developmental and comparative immunology.
167. **Hardie, L. J., L. H. Chappell, and C. J. Secombes.** 1994. Human tumor necrosis factor alpha influences rainbow trout Oncorhynchus mykiss

leucocyte responses. Veterinary immunology and immunopathology **40:**73-84.

168. **Hardie, L. J., K. J. Laing, G. D. Daniels, P. S. Grabowski, C. Cunningham, and C. J. Secombes.** 1998. Isolation of the first piscine transforming growth factor beta gene: analysis reveals tissue specific expression and a potential regulatory sequence in rainbow trout (Oncorhynchus mykiss). Cytokine **10:**555-563.
169. **Harms, C. A., K. E. Howard, J. C. Wolf, S. A. Smith, and S. Kennedy-Stoskopf.** 2003. Transforming growth factor-beta response to mycobacterial infection in striped bass Morone saxatilis and hybrid tilapia Oreochromis spp. Veterinary immunology and immunopathology **95:**155-163.
170. **Harun, N. O., J. Zou, Y. A. Zhang, P. Nie, and C. J. Secombes.** 2008. The biological effects of rainbow trout (Oncorhynchus mykiss) recombinant interleukin-8. Developmental and comparative immunology **32:**673-681.
171. **Heinrich, P. C., J. V. Castell, and T. Andus.** 1990. Interleukin-6 and the acute phase response. The Biochemical journal **265:**621-636.
172. **Heinrich, P. C., F. Horn, L. Graeve, E. Dittrich, I. Kerr, G. Muller-Newen, J. Grotzinger, and A. Wollmer.** 1998. Interleukin-6 and related cytokines: effect on the acute phase reaction. Zeitschrift fur Ernahrungswissenschaft **37 Suppl 1:**43-49.
173. **Heldin, C. H., K. Miyazono, and P. ten Dijke.** 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature **390:**465-471.
174. **Hernandez-Pando, R., D. Aguilar, M. L. Hernandez, H. Orozco, and G. Rook.** 2004. Pulmonary tuberculosis in BALB/c mice with non-functional IL-4 genes: changes in the inflammatory effects of TNF-alpha and in the regulation of fibrosis. European journal of immunology **34:**174-183.
175. **Herwald, H., H. Cramer, M. Morgelin, W. Russell, U. Sollenberg, A. Norrby-Teglund, H. Flodgaard, L. Lindbom, and L. Bjorck.** 2004. M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. Cell **116:**367-379.
176. **Hirono, I., B. H. Nam, T. Kurobe, and T. Aoki.** 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder Paralychthys olivaceus. J Immunol **165:**4423-4427.
177. **Hong, S., S. Peddie, J. J. Campos-Perez, J. Zou, and C. J. Secombes.** 2003. The effect of intraperitoneally administered recombinant IL-1beta on immune parameters and resistance to Aeromonas salmonicida in the rainbow trout (Oncorhynchus mykiss). Developmental and comparative immunology **27:**801-812.
178. **Hong, S., and C. J. Secombes.** 2009. Two peptides derived from trout IL-1beta have different stimulatory effects on immune gene expression after intraperitoneal administration. Comp Biochem Physiol B Biochem Mol Biol **153:**275-280.

179. **Hong, S., J. Zou, B. Collet, N. C. Bols, and C. J. Secombes.** 2004. Analysis and characterisation of IL-1beta processing in rainbow trout, Oncorhynchus mykiss. Fish & shellfish immunology **16:**453-459.
180. **Hong, S., J. Zou, M. Crampe, S. Peddie, G. Scapigliati, N. Bols, C. Cunningham, and C. J. Secombes.** 2001. The production and bioactivity of rainbow trout (Oncorhynchus mykiss) recombinant IL-1 beta. Veterinary immunology and immunopathology **81:**1-14.
181. **Huang, S., S. Yuan, L. Guo, Y. Yu, J. Li, T. Wu, T. Liu, M. Yang, K. Wu, H. Liu, J. Ge, Y. Yu, H. Huang, M. Dong, C. Yu, S. Chen, and A. Xu.** 2008. Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. Genome research **18:**1112-1126.
182. **Huang, S. H., W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, RM., Aguet, M. .** 1993. Immune response in mice that lack the interferon-γ receptor. Science (New York, N.Y **259**.
183. **Huising, M. O., E. Stolte, G. Flik, H. F. Savelkoul, and B. M. Verburg-van Kemenade.** 2003. CXC chemokines and leukocyte chemotaxis in common carp (Cyprinus carpio L.). Developmental and comparative immunology **27:**875-888.
184. **Hunter, C. A., L. A. Ellis-Neyes, T. Slifer, S. Kanaly, G. Grunig, M. Fort, D. Rennick, and F. G. Araujo.** 1997. IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. J Immunol **158:**3311-3316.
185. **Igawa, D., M. Sakai, and R. Savan.** 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Molecular immunology **43:**999-1009.
186. **Ihle, J. N., and I. M. Kerr.** 1995. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet **11:**69-74.
187. **Ingenbleek, Y., and V. Young.** 1994. Transthyretin (prealbumin) in health and disease: nutritional implications. Annual review of nutrition **14:**495-533.
188. **Ingerslev, H. C., A. Ronneseth, E. F. Pettersen, and H. I. Wergeland.** 2009. Differential expression of immune genes in Atlantic salmon (Salmo salar L.) challenged intraperitoneally or by cohabitation with IPNV. Scand J Immunol **69:**90-98.
189. **Inoue, Y., S. Kamota, K. Ito, Y. Yoshiura, M. Ototake, T. Moritomo, and T. Nakanishi.** 2005. Molecular cloning and expression analysis of rainbow trout (Oncorhynchus mykiss) interleukin-10 cDNAs. Fish & shellfish immunology **18:**335-344.
190. **Ischiropoulos, H., L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith, and J. S. Beckman.** 1992. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Archives of biochemistry and biophysics **298:**431-437.

191. **Ishibe, K., T. Yamanishi, Y. Wang, K. Osatomi, K. Hara, K. Kanai, K. Yamaguchi, and T. Oda.** 2009. Comparative analysis of the production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-alpha) from macrophages exposed to high virulent and low virulent strains of Edwardsiella tarda. Fish & shellfish immunology **27:**386-389.
192. **Ishii, A., M. Kawasaki, M. Matsumoto, S. Tochinai, and T. Seya.** 2007. Phylogenetic and expression analysis of amphibian Xenopus Toll-like receptors. Immunogenetics **59:**281-293.
193. **Iyengar, R., D. J. Stuehr, and M. A. Marletta.** 1987. Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. Proceedings of the National Academy of Sciences of the United States of America **84:**6369-6373.
194. **Jagels, M. A., J. D. Chambers, K. E. Arfors, and T. E. Hugli.** 1995. C5a- and tumor necrosis factor-alpha-induced leukocytosis occurs independently of beta 2 integrins and L-selectin: differential effects on neutrophil adhesion molecule expression in vivo. Blood **85:**2900-2909.
195. **Jagels, M. A., and T. E. Hugli.** 1992. Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow. J Immunol **148:**1119-1128.
196. **Janeway, C. A., Jr.** 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology **54 Pt 1:**1-13.
197. **Jang, S. I., L. J. Hardie, and C. J. Secombes.** 1994. Effects of transforming growth factor beta 1 on rainbow trout Oncorhynchus mykiss macrophage respiratory burst activity. Developmental and comparative immunology **18:**315-323.
198. **Jang, S. I., L. J. Hardie, and C. J. Secombes.** 1995. Elevation of rainbow trout Oncorhynchus mykiss macrophage respiratory burst activity with macrophage-derived supernatants. Journal of leukocyte biology **57:**943-947.
199. **Jensen, L. E., M. P. Hiney, D. C. Shields, C. M. Uhlar, A. J. Lindsay, and A. S. Whitehead.** 1997. Acute phase proteins in salmonids: evolutionary analyses and acute phase response. J Immunol **158:**384-392.
200. **Jorgensen, J. B., H. Lunde, L. Jensen, A. S. Whitehead, and B. Robertsen.** 2000. Serum amyloid A transcription in Atlantic salmon (Salmo salar L.) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1 beta, IL-6 and TNF alpha or bacterial lipopolysaccharide. Developmental and comparative immunology **24:**553-563.
201. **Jozsef, L., C. Zouki, N. A. Petasis, C. N. Serhan, and J. G. Filep.** 2002. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit peroxynitrite formation, NF-kappa B and AP-1 activation, and IL-8 gene expression in human leukocytes. Proceedings of the National Academy of Sciences of the United States of America **99:**13266-13271.
202. **Jurecka, P., I. Irnazarow, J. L. Stafford, A. Ruszczyk, N. Taverne, M. Belosevic, H. F. Savelkoul, and G. F. Wiegertjes.** 2009. The induction

of nitric oxide response of carp macrophages by transferrin is influenced by the allelic diversity of the molecule. Fish & shellfish immunology **26:**632-638.

203. **Kadowaki, T., H. Harada, Y. Sawada, C. Kohchi, G. Soma, Y. Takahashi, and H. Inagawa.** 2009. Two types of tumor necrosis factor-alpha in bluefin tuna (Thunnus orientalis) genes: Molecular cloning and expression profile in response to several immunological stimulants. Fish & shellfish immunology **27:**585-594.
204. **Kaplan, D. H., A. C. Greenlund, J. W. Tanner, A. S. Shaw, and R. D. Schreiber.** 1996. Identification of an interferon-gamma receptor alpha chain sequence required for JAK-1 binding. The Journal of biological chemistry **271:**9-12.
205. **Kasama, T., R. M. Strieter, N. W. Lukacs, P. M. Lincoln, M. D. Burdick, and S. L. Kunkel.** 1995. Interferon gamma modulates the expression of neutrophil-derived chemokines. J Investig Med **43:**58-67.
206. **Kasama, T., R. M. Strieter, T. J. Standiford, M. D. Burdick, and S. L. Kunkel.** 1993. Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha. The Journal of experimental medicine **178:**63-72.
207. **Kato, Y., M. Nakao, M. Shimizu, H. Wariishi, and T. Yano.** 2004. Purification and functional assessment of C3a, C4a and C5a of the common carp (Cyprinus carpio) complement. Developmental and comparative immunology **28:**901-910.
208. **Keestra, A. M., M. R. de Zoete, R. A. van Aubel, and J. P. van Putten.** 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J Immunol **178:**7110-7119.
209. **Kerr, I. M., and G. R. Stark.** 1992. The antiviral effects of the interferons and their inhibition. Journal of interferon research **12:**237-240.
210. **Khan, S., J. D. Greenberg, and N. Bhardwaj.** 2009. Dendritic cells as targets for therapy in rheumatoid arthritis. Nat Rev Rheumatol **5:**566-571.
211. **Kim, E. Y., J. J. Priatel, S. J. Teh, and H. S. Teh.** 2006. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. J Immunol **176:**1026-1035.
212. **Kim, E. Y., and H. S. Teh.** 2004. Critical role of TNF receptor type-2 (p75) as a costimulator for IL-2 induction and T cell survival: a functional link to CD28. J Immunol **173:**4500-4509.
213. **Kimura, T., K. Nakayama, J. Penninger, M. Kitagawa, H. Harada, T. Matsuyama, N. Tanaka, R. Kamijo, J. Vilcek, T. W. Mak, and et al.** 1994. Involvement of the IRF-1 transcription factor in antiviral responses to interferons. Science (New York, N.Y **264:**1921-1924.
214. **Kingsley, D. M.** 1994. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev **8:**133-146.

215. **Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph.** 1986. Stimulation of neutrophils by tumor necrosis factor. J Immunol **136:**4220-4225.
216. **Knolle, P., H. Lohr, U. Treichel, H. P. Dienes, A. Lohse, J. Schlaack, and G. Gerken.** 1995. Parenchymal and nonparenchymal liver cells and their interaction in the local immune response. Zeitschrift fur Gastroenterologie **33:**613-620.
217. **Kobayashi, Y., K. Yamamoto, T. Saido, H. Kawasaki, J. J. Oppenheim, and K. Matsushima.** 1990. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. Proceedings of the National Academy of Sciences of the United States of America **87:**5548-5552.
218. **Kodama, H., K. Tijiwa, T. Moritomo, and T. Nakanishi.** 2002. Granulocyte responses to experimental injection of live and formalin-killed bacteria in carp (Cyprinus carpio). Veterinary immunology and immunopathology **90:**101-105.
219. **Kohli, G., S. Hu, E. Clelland, T. Di Muccio, J. Rothenstein, and C. Peng.** 2003. Cloning of transforming growth factor-beta 1 (TGF-beta 1) and its type II receptor from zebrafish ovary and role of TGF-beta 1 in oocyte maturation. Endocrinology **144:**1931-1941.
220. **Kono, H., and K. L. Rock.** 2008. How dying cells alert the immune system to danger. Nature reviews **8:**279-289.
221. **Kono, T., K. Fujiki, M. Nakao, T. Yano, M. Endo, and M. Sakai.** 2002. The immune responses of common carp, Cyprinus carpio L., injected with carp interleukin-1beta gene. J Interferon Cytokine Res **22:**413-419.
222. **Koo, I. C., C. Wang, S. Raghavan, J. H. Morisaki, J. S. Cox, and E. J. Brown.** 2008. ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. Cellular microbiology **10:**1866-1878.
223. **Kotenko, S. V., C. D. Krause, L. S. Izotova, B. P. Pollack, W. Wu, and S. Pestka.** 1997. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. Embo J **16:**5894-5903.
224. **Koul, A., T. Herget, B. Klebl, and A. Ullrich.** 2004. Interplay between mycobacteria and host signalling pathways. Nat Rev Microbiol **2:**189-202.
225. **Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu.** 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell **53:**45-53.
226. **Krishnan, L., L. J. Guilbert, T. G. Wegmann, M. Belosevic, and T. R. Mosmann.** 1996. T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J Immunol **156:**653-662.
227. **Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller.** 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell **75:**263-274.

228. **Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward, and S. Karlsson.** 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proceedings of the National Academy of Sciences of the United States of America **90:**770-774.
229. **Kulkarni, A. B., and S. Karlsson.** 1993. Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. The American journal of pathology **143:**3-9.
230. **Kurata, O., N. Okamoto, and Y. Ikeda.** 1995. Neutrophilic granulocytes in carp, Cyprinus carpio, possess a spontaneous cytotoxic activity. Developmental and comparative immunology **19:**315-325.
231. **Kurt-Jones, E. A., D. I. Beller, S. B. Mizel, and E. R. Unanue.** 1985. Identification of a membrane-associated interleukin 1 in macrophages. Proceedings of the National Academy of Sciences of the United States of America **82:**1204-1208.
232. **Laing, K. J., N. Bols, and C. J. Secombes.** 2002. A CXC chemokine sequence isolated from the rainbow trout Oncorhynchus mykiss resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11. European cytokine network **13:**462-473.
233. **Laing, K. J., P. S. Grabowski, M. Belosevic, and C. J. Secombes.** 1996. A partial sequence for nitric oxide synthase from a goldfish (Carassius auratus) macrophage cell line. Immunology and cell biology **74:**374-379.
234. **Laing, K. J., L. J. Hardie, W. Aartsen, P. S. Grabowski, and C. J. Secombes.** 1999. Expression of an inducible nitric oxide synthase gene in rainbow trout Oncorhynchus mykiss. Developmental and comparative immunology **23:**71-85.
235. **Laing, K. J., M. K. Purcell, J. R. Winton, and J. D. Hansen.** 2008. A genomic view of the NOD-like receptor family in teleost fish: identification of a novel NLR subfamily in zebrafish. BMC evolutionary biology **8:**42.
236. **Laing, K. J., T. Wang, J. Zou, J. Holland, S. Hong, N. Bols, I. Hirono, T. Aoki, and C. J. Secombes.** 2001. Cloning and expression analysis of rainbow trout Oncorhynchus mykiss tumour necrosis factor-alpha. Eur J Biochem **268:**1315-1322.
237. **Laing, K. J., J. J. Zou, T. Wang, N. Bols, I. Hirono, T. Aoki, and C. J. Secombes.** 2002. Identification and analysis of an interleukin 8-like molecule in rainbow trout Oncorhynchus mykiss. Developmental and comparative immunology **26:**433-444.
238. **Le, J., L. F. Reis, and J. Vilcek.** 1988. Tumor necrosis factor and interleukin 1 can act as essential growth factors in a murine plasmacytoma line. Lymphokine research **7:**99-106.
239. **Lee, E. Y., H. H. Park, Y. T. Kim, and T. J. Choi.** 2001. Cloning and sequence analysis of the interleukin-8 gene from flounder (Paralichthys olivaceous). Gene **274:**237-243.

240. **Lee, T. D., M. L. Gonzalez, P. Kumar, P. Grammas, and H. A. Pereira.** 2003. CAP37, a neutrophil-derived inflammatory mediator, augments leukocyte adhesion to endothelial monolayers. Microvasc Res **66:**38-48.
241. **Leibovich, S. J.** 1978. Production of macrophage-dependent fibroblast-stimulating activity (M-FSA) by murine macrophages. Effects on BALBc 3T3 fibroblasts. Experimental cell research **113:**47-56.
242. **Leibovich, S. J., and R. Ross.** 1976. A macrophage-dependent factor that stimulates the proliferation of fibroblasts in vitro. The American journal of pathology **84:**501-514.
243. **Leibovich, S. J., and R. Ross.** 1975. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. The American journal of pathology **78:**71-100.
244. **Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:**973-983.
245. **Leto, T. L., K. J. Lomax, B. D. Volpp, H. Nunoi, J. M. Sechler, W. M. Nauseef, R. A. Clark, J. I. Gallin, and H. L. Malech.** 1990. Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. Science (New York, N.Y **248:**727-730.
246. **Letterio, J. J., and A. B. Roberts.** 1998. Regulation of immune responses by TGF-beta. Annu Rev Immunol **16:**137-161.
247. **Levy, B. D., C. B. Clish, B. Schmidt, K. Gronert, and C. N. Serhan.** 2001. Lipid mediator class switching during acute inflammation: signals in resolution. Nature immunology **2:**612-619.
248. **Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh.** 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nature reviews **7:**678-689.
249. **Li, J., R. Peters, S. Lapatra, M. Vazzana, and J. O. Sunyer.** 2004. Anaphylatoxin-like molecules generated during complement activation induce a dramatic enhancement of particle uptake in rainbow trout phagocytes. Developmental and comparative immunology **28:**1005-1021.
250. **Li, J., M. Zhang, and Y. C. Rui.** 1997. Tumor necrosis factor mediated release of platelet-derived growth factor from bovine cerebral microvascular endothelial cells. Zhongguo Yao Li Xue Bao **18:**133-136.
251. **Li, M. O., M. R. Sarkisian, W. Z. Mehal, P. Rakic, and R. A. Flavell.** 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. Science (New York, N.Y **302:**1560-1563.
252. **Li, S., X. Li, H. Zheng, B. Xie, K. R. Bidasee, and G. J. Rozanski.** 2008. Pro-oxidant effect of transforming growth factor- beta1 mediates contractile dysfunction in rat ventricular myocytes. Cardiovascular research **77:**107-117.
253. **Lieschke, G. J., D. Grail, G. Hodgson, D. Metcalf, E. Stanley, C. Cheers, K. J. Fowler, S. Basu, Y. F. Zhan, and A. R. Dunn.** 1994. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia,

granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood **84:**1737-1746.

254. **Lin, B., S. Chen, Z. Cao, Y. Lin, D. Mo, H. Zhang, J. Gu, M. Dong, Z. Liu, and A. Xu.** 2007. Acute phase response in zebrafish upon Aeromonas salmonicida and Staphylococcus aureus infection: striking similarities and obvious differences with mammals. Molecular immunology **44:**295-301.
255. **Liongue, C., C. J. Hall, B. A. O'Connell, P. Crosier, and A. C. Ward.** 2009. Zebrafish granulocyte colony-stimulating factor receptor signaling promotes myelopoiesis and myeloid cell migration. Blood **113:**2535-2546.
256. **Liu, Y., T. Iwasaki, S. Watarai, and H. Kodama.** 2004. Effect of turpentine oil on C-reactive protein (CRP) production in rainbow trout (Oncorhynchus mykiss). Fish & shellfish immunology **17:**203-210.
257. **Liu, Y., S. H. Wei, A. S. Ho, R. de Waal Malefyt, and K. W. Moore.** 1994. Expression cloning and characterization of a human IL-10 receptor. J Immunol **152:**1821-1829.
258. **Lohmann-Matthes, M. L., B. Luttig, and S. Hockertz.** 1991. Involvement of membrane-associated TNF in the killing of Leishmania donovani parasites by macrophages. Behring Inst Mitt**:**125-132.
259. **Lomedico, P. T., U. Gubler, C. P. Hellmann, M. Dukovich, J. G. Giri, Y. C. Pan, K. Collier, R. Semionow, A. O. Chua, and S. B. Mizel.** 1984. Cloning and expression of murine interleukin-1 cDNA in Escherichia coli. Nature **312:**458-462.
260. **Long, Q., E. Quint, S. Lin, and M. Ekker.** 2000. The zebrafish scyba gene encodes a novel CXC-type chemokine with distinctive expression patterns in the vestibulo-acoustic system during embryogenesis. Mechanisms of development **97:**183-186.
261. **Lopez-Castejon, G., M. P. Sepulcre, I. Mulero, P. Pelegrin, J. Meseguer, and V. Mulero.** 2008. Molecular and functional characterization of gilthead seabream Sparus aurata caspase-1: the first identification of an inflammatory caspase in fish. Molecular immunology **45:**49-57.
262. **Lopez-Castejon, G., M. P. Sepulcre, F. J. Roca, B. Castellana, J. V. Planas, J. Meseguer, and V. Mulero.** 2007. The type II interleukin-1 receptor (IL-1RII) of the bony fish gilthead seabream Sparus aurata is strongly induced after infection and tightly regulated at transcriptional and post-transcriptional levels. Molecular immunology **44:**2772-2780.
263. **Lopez-Castejon, G., M. T. Young, J. Meseguer, A. Surprenant, and V. Mulero.** 2007. Characterization of ATP-gated P2X7 receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin-1 beta. Molecular immunology **44:**1286-1299.
264. **Lopez-Munoz, A., F. J. Roca, M. P. Sepulcre, J. Meseguer, and V. Mulero.** Zebrafish larvae are unable to mount a protective antiviral response against waterborne infection by spring viremia of carp virus. Developmental and comparative immunology **34:**546-552.

265. **Lowenstein, C. J., and E. Padalko.** 2004. iNOS (NOS2) at a glance. Journal of cell science **117:**2865-2867.
266. **Lu, D. Q., J. X. Bei, L. N. Feng, Y. Zhang, X. C. Liu, L. Wang, J. L. Chen, and H. R. Lin.** 2008. Interleukin-1beta gene in orange-spotted grouper, Epinephelus coioides: molecular cloning, expression, biological activities and signal transduction. Molecular immunology **45:**857-867.
267. **Ludviksson, B. R., D. Seegers, A. S. Resnick, and W. Strober.** 2000. The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. European journal of immunology **30:**2101-2111.
268. **Lund, V., and J. A. Olafsen.** 1999. Changes in serum concentration of a serum amyloid P-like pentraxin in Atlantic salmon, Salmo salar L., during infection and inflammation. Developmental and comparative immunology **23:**61-70.
269. **Luo, M., E. A. Fadeev, and J. T. Groves.** 2005. Mycobactin-mediated iron acquisition within macrophages. Nature chemical biology **1:**149-153.
270. **Lutfalla, G., H. Roest Crollius, N. Stange-Thomann, O. Jaillon, K. Mogensen, and D. Monneron.** 2003. Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: the class II cytokine receptors and their ligands in mammals and fish. BMC genomics **4:**29.
271. **MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North, and A. Surprenant.** 2001. Rapid secretion of interleukin-1beta by microvesicle shedding. Immunity **15:**825-835.
272. **MacKenzie, S., J. V. Planas, and F. W. Goetz.** 2003. LPS-stimulated expression of a tumor necrosis factor-alpha mRNA in primary trout monocytes and in vitro differentiated macrophages. Developmental and comparative immunology **27:**393-400.
273. **Magez, S., B. Stijlemans, G. Caljon, H. P. Eugster, and P. De Baetselier.** 2002. Control of experimental Trypanosoma brucei infections occurs independently of lymphotoxin-alpha induction. Infection and immunity **70:**1342-1351.
274. **Markiewski, M. M., and J. D. Lambris.** 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. The American journal of pathology **171:**715-727.
275. **Martin, C., P. C. Burdon, G. Bridger, J. C. Gutierrez-Ramos, T. J. Williams, and S. M. Rankin.** 2003. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity **19:**583-593.
276. **Martin, E., C. Nathan, and Q. W. Xie.** 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. The Journal of experimental medicine **180:**977-984.
277. **Martin, J. H., and S. W. Edwards.** 1993. Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. Reactive oxygen intermediates are involved in monocyte-mediated cytotoxicity, whereas reactive nitrogen intermediates are employed by macrophages in tumor cell killing. J Immunol **150:**3478-3486.

278. **Martin, S. A., B. P. Mohanty, P. Cash, D. F. Houlihan, and C. J. Secombes.** 2007. Proteome analysis of the Atlantic salmon (Salmo salar) cell line SHK-1 following recombinant IFN-gamma stimulation. Proteomics **7:**2275-2286.
279. **Martinez, F. O., A. Sica, A. Mantovani, and M. Locati.** 2008. Macrophage activation and polarization. Front Biosci **13:**453-461.
280. **Massague, J.** 1998. TGF-beta signal transduction. Annu Rev Biochem **67:**753-791.
281. **Massague, J., and F. Weis-Garcia.** 1996. Serine/threonine kinase receptors: mediators of transforming growth factor beta family signals. Cancer Surv **27:**41-64.
282. **Mastellos, D., D. Morikis, S. N. Isaacs, M. C. Holland, C. W. Strey, and J. D. Lambris.** 2003. Complement: structure, functions, evolution, and viral molecular mimicry. Immunologic research **27:**367-386.
283. **Mathew, J. A., Y. X. Guo, K. P. Goh, J. Chan, B. M. Verburg-van Kemenade, and J. Kwang.** 2002. Characterisation of a monoclonal antibody to carp IL-1beta and the development of a sensitive capture ELISA. Fish & shellfish immunology **13:**85-95.
284. **Matsumoto, M., N. Tanaka, H. Harada, T. Kimura, T. Yokochi, M. Kitagawa, C. Schindler, and T. Taniguchi.** 1999. Activation of the transcription factor ISGF3 by interferon-gamma. Biol Chem **380:**699-703.
285. **Mayumi, M., Y. Takeda, M. Hoshiko, K. Serada, M. Murata, T. Moritomo, F. Takizawa, I. Kobayashi, K. Araki, T. Nakanishi, and H. Sumimoto.** 2008. Characterization of teleost phagocyte NADPH oxidase: molecular cloning and expression analysis of carp (Cyprinus carpio) phagocyte NADPH oxidase. Molecular immunology **45:**1720-1731.
286. **McCartney-Francis, N., D. Mizel, H. Wong, L. Wahl, and S. Wahl.** 1990. TGF-beta regulates production of growth factors and TGF-beta by human peripheral blood monocytes. Growth Factors **4:**27-35.
287. **Meda, L., S. Gasperini, M. Ceska, and M. A. Cassatella.** 1994. Modulation of proinflammatory cytokine release from human polymorphonuclear leukocytes by gamma interferon. Cell Immunol **157:**448-461.
288. **Meseguer, J., M. A. Esteban, A. Garcia Ayala, A. Lopez Ruiz, and B. Agulleiro.** 1990. Granulopoiesis in the head-kidney of the sea bass (Dicentrarchus labrax L.): an ultrastructural study. Archives of histology and cytology **53:**287-296.
289. **Metz, J. R., M. O. Huising, K. Leon, B. M. Verburg-van Kemenade, and G. Flik.** 2006. Central and peripheral interleukin-1beta and interleukin-1 receptor I expression and their role in the acute stress response of common carp, Cyprinus carpio L. J Endocrinol **191:**25-35.
290. **Mevorach, D., J. O. Mascarenhas, D. Gershov, and K. B. Elkon.** 1998. Complement-dependent clearance of apoptotic cells by human macrophages. The Journal of experimental medicine **188:**2313-2320.

291. **Milev-Milovanovic, I., S. Long, M. Wilson, E. Bengten, N. W. Miller, and V. G. Chinchar.** 2006. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics **58:**70-80.
292. **Ming, W. J., L. Bersani, and A. Mantovani.** 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J Immunol **138:**1469-1474.
293. **Mizrahi, A., Y. Berdichevsky, Y. Ugolev, S. Molshanski-Mor, Y. Nakash, I. Dahan, N. Alloul, Y. Gorzalczany, R. Sarfstein, M. Hirshberg, and E. Pick.** 2006. Assembly of the phagocyte NADPH oxidase complex: chimeric constructs derived from the cytosolic components as tools for exploring structure-function relationships. Journal of leukocyte biology **79:**881-895.
294. **Mizutani, H., R. Black, and T. S. Kupper.** 1991. Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. The Journal of clinical investigation **87:**1066-1071.
295. **Mori, M.** 2007. Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling. The Journal of nutrition **137:**1616S-1620S.
296. **Moser, B., M. Wolf, A. Walz, and P. Loetscher.** 2004. Chemokines: multiple levels of leukocyte migration control. Trends in immunology **25:**75-84.
297. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol **7:**145-173.
298. **Moss, M. L., S. L. Jin, J. D. Becherer, D. M. Bickett, W. Burkhart, W. J. Chen, D. Hassler, M. T. Leesnitzer, G. McGeehan, M. Milla, M. Moyer, W. Rocque, T. Seaton, F. Schoenen, J. Warner, and D. Willard.** 1997. Structural features and biochemical properties of TNF-alpha converting enzyme (TACE). J Neuroimmunol **72:**127-129.
299. **Mott, J., Y. Rikihisa, and S. Tsunawaki.** 2002. Effects of Anaplasma phagocytophila on NADPH oxidase components in human neutrophils and HL-60 cells. Infection and immunity **70:**1359-1366.
300. **Mukhopadhyay, A., J. Suttles, R. D. Stout, and B. B. Aggarwal.** 2001. Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. The Journal of biological chemistry **276:**31906-31912.
301. **Munger, J. S., J. G. Harpel, P. E. Gleizes, R. Mazzieri, I. Nunes, and D. B. Rifkin.** 1997. Latent transforming growth factor-beta: structural features and mechanisms of activation. Kidney Int **51:**1376-1382.
302. **Murillo, M. M., I. Carmona-Cuenca, G. Del Castillo, C. Ortiz, C. Roncero, A. Sanchez, M. Fernandez, and I. Fabregat.** 2007. Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands

through a nuclear factor-kappaB-dependent mechanism. The Biochemical journal **405:**251-259.

303. **Nahrendorf, M., F. K. Swirski, E. Aikawa, L. Stangenberg, T. Wurdinger, J. L. Figueiredo, P. Libby, R. Weissleder, and M. J. Pittet.** 2007. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. The Journal of experimental medicine **204:**3037-3047.
304. **Naismith, J. H., B. J. Brandhuber, T. Q. Devine, and S. R. Sprang.** 1996. Seeing double: crystal structures of the type I TNF receptor. J Mol Recognit **9:**113-117.
305. **Naismith, J. H., T. Q. Devine, T. Kohno, and S. R. Sprang.** 1996. Structures of the extracellular domain of the type I tumor necrosis factor receptor. Structure **4:**1251-1262.
306. **Nakanishi, Y., H. Kodama, T. Murai, T. Mikami, and H. Izawa.** 1991. Activation of rainbow trout complement by C-reactive protein. American journal of veterinary research **52:**397-401.
307. **Nam, B. H., G. H. An, G. W. Baeck, M. C. Kim, J. W. Kim, H. J. Park, D. C. Lee, and C. I. Park.** 2009. Molecular cloning and expression of cDNAs for two distinct granulocyte colony stimulating factor genes from black rockfish Sebastes schlegelii. Fish & shellfish immunology **27:**360-364.
308. **Nam, H. J., Y. Y. Park, G. Yoon, H. Cho, and J. H. Lee.** Co-treatment with hepatocyte growth factor and TGF-beta1 enhances migration of HaCaT cells through NADPH oxidase-dependent ROS generation. Exp Mol Med **42:**270-279.
309. **Nascimento, D. S., P. J. Pereira, M. I. Reis, A. do Vale, J. Zou, M. T. Silva, C. J. Secombes, and N. M. dos Santos.** 2007. Molecular cloning and expression analysis of sea bass (Dicentrarchus labrax L.) tumor necrosis factor-alpha (TNF-alpha). Fish & shellfish immunology **23:**701-710.
310. **Nathan, C., and Q. W. Xie.** 1994. Nitric oxide synthases: roles, tolls, and controls. Cell **78:**915-918.
311. **Nelson, D. R., G. Y. Lauwers, J. Y. Lau, and G. L. Davis.** 2000. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders. Gastroenterology **118:**655-660.
312. **Neumann, M., and E. Kownatzki.** 1989. The effect of adherence on the generation of reactive oxygen species by human neutrophilic granulocytes. Agents and actions **26:**183-185.
313. **Neumann, N. F., D. R. Barreda, and M. Belosevic.** 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. Fish & shellfish immunology **10:**1-20.
314. **Neumann, N. F., D. Fagan, and M. Belosevic.** 1995. Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric

oxide production in teleost macrophages. Developmental and comparative immunology **19:**473-482.

315. **Neumann, N. F., J. L. Stafford, D. Barreda, A. J. Ainsworth, and M. Belosevic.** 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Developmental and comparative immunology **25:**807-825.
316. **Ng, V. H., J. S. Cox, A. O. Sousa, J. D. MacMicking, and J. D. McKinney.** 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Molecular microbiology **52:**1291-1302.
317. **Nonaka, M.** 2000. Origin and evolution of the complement system. Current topics in microbiology and immunology **248:**37-50.
318. **Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding.** 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J Immunol **167:**910-918.
319. **Nunes, I., P. E. Gleizes, C. N. Metz, and D. B. Rifkin.** 1997. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. The Journal of cell biology **136:**1151-1163.
320. **O'Neill, L. A.** 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE **2000:**re1.
321. **Oladiran, A., and M. Belosevic.** 2009. Trypanosoma carassii hsp70 increases expression of inflammatory cytokines and chemokines in macrophages of the goldfish (Carassius auratus L.). Developmental and comparative immunology **33:**1128-1136.
322. **Olavarria, V. H., L. Gallardo, J. E. Figueroa, and V. Mulero.** Lipopolysaccharide primes the respiratory burst of Atlantic salmon SHK-1 cells through protein kinase C-mediated phosphorylation of p47phox. Developmental and comparative immunology **34:**1242-1253.
323. **Omer, F. M., J. B. de Souza, and E. M. Riley.** 2003. Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal Plasmodium yoelii infections. J Immunol **171:**5430-5436.
324. **Ordas, M. C., M. M. Costa, F. J. Roca, G. Lopez-Castejon, V. Mulero, J. Meseguer, A. Figueras, and B. Novoa.** 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Molecular immunology **44:**389-400.
325. **Oshiumi, H., A. Matsuo, M. Matsumoto, and T. Seya.** 2008. Pan-vertebrate toll-like receptors during evolution. Current genomics **9:**488-493.
326. **Oswald, I. P., T. A. Wynn, A. Sher, and S. L. James.** 1992. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous

production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. Proc Natl Acad Sci U S A **89:**8676-8680.

327. **Pagan-Ramos, E., S. S. Master, C. L. Pritchett, R. Reimschuessel, M. Trucksis, G. S. Timmins, and V. Deretic.** 2006. Molecular and physiological effects of mycobacterial oxyR inactivation. Journal of bacteriology **188:**2674-2680.
328. **Pahlman, L. I., M. Morgelin, J. Eckert, L. Johansson, W. Russell, K. Riesbeck, O. Soehnlein, L. Lindbom, A. Norrby-Teglund, R. R. Schumann, L. Bjorck, and H. Herwald.** 2006. Streptococcal M protein: a multipotent and powerful inducer of inflammation. J Immunol **177:**1221-1228.
329. **Palic, D., C. B. Andreasen, J. Ostojic, R. M. Tell, and J. A. Roth.** 2007. Zebrafish (Danio rerio) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. Journal of immunological methods **319:**87-97.
330. **Parameswaran, N., and S. Patial.** 2010. Tumor necrosis factor-alpha signaling in macrophages. Critical reviews in eukaryotic gene expression **20:**87-103.
331. **Parekh, T., B. Saxena, J. Reibman, B. N. Cronstein, and L. I. Gold.** 1994. Neutrophil chemotaxis in response to TGF-beta isoforms (TGF-beta 1, TGF-beta 2, TGF-beta 3) is mediated by fibronectin. J Immunol **152:**2456-2466.
332. **Park, Y. K., B. Bearson, S. H. Bang, I. S. Bang, and J. W. Foster.** 1996. Internal pH crisis, lysine decarboxylase and the acid tolerance response of Salmonella typhimurium. Molecular microbiology **20:**605-611.
333. **Parker, L. C., L. R. Prince, and I. Sabroe.** 2007. Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity. Clin Exp Immunol **147:**199-207.
334. **Parkos, C. A., R. A. Allen, C. G. Cochrane, and A. J. Jesaitis.** 1987. Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. The Journal of clinical investigation **80:**732-742.
335. **Parkos, C. A., R. A. Allen, C. G. Cochrane, and A. J. Jesaitis.** 1988. The quaternary structure of the plasma membrane b-type cytochrome of human granulocytes. Biochimica et biophysica acta **932:**71-83.
336. **Parkos, C. A., M. C. Dinauer, L. E. Walker, R. A. Allen, A. J. Jesaitis, and S. H. Orkin.** 1988. Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b. Proceedings of the National Academy of Sciences of the United States of America **85:**3319-3323.
337. **Peatman, E., P. Baoprasertkul, J. Terhune, P. Xu, S. Nandi, H. Kucuktas, P. Li, S. Wang, B. Somridhivej, R. Dunham, and Z. Liu.** 2007. Expression analysis of the acute phase response in channel catfish

(Ictalurus punctatus) after infection with a Gram-negative bacterium. Developmental and comparative immunology **31:**1183-1196.

338. **Peatman, E., and Z. Liu.** 2006. CC chemokines in zebrafish: evidence for extensive intrachromosomal gene duplications. Genomics **88:**381-385.
339. **Peddie, S., P. E. McLauchlan, A. E. Ellis, and C. J. Secombes.** 2003. Effect of intraperitoneally administered IL-1beta-derived peptides on resistance to viral haemorrhagic septicaemia in rainbow trout Oncorhynchus mykiss. Diseases of aquatic organisms **56:**195-200.
340. **Peddie, S., J. Zou, C. Cunningham, and C. J. Secombes.** 2001. Rainbow trout (Oncorhynchus mykiss) recombinant IL-1beta and derived peptides induce migration of head-kidney leucocytes in vitro. Fish & shellfish immunology **11:**697-709.
341. **Pelegrin, P., E. Chaves-Pozo, V. Mulero, and J. Meseguer.** 2004. Production and mechanism of secretion of interleukin-1beta from the marine fish gilthead seabream. Developmental and comparative immunology **28:**229-237.
342. **Pennica, D., W. J. Kohr, B. M. Fendly, S. J. Shire, H. E. Raab, P. E. Borchardt, M. Lewis, and D. V. Goeddel.** 1992. Characterization of a recombinant extracellular domain of the type 1 tumor necrosis factor receptor: evidence for tumor necrosis factor-alpha induced receptor aggregation. Biochemistry **31:**1134-1141.
343. **Perez, C., I. Albert, K. DeFay, N. Zachariades, L. Gooding, and M. Kriegler.** 1990. A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell **63:**251-258.
344. **Perretti, M., J. D. Croxtall, S. K. Wheller, N. J. Goulding, R. Hannon, and R. J. Flower.** 1996. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. Nature medicine **2:**1259-1262.
345. **Perretti, M., and F. D'Acquisto.** 2006. Novel aspects of annexin 1 and glucocorticoid biology: intersection with nitric oxide and the lipoxin receptor. Inflamm Allergy Drug Targets **5:**107-114.
346. **Perussia, B.** 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. Current opinion in immunology **3:**49-55.
347. **Pettersen, E. F., R. Bjerknes, and H. I. Wergeland.** 2000. Studies of Atlantic salmon (Salmo salar L.) blood, spleen and head kidney leucocytes using specific monoclonal antibodies, immunohistochemistry and flow cytometry. Fish & shellfish immunology **10:**695-710.
348. **Pilcher, B. K., J. Dumin, M. J. Schwartz, B. A. Mast, G. S. Schultz, W. C. Parks, and H. G. Welgus.** 1999. Keratinocyte collagenase-1 expression requires an epidermal growth factor receptor autocrine mechanism. The Journal of biological chemistry **274:**10372-10381.
349. **Pilcher, B. K., J. A. Dumin, B. D. Sudbeck, S. M. Krane, H. G. Welgus, and W. C. Parks.** 1997. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. The Journal of cell biology **137:**1445-1457.

350. **Pilcher, B. K., B. D. Sudbeck, J. A. Dumin, H. G. Welgus, and W. C. Parks.** 1998. Collagenase-1 and collagen in epidermal repair. Archives of dermatological research **290 Suppl:**S37-46.
351. **Pilcher, B. K., M. Wang, X. J. Qin, W. C. Parks, R. M. Senior, and H. G. Welgus.** 1999. Role of matrix metalloproteinases and their inhibition in cutaneous wound healing and allergic contact hypersensitivity. Annals of the New York Academy of Sciences **878:**12-24.
352. **Pinson, D. M., R. D. LeClaire, R. B. Lorsbach, M. J. Parmely, and S. W. Russell.** 1992. Regulation by transforming growth factor-beta 1 of expression and function of the receptor for IFN-gamma on mouse macrophages. J Immunol **149:**2028-2034.
353. **Pinto, R. D., D. S. Nascimento, M. I. Reis, A. do Vale, and N. M. Dos Santos.** 2007. Molecular characterization, 3D modelling and expression analysis of sea bass (Dicentrarchus labrax L.) interleukin-10. Mol Immunol **44:**2056-2065.
354. **Pleguezuelos, O., J. Zou, C. Cunningham, and C. J. Secombes.** 2000. Cloning, sequencing, and analysis of expression of a second IL-1beta gene in rainbow trout (Oncorhynchus mykiss). Immunogenetics **51:**1002-1011.
355. **Poss, K. D., M. T. Keating, and A. Nechiporuk.** 2003. Tales of regeneration in zebrafish. Dev Dyn **226:**202-210.
356. **Praveen, K., D. L. Evans, and L. Jaso-Friedmann.** 2006. Constitutive expression of tumor necrosis factor-alpha in cytotoxic cells of teleosts and its role in regulation of cell-mediated cytotoxicity. Molecular immunology **43:**279-291.
357. **Qin, H., C. A. Wilson, K. L. Roberts, B. J. Baker, X. Zhao, and E. N. Benveniste.** 2006. IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. J Immunol **177:**7761-7771.
358. **Raivio, T. L.** 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. Molecular microbiology **56:**1119-1128.
359. **Reboul, J., K. Gardiner, D. Monneron, G. Uze, and G. Lutfalla.** 1999. Comparative genomic analysis of the interferon/interleukin-10 receptor gene cluster. Genome research **9:**242-250.
360. **Reed, S. G., C. E. Brownell, D. M. Russo, J. S. Silva, K. H. Grabstein, and P. J. Morrissey.** 1994. IL-10 mediates susceptibility to Trypanosoma cruzi infection. J Immunol **153:**3135-3140.
361. **Reibman, J., S. Meixler, T. C. Lee, L. I. Gold, B. N. Cronstein, K. A. Haines, S. L. Kolasinski, and G. Weissmann.** 1991. Transforming growth factor beta 1, a potent chemoattractant for human neutrophils, bypasses classic signal-transduction pathways. Proceedings of the National Academy of Sciences of the United States of America **88:**6805-6809.
362. **Rennick, D. M., and M. M. Fort.** 2000. Lessons from genetically engineered animal models. XII. IL-10-deficient (IL-10(-/-) mice and intestinal inflammation. Am J Physiol Gastrointest Liver Physiol **278:**G829-833.

363. **Rennick, D. M., M. M. Fort, and N. J. Davidson.** 1997. Studies with IL-10-/- mice: an overview. Journal of leukocyte biology **61:**389-396.
364. **Rieger, A. M., B. E. Hall, and D. R. Barreda.** 2010. Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms. Developmental and comparative immunology **34:**1144-1159.
365. **Riley, J. K., K. Takeda, S. Akira, and R. D. Schreiber.** 1999. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. J Biol Chem **274:**16513-16521.
366. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America **102:**9577-9582.
367. **Robertsen, B.** 2006. The interferon system of teleost fish. Fish & shellfish immunology **20:**172-191.
368. **Robinson, J. M.** 2009. Phagocytic leukocytes and reactive oxygen species. Histochemistry and cell biology **131:**465-469.
369. **Robinson, J. M.** 2008. Reactive oxygen species in phagocytic leukocytes. Histochemistry and cell biology **130:**281-297.
370. **Robinson, N., M. Wolke, K. Ernestus, and G. Plum.** 2007. A mycobacterial gene involved in synthesis of an outer cell envelope lipid is a key factor in prevention of phagosome maturation. Infection and immunity **75:**581-591.
371. **Roca, F. J., I. Mulero, A. Lopez-Munoz, M. P. Sepulcre, S. A. Renshaw, J. Meseguer, and V. Mulero.** 2008. Evolution of the inflammatory response in vertebrates: fish TNF-alpha is a powerful activator of endothelial cells but hardly activates phagocytes. J Immunol **181:**5071-5081.
372. **Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, F. Bussolino, V. Poli, G. Ciliberto, and A. Mantovani.** 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity **6:**315-325.
373. **Rombouts, Y., A. Burguiere, E. Maes, B. Coddeville, E. Elass, Y. Guerardel, and L. Kremer.** 2009. Mycobacterium marinum lipooligosaccharides are unique caryophyllose-containing cell wall glycolipids that inhibit tumor necrosis factor-alpha secretion in macrophages. The Journal of biological chemistry **284:**20975-20988.
374. **Rook, A. H., J. H. Kehrl, L. M. Wakefield, A. B. Roberts, M. B. Sporn, D. B. Burlington, H. C. Lane, and A. S. Fauci.** 1986. Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J Immunol **136:**3916-3920.
375. **Roque, S., C. Nobrega, R. Appelberg, and M. Correia-Neves.** 2007. IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to

Mycobacterium avium infection and influences efficacy of antibiotic therapy. J Immunol **178:**8028-8035.

376. **Rotllant, J., D. Parra, R. Peters, H. Boshra, and J. O. Sunyer.** 2004. Generation, purification and functional characterization of three C3a anaphylatoxins in rainbow trout: role in leukocyte chemotaxis and respiratory burst. Developmental and comparative immunology **28:**815-828.
377. **Rubbo, H., A. Denicola, and R. Radi.** 1994. Peroxynitrite inactivates thiol-containing enzymes of Trypanosoma cruzi energetic metabolism and inhibits cell respiration. Archives of biochemistry and biophysics **308:**96-102.
378. **Ruddle, N. H.** 1986. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J Immunol **136:**2335-2336.
379. **Rudin, W., N. Favre, G. Bordmann, and B. Ryffel.** 1997. Interferon-gamma is essential for the development of cerebral malaria. European journal of immunology **27:**810-815.
380. **Russo, R., C. A. Shoemaker, V. S. Panangala, and P. H. Klesius.** 2009. In vitro and in vivo interaction of macrophages from vaccinated and non-vaccinated channel catfish (Ictalurus punctatus) to Edwardsiella ictaluri. Fish & shellfish immunology **26:**543-552.
381. **Ryckaert, J., P. Bossier, K. D'Herde, A. Diez-Fraile, P. Sorgeloos, F. Haesebrouck, and F. Pasmans.** 2010. Persistence of Yersinia ruckeri in trout macrophages. Fish & shellfish immunology **29:**648-655.
382. **Rycyzyn, M. A., M. R. Wilson, E. Bengten, G. W. Warr, L. W. Clem, and N. W. Miller.** 1998. Mitogen and growth factor-induced activation of a STAT-like molecule in channel catfish lymphoid cells. Molecular immunology **35:**127-136.
383. **Sad, S., R. Marcotte, and T. R. Mosmann.** 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity **2:**271-279.
384. **Saeij, J. P., R. J. Stet, B. J. de Vries, W. B. van Muiswinkel, and G. F. Wiegertjes.** 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Developmental and comparative immunology **27:**29-41.
385. **Saeij, J. P., R. J. Stet, A. Groeneveld, L. B. Verburg-van Kemenade, W. B. van Muiswinkel, and G. F. Wiegertjes.** 2000. Molecular and functional characterization of a fish inducible-type nitric oxide synthase. Immunogenetics **51:**339-346.
386. **Sangrador-Vegas, A., S. A. Martin, P. G. O'Dea, and T. J. Smith.** 2000. Cloning and characterization of the rainbow trout (Oncorhynchus mykiss) type II interleukin-1 receptor cDNA. Eur J Biochem **267:**7031-7037.
387. **Santos, M. D., M. Yasuike, I. Hirono, and T. Aoki.** 2006. The granulocyte colony-stimulating factors (CSF3s) of fish and chicken. Immunogenetics **58:**422-432.

388. **Savan, R., D. Igawa, and M. Sakai.** 2003. Cloning, characterization and expression analysis of interleukin-10 from the common carp, Cyprinus carpio L. Eur J Biochem **270:**4647-4654.
389. **Savan, R., T. Kono, A. Aman, and M. Sakai.** 2003. Isolation and characterization of a novel CXC chemokine in common carp (Cyprinus carpio L.). Molecular immunology **39:**829-834.
390. **Savan, R., T. Kono, D. Igawa, and M. Sakai.** 2005. A novel tumor necrosis factor (TNF) gene present in tandem with theTNF-alpha gene on the same chromosome in teleosts. Immunogenetics **57:**140-150.
391. **Savan, R., S. Ravichandran, J. R. Collins, M. Sakai, and H. A. Young.** 2009. Structural conservation of interferon gamma among vertebrates. Cytokine & growth factor reviews **20:**115-124.
392. **Savan, R., and M. Sakai.** 2004. Presence of multiple isoforms of TNF alpha in carp (Cyprinus carpio L.): genomic and expression analysis. Fish & shellfish immunology **17:**87-94.
393. **Scaffidi, P., T. Misteli, and M. E. Bianchi.** 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature **418:**191-195.
394. **Scannell, M., M. B. Flanagan, A. deStefani, K. J. Wynne, G. Cagney, C. Godson, and P. Maderna.** 2007. Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. J Immunol **178:**4595-4605.
395. **Schindler, R., J. Mancilla, S. Endres, R. Ghorbani, S. C. Clark, and C. A. Dinarello.** 1990. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood **75:**40-47.
396. **Schirren, C. G., K. Scharffetter, R. Hein, O. Braun-Falco, and T. Krieg.** 1990. Tumor necrosis factor alpha induces invasiveness of human skin fibroblasts in vitro. The Journal of investigative dermatology **94:**706-710.
397. **Schmidtchen, A., I. M. Frick, E. Andersson, H. Tapper, and L. Bjorck.** 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Molecular microbiology **46:**157-168.
398. **Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume.** 2004. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of leukocyte biology **75:**163-189.
399. **Schweitzer, K. M., A. M. Drager, P. van der Valk, S. F. Thijsen, A. Zevenbergen, A. P. Theijsmeijer, C. E. van der Schoot, and M. M. Langenhuijsen.** 1996. Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. The American journal of pathology **148:**165-175.
400. **Secombes, C., J. Zou, G. Daniels, C. Cunningham, A. Koussounadis, and G. Kemp.** 1998. Rainbow trout cytokine and cytokine receptor genes. Immunological reviews **166:**333-340.

401. **Secombes, C. J.** 1987. Lymphokine-release from rainbow trout leucocytes stimulated with concanavalin A. Effects upon macrophage spreading and adherence. Developmental and comparative immunology **11:**513-520.
402. **Sehgal, P. B., D. C. Helfgott, U. Santhanam, S. B. Tatter, R. H. Clarick, J. Ghrayeb, and L. T. May.** 1988. Regulation of the acute phase and immune responses in viral disease. Enhanced expression of the beta 2-interferon/hepatocyte-stimulating factor/interleukin 6 gene in virus-infected human fibroblasts. The Journal of experimental medicine **167:**1951-1956.
403. **Semerad, C. L., F. Liu, A. D. Gregory, K. Stumpf, and D. C. Link.** 2002. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. Immunity **17:**413-423.
404. **Seppola, M., A. N. Larsen, K. Steiro, B. Robertsen, and I. Jensen.** 2008. Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). Mol Immunol **45:**887-897.
405. **Sepulcre, M. P., F. Alcaraz-Perez, A. Lopez-Munoz, F. J. Roca, J. Meseguer, M. L. Cayuela, and V. Mulero.** 2009. Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-kappaB activation. J Immunol **182:**1836-1845.
406. **Serhan, C. N., N. Chiang, and T. E. Van Dyke.** 2008. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nature reviews **8:**349-361.
407. **Sheppard, F. R., M. R. Kelher, E. E. Moore, N. J. McLaughlin, A. Banerjee, and C. C. Silliman.** 2005. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. Journal of leukocyte biology **78:**1025-1042.
408. **Shull, M. M., I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, and et al.** 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature **359:**693-699.
409. **Sica, A., and V. Bronte.** 2007. Altered macrophage differentiation and immune dysfunction in tumor development. The Journal of clinical investigation **117:**1155-1166.
410. **Sieger, D., C. Stein, D. Neifer, A. M. van der Sar, and M. Leptin.** 2009. The role of gamma interferon in innate immunity in the zebrafish embryo. Dis Model Mech **2:**571-581.
411. **Sigel, M. M., B. A. Hamby, and E. M. Huggins, Jr.** 1986. Phylogenetic studies on lymphokines. Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1 like factor. Veterinary immunology and immunopathology **12:**47-58.
412. **Sillett, H. K., S. M. Cruickshank, J. Southgate, and L. K. Trejdosiewicz.** 2001. Transforming growth factor-beta promotes 'death by neglect' in post-activated human T cells. Immunology **102:**310-316.

413. **Silva, M. T.** 2009. When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. Journal of leukocyte biology.
414. **Simmons, P. J., B. Masinovsky, B. M. Longenecker, R. Berenson, B. Torok-Storb, and W. M. Gallatin.** 1992. Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood **80:**388-395.
415. **Sims, J. E.** 2002. IL-1 and IL-18 receptors, and their extended family. Current opinion in immunology **14:**117-122.
416. **Sims, J. E., M. J. Nicklin, J. F. Bazan, J. L. Barton, S. J. Busfield, J. E. Ford, R. A. Kastelein, S. Kumar, H. Lin, J. J. Mulero, J. Pan, Y. Pan, D. E. Smith, and P. R. Young.** 2001. A new nomenclature for IL-1-family genes. Trends in immunology **22:**536-537.
417. **Singer, K. H., R. M. Scearce, D. T. Tuck, L. P. Whichard, S. M. Denning, and B. F. Haynes.** 1989. Removal of fibroblasts from human epithelial cell cultures with use of a complement fixing monoclonal antibody reactive with human fibroblasts and monocytes/macrophages. The Journal of investigative dermatology **92:**166-170.
418. **Singer, S. J., and A. Kupfer.** 1986. The directed migration of eukaryotic cells. Annual review of cell biology **2:**337-365.
419. **Smith, D. E., B. R. Renshaw, R. R. Ketchem, M. Kubin, K. E. Garka, and J. E. Sims.** 2000. Four new members expand the interleukin-1 superfamily. The Journal of biological chemistry **275:**1169-1175.
420. **Smith, J., J. Manoranjan, M. Pan, A. Bohsali, J. Xu, J. Liu, K. L. McDonald, A. Szyk, N. LaRonde-LeBlanc, and L. Y. Gao.** 2008. Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in Mycobacterium marinum escape from the vacuole. Infection and immunity **76:**5478-5487.
421. **Soehnlein, O.** 2009. Direct and alternative antimicrobial mechanisms of neutrophil-derived granule proteins. J Mol Med **87:**1157-1164.
422. **Soehnlein, O., Y. Kai-Larsen, R. Frithiof, O. E. Sorensen, E. Kenne, K. Scharffetter-Kochanek, E. E. Eriksson, H. Herwald, B. Agerberth, and L. Lindbom.** 2008. Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. The Journal of clinical investigation **118:**3491-3502.
423. **Soehnlein, O., S. Oehmcke, X. Ma, A. G. Rothfuchs, R. Frithiof, N. van Rooijen, M. Morgelin, H. Herwald, and L. Lindbom.** 2008. Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. Eur Respir J **32:**405-412.
424. **Sorensen, O. E., P. Follin, A. H. Johnsen, J. Calafat, G. S. Tjabringa, P. S. Hiemstra, and N. Borregaard.** 2001. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood **97:**3951-3959.
425. **Spencer, S. D., F. Di Marco, J. Hooley, S. Pitts-Meek, M. Bauer, A. M. Ryan, B. Sordat, V. C. Gibbs, and M. Aguet.** 1998. The orphan receptor

CRF2-4 is an essential subunit of the interleukin 10 receptor. The Journal of experimental medicine **187:**571-578.

426. **St John, G., N. Brot, J. Ruan, H. Erdjument-Bromage, P. Tempst, H. Weissbach, and C. Nathan.** 2001. Peptide methionine sulfoxide reductase from Escherichia coli and Mycobacterium tuberculosis protects bacteria against oxidative damage from reactive nitrogen intermediates. Proceedings of the National Academy of Sciences of the United States of America **98:**9901-9906.
427. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Adv Virus Res **38:**147-200.
428. **Stafford, J. L., E. C. Wilson, and M. Belosevic.** 2004. Recombinant transferrin induces nitric oxide response in goldfish and murine macrophages. Fish & shellfish immunology **17:**171-185.
429. **Stamm, L. M., J. H. Morisaki, L. Y. Gao, R. L. Jeng, K. L. McDonald, R. Roth, S. Takeshita, J. Heuser, M. D. Welch, and E. J. Brown.** 2003. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. The Journal of experimental medicine **198:**1361-1368.
430. **Stamm, L. M., M. A. Pak, J. H. Morisaki, S. B. Snapper, K. Rottner, S. Lommel, and E. J. Brown.** 2005. Role of the WASP family proteins for Mycobacterium marinum actin tail formation. Proceedings of the National Academy of Sciences of the United States of America **102:**14837-14842.
431. **Stevenson, F. T., S. L. Bursten, C. Fanton, R. M. Locksley, and D. H. Lovett.** 1993. The 31-kDa precursor of interleukin 1 alpha is myristoylated on specific lysines within the 16-kDa N-terminal propiece. Proceedings of the National Academy of Sciences of the United States of America **90:**7245-7249.
432. **Stevenson, M. M., M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba.** 1990. Role of endogenous gamma interferon in host response to infection with blood-stage Plasmodium chabaudi AS. Infection and immunity **58:**3225-3232.
433. **Stolte, E. H., H. F. Savelkoul, G. Wiegertjes, G. Flik, and B. M. Lidy Verburg-van Kemenade.** 2008. Differential expression of two interferon-gamma genes in common carp (Cyprinus carpio L.). Developmental and comparative immunology **32:**1467-1481.
434. **Strieter, R. M., P. J. Polverini, S. L. Kunkel, D. A. Arenberg, M. D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, and et al.** 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. The Journal of biological chemistry **270:**27348-27357.
435. **Stuehr, D. J., and C. F. Nathan.** 1989. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. The Journal of experimental medicine **169:**1543-1555.
436. **Su, J., T. Huang, J. Dong, J. Heng, R. Zhang, and L. Peng.** 2010. Molecular cloning and immune responsive expression of MDA5 gene, a

pivotal member of the RLR gene family from grass carp Ctenopharyngodon idella. Fish & shellfish immunology **28:**712-718.

437. **Subbian, S., P. K. Mehta, S. L. Cirillo, L. E. Bermudez, and J. D. Cirillo.** 2007. A Mycobacterium marinum mel2 mutant is defective for growth in macrophages that produce reactive oxygen and reactive nitrogen species. Infection and immunity **75:**127-134.
438. **Subbian, S., P. K. Mehta, S. L. Cirillo, and J. D. Cirillo.** 2007. The Mycobacterium marinum mel2 locus displays similarity to bacterial bioluminescence systems and plays a role in defense against reactive oxygen and nitrogen species. BMC microbiology **7:**4.
439. **Subramaniam, P. S., B. A. Torres, and H. M. Johnson.** 2001. So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. Cytokine **15:**175-187.
440. **Subramaniam, S., C. Stansberg, L. Olsen, J. Zou, C. J. Secombes, and C. Cunningham.** 2002. Cloning of a Salmo salar interleukin-1 receptor-like cDNA. Developmental and comparative immunology **26:**415-431.
441. **Sullivan, C., J. Charette, J. Catchen, C. R. Lage, G. Giasson, J. H. Postlethwait, P. J. Millard, and C. H. Kim.** 2009. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. J Immunol **183:**5896-5908.
442. **Sunyer, J. O., H. Boshra, and J. Li.** 2005. Evolution of anaphylatoxins, their diversity and novel roles in innate immunity: insights from the study of fish complement. Veterinary immunology and immunopathology **108:**77-89.
443. **Sunyer, J. O., L. Tort, and J. D. Lambris.** 1997. Diversity of the third form of complement, C3, in fish: functional characterization of five forms of C3 in the diploid fish Sparus aurata. The Biochemical journal **326 (Pt 3):**877-881.
444. **Sunyer, J. O., L. Tort, and J. D. Lambris.** 1997. Structural C3 diversity in fish: characterization of five forms of C3 in the diploid fish Sparus aurata. J Immunol **158:**2813-2821.
445. **Sunyer, J. O., I. K. Zarkadis, A. Sahu, and J. D. Lambris.** 1996. Multiple forms of complement C3 in trout that differ in binding to complement activators. Proceedings of the National Academy of Sciences of the United States of America **93:**8546-8551.
446. **Suvas, S., A. K. Azkur, B. S. Kim, U. Kumaraguru, and B. T. Rouse.** 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. J Immunol **172:**4123-4132.
447. **Syto, R., N. J. Murgolo, E. H. Braswell, P. Mui, E. Huang, and W. T. Windsor.** 1998. Structural and biological stability of the human interleukin 10 homodimer. Biochemistry **37:**16943-16951.
448. **Taekema-Roelvink, M. E., C. Kooten, S. V. Kooij, E. Heemskerk, and M. R. Daha.** 2001. Proteinase 3 enhances endothelial monocyte chemoattractant protein-1 production and induces increased adhesion of neutrophils to endothelial cells by upregulating intercellular cell adhesion molecule-1. J Am Soc Nephrol **12:**932-940.

449. **Tafalla, C., R. Aranguren, C. J. Secombes, J. L. Castrillo, B. Novoa, and A. Figueras.** 2003. Molecular characterisation of sea bream (Sparus aurata) transforming growth factor beta1. Fish & shellfish immunology **14:**405-421.
450. **Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi.** 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. Science (New York, N.Y **288:**2357-2360.
451. **Takaoka, A., and H. Yanai.** 2006. Interferon signalling network in innate defence. Cellular microbiology **8:**907-922.
452. **Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira.** 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity **10:**39-49.
453. **Tal, T. L., J. A. Franzosa, and R. L. Tanguay.** Molecular signaling networks that choreograph epimorphic fin regeneration in zebrafish - a mini-review. Gerontology **56:**231-240.
454. **Tan, J. C., S. Braun, H. Rong, R. DiGiacomo, E. Dolphin, S. Baldwin, S. K. Narula, P. J. Zavodny, and C. C. Chou.** 1995. Characterization of recombinant extracellular domain of human interleukin-10 receptor. J Biol Chem **270:**12906-12911.
455. **Tan, J. C., S. R. Indelicato, S. K. Narula, P. J. Zavodny, and C. C. Chou.** 1993. Characterization of interleukin-10 receptors on human and mouse cells. J Biol Chem **268:**21053-21059.
456. **Tan, T., W. L. Lee, D. C. Alexander, S. Grinstein, and J. Liu.** 2006. The ESAT-6/CFP-10 secretion system of Mycobacterium marinum modulates phagosome maturation. Cellular microbiology **8:**1417-1429.
457. **Tartaglia, L. A., T. M. Ayres, G. H. Wong, and D. V. Goeddel.** 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell **74:**845-853.
458. **Temperley, N. D., S. Berlin, I. R. Paton, D. K. Griffin, and D. W. Burt.** 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC genomics **9:**62.
459. **Terashima, T., D. English, J. C. Hogg, and S. F. van Eeden.** 1998. Release of polymorphonuclear leukocytes from the bone marrow by interleukin-8. Blood **92:**1062-1069.
460. **Theiss, A. L., J. G. Simmons, C. Jobin, and P. K. Lund.** 2005. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. The Journal of biological chemistry **280:**36099-36109.
461. **Thomson, L., A. Denicola, and R. Radi.** 2003. The trypanothione-thiol system in Trypanosoma cruzi as a key antioxidant mechanism against peroxynitrite-mediated cytotoxicity. Archives of biochemistry and biophysics **412:**55-64.
462. **Towne, J. E., K. E. Garka, B. R. Renshaw, G. D. Virca, and J. E. Sims.** 2004. Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-

1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. The Journal of biological chemistry **279:**13677-13688.

463. **Tsukazaki, T., T. A. Chiang, A. F. Davison, L. Attisano, and J. L. Wrana.** 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell **95:**779-791.
464. **Tsunawaki, S., S. Kagara, K. Yoshikawa, L. S. Yoshida, T. Kuratsuji, and H. Namiki.** 1996. Involvement of p40phox in activation of phagocyte NADPH oxidase through association of its carboxyl-terminal, but not its amino-terminal, with p67phox. The Journal of experimental medicine **184:**893-902.
465. **Tsunawaki, S., H. Mizunari, M. Nagata, O. Tatsuzawa, and T. Kuratsuji.** 1994. A novel cytosolic component, p40phox, of respiratory burst oxidase associates with p67phox and is absent in patients with chronic granulomatous disease who lack p67phox. Biochemical and biophysical research communications **199:**1378-1387.
466. **Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan.** 1988. Deactivation of macrophages by transforming growth factor-beta. Nature **334:**260-262.
467. **Uenobe, M., C. Kohchi, N. Yoshioka, A. Yuasa, H. Inagawa, K. Morii, T. Nishizawa, Y. Takahashi, and G. Soma.** 2007. Cloning and characterization of a TNF-like protein of Plecoglossus altivelis (ayu fish). Molecular immunology **44:**1115-1122.
468. **Uran, P. A., A. A. Goncalves, J. J. Taverne-Thiele, J. W. Schrama, J. A. Verreth, and J. H. Rombout.** 2008. Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.). Fish Shellfish Immunol **25:**751-760.
469. **van den Berg, J. M., S. Weyer, J. J. Weening, D. Roos, and T. W. Kuijpers.** 2001. Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. Journal of leukocyte biology **69:**467-473.
470. **van der Aa, L. M., M. Chadzinska, E. Tijhaar, P. Boudinot, and B. M. Verburg-van Kemenade.** 2010. CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation. PloS one **5:**e12384.
471. **van der Wel, N., D. Hava, D. Houben, D. Fluitsma, M. van Zon, J. Pierson, M. Brenner, and P. J. Peters.** 2007. M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells. Cell **129:**1287-1298.
472. **van Miert, A. S.** 1995. Pro-inflammatory cytokines in a ruminant model: pathophysiological, pharmacological, and therapeutic aspects. The Veterinary quarterly **17:**41-50.
473. **van Strijp, J. A., M. E. van der Tol, L. A. Miltenburg, K. P. van Kessel, and J. Verhoef.** 1991. Tumour necrosis factor triggers granulocytes to internalize complement-coated virus particles. Immunology **73:**77-82.
474. **Vandal, O. H., L. M. Pierini, D. Schnappinger, C. F. Nathan, and S. Ehrt.** 2008. A membrane protein preserves intrabacterial pH in

intraphagosomal Mycobacterium tuberculosis. Nature medicine **14:**849-854.

475. **Verburg-van Kemenade, B. M., F. A. Weyts, R. Debets, and G. Flik.** 1995. Carp macrophages and neutrophilic granulocytes secrete an interleukin-1-like factor. Developmental and comparative immunology **19:**59-70.
476. **Vigers, G. P., L. J. Anderson, P. Caffes, and B. J. Brandhuber.** 1997. Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1beta. Nature **386:**190-194.
477. **Voll, R. E., M. Herrmann, E. A. Roth, C. Stach, J. R. Kalden, and I. Girkontaite.** 1997. Immunosuppressive effects of apoptotic cells. Nature **390:**350-351.
478. **Volpp, B. D., W. M. Nauseef, J. E. Donelson, D. R. Moser, and R. A. Clark.** 1989. Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. Proceedings of the National Academy of Sciences of the United States of America **86:**7195-7199.
479. **Wahl, S. M., N. McCartney-Francis, J. B. Allen, E. B. Dougherty, and S. F. Dougherty.** 1990. Macrophage production of TGF-beta and regulation by TGF-beta. Annals of the New York Academy of Sciences **593:**188-196.
480. **Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin.** 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol **17:**331-367.
481. **Walter, M. R., and T. L. Nagabhushan.** 1995. Crystal structure of interleukin 10 reveals an interferon gamma-like fold. Biochemistry **34:**12118-12125.
482. **Walter, M. R., W. T. Windsor, T. L. Nagabhushan, D. J. Lundell, C. A. Lunn, P. J. Zauodny, and S. K. Narula.** 1995. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. Nature **376:**230-235.
483. **Wang, T., S. Bird, A. Koussounadis, J. W. Holland, A. Carrington, J. Zou, and C. J. Secombes.** 2009. Identification of a novel IL-1 cytokine family member in teleost fish. J Immunol **183:**962-974.
484. **Wang, T., J. W. Holland, A. Carrington, J. Zou, and C. J. Secombes.** 2007. Molecular and functional characterization of IL-15 in rainbow trout Oncorhynchus mykiss: a potent inducer of IFN-gamma expression in spleen leukocytes. J Immunol **179:**1475-1488.
485. **Wang, T., M. Ward, P. Grabowski, and C. J. Secombes.** 2001. Molecular cloning, gene organization and expression of rainbow trout (Oncorhynchus mykiss) inducible nitric oxide synthase (iNOS) gene. The Biochemical journal **358:**747-755.
486. **Wang, Y., Q. Wang, P. Baoprasertkul, E. Peatman, and Z. Liu.** 2006. Genomic organization, gene duplication, and expression analysis of interleukin-1beta in channel catfish (Ictalurus punctatus). Molecular immunology **43:**1653-1664.

487. **Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley.** 1994. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. The Journal of experimental medicine **179:**1367-1371.
488. **Warner, S. J., and P. Libby.** 1989. Human vascular smooth muscle cells. Target for and source of tumor necrosis factor. J Immunol **142:**100-109.
489. **Weber-Nordt, R. M., J. K. Riley, A. C. Greenlund, K. W. Moore, J. E. Darnell, and R. D. Schreiber.** 1996. Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. J Biol Chem **271:**27954-27961.
490. **Weiss, T., M. Grell, K. Siemienski, F. Muhlenbeck, H. Durkop, K. Pfizenmaier, P. Scheurich, and H. Wajant.** 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. J Immunol **161:**3136-3142.
491. **Wheelock, E. F.** 1965. Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. Science (New York, N.Y **149:**310-311.
492. **Wilson, E. H., U. Wille-Reece, F. Dzierszinski, and C. A. Hunter.** 2005. A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis. J Neuroimmunol **165:**63-74.
493. **Winkelhake, J. L., M. J. Vodicnik, and J. L. Taylor.** 1983. Induction in rainbow trout of an acute phase (C-reactive) protein by chemicals of environmental concern. Comp Biochem Physiol C **74:**55-58.
494. **Wiseman, D. M., P. J. Polverini, D. W. Kamp, and S. J. Leibovich.** 1988. Transforming growth factor-beta (TGF beta) is chemotactic for human monocytes and induces their expression of angiogenic activity. Biochemical and biophysical research communications **157:**793-800.
495. **Wrana, J. L.** 1998. TGF-beta receptors and signalling mechanisms. Miner Electrolyte Metab **24:**120-130.
496. **Wrana, J. L., L. Attisano, R. Wieser, F. Ventura, and J. Massague.** 1994. Mechanism of activation of the TGF-beta receptor. Nature **370:**341-347.
497. **Wu, Y., Q. H. Wang, L. Zheng, H. Feng, J. Liu, S. H. Ma, and Y. M. Cao.** 2007. Plasmodium yoelii: distinct CD4(+)CD25(+) regulatory T cell responses during the early stages of infection in susceptible and resistant mice. Experimental parasitology **115:**301-304.
498. **Wynn, T. A.** 2004. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nature reviews **4:**583-594.
499. **Wynn, T. A., M. Hesse, N. G. Sandler, M. Kaviratne, K. F. Hoffmann, M. G. Chiaramonte, R. Reiman, A. W. Cheever, J. P. Sypek, and M. M. Mentink-Kane.** 2004. P-selectin suppresses hepatic inflammation and fibrosis in mice by regulating interferon gamma and the IL-13 decoy receptor. Hepatology **39:**676-687.
500. **Xu, J., O. Laine, M. Masciocchi, J. Manoranjan, J. Smith, S. J. Du, N. Edwards, X. Zhu, C. Fenselau, and L. Y. Gao.** 2007. A unique

Mycobacterium ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. Molecular microbiology **66:**787-800.

501. **Yamasaki, S., E. Ishikawa, M. Sakuma, H. Hara, K. Ogata, and T. Saito.** 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. Nature immunology **9:**1179-1188.
502. **Yaswen, L., A. B. Kulkarni, T. Fredrickson, B. Mittleman, R. Schiffman, S. Payne, G. Longenecker, E. Mozes, and S. Karlsson.** 1996. Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. Blood **87:**1439-1445.
503. **Yin, Z., and J. Kwang.** 2000. Carp interleukin-1 beta in the role of an immuno-adjuvant. Fish & shellfish immunology **10:**375-378.
504. **Yonemaru, M., K. E. Stephens, A. Ishizaka, H. Zheng, R. S. Hogue, J. J. Crowley, J. R. Hatherill, and T. A. Raffin.** 1989. Effects of tumor necrosis factor on PMN chemotaxis, chemiluminescence, and elastase activity. J Lab Clin Med **114:**674-681.
505. **Young, H. A., and K. J. Hardy.** 1995. Role of interferon-gamma in immune cell regulation. Journal of leukocyte biology **58:**373-381.
506. **Young, J. D., C. C. Liu, G. Butler, Z. A. Cohn, and S. J. Galli.** 1987. Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. Proceedings of the National Academy of Sciences of the United States of America **84:**9175-9179.
507. **Zdanov, A., C. Schalk-Hihi, A. Gustchina, M. Tsang, J. Weatherbee, and A. Wlodawer.** 1995. Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. Structure **3:**591-601.
508. **Zhan, Y., and K. Jimmy.** 2000. Molecular isolation and characterisation of carp transforming growth factor beta 1 from activated leucocytes. Fish & shellfish immunology **10:**309-318.
509. **Zhang, D. C., Y. Q. Shao, Y. Q. Huang, and S. G. Jiang.** 2005. Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (Danio rerion). J Biochem Mol Biol **38:**571-576.
510. **Zhang, M., Z. Z. Xiao, and L. Sun.** 2011. Suppressor of cytokine signaling 3 inhibits head kidney macrophage activation and cytokine expression in Scophthalmus maximus. Developmental and comparative immunology **35:**174-181.
511. **Zhang, Y., X. H. Feng, and R. Derynck.** 1998. Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-beta-induced transcription. Nature **394:**909-913.
512. **Zhao, C., H. Zhang, W. C. Wong, X. Sem, H. Han, S. M. Ong, Y. C. Tan, W. H. Yeap, C. S. Gan, K. Q. Ng, M. B. Koh, P. Kourilsky, S. K. Sze, and S. C. Wong.** 2009. Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. J Proteome Res **8:**4028-4038.

513. **Zhao, X., M. Mohaupt, J. Jiang, S. Liu, B. Li, and Z. Qin.** 2007. Tumor necrosis factor receptor 2-mediated tumor suppression is nitric oxide dependent and involves angiostasis. Cancer Res **67:**4443-4450.
514. **Zhonghua, C., G. Chunpin, Z. Yong, X. Kezhi, and Z. Yaou.** 2008. Cloning and bioactivity analysis of a CXC ligand in black seabream Acanthopagrus schlegeli: the evolutionary clues of ELR+CXC chemokines. BMC immunology **9:**66.
515. **Zhu, L., C. Gunn, and J. S. Beckman.** 1992. Bactericidal activity of peroxynitrite. Archives of biochemistry and biophysics **298:**452-457.
516. **Ziegler-Heitbrock, L.** 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. Journal of leukocyte biology **81:**584-592.
517. **Zou, J., A. Carrington, B. Collet, J. M. Dijkstra, Y. Yoshiura, N. Bols, and C. Secombes.** 2005. Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. J Immunol **175:**2484-2494.
518. **Zou, J., M. Chang, P. Nie, and C. J. Secombes.** 2009. Origin and evolution of the RIG-I like RNA helicase gene family. BMC evolutionary biology **9:**85.
519. **Zou, J., M. S. Clark, and C. J. Secombes.** 2003. Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, Fugu rubripes. Immunogenetics **55:**325-335.
520. **Zou, J., P. S. Grabowski, C. Cunningham, and C. J. Secombes.** 1999. Molecular cloning of interleukin 1beta from rainbow trout Oncorhynchus mykiss reveals no evidence of an ice cut site. Cytokine **11:**552-560.
521. **Zou, J., S. Peddie, G. Scapigliati, Y. Zhang, N. C. Bols, A. E. Ellis, and C. J. Secombes.** 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, Oncorhynchus mykiss. Developmental and comparative immunology **27:**813-822.
522. **Zou, J., C. J. Secombes, S. Long, N. Miller, L. W. Clem, and V. G. Chinchar.** 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). Developmental and comparative immunology **27:**845-858.
523. **Zou, J., T. Wang, I. Hirono, T. Aoki, H. Inagawa, T. Honda, G. I. Soma, M. Ototake, T. Nakanishi, A. E. Ellis, and C. J. Secombes.** 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, Oncorhynchus mykiss. Developmental and comparative immunology **26:**161-172.
524. **Zou, J., Y. Yoshiura, J. M. Dijkstra, M. Sakai, M. Ototake, and C. Secombes.** 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish & shellfish immunology **17:**403-409.

CHAPTER II: MATERIALS AND METHODS

1. CELL CULTURE

1.1. Fish

Goldfish (*Carassius auratus* L.) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were maintained at 20°C in a flow-through water system on a simulated natural photoperiod (Edmonton, Alberta). The fish were fed to satiation daily with trout pellets, and acclimatized to this environment for at least 3 weeks prior to use.

1.2. Fish serum

Fish serum was obtained by bleeding common carp (*Cyprinus carpio*). Carp were anaesthetized with tricane methanosulfonate (TMS: approximately 40 mg/L, Syndel Laboratories) and bled from caudal venipuncture using 1 mL syringes and 23G needles. Carp were maintained strictly for serum extraction purposes and were bled every 4-8 weeks. Collected carp blood was allowed to clot overnight at 4°C, centrifuged at 1000 x *g* for 25 min and serum removed and heat-inactivated for 30 min at 56°C, filter sterilized (0.22 μm), and stored at -20°C until used in the experiments.

1.3. Goldfish kidney-derived monocyte/macrophage culture medium and conditions

The incomplete medium for the culture of goldfish leukocytes (MGFL-15) used in all studies has been previously described (12). The medium constituents are listed in Tables 2.1-2.4. Complete medium (C-MGFL-15) comprised of MGFL containing 100 U/mL penicillin / 100 μg/mL streptomycin (Gibco), 100 μg/mL gentamicin (Gibco), 10% newborn calf serum (Hyclone) and 5% carp serum. All goldfish cell cultures were grown and maintained at 21°C.

1.4. Isolation of goldfish kidney leukocytes

Leukocytes were isolated from goldfish kidneys using previously described procedures (1, 10, 11). Briefly, fish were anesthetized using tricane methanosulfonate (TMS; approximately 40 mg/mL), the kidneys were aseptically removed and placed in ice-cold MGFL-15. The kidneys were gently passed through sterile stainless steel screens using medium containing antibiotics (100 U/mL penicillin / 100 μg/mL streptomycin, Gibco) and heparin (50 U/mL, Sigma). The resulting cell suspension was layered over 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 minutes at 4°C. Cells at the medium-51% percoll interface were transferred into clean tubes and washed twice with MGFL-15 by centrifugation at 200 x *g* for 10 min at 4°C. The resulting cell pellets were re-suspended in C-MGFL-15 and the number of viable leukocytes was determined using trypan blue exclusion method (Gibco). A typical yield of 1.5 x 10^7 to 3.5 x10^7 leukocytes was obtained per fish.

1.5. Generation of mitogen-stimulated goldfish kidney leukocyte conditioned supernatants (MAF)

MAF supernatants were prepared using protocols described previously (11). Briefly, goldfish kidney leukocytes (4×10^6/mL, 20 mL cultures) were incubated overnight (18h) in medium containing 10% newborn calf serum (Hyclone) and 5% carp serum. The following day, the cells were treated with 10 μg/mL concanavalin A (Boehringer Manheim), 10 ng/mL PMA (Sigma), and Ca^{2+} ionophore A23187 (Sigma) for an additional 6 hours and washed 3 times with 10 mL of Hank's balanced salt solution. The cells were sub-cultured in fresh medium and incubated for 72 hours at 20°C before the supernatants were harvested. These supernatants contained a complex mixture of factors that have been functionally characterized and shown to induce antimicrobial responses of goldfish macrophages (11). The supernatants were pooled, concentrated ten fold against polyethylene glycol (Sigma) and dialyzed against PBS, prior to use in the experiments.

1.6. Establishment of primary kidney macrophage (PKM) cultures

Goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (18–2 x 10^6 cells/flask from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium (C-MGFL-15) and 5 mL of cell-conditioned medium (CCM) from previous cultures. The PKM cultures consisted of heterogeneous populations of cells including early progenitor cells, monocytes and mature macrophages as determined by flow cytometry, morphology, cytochemistry and function (10). Less aged cultures (2-4 days) consisted

predominantly of monocytic cells while older cultures (6-8 days) primarily comprised of mature macrophages.

1.7. Collection of cell-conditioned medium

Goldfish PKM cultures aged 6-8 days were centrifuged at 200 x *g* for 10 min at 4°C and the resulting cell conditioned media (CCM) from individual cultures were pooled, filter-sterilized (0.22 μm) and stored at 4°C until use.

1.8. Isolation of goldfish splenocytes

Spleens from individual fish were aseptically removed, passed through stainless steel screens and re-suspended in incomplete (no serum) MGFL-15 medium containing 100 U/mL penicillin /100 μg/mL streptomycin. The cell suspensions were than layered over 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 minutes. The cells at the 51% Percoll-medium interface were carefully removed and washed twice in MGFL-15 medium (400 x *g* for 10 minutes) and re-suspended in complete medium (C-MGFL-15) prior to use in the experiments.

1.9. Isolation of goldfish peripheral blood leukocytes

Individual fish were bled from the caudal vein and 2 mL of whole blood was diluted in 10 mL of MGFL-15 medium containing heparin 50,000 U/mL (Sigma) and 100 U/mL penicillin /100 μg/mL streptomycin. The blood cells were centrifuged at 400 x *g* for 10 minutes and the red blood cells were removed by hypotonic lysis using 9 mL of Milli-Q® water for one minute, after which 1 mL

of 10X PBS was added to the cell suspension. The peripheral blood leukocyte suspensions (PBLs) were then washed twice in MGFL-15 medium and re-suspended in the C-MGFL-15 medium prior to use in the experiments.

1.10. Isolation of goldfish kidney granulocytes

Kidneys from individual fish were passed through stainless steal screens, layered over 51% Percoll (Sigma) and centrifuged at 400 x *g* for 25 minutes. The pellets containing kidney derived granulocytes and red blood cells was re-suspended in 1 mL of medium. The red blood cells were removed by hypotonic lysis using 9 mL of Milli-Q® water for one minute, after which 1 mL of 10X PBS was added to the cell suspension. This cell suspension contained greater than 98% granulocytes based on cytochemical staining, and the cells were washed twice in MGFL-15 medium (400 x *g* for 10 minutes) and re-suspended in the C-MFGL-15 medium prior to use in the experiments.

2. MYCOBACTERIA

2.1. Mycobacterium growth conditions

The *Mycobacterium marinum* strain ATCC 927 (fish isolate) was a kind gift from Dr. Lourens Robberts, School of Public Health, University of Alberta. Bacteria were grown with shaking at 30°C as a dispersed culture in 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.5% glycerol and 10% albumin-dextrose complex for 7 to 10 days. The number of colony forming units (cfu) per milliliter was determined by plating on Middlebrook 7H10 agar (Difco). Before

all experiments, bacterial cultures were dispersed by 10-15 passages through a 25-gauge needle. When required, bacteria were heat-killed by incubation of enumerated bacterial cultures at 80°C water bath for 30 minutes. Loss of bacterial viability following heat-killing was confirmed by a plating resulting cultures on Middlebrook 7H10 agar (Difco) and observing no growth.

2.2. Analysis of *M. marinum* survival in cytokine-treated goldfish phagocytes

Day 3 primary kidey macrophage (PKM) cultures enriched for monocytes or day 6-8 cultures enriched for mature macrophages (8, 10) were incubated for 5 hours with 10^5, 10^6 or 10^7 cfu/mL of viable *M. marinum*, and then treated with 100 ng/mL of rgTNFα2, rgIFNγ, rgIFNγrel or 500 ng/mL of rgIL-10 and incubated for an additional 12 hours. In certain experiments, monocytes and macrophages were pre-treated for 5 hours with 100 ng/mL of rgTNFα2, rgIFNγ, rgIFNγrel or 500 ng/mL of rgIL-10 prior to infection with 10^5, 10^6 or 10^7 cfu/mL of viable *M. marinum* and further incubated for 12 hours. Following incubation, cells were washed twice MGFL-15 in order to remove any cell-free bacteria, the medium was removed and 50 μL sterile 0.1% Tween-20 in distilled water was added for 10 minutes to lyse the phagocytes. After lysis, 150 μL of Middlebrook 7H9 (Difco, Detroit, Mich.) broth was added to each well. The contents of individual wells were serially diluted and plated in duplicate on Middlebrook 7H10 agar (Difco) plates. The plates were then incubated at 30°C in a humid chamber for 4 days and the resulting colonies scored, averaged and represented as

cfu/mL.

3. MOLECULAR IDENTIFICATION OF GOLDFISH IMMUNE AND INFLAMMATORY GENES

3.1. Goldfish tumor necrosis factor-alpha isoforms (TNFα1 and TNFα2)

Degenerate primers were designed against the carp TNFα sequences and used to obtain amplicons corresponding to goldfish TNFα1 and TNFα2. RACE PCR was performed using the SMART RACE PCR system (Clonetech) according to manufacturers specifications, using the primers designed against the partial TNFα1 and TNFα2 amplicons and the full ORF for goldfish TNFα2 was obtained. Several attempts to generate the full ORF for TNFα1 were not successful, and only the portion of the sequence representing the cleaved active fragment of TNFα1 was identified.

3.2. Goldfish tumor necrosis factor receptors (TNF-R1 and TNF-R2)

Primers designed against the zebrafish TNF-R1 and TNF-R2 sequences were used to obtain partial sequences of corresponding goldfish cDNA transcripts. These were then blasted against the NCBI database and identified as the likely homologues of goldfish TNF-R1 and TNF-R2. RACE PCR (Clonetech) was then conducted based on the partial sequences and the subsequent RACE products were sequenced to yield the remaining open reading frame and untranslated regions of the transcript.

3.3. Goldfish interferon-gamma (IFNγ)

Primers designed against the zebrafish IFNγ sequence were used to obtain partial sequences of the goldfish cDNA transcript. These were than blasted against the NCBI database and identified as the likely homologues of goldfish IFNγ. RACE PCR (Clonetech) was then conducted based on the partial sequences and the subsequent RACE products were sequenced to yield the remaining open reading frame and untranslated regions of the transcript.

3.4. Goldfish interferon-gamma related (IFNγrel)

Primers designed against the carp IFNγrel sequence were used for identification of partial sequences of the goldfish IFNγrel cDNA transcript. These were then blasted against the NCBI database and identified as the likely homologues of goldfish IFNγrel. RACE PCR (Clonetech) was then conducted based on the partial sequences and the subsequent RACE products were sequenced to yield the remaining open reading frame and untranslated regions of the goldfish IFNγrel transcript.

3.5. Goldfish interferon-gamma receptors (IFNGR1-1 and IFNGR1-2)

Primers designed against the zebrafish IFNGR1-1 and IFNGR1-2 sequences were used to obtain partial sequences of corresponding goldfish cDNA transcripts. These were then blasted against the NCBI database and identified as the likely homologues of goldfish IFNGR1-1 and IFNGR1-2. RACE PCR (Clonetech) was then conducted based on the partial sequences and the

subsequent RACE products were sequenced to yield the remaining open reading frame and untranslated regions of the transcript.

3.6. Goldfish interleukin-10 (IL-10)

Partial goldfish IL-10 sequence was obtained using primers designed against the carp and zebrafish interleukin-10 sequences. RACE PCR was performed using the SMART RACE PCR system (Clonetech) according to manufacturers specifications, using the primers designed against the partial goldfish IL-10 sequences. By these means, the full ORF and UTRs of the goldfish IL-10 cDNA transcript were obtained.

3.7. Identification of goldfish immune and inflammatory gene transcripts

Throughout the course of this thesis work I identified several additional goldfish immune and inflammatory genes. In general, the goldfish immune gene cDNA transcripts were identified using PCR primers designed against the closest phylogenetically related species with the corresponding gene sequences available in GenBank. For a full list of the goldfish genes identified during the preparation of this thesis and their corresponding GenBank accession numbers please refer to Table 2.5.

4. *IN SILICO* ANALYSIS OF INFLAMMATORY CYTOKINES AND CYTOKINE RECEPTORS

4.1. Sequence alignments and phylogenetic analysis

All nucleotide and protein alignments were performed using the Clustal W

software (http://www.ebi.ac.uk/clustalw/). All phylogenetic analyses were conducted using the neighbor joining method for relationship calculations, bootstrapped 10,000 times and visualized using the njplot program (http://pbil.univ-lyon1.fr/software/njplot.html). All nucleotide and protein sequences used in this thesis have been submitted to GenBank.

4.2. Synteny analysis of zebrafish IFNGR1-1 and IFNGR1-2

Synteny analysis was performed using NCBI server, map viewer option. Zebrafish IFNGR1-1 nucleotide and protein sequences were derived from the NCBI database. The identified clones corresponding to the zebrafish IFNGR1-2 were assessed using the GENESCAN Web Server and the deduced exons were compiled into a continuous sequence corresponding to the zebrafish IFNGR1-2 open reading frame (ORF).

5. QUANTITATIVE EXPRESSION ANALYSIS OF GOLDFISH IMMUNE GENE EXPRESSION

Note that all Q-PCR primer sequences employed in this thesis are listed in Table 2.6

5.1. Analysis of TNFα1 and TNFα2 expression in tissues of healthy goldfish

Partial cDNA fragments of the goldfish TNFα-1 and TNFα-2 were PCR amplified and inserted into TOPO TA vector (Invitrogen). Vectors bearing the TNFα1 and TNFα2 inserts were serially diluted and used to generate standard

curves for comparisons of transcript copies present in cDNA samples during real-time PCR analysis. Four micrograms of total RNA from kidney, spleen, brain, gill, muscle, intestine, and heart tissue were reverse transcribed into cDNA using Superscript First Strand Synthesis System (Invitrogen). These were subsequently diluted 1 in 20 and used for real-time PCR analyses. Absolute-real-time PCR analyses was performed using the Applied Biosystems 7500 fast real-time PCR equipment7500. Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative tissue expressions of TNFα-1 and TNFα-2 mRNA were done for tissues obtained from five fish (n=5) and were analyzed using the 7500 Fast software (Applied Biosystems).

5.2. Analysis of TNFα1 and TNFα2 expression in FACS-sorted goldfish monocytes and macrophages

Monocytes and macrophages were sorted using a FACS Calibur flow cytometer (Becton/Dickinson) using a previously described protocols (10) and RNA was extracted either immediately following sorting or subsequent to treatment of cells for 4 hours with MAF. Four hundred nanograms of total RNA from different cell subpopulations (monocytes or macrophages) was reverse transcribed into cDNA using Superscript First Strand Synthesis System (Invitrogen), diluted 1 in 10 and used for real-time PCR analysis. Real-time PCR analysis was carried out as described above.

5.3. Analysis of TNF-R1 and TNF-R2 expression in tissues of healthy goldfish

Total RNA was isolated from kidney, spleen, brain, heart, muscle, intestine, and gill tissues of healthy fish using TRIzol (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Primers specific for goldfish TNF-R1 and TNF-R2 were designed using Primer Express software (Applied Biosystems) and the expression of goldfish TNF-R1 and TNF-R2 in relation to the endogenous control gene, elongation factor 1 alpha (EF-1α), was determined using quantitative PCR. Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Analyses of the relative tissue expression data for five fish (n=5) were carried out using the 7500 Fast software (Applied Biosystems). Direct comparison of receptor expression was achieved by performing the ddCT analysis using the lowest expression (highest delta CT, TNF-R1 in muscle) as the standard for the expression of both receptors. The RQ values were normalized against the expression seen in the lowest tissue group for TNF-R1 (muscle).

5.4. Analysis of TNF-R1 and TNF-R2 expression in goldfish immune cell populations

Monocytes and macrophages were sorted using a FACS Calibur flow cytometer (Becton/Dickinson) using a previously described protocol and cytometer parameters (10). Cell populations including goldfish kidney

leukocytes, PBLs and kidney granulocytes were isolated as described above. Cells from five individual fish (*n*=5) were used for all experiments. Total RNA was isolated from cell subpopulations using TRIzol either immediately after cell isolation or following treatment for 4 hours with 100 ng/mL of rgTNFα2 and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Relative expression analysis of goldfish IFNγ was performed on the respective cDNAs as described above for tissues. Direct comparisons for the expression of both receptors were achieved by performing the ddCT using the lowest expression (highest CT, TNF-R1 in PBLs) as the standard for the expression of both receptors. The RQ values were normalized against the lowest observed expression TNF-R1 (PBLs).

5.5. Analysis of TNF-R1 and TNF-R2 expression in goldfish macrophages treated with rgTNFα2, rgIFNγ, or rgTGFβ

Six to 8 day-old primary macrophage cultures derived from 5 individual fish (*n*=5) were either left untreated or treated with 100 ng/mL of either rgTNFα2, rgIFNγ, or rgTGFβ for 0, 2, 6, 12, 24, 48 and 72 hours. Each treatment group consisted of 1x 10^6 cells in a final volume of 400 μL of complete medium. Immediately following the indicated incubation times, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit in accordance to manufacturer's directions. Expression analysis of goldfish TNF-R1 and TNF-R2 was performed relative to EF-1α, using the ddCT value of TNF-R1 at 0h to standardize the expression of

both receptors. The RQ values were normalized against the TNF-R1 for baseline expression (0h).

5.6. Analysis of IFNγ expression in tissues of healthy goldfish Total RNA was isolated from kidney, spleen, brain, heart, muscle, intestine, and gill tissues of healthy fish using TRIzol (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Primers specific for goldfish IFNγ were designed using Primer Express software (Applied Biosystems) and the expression of goldfish IFNγ in relation to the endogenous control gene, elongation factor 1 alpha (EF-1α) was determined. Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Analyses of the relative tissue expression data for five fish (n=5) were carried out using the 7500 Fast software (Applied Biosystems). Resulting RQ values were normalized against the expression seen in the lowest tissue group (muscle).

5.7. Analysis of IFNγ expression in activated goldfish immune cell populations

Freshly isolated kidney leukocytes from 5 individual fish (n = 5) were treated with either 10 μg/mL PHA (Sigma) or 50 μg/mL Poly I:C (Sigma). To induce mixed leukocyte reaction (MLR), equal number of cells from 5 fish were combined and cultured for various periods. At 0, 2, 6, 12, 24, 48 and 72 hours after treatment with the reagents or post MLR, samples were harvested and total

RNA was isolated using TRIzol (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Expression analysis of goldfish IFNγ was done using the procedures described above. The MLR expression analysis was performed in triplicate for each time point. The expression data was normalized using the baseline expression at 0 hour time points.

Monocytes and macrophages were sorted using a FACS Calibur flow cytometer (Becton/Dickinson) using a previously described protocol and cytometer parameters (10). Cell populations including goldfish kidney leukocytes, PBLs and kidney granulocytes were isolated as described above. Cells from five individual fish (*n*=5) were used for all experiments. Total RNA was isolated from cell subpopulations using TRIzol either immediately after cell isolation or following treatment for 4 hours with 100 ng/mL of rgTNFα2 and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Relative expression analysis of goldfish IFNγ was performed on the respective cDNAs as described above for tissues. All the results were normalized against the lowest expression seen, which was that of resting kidney leukocytes.

5.8. Analysis of pro-inflammatory cytokine and chemokine expression in goldfish macrophages activated with recombinant goldfish IFNγ

Six to 8 day-old macrophage cultures derived from 5 individual fish (*n*=5) were treated with 100 ng/mL of rgIFNγ for 0, 2, 6, 12, 24, 48 and 72 hours. Each

treatment group consisted of 1 x 10^6 cells in a final volume of 400 µL of complete medium. Immediately following the indicated incubation times, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit in accordance to manufacturer's directions. Primers for goldfish, IL-1β1, IL1-β2, TNFα1, TNFα2, IL-12-p35, IL-12-p40, RSAD-2 (=viperin) CXCL-8 (=IL-8), CCL-1, TLR-3, iNOS isoforms A and B, TGF β and IFNγ were designed using Primer Express software (Applied Biosystems) and their relative expression determined in relation to EF-1α. All expression data were normalized using the baseline expression at 0 hour time point.

5.9. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in zebrafish and goldfish tissues

Total RNA was isolated from kidney, spleen, brain, heart, muscle, intestine, and gill tissues of healthy zebrafish and goldfish using TRIzol (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Primers specific for zebrafish and goldfish IFNGR1-1 and IFNGR1-2 were designed using Primer Express software (Applied Biosystems) and the expression of IFNGR1-1 and IFNGR1-2 in relation to the endogenous control gene, elongation factor 1 alpha (EF-1α) was determined in both fish species using quantitative PCR. Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Analyses of the relative tissue

expression in zebrafish were done using pooled tissues of six fish (performed in triplicate).

Tissues from five goldfish (*n*=5) were used for the Q-PCR analysis that was carried out using the 7500 Fast software (Applied Biosystems). Direct comparison of receptor expression was achieved by performing the ddCT analysis using the lowest expression (highest delta CT, IFNGR1-2: zebrafish intestine, goldfish muscle) as the standard for the expression of both receptors. The RQ values were normalized against the expression seen in the lowest tissue group for IFNGR1-2 (zebrafish intestine, goldfish muscle).

5.10. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in goldfish immune cell populations

Monocytes and macrophages were sorted using a FACS Calibur flow cytometer (Becton/Dickinson) using a previously described protocol and cytometer parameters (10). Cell populations including goldfish kidney leukocytes, PBLs and kidney neutrophils were isolated as described above. Cells from five individual fish (*n*=5) were used for all experiments. Total RNA was isolated from cell subpopulations using TRIzol either immediately after cell isolation and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. Direct comparisons for the expression of both receptors were achieved by performing the ddCT using the lowest expression (highest CT, IFNGR1-2 in PBLs) as the

standard for the expression of both receptors. The RQ values were normalized against the lowest observed expression IFNGR1-2 (PBLs).

5.11. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in activated goldfish macrophages

Primary macrophage cultures were derived from five individual fish (*n*=5) and cells from 6 to 8 day old cultures were either left untreated or treated with 100 ng/mL of either rgTNFα2, rgIFNγ2, or rgTGFβ for 0, 2, 6, 12, 24, 48 and 72 hours. Each treatment group consisted of 1 x 10^6 cells in a final volume of 400 µL of complete medium. Immediately following the indicated incubation times, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit according to manufacturer's directions. Expression analysis of goldfish IFNGR1-1 and IFNGR1-2 was performed relative to EF-1α, using the ddCT value of IFNGR1-2 at 0h to standardize the expression of both receptors. The RQ values were normalized against the IFNGR1-2 for baseline expression (0h).

5.12. Analysis of IFNγ and IFNγrel gene expression in goldfish tissues and immune cell populations

Preparation of cDNA corresponding to goldfish tissues and immune cell populations and the Q-PCR thermocycling parameters were previously described (5). Goldfish specific IFNγ and IFNγrel primers were designed using Primer Express software (Applied Biosystems) and the expression was assessed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). Tissues from

five goldfish (*n*=5) and cell populations from four goldfish (*n*=4) were used for the Q-PCR analysis carried out using 7500 Fast software (Applied Biosystems). Direct comparisons of IFNγ and IFNγrel expression was achieved by performing ddCT analysis using lowest expression (highest delta CT, IFNγrel: muscle and monocytes) as the standard for the expression for both cytokines. The RQ values were normalized against the lowest observed tissue and cell expression (IFNγrel, muscle and monocytes, respectively).

5.13. Analysis of immune gene expression in rgIFNγrel- and rgIFNγ-stimulated goldfish monocytes and macrophages

Day 3 cultures enriched for monocytes and day 8 cultures abundant in mature macrophages (23,29), were treated for 12 hours with medium,100 ng/mL of rgIFNγrel, 100 ng/mLof rgIFNγ or 100ng/mL rgIFNγrel + 100 ng/mL rgIFNγ. Each treatment group consisted of 1 x 10^6 cells in a final volume of 500 μL of complete medium using cells obtained from cultures established using kidney leukocytes isolated from individual fish (*n*=5). Following indicated treatments, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit according to manufacturer's directions. The genes examined included: IFNGR1-1 and IFNGR1-2; p40 ^{phox}; p47^{phox}; p67 ^{phox}; p22 ^{phox}; gp91^{phox}; IL-1β-1 and IL-1β-2; TNFα1 and TNFα2; CXCL8; CCL1; iNOSA and iNOSB, TGFβ and ceruloplasmin. Goldfish IRF expression analysis was performed by treating goldfish monocytes in a final volume of 500 μL for 0, 15, 30 or 90 minutes with

100 ng/mL of rgIFNγ or rgIFNγrel. RNA isolation and cDNA synthesis were performed as described above. Expression analysis of all genes was performed using the delta CT method relative to EF-1α and derived RQ values were normalized against respective untreated controls.

5.14. Analysis of interleukin-10 gene expression in goldfish tissues and immune cell populations

Preparation of cDNA of goldfish tissues and immune cell populations and the Q-PCR thermocycling parameters were previously described (5). Goldfish specific IL-10 primers were designed using Primer Express software (Applied Biosystems) and the expression was assessed relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). Tissues from five goldfish (n=5) and cell populations from four individual goldfish (n=4) were used for the Q-PCR analysis, carried out using 7500 Fast software (Applied Biosystems). Total RNA was extracted using TRIzol either immediately after cell isolation or following treatment for 4 h with 100 ng/mL rgTNFα2 and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit according to manufacturer's directions. The RQ values were normalized against the lowest observed tissue and cell expression (muscle and monocytes, respectively).

5.15. Analysis of rgIL-10 down-regulation of immune gene expression in cells stimulated with heat-killed *Aeromonas salmonicida*

Day 3 macrophage cultures enriched for monocytes (8, 10) as well as freshly-isolated splenocytes were pre-treated for 2 hours with medium only or with 500 ng/mL of rgIL-10. Subsequently either medium or heat-killed *A. salmonicida* (2 μg/mL) was added to the cultures and the cells incubated for additional 6 hours. Each treatment group consisted of 1 x 10^6 cells in a final volume of 500 μL of complete medium using cells obtained from cultures established using kidney leukocytes isolated from individual fish (*n*=5). Following indicated treatments, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit according to manufacturer's directions. The genes examined included: TNFα1 and TNFα2; IL-1β-1 and IL-1β-2; IL-10; TGFβ; CCL1; CXCL8; $p40^{phox}$; $p47^{phox}$; $p67^{phox}$; $p22^{phox}$; $gp91^{phox}$; IFNγ and IFNγrelated (IFNγrel). Goldfish SOCS-3 expression analysis was performed by treating 1 x 10^6 goldfish monocytes in a final volume of 500 μL for 2, 6, 12 or 24 hours with 500 ng/mL of rgIL-10. The RNA isolation and cDNA synthesis were performed as described above. Expression analysis of all genes was performed using the delta CT method relative to EF-1α and derived RQ values were normalized against respective untreated controls.

5.16. Analysis of *M. marinum*-induced gene expression in goldfish monocytes and macrophages

Day 3 PKM cultures enriched for monocytes and day 6-8 cultures enriched for mature macrophages (8, 10) were incubated for 12 hours with viable

or heat-killed *M. marinum*. All treatment groups consisted of 2 x 10^6 cells in a final volume of 500 μL of complete medium using cells from cultures established from individual fish (*n*=5). Following exposure to mycobacteria, the total RNA was isolated from the cells using TRIzol and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit according to manufacturer's directions. The genes examined included: CCL1; CXCL8 (IL-8); $gp91^{phox}$; IDO; IFNγ; IFNγrelated (IFNγrel); IFNGR1-1; IFNGR1-2; IL-1β-1 and IL-1β-2; IL-12 p35 and p40; iNOS A and B; NRAMP; $p22^{phox}$; $p40^{phox}$; $p47^{phox}$; $p67^{phox}$; SOCS-3; TGFβ; TNFα1 and TNFα2; TNFR1 and TNFR2. Expression analysis of all genes was performed using the delta CT method relative to endogenous control gene, elongation factor -1 alpha (EF-1α) and the derived RQ values were normalized against respective non-treated controls.

6. PROCARYOTIC EXPRESSION OF GOLDFISH RECOMBINANT CYTOKINES AND CYTOKINE RECEPTORS

6.1. Cloning of goldfish cytokines and cytokine receptors into pET SUMO vectors

The production of the recombinant goldfish cytokines and cytokine receptors (rgTNFα2, rgIFNγ, rgIFNγrel, rgIFNGR1-1, rgIFNGR1-2, rgTNF-R1, rgTNF-R2 and rgIL-10) characterized in this thesis has been described previously (3-9). Briefly, pET SUMO vectors (Invitrogen) encoding the mature, signal sequence-cleaved goldfish TNFα2, IFNγ, IFNγrel or IL-10 or pET SUMO vectors encoding the mature extracellular domains of the goldfish TNF-R1, TNF-R2,

IFNGR1-1 or IFNGR1-2 were transformed into BL21 Star One Shot *E. coli* (Invitrogen). The pET SUMO vector encoded a His^6 tag, N-terminally of the above recombinant molecules.

6.2. Recombinant goldfish cytokine and cytokine receptor pilot expression studies

Preliminary pilot expression studies were performed where bacterial cultures expressing the desired recombinants were grown for 2 hours, induced with 1mM IPTG and sampled every subsequent 2 hours to determine the optimal induction and protein expression times for each respective recombinant protein. Following the sampling period, bacterial lysate supernatants and pelleted bacterial fractions were resolved by SDS-PAGE and visualized by western blotting against the His^6 N-terminal tags on the recombinant proteins. By assaying lysates as well as pelleted bacterial fractions I was able to determine the optimal induction time and whether the respective recombinants were being retained in bacterial inclusion bodies and would require denaturing conditions during lysis. Please refer to Table 2.7 for a list of optimal induction conditions determined from these preliminary studies.

6.3. Scale-up production of goldfish recombinant cytokines and cytokine receptors

Following the above preliminary expression studies, overnight starter cultures of *E. coli* encoding the respective cytokines were scaled up and grown for

2 hours, induced and grown in the presence of 1mM IPTG for respective optimal times (as indicated in Table 2.7), pelleted, frozen at –20ºC for 30 minutes and lysed (5 mL of 10X FastBreak cell lysis reagent (Promega) in 45 mL of wash buffer (100 mM Hepes, 10 mM imidazole, pH 7.5) or denaturing wash buffer (100 mM Hepes, 10 mM imidazole, 7.5 mM Urea, pH 7.5) for 15 minutes. These lysates were pelleted by centrifugation at 16000 x *g* for 10 min at 4ºC and the resulting supernatants were incubated with MagneHis Ni-Particles (Promega) for 20-30 minutes. Ni-particles bound to the recombinant proteins were retained using a PolyATtrack System 1000 magnet (Promega), were washed extensively with the wash buffer described above. The recombinant cytokines were then eluted using 500 mM imidazole. The proteins were re-natured in 10 volumes of re-naturation buffer(s) (4 mM reduced glutathione, 2 mM oxidized glutathione, 50 mM sodium borate, 5 mM EDTA, pH 8.5 for rgIFNγ, rgIFNγrel, rgIFNGR1-1, rgIFNGR1-2 and rgIL-10 or 40 mM Tris, pH 8 for rgTNFα2, rgTNF-R1 and rgTNF-R2) overnight and dialyzed against 1X PBS. The proteins were subsequently concentrated against polyethylene glycol flakes and further dialyzed overnight against 1X PBS. The recombinant proteins were passed through EndoTrap Red endotoxin removal columns (Cambrex) to remove potential traces of endotoxin and successful LPS removal was confirmed by Limulus Amebocyte Lysate (LAL) Endosafe kits (Charles River). Purification of the recombinant cytokines was confirmed by Western blot, and the identity of the proteins was confirmed by mass spectrometry. The protein concentrations were determined using a Micro BCA Protein Assay Kit (Pierce). A vector control was prepared by

growing *E. coli* cells expressing an empty pET SUMO vector and following the exact protocols described for rgTNFα2 preparations. The vector control had no protein contents as examined by the Micro BCA Protein Assay Kit (Pierce). Note that while monocytes primed with 100 ng/mL of rgTNFα2 or rgIFNγ produced statistically significant ROI ($P < 0.05$), cell primed with 100 ng/mL of rgIL-10 or vector control (volumes equivalent to those used for 100 ng/mL rgTNFα2) did not, suggesting an absence of confounding contaminants (Table 2.8).

7. FUNCTIONAL ANALYSIS OF CYTOKINE REGULATION OF GOLDFISH MONOCYTE AND MACROPHAGE ANTIMICROBIAL RESPONSES

7.1. Assessment of rgTNFα2 ability to elicit goldfish monocyte and macrophage chemotactic responses

The chemotaxis assay was performed using blind well leucite chemotaxis chambers (Nucleoprobe Corp.). Medium, LPS (33ng/mL) or different concentrations of the rgTNFα2 (0.007, 0.07, 0.7, 7, 70, 700 or 7000ng/mL) were applied to the lower wells of a leucite chemotaxis chamber and overlaid with polycarbonate membrane filters (5μm pore size; Nucleopore Corp.). To the upper well of the chemotaxis apparatus, 5×10^5 of four to six-day-old kidney-derived macrophages were added and the chambers incubated for 4 hours at 20°C. After incubation, the contents of the top wells were aspirated and the filters were removed and mounted bottom-side-up on microscope slides. The filters were air-dried, fixed using absolute methanol and stained using Wright's hematoxylin

solution. Chemotactic activity was determined by counting the total number of cells present in twenty randomly selected fields of view under oil immersion (magnification 1000X). To assess chemokinesis, the highest concentrations of chemo-attractant that induced a response as determined in previous experiments (70 ng/mL of rgTNFα2 or 33 ng/mL of LPS positive control) were applied to both the upper and lower wells of the chemotaxis apparatus, and cell migration determined as described above.

7.2. Assessment of rgTNFα2 ability to enhance the goldfish monocyte phagocytosis

Four day-old kidney-derived macrophages were seeded into individual wells of 96 well plates at a density of 3 x 10^5 cells/well. The cell cultures were treated with medium control, MAF, or rgTNFα2 (20, 90, 370 or 1500ng/mL) and incubated with fluorescent beads (2.0 μm diameter YG, Polysciences) at a 10:1 ratio of beads to cell in a final volume of 100 μL/well. The plates were incubated for 18 hours at 20^{o}C. After incubation 40μL trypsin-EDTA (0.05% Trypsin, Gibco) was added to each well for 5 min. The cells were removed from individual wells, suspended in 850μL of incomplete medium to inactivate trypsin, layered over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose and centrifuged at 100 x *g* for 15min at 4°C. Flow cytometry analysis was done using pre-optimized instrument settings using FACSCalibur apparatus (Becton/Dickinson).

7.3. Assessment of rgTNFα2 ability to prime the goldfish monocyte respiratory burst response

DHR (Molecular Probes) was dissolved in DMSO at a concentration of 29mM and stored at -80°C until use. Four to six day old kidney-derived macrophages were seeded into 5mL polystyrene tubes at a final density of 2.5 x 10^5 cells/mL and incubated with medium, MAF or rgTNFα2 (0.25, 25, 2500 ng/mL) for 3 hours. After incubation, the cells were exposed to DHR (final concentration = 10μM) + PMA (final concentration = 100ng/mL) in PBS or DHR (final concentration = 10μM) in PBS (without PMA). The cells were allowed to take up the DHR and undergo any DHR oxidation for 30 min after which samples were analyzed using FACS Calibur flow cytometer. All experiments were staggered with respect to time in order to facilitate the transient state of oxidized DHR (rhodamine) fluorescence.

A time course of rgTNFα2-mediated priming of the macrophage respiratory burst response was determined using a nitro blue tetrazolium (NBT) assay. Briefly, 4 to 6 day old kidney-derived macrophages were seeded into 96 well plates at a density of 3 x 10^5 cells/well and incubated with either medium, MAF, or rgTNFα2 (30 or 3000 ng/mL) for 1, 6, 12, 24, 48 and 72 hours. After incubation, NBT (2 mg/mL, Sigma) / PMA (final concentration = 100ng/mL, Sigma) in 1X PBS or NBT in 1X PBS (without PMA) were added to the wells and the cultures incubated at room temperature for 30 min. The plates were centrifuged, the supernatants removed and cells fixed with 70% methanol. Non-reduced NBT was removed by washing with 70% methanol and reduced NBT

was dissolved with 2M KOH. DMSO was added to induce the colorimetric conversion and the plates were read at 630nm. Readings from cells alone (no PMA) were subtracted from treatment values to factor in background NBT reduction.

7.4. Assessment of rgTNFα2 ability to elicit goldfish macrophage nitric oxide production

Macrophages were isolated from kidneys of 6 fish and cultures from individual fish established using previously described protocols (10). The nitric oxide assay was performed as previously described (10). Briefly, six-day old cultured macrophages were seeded into 96 well plates at a density of 3×10^5 cells/well and treated with medium, heat-killed *A. salmonicida*, or rgTNFα2 (2.5, 25 or 250ng/mL) in a final volume of 100μL. The cells were incubated at 20°C for 48 and 72 hours and nitric oxide production measured using Griess reaction.

7.5. Assessment of the ability of rgTNF-R1 and rgTNF-R2 to ablate the rgTNFα1 and rgTNFα2 mediated priming of the monocyte ROI responses

Four day-old PKMs or freshly isolated goldfish kidney macrophages were seeded into 96 well plates at a density of 3×10^5 cells per well and incubated in culture medium alone (control) or treated with the following: rgIFNγ (100 ng/mL), rgTNFα1 (100 ng/mL), rgTNFα2 (100 ng/mL), rgTNF-R1 (100 ng/mL), rgTNF-R2 (100 ng/mL) or a combination of rgIFNγ, rgTNFα-1 or rgTNFα2 (100

ng/mL each) and rgTNF-R1 or rgTNF-R2 (1, 100 or 10000 ng/mL). The cell cultures were incubated for 18 hours at 20^{o}C after which NBT (2 mg/mL, Sigma) and PMA (final conc.100 ng/mL, Sigma) in PBS were added to the cultures and incubate at room temp for an additional 30 minutes. The plates were than centrifuged at 400 x *g* for 10 min, the supernatants aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Readings from cells alone (no PMA) were subtracted from treatment values to factor in background NBT reduction. Experiments were done using PKMs from five individual fish (*n*=5).

7.6. Assessment of rgIFNγ ability to enhance the goldfish monocyte phagocytosis

Four day-old PKMs were seeded into individual wells of 96 well plates at a density of 3 x 10^5 cells per well. These were incubated in culture medium alone (control) or treated with the following: MAF, rgTNFα2 (10 or 100 ng/mL), rgIFNγ (1, 10, 100 or 1000 ng/mL) or a combination of rgIFNγ and rgTNFα2 (1, 10, 100 or 1000 ng/mL and 10 ng/mL, respectively). To each well fluorescent beads at a 10:1 ratio (fluorescent beads:cell) (2.0μm diameter, YG, Polysciences) were added in a final volume of 100 μL. The plates were incubated for 18 hours and then treated with trypsin-EDTA (0.05% Trypsin, Gibco) (40 μL /well) for 5 minutes at room temperature in order to remove cells from the bottom of the wells

and non-ingested beads. Cells were then re-suspended in 850 μL of incomplete medium to inactivate trypsin and centrifuged (100 x *g* for 15minutes at 4°C) over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose in order further dissociate cells from non-ingested beads. FACSs analysis was than performed under pre-optimized instrument settings using a FACSCalibur flow cytometer (Becton/Dickinson). Phagocytosis assays were done using monocyte cultures established from 5 individual fish (*n*=5).

7.7. Assessment of rgIFNγ ability to prime the goldfish monocyte respiratory burst response

Four day-old PKMs or freshly isolated goldfish kidney granulocytes were seeded into 96 well plates at a density of 3 x 10^5 cells per well and incubated in culture medium alone (control) or treated with the following: MAF, rgTNFα2 (10 or 100 ng/mL), rgIFNγ (1, 10, 100 or 1000 ng/mL) or a combination of rgIFNγ and rgTNFα2 (1, 10, 100 or 1000 ng/mL and 10ng/mL, respectively). The cell cultures were incubated for 18 hours at 20^{o}C after which NBT (2 mg/mL, Sigma) and PMA (final conc.100 ng/mL, Sigma) in PBS was added to the cultures and incubate at room temp for an additional 30 minutes. The plates were than centrifuged at 400 x *g* for 10 minutes, the supernatants aspirated and cells in the pellet fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2 M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Readings from cells alone (no PMA) were subtracted from treatment values to

factor in background NBT reduction. Experiments were done using PKMs from five individual fish (*n*=5) or in triplicate for each treatment using granulocytes obtained from ten fish.

7.8. Assessment of rgIFNγ ability to elicit goldfish macrophage nitric oxide production

Macrophages were isolated from goldfish kidneys and cultured until day 6. Cells from 5 fish (*n*=5) were isolated and seeded into individual wells of 96 well plates at a density of 3 x 10^5 cells per well. These were incubated in culture medium alone (control) or treated with the following: heat-killed *A. salmonicida*, rgTNFα2 (10 or 100 ng/mL), rgIFNγ (1, 10, 100 or 1000 ng/mL) or a combination of rgIFNγ and rgTNFα2 (1, 10, 100 or 1000 ng/mL and 10 ng/mL, respectively) and incubated at 20°C for 72 hours after which nitrite production was determined using the Griess reaction. Nitrite production was determined colorimetrically at 540 nm and a nitrite standard curve.

7.9. Comparison of rgIFNγ and rgIFNγrel abilities to enhance the phagocytic response of goldfish monocytes

Monocytes from cultures established from kidney leukocytes isolated from individual fish (n=5) were seeded into wells of 96 well plates at a density of 3 x 10^5 cells per well and were treated with either medium only, rgIFNγ (100 ng/mL), rgIFNγrel (1,10 or 100 ng/mL), a combination of rgIFNγ (100 ng/mL) and rgIFNγrel (1,10 or 100 ng/mL) with or without α-rgIFNγrel IgG (5 μg/mL). To

each well fluorescent beads (2.0 μm diameter YG, Polysciences) were added at a ratio of 10 beads :1 cell, in a final volume of 100 μL. The phagocytosis assay was performed as described previously (5, 8).

7.10. Comparison of rgIFNγ and rgIFNγrel abilities to prime the ROI response of goldfish monocytes

Goldfish monocytes were seeded into 96 well plates at a density of 3 x 10^5 cells per well. Cells were primed with either medium only, rgTNα2 (100 ng/mL), rgIFNγ (100 ng/mL), rgIFNγrel (0.001, 0.1, 10 ng/mL), rgTNFα2 (100 ng/mL) in combination with 0.001, 0.1, or 10 ng/mL of rgIFNγrel, or rgIFNγ (100 ng/mL) in combination with 0.001, 0.1, or 10 ng/mL of rgIFNγrel with or without α-rgIFNγrel IgG (5 μg/mL)in a total volume of 100 μL/well. All cultures were incubated for 1, 9 or 16 hours after which phorbol ester (PMA) was used to trigger the ROI production. Medium only-treated, PMA-triggered cells were negative controls. The nitroblue tetrazolium (NBT) assay was performed as described previously (5, 8).

7.11. Comparison of rgIFNγ and rgIFNγrel abilities to elicit nitric oxide response of goldfish macrophages

Eight-day-old goldfish macrophage cultures established from kidney leukocytes of individual fish (n=5) were seeded into wells of 96 well plates at a density of 3 x 10^5 cells/well and treated with medium, rgIFNγ (100 ng/mL), rgIFNγrel (1,10 or 100 ng/mL), and a combination of rgIFNγ (100 ng/mL) and

rgIFNγrel (1,10 or 100 ng/mL) with or without α-rgIFNγrel IgG (5 μg/mL). All cultures were incubated for 72 hours before assessing nitrite production using the Griess reaction as described previously (5, 8).

7.12. Assessment of rgIL-10 ability to abrogate the *A. salmonicida*-induced goldfish monocyte ROI response

The nitroblue tetrazolium (NBT) assay was performed as described previously (5, 8). Briefly, Goldfish monocytes were seeded into 96 well plates at a density of 3×10^5 cells per well. Cells were pre-treated for 2 hours with either medium only or 10, 100 or 1000 ng/mL of rgIL-10. Subsequently medium or heat-killed *Aeromonas salmonicida* (2 μg/mL) was added to the cells and the treatments were allowed to incubate for additional 6 hours after which NBT (2mg/mL, Sigma) and PMA (100 ng/mL, Sigma) in PBS was added to the cultures and incubated for an additional 30 min. The plates were then centrifuged at 400 x *g* for 10 min, the supernatants aspirated and the cells fixed with absolute methanol. Non-reduced NBT was removed by washing with methanol and reduced NBT was dissolved in 2M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630 nm. Absorbances from cells alone (no PMA) were subtracted from treatment values to factor in background NBT reduction. Medium-treated, PMA-triggered cells were negative controls. Experiments were performed using cells from five individual fish ($n = 5$).

7.13. Analysis of the effects of *M. marinum* on the respiratory burst responses of resting and recombinant cytokine-primed goldfish monocytes

The nitroblue tetrazolium (NBT) assay was performed as described previously (5, 8). Briefly, goldfish monocytes were seeded into 96 well plates at a density of 3 x 10^5 cells per well. Cells were pre-incubated for 5 hours with medium only, 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum*, and then treated with 100 ng/mL of rgTNFα2 or rgIFNγ and incubated for an additional 12 hours. Alternatively cell were pre-treated for 5 hours with 100 ng/mL of rgTNFα2 or rgIFNγ, before being incubated with 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum* for an additional 12 hours. Absorbances (@ 630nm) from cells alone (no PMA) were subtracted from those of treatment groups to account for background NBT reduction. Medium only-treated, PMA-triggered cells were negative controls. Monocytes were obtained from cultures established from individual fish (*n*=5).

7.14. Analysis of the effects of *M. marinum* on the nitric oxide responses of resting and recombinant cytokine-stimulated goldfish macrophages

Six-to-eight-day-old goldfish macrophage obtained from cultures established from individual fish (*n*=5), were seeded into wells of 96 well plates at a density of 3 x 10^5 cells/well and pre-incubated for 5 hours with medium only, 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum*, subsequently treated with 100 ng/mL of rgTNFα2 or rgIFNγrel and incubated for an additional

72h. Alternatively cell were pre-treated for 5 hours with medium alone or 100 ng/mL of rgTNFα2 or rgIFNγrel, before being incubated with 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum* for an additional 72 hours. Nitrite production was determined via Griess reaction by the addition of 1% sulfanilamide and 0.1% N-naphthyl-ethylenediamine to aspirated supernatants from the treated cells, measuring absorbance at 540 nm and the amount of nitrite in culture supernatants determined using a nitrite standard curve.

8. WESTERN BLOT ANALYSIS

8.1. Western blot analysis of recombinant cytokine expression

Following the production and isolation of all of the recombinant goldfish cytokines described here, these proteins were resolved by reducing SDS-PAGE and western blot (α-polyHis^6, Sigma) against poly Histidine tags present on the recombinants and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.).

8.2. *In vitro* rgTNF-R1, rgTNF-R2, rgTNFα1 and rgTNFα2 binding studies

For all binding studies, 2.5 μg of each recombinant protein was incubated in conjugation buffer (20 mM Hepes buffer) for one hour. The rgTNFα1, rgTNFα2, rgTNF-R1 and rgTNF-R2 were incubated individually or in ligand-receptor combinations. Following the initial conjugation period, the ligands, receptors and ligand-receptor combinations were cross-linked using

disuccinimidyl suberate (DSS, Therom Scientific). All studies using TNF-R1 were done using a final concentration of 5 mM DSS. Because at 5mM DSS, the cross-linked TNF-R2 displayed smearing when resolved, a final concentration of 2.5 mM DSS was employed for all cross-linking studies with TNF-R2. The cross-linking reactions were allowed to proceed for an additional half hour and terminated for 15 min by the addition of 50 mM Tris (final concentration). The reactions were then resolved using reducing SDS-PAGE and Western blot against poly histidine tags (α-polyHis^6, Sigma) present on the recombinants and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

8.3. *In vitro* rgIFNGR1-1, rgIFNGR1-2, rgIFNγ and rgIFNγrel binding studies

For all binding studies, 2.5 μg of each recombinant protein was incubated in conjugation buffer (20 mM Hepes) for half an hour. The rgIFNγ, rgIFNγrel, rgIFNGR1-1 and rgIFNGR1-2 were incubated individually or in ligand-receptor combinations. Following the initial conjugation period, the ligands, receptors and ligand-receptor combinations were cross-linked using disuccinimidyl suberate (DSS, Therom Scientific). All studies were done using a final concentration of 1 mM DSS. The cross-linking reactions were allowed to proceed for an additional half hour and terminated for 15 min by the addition of 50 mM Tris (final concentration). The reactions were then resolved using reducing SDS-PAGE and western blot against poly histidine tags (α-polyHis^6, Sigma) present on the

recombinants and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

8.4. Immunodetection of rgIFNγrel

The purified rgIFNγrel was used for generation of rabbit polyclonal α-rgIFNγrel immunization. The primary immunization was performed by combining equal volumes of rgIFNγrel (50 mg in 750 mL) with Freud's complete adjuvant (750 mL). Booster injections were carried out as above but using Freud's incomplete adjuvant. The IgG fraction was affinity-purified using HiTrap protein A HP column (Amersham Biosciences) in accordance with the manufacturer's protocol. The isolated α-rgIFNγrel IgG was filter-sterilized (0.22 mm filter, Milipore) and assessed for reactivity against rgIFNγrel using Western blot.

8.5. Western blot analyses of cell lysates and isolated nuclei of macrophages incubated with rgIFNγrel or rgIFNγ

Five million monocytes were incubated with medium alone, rgIFNγrel or rgIFNγ. For ligand association/internalization experiments, cells were incubated with 5 μg of each recombinant protein and assessed at 0, 15, 30 or 90 minutes after treatment. For phospho-Stat1 experiments, cells were treated with 100 ng/mL of IFNγrel or IFNγ and assessed at 0, 15, 30, 90 minutes. For all experiments, cells were pelleted by centrifugation and either immediately re-suspended in Laemmli buffer and boiled at 95 °C or prepared for isolation of

nuclei. The nuclei isolation protocol was adopted from Garcia *et al.*(2). Briefly, pelleted cells were flash frozen on dry ice-ethanol bath for 10 minutes and disrupted by re-suspending them in hypotonic buffer (10mM Hepes, 10mM KCl, 1.5 mM $MgCl_2$, 1mM freshly added DTT, pH 7.9). Nuclei were recovered by centrifugation at 800 x *g* for 10 minutes in a cooled microcentrifuge, re-suspended in Laemmli buffer and boiled at 95 °C. All samples were resolved on freshly cast 10% SDS gels, transferred onto nitrocellulose membranes, blocked for 1 hour and incubated overnight at 4 °C in appropriate primary antibody (α-polyHistidine, Sigma and α-phospho-Stat1(Tyr), Cell Signaling Technology Inc.). The following day the membranes were washed, incubated for 1 hour with appropriate secondary antibody (goat-anti rabbit or goat anti-mouse IgG, Bio Rad) and developed using ECL developing substrate (Pierce).

8.6. *In vitro* cross-linking studies of rgIL-10

The rgIL-10 (2.5 μg) was cross-linked using 1 mM disuccinimidyl suberate (DSS, Therom Scientific) for 30 min before terminating the reaction for 15 min by the addition of 50 mM Tris (final concentration). The reaction was then resolved using reducing SDS-PAGE and Western blot against polyHis tag present on the recombinant and developed using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

8.7. Western blot assessments of cell lysates and isolated nuclei of monocytes incubated with rgIL-10

Five million monocytes were incubated with either medium alone or rgIL-10. For ligand association/internalization experiments, cells were incubated with 5 μg of rgIL-10 and assessed at 0, 15, 30 or 90 minutes after treatment. For phospho-Stat3 experiments, cells were treated with 500 ng/mL of rgIL-10 and assessed at 0, 15, 30, 90 minutes. For all experiments, cells were pelleted by centrifugation and either immediately re-suspended in Laemmli buffer and boiled at 99 °C or prepared for isolation of nuclei. The nuclei isolation protocol has been described previously (6). Briefly, pelleted cells were flash frozen on dry ice-ethanol bath for 10 minutes and disrupted by re-suspending them in hypotonic buffer (10mM Hepes, 10mM KCl, 1.5 mM $MgCl_2$, 1mM freshly added DTT, pH 7.9). Nuclei were recovered by centrifugation at 800 x *g* for 10 minutes in a cooled microcentrifuge, re-suspended in Laemmli buffer and boiled at 99°C. All samples were resolved on freshly cast 10% SDS gels, transferred onto nitrocellulose membranes, blocked for 1 hour and incubated overnight at 4°C in appropriate primary antibody (α-polyHistidine, Sigma and α-phospho-Stat3(Tyr), Epitomics Inc.). The following day the membranes were washed, incubated for 1 hour with secondary antibody (goat-anti mouse or goat-anti rabbit IgG, Bio Rad) and developed using using ECL (Pierce) on X-ray film (Eastman Kodak Co.)

9. STATISTICAL ANALYSIS

Data from chemotaxis, phagocytosis, nitric oxide and reactive oxygen production assays were analyzed using one-way ANOVA. The quantitative PCR expression studies described in Chapter III were analyzed using repeated

measures 2-way ANOVA. The results of quantitative gene expression analysis described in Chapters IV-IX were assessed using one-way ANOVA. Post hoc testing was done using Tukey's test. Probability level of $P < 0.05$ was considered significant.

Table 2.1. Constituents of GFL-15 medium

CONSTITUENT	AMOUNT PER 2L
Leibovitz's L-15 medium	1 package of dry powder
Delbecco's modified eagle medium	1 package of dry powder

Table 2.2. Constituents of Hank's balanced salt solution (10x)

CONSTITUENT	AMOUNT PER 1L
KCl	4g
KH_2PO_4	0.6g
NaCl	80g
Na_2HPO_4-$7H_2O$	0.9g
D-Glucose	10g
Phenol red	0.1g

Table 2.3. Constituents of Nucleic Acids precursor solution

CONSTITUENT	AMOUNT PER 100mL
Adenosine	0.067g
Cytidine	0.061g
Hypoxanthine	0.034g
Thymidine	0.061g
Uridine	0.061g

Table 2.4. Constituents of MGFL-15 (pH 7.4) medium

CONSTITUENT	AMOUNT PER 2L
Hepes	7g
KH_2PO_4	0.688g
K_2PO_4	0.570g
NaOH	0.750g
$NaHCO_3$	0.340g
L-Glutamine	1000mL
Insulin	0.584g
10xHank's balanced salt solution	0.01g
MEM amino acid solution	80mL
MEM non-essential amino acid solution	25mL
MEM sodium pyruvate solution	25mL
MEM vitamin solution	25mL
Nucleic acid precursor solution	20mL
2-mercaptoethanol solution	20mL
GFL-15 medium	7µL

Table 2.5. List of identified goldfish (*Carassius auratus* L.) immune gene cDNAs

GENE	ACCESSION NO.
CC chemokine (CCL-1)	HM355574
Ceruloplasmin	HM355584
CXCL-8/Intereleukin-8 (CXCL-8/IL-8)	HM355573
Cytochrome b245 alpha polypeptide ($p22^{phox}$)	HM355579
Cytochrome b245 beta polypeptide ($gp91^{phox}$)	HM355580
Indoleamine 2,3-dioxigenase (IDO)	JF441269
Interferon gamma (IFNγ)	EU909368
Interferon gamma related (IFNγrel)	GQ149696
Interferon gamma receptor 1-1 (IFNGR1-1)	GQ149697
Interferon gamma receptor 1-1 (IFNGR1-2)	GQ149698
Interferon regulatory factor 2 (IRF-2)	HM355575
Interferon regulatory factor 5 (IRF-5)	HM355576
Interferon regulatory factor 8 (IRF-8)	HM355577
Interferon regulatory factor 9 (IRF-9)	HM355578
Interleukin-10 (IL-10)	HQ259106
Natural resistance associated macrophage protein (NRAMP)	JF441268
Neutrophil cytosolic factor 1 ($p40^{phox}$)	HM355581
Neutrophil cytosolic factor 2 ($p47^{phox}$)	HM355582
Neutrophil cytosolic factor 4 ($p67^{phox}$)	HM355583
Supressor of cytokine signaling-3 (SOCS-3)	HQ259107
Tumor necrosis factor alpha 1 (TNFα1)	EU069818
Tumor necrosis factor alpha 2 (TNFα2)	EU069817
Tumor necrosis factor receptor 1 (TNF-R1)	FJ905476
Tumor necrosis factor receptor 2 (TNF-R2)	FJ905477

Table 2.6. List of Q-PCR primer sequences

PRIMER	SEQUENCE (5'-3')
CCL-1 forward	AAG GTC ACC GAA CCC ATC AG
CCL-1 reverse	TCG TCA CAT GAT GGC CTT CA
CXCL-8 forward	CTG AGA GTC GAC GCA TTG GAA
CXCL-8 reverse	TGG TGT CTT TAC AGT GTG AGT TTG G
EF-1α forward	CCG TTG AGA TGC ACC ATG AGT
EF-1α reverse	TTG ACA GAC ACG TTC TTC ACG TT
gp91 ^{phox} forward	CCC ATC ACC TGT TCA TCG TCT T
gp91^{phox} reverse	TAG TCT GGC CTC GCA CGA TA
IDO forward	CCA TCT CTG CGC AGC TTT G
IDO reverse	GCG AGC CAC ACA CAG ATC AA
IFNγ forward	GAA ACC CTA TGG GCG ATC AA
IFNγ reverse	GTA GAC ACG CTT CAG CTC AAA CA
IFNγrel forward	TGT CGG AGC CAG ACT TCC A
IFNγrel reverse	GAC TCG ATT TTT TCT CGT ACG TTC T
IFNGR1-1 forward	TTT TAC GAC TGC CCA CAT GCT
IFNGR1-1 reverse	GGG TCC GTA ACT ATC TAC CGT ATC T
IFNGR1-2 forward	CAG TAA CCC AAC TGA ACA GAC GAA
IFNGR1-2 reverse	CAC TGT TTG GGA AGG ACT TTC AT
IL-1β-1 forward	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 reverse	GTG ACA CAT TAA GCG GCT TCA C
IL-1β-2 forward	GAT GCG CTG CTC AGC TTC T
IL-1β-2 reverse	AGT GGG TGC TAC ATT AAC CAT ACG
IL-10 forward	CAA GGA GCT CCG TTC TGC AT
IL-10 reverse	TCG AGT AAT GGT GCC AAG TCA TCA
IL-12 p35 forward	TGT TTT ACG TGC ATT CCT TTG G
IL-12 p35 reverse	GGC GCC TGA AAA AAA TAC GA
IL-12 p40 forward	CTT CAG AAG CAG CTT TGT TGT TG
IL-12 p40 reverse	CAG TTT TTG AGA GCT CACCGA TAT C
iNOS A forward	TTG GTA CAT GGG CAC TGA GAT T
iNOS A reverse	CCA ACC CGC TCA AGA ACA TT
iNOS B forward	CAT CTT CCA TCC GAC CCT AGT G
iNOS B reverse	AAA GCT ACG GAA GGG AGC AAT
NRAMP forward	TCT GCC CTG CTC TCC ATC AC
NRAMP reverse	GAC GCC CAG AGC GAT CAG
p22 ^{phox} forward	TGG ACC CCT GAC CAG AAA CT
p22 ^{phox} reverse	AAC ATG AAC CCC CCT GGA A
p40 ^{phox} forward	TCC AAG AGC GGG AAT CAT G
p40 ^{phox} reverse	GTC GAT GCC CTC TGG CTG TA
p47 ^{phox} forward	CCA GGA ATG GGA CAC GAT CT
p47 ^{phox} reverse	GAG GAG AGC CTG AGT TTG CAA
p67 ^{phox} forward	TGC CTG GCA ACA TTG TCT TC
p67 ^{phox} reverse	CCC GCT TCT CAT TGA AAA CAA
SOCS-3 forward	CGA GTC GGG CAC CAA GAA

SOCS-3 reverse	AAG CTC TGG AGT CCG TCT GAA
TGFβ forward	GTA CAC TAC GGC GGA GGA TTG
TGFβ reverse	CGC TTC GAT TCG CTT TCT CT
TLR3 forward	GGA TCC ATG GTG TAG GCA ATT C
TLR3 reverse	ATC AGG GAG TCACGA TTG TTC TC
TNFα1 forward	CAT TCC TAC GGA TGG CAT TTA CTT
TNFα1 reverse	CCT CAG GAA TGT CAG TCT TGC AT
TNFα2 forward	TCA TTC CTT ACG ACG GCA TTT
TNFα2 reverse	CAG TCA CGT CAG CCT TGC AG
TNFR1 forward	GCC CCC TGA CTC AAA AGA AAT
TNFR1 reverse	GCC AGC AAC GTC AGG AAA
TNFR2 forward	CCA AAA CAA CCG CGT GAA T
TNFR2 reverse	CA GAGA TGT GGT GAA GGT CGT ATC
Viperin (RSAD-2) forward	CTT GGA CAT TCT GGC AGT ATC TTG
Viperin (RSAD-2) reverse	TTG CCC TGACCT CGA CCT AT

Table 2.7. Recombinant goldfish protein production conditions

PROTEIN	INDUCTION TIME	LYSIS CONDITIONS
rgIFNγ	4h	denaturing
rgIFNγrel	2h	non-denaturing
rgIFNGR1-1	2h	non-denaturing
rgIFNGR1-2	2h	non-denaturing
rgIL-10	2h	non-denaturing
rgTNFα1	4h	non-denaturing
rgTNFα2	4h	denaturing
rgTNF-R1	2h	non-denaturing
rgTNF-R2	2h	non-denaturing

Table 2.8. Recombinant protein-primed reactive oxygen production

Treatment	Absorbance 630 nm (mean ± SEM)
PMA	0.033 ± 0.008
rgTNFα2	0.115 ± 0.014*
rgIFNγ	0.101 ± 0.022*
rgIL-10	0.036 ± 0.016
vector control	0.034 ± 0.016

Monocyte cultures from five individual fish (*n*=5) were incubated for 16 hours with medium only, 100 ng/mL of rgTNFα2, rgIFNγ, rgIL-10 or vector control (volumes equivalent to those used for 100 ng/mL rgTNFα2). The ROI response was induced with PMA (100 ng/mL). Statistical analysis was performed using one-way ANOVA and the results were considered significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the PMA only treated controls.

10. REFERENCES

1. **Barreda, D. R., N. F. Neumann, and M. Belosevic.** 2000. Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Developmental and comparative immunology **24:**395-406.
2. **Garcia-Garcia, E., and C. Rosales.** 2007. Nuclear factor activation by FcgammaR in human peripheral blood neutrophils detected by a novel flow cytometry-based method. Journal of immunological methods **320:**104-118.
3. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of novel interferon gamma receptor 1 isoforms in zebrafish (Danio rerio) and goldfish (Carassius auratus L.). Molecular immunology **46:**3050-3059.
4. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of tumor necrosis factor receptors 1 and 2 of the goldfish (Carassius auratus L.). Molecular immunology **46:**2190-2199.
5. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization, expression and functional analysis of goldfish (Carassius auratus L.) interferon gamma. Dev Comp Immunol **33:**235-246.
6. **Grayfer, L., E. G. Garcia, and M. Belosevic.** Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (Carassius auratus L.). J Biol Chem **285:**23537-23547.
7. **Grayfer, L., J. W. Hodgkinson, S. J. Hitchen, and M. Belosevic.** Characterization and functional analysis of goldfish (Carassius auratus L.) interleukin-10. Molecular immunology **48:**563-571.
8. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Dev Comp Immunol **32:**532-543.
9. **Haddad, G., P. C. Hanington, E. C. Wilson, L. Grayfer, and M. Belosevic.** 2008. Molecular and functional characterization of goldfish (Carassius auratus L.) transforming growth factor beta. Developmental and comparative immunology **32:**654-663.
10. **Neumann, N. F., D. R. Barreda, and M. Belosevic.** 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. Fish & shellfish immunology **10:**1-20.
11. **Neumann, N. F., D. Fagan, and M. Belosevic.** 1995. Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. Dev Comp Immunol **19:**473-482.
12. **Wang, R., and M. Belosevic.** 1995. The in vitro effects of estradiol and cortisol on the function of a long-term goldfish macrophage cell line. Developmental and comparative immunology **19:**327-336.

CHAPTER III: CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF THE GOLDFISH (*Carassius auratus* L.) TUMOR NECROSIS FACTOR-ALPHA[1]

1. INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a pleiotropic pro-inflammatory cytokine, produced by several cell types including macrophages, monocytes, polymorphonuclear leukocytes, mast cells and smooth muscle cells (5, 24, 25). This cytokine has been demonstrated to up-regulate the expression of other inflammatory cytokines and enhance a variety of cellular responses including phagocytosis, chemotaxis and generation of reactive oxygen and nitrogen intermediates. The diverse biological activities of TNFα as a type II trans-membrane protein, and also as a soluble peptide produced following cleavage by metalloproteinase have been reported (8, 17).

TNFα has been identified in different bony fishes including Japanese flounder (10), trout (28), carp (20, 21), catfish (27), sea bream (6, 7) and turbot (16). Certain teleosts such as carp and trout have multiple isoforms of this cytokine and share a high degree of sequence identity but differ in expression patterns (20, 21). Relatively high constitutive expression of TNFα in the tissues of healthy fish has been reported (7, 21, 27). Similar to mammals, an increase in

[1] A version of this chapter has been published: Grayfer, L., J. G. Walsh, and M. Belosevic. 2008. Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. Developmental and Comparative Immunology 32:532-543.

TNFα mRNA was also observed after treatment of fish leukocytes with LPS or with phorbol esters (17, 26).

The recombinant turbot TNFα has been shown to enhance the expression of IL-1β, IL-8 and TNFα (16), while a trout TNFα induced leukocyte migration and phagocytosis (28). Similarly, the recombinant TNFα from the gilthead sea bream increased phagocyte mobilization and primed their respiratory burst responses (6). In contrast, turbot recombinant TNFα failed to trigger a substantial respiratory burst response but did induce significant nitric oxide production of phagocytes (16). The addition of pentoxifylline (PTX), a TNFα inhibitor, was also shown to abrogate the nitric oxide response of carp phagocytes induced by LPS (17), suggesting a possible regulatory role for TNFα in teleost antimicrobial responses.

In this chapter, I report on the molecular characterization of two goldfish TNFα isoforms and functional analysis of goldfish TNFα2. The quantitative PCR analysis indicated that the two isoforms of this pro-inflammatory cytokine were differentially expressed in tissues and sorted kidney-derived macrophages of the goldfish. The recombinant goldfish TNFα2 induced a dose-dependent chemotaxis, phagocytosis, respiratory burst and nitric oxide responses of kidney-derived monocytes and macrophages.

2. RESULTS

2.1. Goldfish TNFα1 and TNFα2 *in silico* analysis

The complete open reading frame (ORF) of goldfish TNFα2 (Acc. No.: **EU069817**) and a partial ORF of goldfish TNFα1 (Acc. No.: **EU069818**) were obtained. Repeated RACE attempts to identify the full ORF for TNFα1 were not successful, and only the portion of the sequence representing the cleaved active fragment of TNFα1 was identified.

A search of goldfish TNFα2 against the NCBI GenBank database identified a carp TNFα2 sequence as having the highest blast score. Likewise goldfish TNFα1 shared the greatest similarity with carp TNFα1. Goldfish TNFα1 was 75% identical to carp TNFα1 and goldfish TNFα2 was 87% identical to carp TNFα2. Lower per cent identities were observed between goldfish TNFα1 and TNFα2 and other TNFs and were as follows: 51% and 54% (channel catfish), 42% and 38% (Japanese flounder), 25% and 28% (mouse) for TNFα1 and TNFα2, respectively.

The goldfish TNFα2 ORF was 687 base pairs long and encoded a 228 amino acid protein with a predicted size of approximately 25 kD. The translated goldfish sequence contained all the basic elements of the TNF gene family characteristic of fish and higher vertebrate TNFs. These included a trans-membrane domain, the TNF family signature [VL]-x-[LIVM]-x3- G-[LIVMF]-Y-[LIVMFY]2-X2-[QEKHL] (with the exception of I instead of V or L at position 1) and two conserved cysteine residues that are known to influence TNFα tertiary structure (19) (Fig. 3.1). As in several other fish TNF species, the goldfish

TNFα2 also contained the Thr-Leu motif (Fig. 3.1), which was demonstrated to be the TACE metalloproteinase cleavage site for the release of the mature soluble mouse TNFα (16). The amino acid alignment of the goldfish TNFα1 and TNFα2 with those of other fish and higher vertebrate TNF species indicated that goldfish and carp TNF isoforms shared a high degree of identity (Fig. 3.1). a

Phylogenetic analysis of both goldfish TNFα-1 and TNFα-2 and TNFs from other fish and higher vertebrate species confirmed the close relationship between the goldfish and carp TNFα isoforms (Fig. 3.2). Goldfish TNFα-1 was more closely related to the carp TNFα1 than to goldfish TNFα2, and goldfish TNFα2 grouped closer to the carp TNFα2 and carp TNFα3. The salmoniforme and the perciforme TNF species were most closely related to each other and branched separately from those of the cypriniforme species (including goldfish) (Fig. 3.2). All fish TNFα species branched separately from TNFα and TNFβ of the higher vertebrate species (Fig. 3.2).

2.2. Quantitative gene expression analysis of TNFα1 and TNFα2 in goldfish tissues

Specific primers designed against the most divergent portions of both the TNFα1 and TNFα2 sequences were used to determine the expression of these two isoforms. Statistical analysis by repeated measures ANOVA indicated a significant difference in expression between the two isoforms in tissues (Fig. 3.3). In the gill and intestine, TNFα2 mRNA levels were significantly higher ($P < 0.05$) than those of TNFα1 (Fig. 3.3). The mRNA levels of the two goldfish TNFα

isoforms were consistently higher, but not significantly different in kidney, spleen, brain, gill and heart, compared to those in muscle and intestine (Fig. 3.3).

2.3. Quantitative gene expression analysis of TNFα1 and TNFα2 in activated kidney-derived goldfish macrophage subpopulations

Constitutive expression levels of the two goldfish TNF isoforms were similar in non-stimulated sorted monocytes and macrophages. However, when mature macrophages were activated with MAF, the mRNA levels of goldfish TNFα1 and TNFα2 were significantly ($P < 0.05$) higher than those in non-activated macrophages (Fig. 3.4). The expression of TNFα1 and TNFα2 was also higher in MAF-treated monocytes, but this increase in expression after activation was not statistically significant (Fig. 3.4).

2.4. Recombinant goldfish TNFα2 induced chemotaxis of macrophages

A recombinant goldfish tumor necrosis factor alpha 2 (rgTNFα2) was produced for the purpose of functional characterization. This rgTNFα2 induced a chemotactic response in macrophages in a dose-dependent manner (Fig. 3.5). The highest chemotactic response was observed at a concentration of 70 ng/mL of rgTNFα2. This response was 4 times higher than that of the negative control (medium alone), and similar to that induced by LPS (positive control). As expected, chemotaxis was abrogated at higher doses of rgTNFα2 (Fig. 3.5), where 7 μg/mL of the cytokine down-regulated the chemotactic response of macrophages.

To confirm that the observed migration was gradient dependent and not a result of chemokinesis (non-directional migration), equal amounts of rgTNFα2 or LPS (70 ng/mL and 33 ng/mL, respectively) were added to both lower and upper wells of the chemotaxis apparatus. This eliminated the presence of gradients such that any observed migration would be due to chemokinesis. As shown in Figure 3.5, rgTNFα2- or LPS-induced migration was not significantly different ($P > 0.05$) compared to the medium control.

2.5. Recombinant goldfish TNFα2 enhances monocyte phagocytosis

The potential of the rgTNFα2 to enhance the goldfish monocyte phagocytic response was investigated using a flow cytometry based assay described by Li *et al.* (17) which was modified as follows. The assay was optimized to measure an enhanced phagocytic response by determining the number of cells ingesting 3 or more fluorescent beads. MAF was used as a positive control in these assays. Figure 3.6A depicts representative histograms obtained using cells from a single fish showing the relative fluorescence profiles following treatment with medium, MAF or different amounts of rgTNFα2. After treatment with MAF, compared to medium (negative control), a shift in fluorescence intensity corresponding to an enhanced phagocytic response was observed (Fig. 3.6A). A progressively greater shift in fluorescence intensity was also observed after addition of increasing amounts of rgTNFα2 (Fig. 3.6A). Figure 3.6B represents a combined phagocytosis response for kidney-derived monocytes obtained from 6 different fish (mean ± SEM), showing that rgTNFα2

elicited a significantly enhanced phagocytic response in all primary monocyte cultures (Fig. 3.6B).

2.6. Recombinant goldfish TNFα2 induces macrophage nitric oxide responses

Recombinant goldfish TNFα2 was tested for its ability to induce nitric oxide response in goldfish macrophages. Cell cultures obtained from individual fish were treated with different amounts of rgTNFα2 or with heat-killed *Aeromonas salmonicida* (positive control), and nitric oxide response determined at 48 and 72 hours after stimulation. The rgTNFα2 induced the nitric response in a concentration dependent manner where 25 and 250 ng/mL induced significant ($P < 0.05$, repeated measures one-way ANOVA) nitrite production compared to medium treated cells (Fig. 3.7).

2.7. Recombinant goldfish TNFα2 induces monocyte reactive oxygen responses

A flow cytometry assay described previously (15) was employed to investigate the ability of rgTNFα2 to prime kidney-derived monocyte/macrophage cultures for ROI response. Shown in Figure 3.8A are representative fluorescence histograms for non-triggered cells (medium alone) and those treated with medium + PMA, MAF + PMA or rgTNFα2 (0.25, 25 or 2500 ng/ml) + PMA. There was an increase (shift) in fluorescence intensity between medium alone and medium + PMA treated cells, indicating that the cells

in these cultures were triggered to produce ROI (Fig. 3.8B). When the monocyte/macrophage cultures were treated with MAF + PMA or rTNFA2 + PMA there was a further shift in fluorescence intensity indicating a further increase in ROI production. The area with the highest fluorescence intensity ($>10^3$ fluorescence units) was gated using FACS Calibur Cell Quest settings and used for further analysis of the subpopulations of macrophages that were responsible for the maximal ROI response (Fig. 3.8A).

Goldfish kidney-derived macrophage cultures are complex, consisting of three major cell subpopulations, which we have characterized as the progenitor cells (R1-gate), the monocytes (R3-gate) and mature macrophages (R2-gate) (1, 15). Whole cultures were analyzed using fluorescence intensity for ROI production (Fig. 3.8A) and the cell subpopulations that were responsible for the ROI responses were determined by "back-gating" from the fluorescence histograms onto size and internal complexity scatter plots the cells emitting increased fluorescence (Fig. 3.8B). The cells treated with medium alone (negative control) displayed little fluorescence and correspondingly very few cells were seen in the three subpopulation flow cytometer gates (Fig. 3.8A,B). In contrast, the scatter plot corresponding to MAF (+ triggered by PMA) showed substantially more monocytes in the R3-gate (Fig. 3.8B) in line with the increased response seen in the MAF fluorescence histogram (Fig. 3.8A). Similarly, with increasing amount of rgTNFα2 added to the cultures and then triggered by PMA, progressively more monocytes producing ROI were observed, as judged by the increasing shifts in fluorescence and increased number of cells in the R3-gate

(Fig. 3.8A,B). Taken together, my results indicate that the monocyte subpopulation was responsible for maximal ROI response following rgTNFα2 treatment.

Using the optimized DHR assay I then examined the induction of ROI in kidney-derived macrophages obtained from different fish (n=5). The cultures were treated with different amounts of rgTNFα2 for 3 hours, which caused a significant dose-dependent increase of the PMA-triggered (mean ± SEM) ROI response compared to the PMA alone treatment group (Fig. 3.9). Priming with lower amounts of rgTNFα2 (0.25ng/mL) did not elicit a significant ROI response (Fig. 3.9).

An NBT based ROI assay was employed to determine the time course of the rTNFα2- mediated priming of the ROI response. When the priming agent used was MAF, the ROI response peaked at 12 hours (Fig. 3.10). However, the priming effect although not as robust as that observed for MAF, was of longer duration (as much as 48 hours longer) when cell cultures were treated with rgTNFα2 (Fig. 3.10).

3. DISCUSSION

Two isoforms of TNFα were identified in the goldfish. The goldfish TNFα isoforms were shared high degree of identity with the carp TNFα1 and TNFα2. The complete open reading frame of goldfish TNFα2 was identified and the predicted amino acid sequence shared hallmark features with other previously described fish and mammalian TNFs (7, 10, 21).

The constitutive tissue expression of TNFα in bony fish has been shown to be highly variable. When the first TNF gene was identified in the Japanese flounder, little or no constitutive tissue expression was reported (10). However, more recent TNFα expression studies in carp, catfish, sea bream and sea bass reported significant constitutive expression in different tissues (6, 14, 21, 27). In carp, a broad pattern of tissue expression was attributed to only one isoform (TNFα3), while the other isoforms (TNFα1 and TNFα2) were constitutively expressed only in the gill (21). The results of my expression analysis were different from that reported in carp, since I observed high constitutive expression of the goldfish TNFα1 and TNFα2 in most tissues examined. This discrepancy could be due to different assays used (RT-PCR versus quantitative PCR).

Mononuclear phagocytes of mammals have been identified as the primary producers of TNFα (12) and my results and those of others suggested that the same was true in fish (10, 16, 21). Although higher mRNA levels of both goldfish TNFα isoforms were observed in MAF-activated mature macrophages, compared to monocytes, the rgTNFα2-mediated effects appear to be segregated. Monocytes appeared to be major producers of rgTNFα2-induced ROI as shown in this study. In contrast, goldfish mature macrophages produced nitrogen intermediates in response to different stimuli (9, 22). It has been reported that mammalian TNFα isoforms were differentially induced by LPS or GM-CSF in monocytes compared to the monocyte-derived macrophages (2). These mammalian isoforms are distinct from those reported in fish, since they are generated by post-transcriptional/ translational modifications (2) rather than by

being encoded by different genes as in teleosts (21, 28). Presumably the role of different TNFα isoforms in mammals is to finely tune the pro-inflammatory responses, and the fish TNFα isoforms being encoded by separate genes may have similar role.

Goldfish TNFα1 and TNFα2 isoforms were up regulated in response to MAF. In trout, multiple TNFα isoforms exhibited differential expression in stimulated macrophages (28). The trout TNFα1 was only expressed at very low levels in stimulated trout macrophages compared to the TNFα2 (28). In addition, the recombinant trout TNFα1 did not upregulate native trout TNFα1, while recombinant TNFα2 induced increased expression of both native TNFα isoforms (28). Given the evolutionary distances between different teleost groups, it is likely that TNF isoforms among teleosts may be regulated by and are differentially responsive to various stimuli. Since MAF supernatants contain a plethora of potential immunomodulating agents, the differences in expression profiles of the two goldfish TNFα isoforms after activation monocyte/macrophage and their role in the regulation of inflammation remain to be elucidated.

TNFα has been thoroughly characterized in mammals and is one of the central regulatory as well as effector cytokines in inflammation. Mammalian TNFα has been shown to promote macrophage migration, phagocytosis and production of reactive oxygen and nitrogen intermediates. It is clear from our results and those of others (10, 21, 28) that the pro-inflammatory roles of TNFα have been evolutionarily conserved in teleosts. For example, it has been reported

that trout recombinant TNFα induced a gradient dependent migration response of head kidney leukocytes (26). Similarly, my results indicate that rgTNFα2 induced a chemotactic response of goldfish kidney-derived macrophages. It has been established that the mammalian TNFα does not induce a direct chemotactic effect; instead it promotes chemotaxis by inducing production of chemokines by various cell types (3, 13). In contrast, in both trout and goldfish, rgTNFα was chemotactic to kidney-derived macrophages in a dose-dependent manner. The elucidation of precise mechanisms of teleost TNFα-mediated chemotaxis, and whether they differ from those of mammals, must await further understanding of TNFα/TNFα receptor interactions in teleosts.

It was demonstrated in this study and elsewhere (26) that recombinant teleost TNFα induced an enhanced phagocytic response of kidney-derived phagocytes. I employed a flow cytometry-based phagocytosis assays that allowed the examination of TNFα-induced phagocytosis at both cell subpopulation and individual cell level. My results showed that the rgTNFα2 induced phagocytosis of kidney-derived macrophages.

Mammalian TNFα has been shown to induce reactive oxygen production by neutrophils including production of super oxide anions (23) as well as hydrogen peroxide (11). Clearly, TNFα is one of the central cytokines responsible for induction of macrophage antimicrobial responses in higher vertebrates (4, 18). Like mammalian TNFα, goldfish rTNFα2 also induced dose-dependent production of reactive oxygen and nitrogen intermediates in kidney-derived macrophages.

My results indicate that the goldfish monocyte subpopulation was selectively induced to generate an ROI response by either MAF or rgTNFα2, whereas the mature macrophages were induced by MAF (12, 19) and rgTNFα2 to generate nitrogen intermediates. The elaboration of segregated antimicrobial functions in monocytes and mature macrophages previously observed in the goldfish (12, 19) and in this study, may be similar to those observed in higher vertebrates where activated human monocytes have been reported to produce copious amounts of reactive oxygen intermediates, whereas monocyte-derived macrophages upon further differentiation acquired the capacity to produce reactive nitrogen intermediates upon activation (26).

The findings of this study indicate that TNFα plays a central role in the inflammatory response of lower vertebrate species such as bony fish. The goldfish version of this cytokine is a potent inducer of several hallmark inflammatory response pathways and as such is an important tool for further delineation of the regulatory mechanisms of inflammation in teleosts.

Figure 3.1. Protein sequence alignment of goldfish TNFα1 and TNFα2 with sequences from other fish and higher vertebrate species. Asterisks (*) indicate fully conserved residues, "." and ":" indicate partially conserved and semi-conserved substitutions, respectively. Arrows denote conserved cysteines important for TNFα structure. The putative TACE cleavage site is underlined and the TNFα family signature is identified by a line above it. The trans membrane domain of TNFα2 is indicated by a

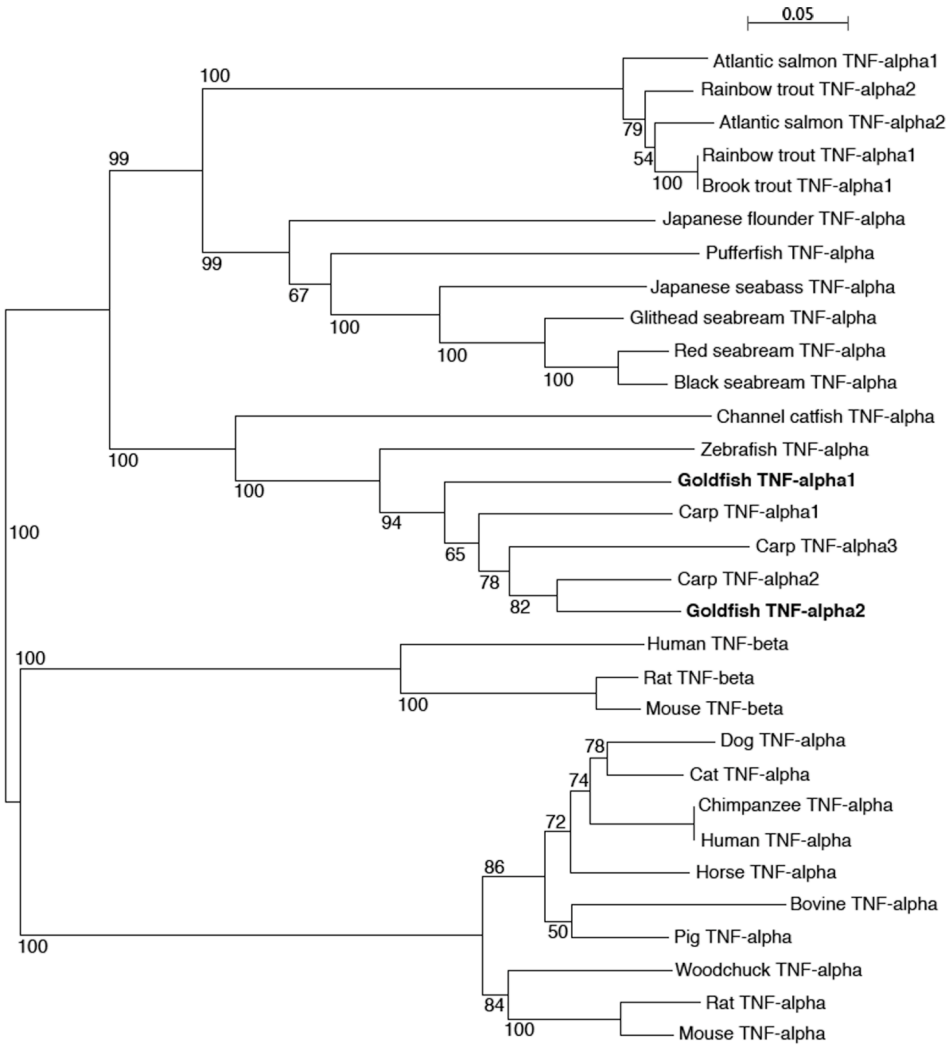

Figure 3.2. Phylogenetic analysis of goldfish TNFα1 and TNFα2 in relation to other fish and mammalian TNFα and TNFβ protein sequences. The analysis was conducted using the neighbor joining method and was bootstrapped 10,000 times.

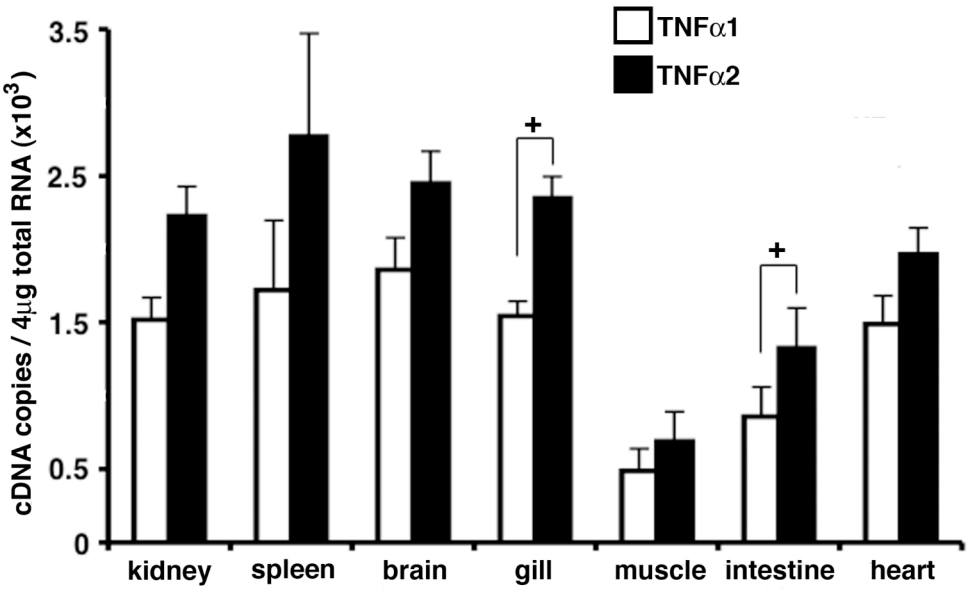

Figure 3.3. Quantitative gene expression analysis (Absolute real-time PCR) of TNFα1 and TNFα2 in goldfish tissues. Tissues from 5 fish were used (n=5). Asterisks (+) indicate statistical difference ($P < 0.05$), repeated measures 2-way ANOVA).

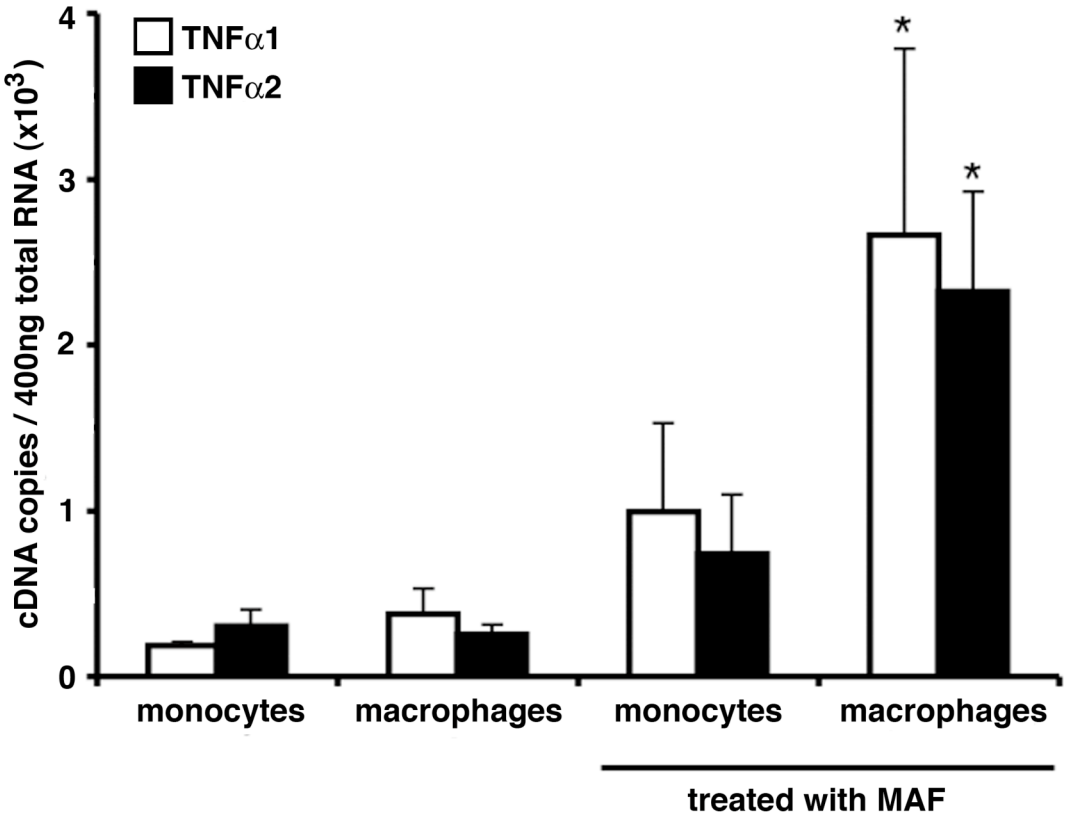

Figure 3.4. Quantitative gene expression analysis (Absolute real-time PCR) of TNFα1 and TNFα2 in goldfish kidney-derived monocytes and macrophages. Monocytes and macrophages were obtained from individual cultures established from 5 fish (n=5) by sorting using FACS Calibur flow cytometer. Results were expressed as mean ± SEM Asterisks denotes statistical significance in expressions of both isoforms from those in other treatment groups ($P < 0.05$, repeated measures 2-way ANOVA).

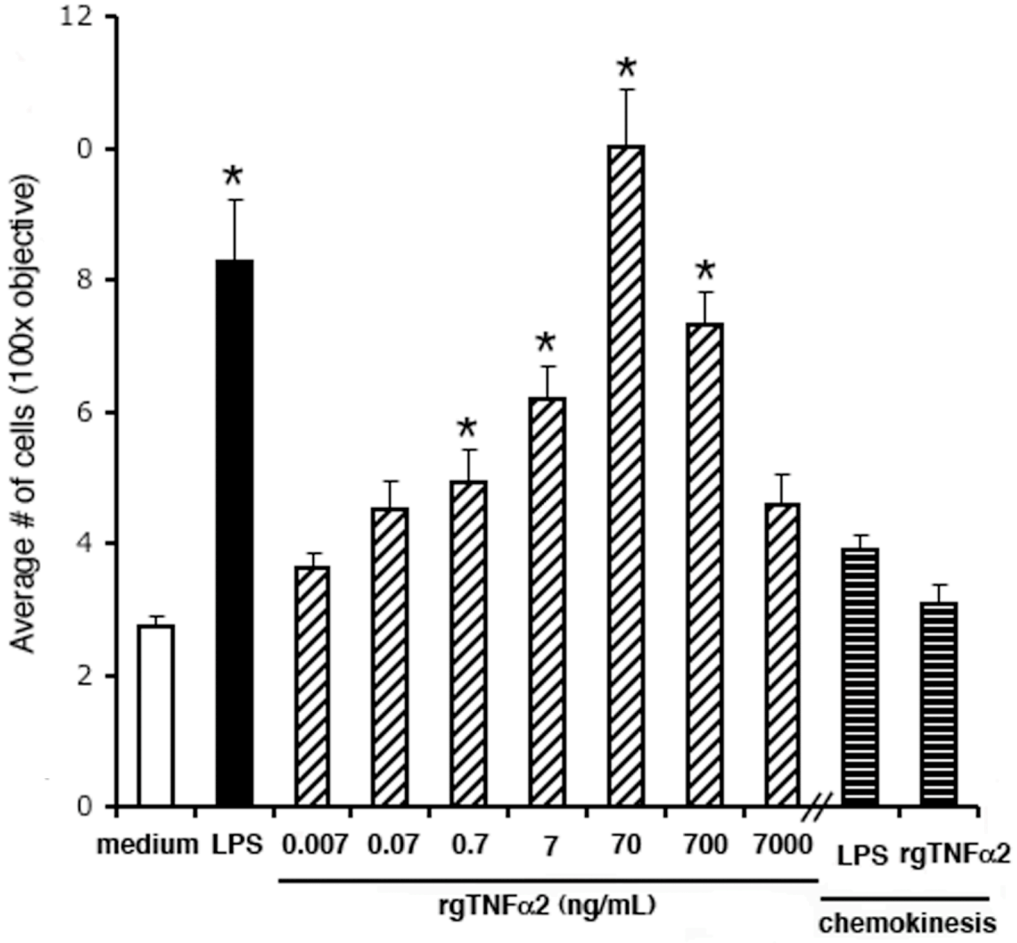

Figure 3.5. Chemotactic response of goldfish macrophages induced by recombinant goldfish TNFα2 (rgTNFα2). Chemotactic activity was determined as the mean ± SEM number of cells in twenty randomly selected fields of view under oil immersion (1,000X magnification). Kidney-derived macrophage cultures were established from individual fish (n =6). Asterisks denote significant differences when compared to medium control ($P < 0.05$, one-way ANOVA and Tukey's test).

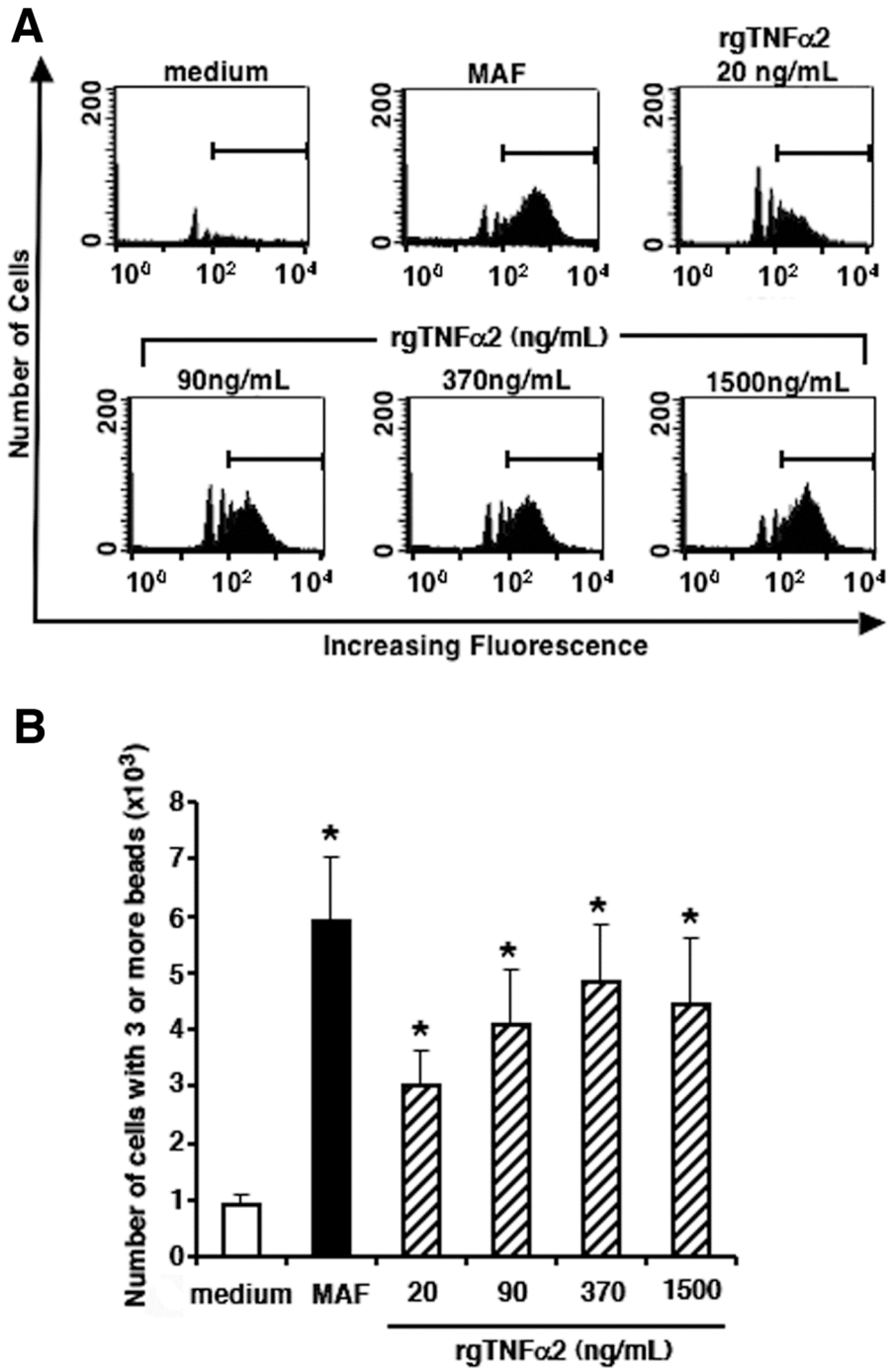

Figure 3.6. Phagocytic response by goldfish monocytes induced by recombinant goldfish TNFα2 (rgTNFα2). (A) Representative histogram plots of the phagocytic response of treated monocytes isolated from a single fish. **(B)** Combined data for cells isolated from 6 different fish mean ± SEM (n=6). Asterisks denote significantly different from medium control ($P < 0.05$, one-way ANOVA).

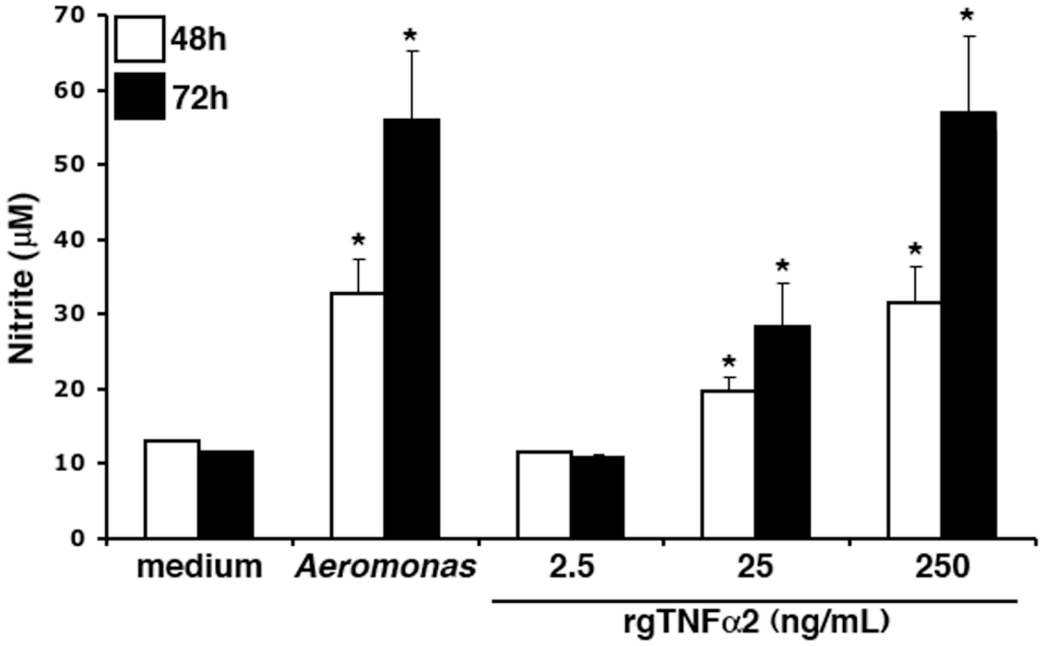

Figure 3.7. Nitric oxide responses of goldfish macrophages induced by recombinant goldfish TNFα2 (rgTNFα2). Kidney-derived macrophage cultures were established from individual fish and nitric oxide determined using the Griess reaction. The results are presented as mean ± SEM (n = 6). Asterisks denote significantly different from medium control ($P < 0.05$, repeated measures one-way ANOVA).

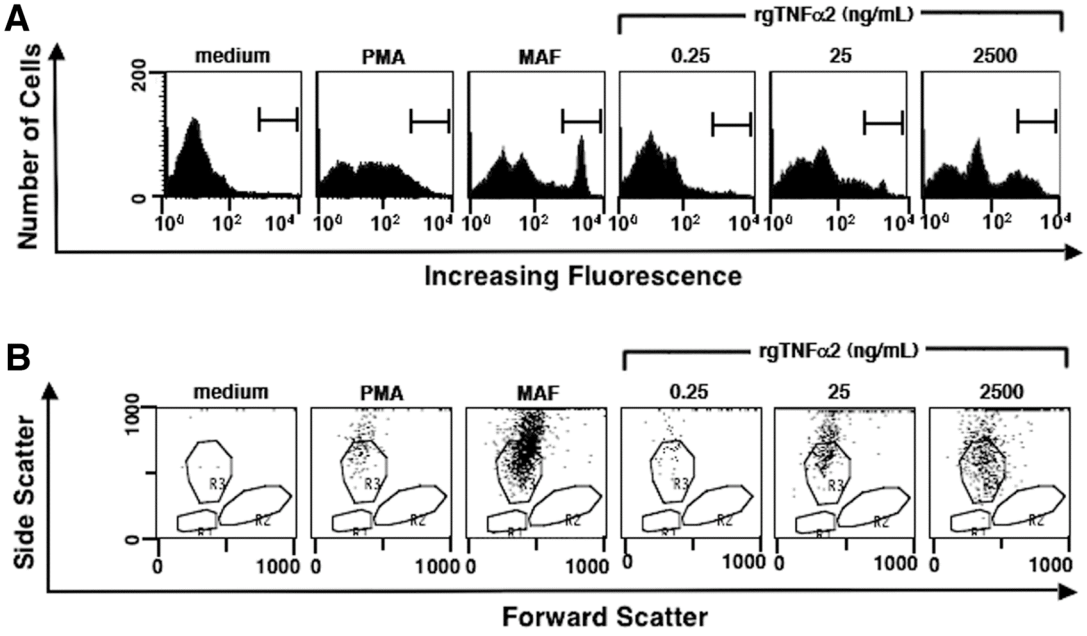

Figure 3.8. The induction of reactive oxygen intermediates by goldfish kidney-derived monocytes and macrophages by recombinant goldfish TNFα2 (rgTNFα-2). The ROI response was measured using DHR assay, FACS Calibur and Cell Quest software. (a) Fluorescence histograms of cells labeled with DHR and treated with medium alone, medium + PMA, MAF + PMA, or rTNFα-2 (0.25, 25 or 2500ng/mL) + PMA. (b) Corresponding size and internal complexity parameter scatter plots of cells identified as ROI producers. R1- progenitor cell subpopulation gate; R2- mature macrophage gate; R3- monocyte gate.

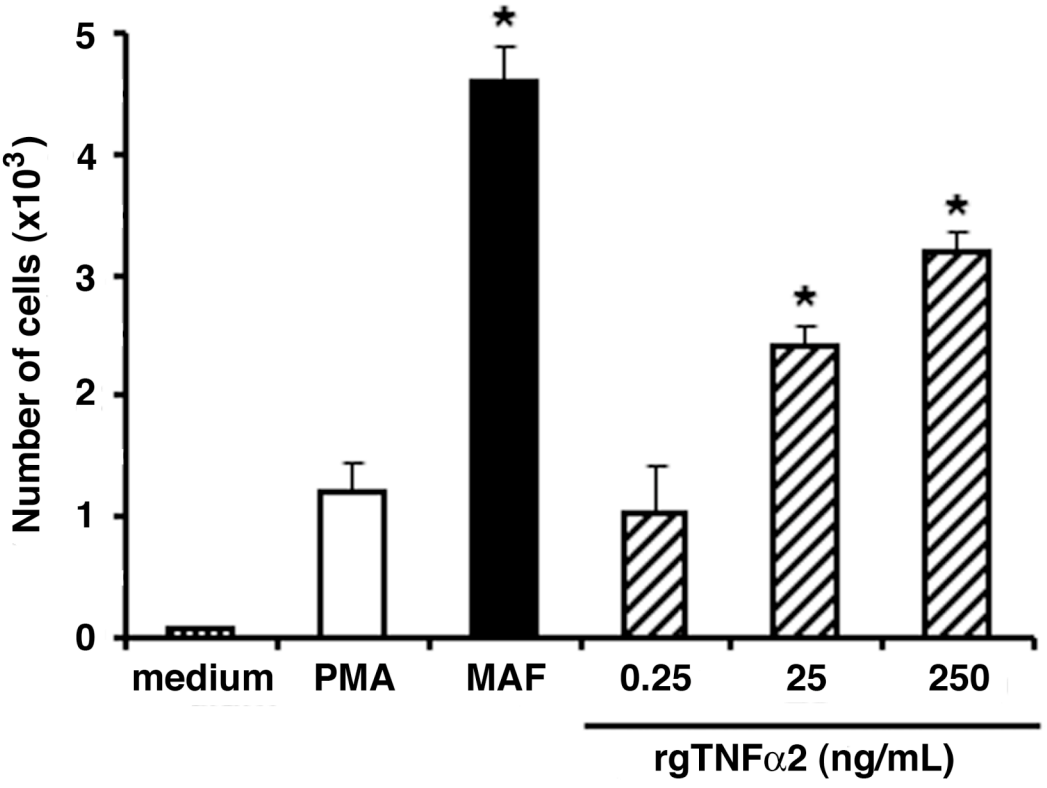

Figure 3.9. The priming of respiratory burst responses in goldfish kidney-derived monocytes treated with different concentrations of rgTNFα2. Macrophage cultures were established from individual fish. Results are presented as mean ± SEM (n= 5) number of cells exhibiting enhanced priming of the respiratory burst response following treatment with different doses of rTNFα-2. Asterisks (*) denote statistical difference between experimental groups compared to medium + PMA group ($P < 0.05$, one-way ANOVA).

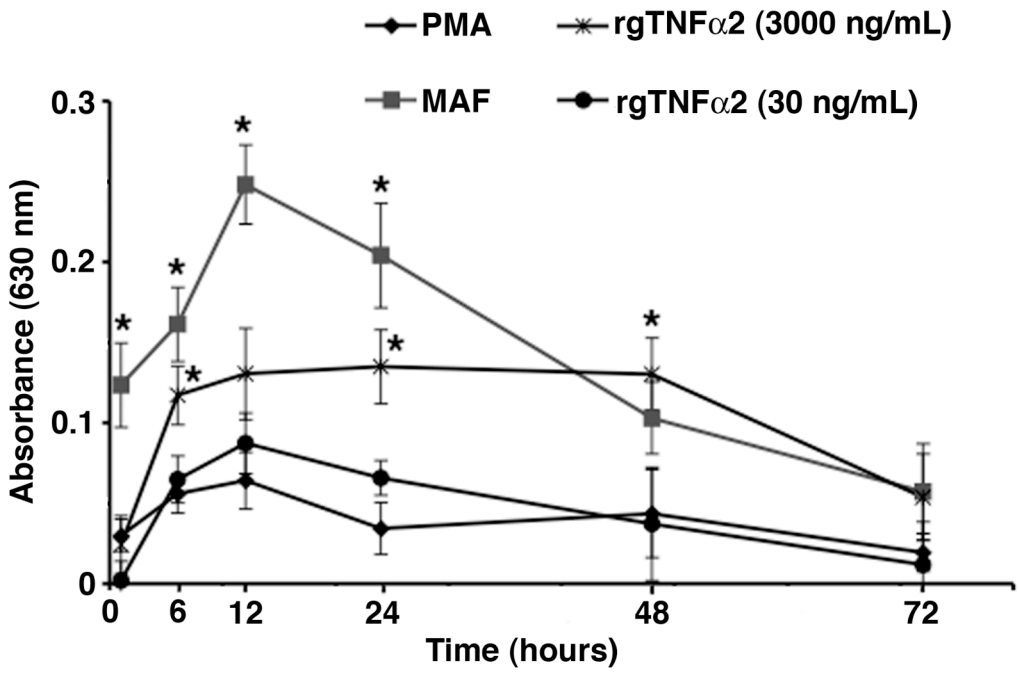

Figure 3.10. Time course of the respiratory burst response in goldfish kidney derived monocytes treated with rgTNFα2. ROI production was assessed using the NBT reduction assay. Results are presented as mean ± SEM (n=5). The values obtained from cells alone group were subtracted from those from experimental groups to factor in background NBT reduction. Asterisks denote significant differences from medium + PMA treated cells ($P < 0.05$, one-way ANOVA).

4. REFERENCES

1. **Belosevic, M., P. C. Hanington, and D. R. Barreda.** 2006. Development of goldfish macrophages in vitro. Fish & shellfish immunology **20:**152-171.
2. **Branch, D. R., and L. J. Guilbert.** 1996. Differential expression of tumor necrosis factor-alpha isoforms from lipopolysaccharide- and cytokine-stimulated mouse macrophages. The international journal of biochemistry & cell biology **28:**949-955.
3. **Chen, M. C., P. Keshavan, G. D. Gregory, and D. J. Klumpp.** 2007. RANTES mediates TNF-dependent lamina propria mast cell accumulation and barrier dysfunction in neurogenic cystitis. American journal of physiology **292:**F1372-1379.
4. **De Titto, E. H., J. R. Catterall, and J. S. Remington.** 1986. Activity of recombinant tumor necrosis factor on Toxoplasma gondii and Trypanosoma cruzi. J Immunol **137:**1342-1345.
5. **Dubravec, D. B., D. R. Spriggs, J. A. Mannick, and M. L. Rodrick.** 1990. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. Proceedings of the National Academy of Sciences of the United States of America **87:**6758-6761.
6. **Garcia-Castillo, J., E. Chaves-Pozo, P. Olivares, P. Pelegrin, J. Meseguer, and V. Mulero.** 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci **61:**1331-1340.
7. **Garcia-Castillo, J., P. Pelegrin, V. Mulero, and J. Meseguer.** 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics **54:**200-207.
8. **Goetz, F. W., J. V. Planas, and S. MacKenzie.** 2004. Tumor necrosis factors. Developmental and comparative immunology **28:**487-497.
9. **Hanington, P. C., and M. Belosevic.** 2007. Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (Carassius auratus L.) macrophages. Developmental and comparative immunology **31:**817-829.
10. **Hirono, I., B. H. Nam, T. Kurobe, and T. Aoki.** 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder Paralychthys olivaceus. J Immunol **165:**4423-4427.
11. **Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph.** 1986. Stimulation of neutrophils by tumor necrosis factor. J Immunol **136:**4220-4225.
12. **Li, J., R. Peters, S. Lapatra, M. Vazzana, and J. O. Sunyer.** 2004. Anaphylatoxin-like molecules generated during complement activation induce a dramatic enhancement of particle uptake in rainbow trout phagocytes. Developmental and comparative immunology **28:**1005-1021.

13. **Lin, S. K., S. H. Kok, C. T. Shun, C. Y. Hong, C. C. Wang, M. C. Hsu, and C. M. Liu.** 2007. Tumor necrosis factor-alpha stimulates the expression of C-C chemokine ligand 2 gene in fibroblasts from the human nasal polyp through the pathways of mitogen-activated protein kinase. American journal of rhinology **21:**251-255.
14. **Nascimento, D. S., P. J. Pereira, M. I. Reis, A. do Vale, J. Zou, M. T. Silva, C. J. Secombes, and N. M. dos Santos.** 2007. Molecular cloning and expression analysis of sea bass (Dicentrarchus labrax L.) tumor necrosis factor-alpha (TNF-alpha). Fish & shellfish immunology **23:**701-710.
15. **Neumann, N. F., D. R. Barreda, and M. Belosevic.** 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. Fish & shellfish immunology **10:**1-20.
16. **Ordas, M. C., M. M. Costa, F. J. Roca, G. Lopez-Castejon, V. Mulero, J. Meseguer, A. Figueras, and B. Novoa.** 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Molecular immunology **44:**389-400.
17. **Pfeffer, K.** 2003. Biological functions of tumor necrosis factor cytokines and their receptors. Cytokine & growth factor reviews **14:**185-191.
18. **Philip, R., and L. B. Epstein.** 1986. Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature **323:**86-89.
19. **Rink, L., and H. Kirchner.** 1996. Recent progress in the tumor necrosis factor-alpha field. International archives of allergy and immunology **111:**199-209.
20. **Saeij, J. P., R. J. Stet, B. J. de Vries, W. B. van Muiswinkel, and G. F. Wiegertjes.** 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Developmental and comparative immunology **27:**29-41.
21. **Savan, R., and M. Sakai.** 2004. Presence of multiple isoforms of TNF alpha in carp (Cyprinus carpio L.): genomic and expression analysis. Fish & shellfish immunology **17:**87-94.
22. **Stafford, J. L., E. C. Wilson, and M. Belosevic.** 2004. Recombinant transferrin induces nitric oxide response in goldfish and murine macrophages. Fish & shellfish immunology **17:**171-185.
23. **Tsujimoto, M., S. Yokota, J. Vilcek, and G. Weissmann.** 1986. Tumor necrosis factor provokes superoxide anion generation from neutrophils. Biochemical and biophysical research communications **137:**1094-1100.
24. **Warner, S. J., and P. Libby.** 1989. Human vascular smooth muscle cells. Target for and source of tumor necrosis factor. J Immunol **142:**100-109.
25. **Young, J. D., C. C. Liu, G. Butler, Z. A. Cohn, and S. J. Galli.** 1987. Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. Proceedings of the National Academy of Sciences of the United States of America **84:**9175-9179.

26. **Zou, J., S. Peddie, G. Scapigliati, Y. Zhang, N. C. Bols, A. E. Ellis, and C. J. Secombes.** 2003. Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, Oncorhynchus mykiss. Developmental and comparative immunology **27:**813.822.

27. **Zou, J., C. J. Secombes, S. Long, N. Miller, L. W. Clem, and V. G. Chinchar.** 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). Developmental and comparative immunology **27:**845-858.

28. **Zou, J., T. Wang, I. Hirono, T. Aoki, H. Inagawa, T. Honda, G. I. Soma, M. Ototake, T. Nakanishi, A. E. Ellis, and C. J. Secombes.** 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, Oncorhynchus mykiss. Developmental and comparative immunology **26:**161-172.

CHAPTER IV: MOLECULAR CHARACTERIZATION OF TUMOR NECROSIS FACTOR RECEPTORS 1 AND 2 OF THE GOLDFISH (*Carassius auratus* L.)[2]

1. INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a highly pleotropic cytokine involved in a spectrum of physiological processes that control inflammation, anti-tumor responses and homeostasis. This pro-inflammatory mediator can be either membrane bound or secreted by cells that participate in host defense. The effects elicited by TNFα result from its trimerization and binding to one of two cognate receptors, TNF-R1 or TNF-R2 (4, 8, 20), with TNF-R1 being present on most cells, while the surface expression of TNF-R2 in higher vertebrates being restricted to endothelial cells and cells of the immune system (5, 35).

To date, there is no consensus as to the exact contribution of the receptors in the plethora of biological effects induced by TNFα. Some work suggests that TNF-R1 is the receptor for the soluble ligand, whereas the membrane bound TNFα mediates its effects exclusively through TNF-R2 (14). The results of some studies suggest that TNF-R1 mediates apoptosis and that TNF-R2 mediates proliferation of cells, while other studies provide evidence that TNFα signaling requires cooperation of the two receptors (21, 38). Until recently, the prevailing theory was that the majority of the biological effects mediated by TNFα are

[2] A version of this chapter has been published: Grayfer, L., and M. Belosevic. 2009. Molecular characterization of tumor necrosis factor receptors 1 and 2 of the goldfish (*Carassius auratus* L.). Molecular Immunology 46:2190-2199.

achieved through its interaction with TNF-R1 where the TNF-R2 plays a minor role in binding and redistributing the ligand to TNF-R1 in a process coined "ligand passing" (7, 9, 36). However, there is accumulating evidence that signaling through the TNF-R2 influences a number of pro-inflammatory responses, including the activation of T cells (16, 17, 34), myofibroblasts (32), inhibition of angiogenesis and tumor suppression (41).

Homologues of TNFα have been identified in a number of bony fish (teleosts) including Japanese flounder (15), rainbow trout (19), gilthead seabream (11), carp (28), catfish (42), tilapia (27), turbot (25), mandarin fish (40) and goldfish (13). In contrast, relatively little is known about the teleost TNF receptors. Homologues of TNF-R1 and TNF-R2 have been identified in the Japanese flounder where both receptors were expressed in tissues of healthy fish and were up-regulated in peripheral blood leukocytes (PBLs) in response to various stimuli (26). A death domain-containing TNF receptor has been identified in zebrafish and has been coined the "ovarian TNF receptor" (OTR) because it was found to be highly expressed in the ovaries compared to the other tissues (3). Predicted zebrafish sequences for TNF-R1 and TNF-R2 in the NCBI database, show that zebrafish TNF-R1 has very high sequence homology to the zebrafish OTR.

In this chapter, I report on the cloning and characterization of goldfish tumor necrosis factor receptors 1 and 2. Comprehensive Q-PCR analysis revealed that the TNF-R2 gene expression was substantially higher than that of TNF-R1 in all tissues of healthy fish. Both receptors were most robustly expressed in

monocytes; the gene expression of TNF-R1 was lowest in mature macrophages while that of TNF-R2 was lowest in PBLs. Incubation of goldfish macrophages with recombinant goldfish TNFα2 (rgTNFα2) up-regulated gene expression of TNF-R2 while it down-regulated the expression of TNF-R1. Treatment of macrophages with rgIFNγ increased the gene expression of both receptors while treatment with rgTGFβ caused a time dependent decrease in the expression of both receptors. *In vitro* experiments using recombinant TNFα1 and TNFα2 demonstrated that both cytokine isoforms were capable of binding to either recombinant goldfish TNF-R1 or TNF-R2, and that the addition of known amounts of recombinant receptors (rgTNF-R1 or rgTNF-R2) down-regulated rgTNFα-primed induction of respiratory burst by activated monocytes.

2. RESULTS

2.1. Goldfish TNF-R1 and TNF-R2 *in silico* analysis

The complete open reading frames (ORFs) and the untranslated regions (UTRs) of the goldfish TNF-R1 and TNF-R2 cDNA transcripts were obtained. BLASTx analysis of the goldfish TNF-R1 sequence indicated the highest homology to a zebrafish TNF-R1 as well as to an ovarian TNF receptor, both of which encode for the same protein. BLASTx assessment of the TNF-R2 sequence demonstrated that it most closely resembled the zebrafish clone MGC:163064 the predicted protein sequence sharing all hallmarks of TNF-R2 described for other species.

Alignment of goldfish TNF-R1 with other known fish and higher

vertebrate TNF-R1 protein sequences demonstrated the close relationship between the goldfish TNF-R1 and other TNF-R1 proteins (Fig. 4.1). The goldfish TNF-R1 protein contained a predicted signal sequence (overhead line), a transmembrane domain (bolded) as well as cystein rich domains otherwise known as TNFR homology domains (THDs, boxed) that are characteristic of all members of the TNF receptor superfamily (Fig. 4.1). These domains are comprised of six conserved cysteins each, with four domains present on the extracellular portions of all known higher vertebrate TNF-R1 proteins. Interestingly, goldfish and all other fish TNF-R1 proteins examined, contained only three of these domains in addition to having a conserved pair of cysteins (denote by * above them) that are absent from all higher vertebrate TNF-R1 proteins examined (Fig. 4.1). The intracellular portion of the goldfish TNF-R1 contained a predicted death domain characteristic of TNF-R1s. This domain included the highly conserved motif of $(W/E)X_{31}LX_2WX_{12}LX_3L$ (with R in the W/E position) and six conserved or semi-conserved residues (denoted by double overhead lines and arrows) that are crucial for the TNF-R1 mediated cytotoxicity (31) (Fig. 4.1). The goldfish TNF-R1 also had a predicted TNF Receptor Association Factor (TRAF) 6 binding site, suggesting that the downstream signaling nature of this receptor may be pleotropic.

Alignment of goldfish TNF-R2 with other known fish and higher vertebrate TNF-R2 protein sequences demonstrated the close homology shared between the goldfish TNF-R2 and predicted zebrafish TNF-R2 protein (Fig. 4.2). The goldfish TNF-R2 protein was predicted to contain a characteristic signal

sequence (overhead line), trans-membrane domain (bold) and cystein-rich areas comprising of four highly conserved THDs (boxed) in the protein's extracellular region (Fig. 4.2). Also present in the intracellular region of this receptor were 3 predicted TRAF2 binding sites (Fig. 4.2) presumably employed in signaling.

Phylogenetic analysis confirmed the close relationships between the goldfish and zebrafish TNF-R1 and TNF-R2 (Fig. 4.3). The goldfish and zebrafish TNF-R1 proteins branched separately from the zebrafish hematopoietic death receptor (HDR) and from the halibut TNF-R1 sequences (Fig. 4.3). Interestingly, the fish TNF-R1 proteins branched independently from the higher vertebrate TNF-R1s and closer to Fas proteins (Fig. 4.3). All fish TNF-R2 sequences branched separately from but closest to TNF-R2 proteins of higher vertebrates (Fig. 4.3).

2.2. Analysis of TNF-R1 and TNF-R2 gene expression in goldfish tissues

Quantitative gene expression analysis of goldfish TNF-R1 and TNF-R2 in tissues of healthy fish revealed that gene expression of TNF-R2 was significantly higher for all tissues examined compared to that of TNF-R1 (Fig. 4.4A). The highest observed gene expression of TNF-R1 was in the brain, followed by heart and than intestine (Fig. 4.4A). The gene expression of TNF-R2 was substantially higher in the spleen than other tissues, followed by brain, gill and kidney (Fig. 4.4A).

2.3. Analysis of TNF-R1 and TNF-R2 gene expression in goldfish immune cell populations

Quantitative gene expression of TNF-R1 and TNF-R2 was assessed in different goldfish immune cell populations. The cell populations examined included PBL, kidney-derived granulocytes, splenocytes as well as FACS-sorted goldfish monocytes and macrophages. The expression of TNF-R2 was significantly higher than that of TNF-R1 in all cell populations examined (Fig. 4.4B). TNF-R1 was expressed highly in monocytes, followed by granulocytes, splenocytes, and mature macrophages. The lowest mRNA levels of TNF-R1 were observed in PBL (Fig. 4.4B). The expression of TNF-R2 was similar in all cell populations examined with the exception of mature macrophages, where the expression of TNF-R2 was found to be significantly lower (Fig. 4B).

2.4. Analysis of TNF-R1 and TNF-R2 gene expression in activated goldfish macrophages

In order to examine possible cytokine-induced changes in goldfish macrophage TNF-R1 and TNF-R2 gene expression, mature macrophage cultures were either left untreated or stimulated with 100 ng/mL of rgTNFα2, rgIFNγ, or rgTGFβ. The addition of rgTNFα2 to macrophage cultures caused a significant increase in the expression of TNF-R2 from 12 to 48 hrs after treatment (Fig. 4.5A). In contrast, TNF-R1 expression was modestly but significantly decreased at 6, 24, 48 and 72 hrs post treatment (Fig. 4.5A). Treatment of macrophages with rgIFNγ resulted in significant increases in the mRNA levels of both receptors

(Fig. 4.5B). The TNF-R1 gene expression was significantly up-regulated at 2 and 12 hrs and TNF-R2 gene expression at 12 to 72 hrs after treatment (Fig. 4.5B). As predicted, the addition of rgTGFβ to goldfish macrophage cultures caused a time-dependent decrease in the gene expression of both receptors, where TNF-R1 expression was down-regulated at 2, 6 and 12 hrs, and that TNF-R2 at 24, 48 and 72 hrs after treatment (Fig. 4.5C). The gene expression of both receptors in non-treated cells, although slightly variable, was not significant for either TNF-R1 or TNF-R2 during the study period (Fig. 4.5D).

2.5. *In vitro* binding analysis of rgTNFα1 and rgTNFα2 to rgTNF-R1 and rgTNF-R2

To determine whether goldfish TNF-R1 and TNF-R2 interacted with their predicted ligands, TNFα1 and TNFα2, the extracellular domains of both receptors and the active forms of the two ligands were produced as recombinant proteins. When incubated with the cross-linker DSS, the rgTNFα1, rgTNFα2 as well as rgTNF-R1 displayed band shifts indicative of dimerization (Figs. 4.6A and B, respectively). When rgTNF-R1 was incubated with rgTNFα1 or rgTNFα2 in the presence of the cross-linker two additional bands of approximately 90 and 120 KDa were observed, suggesting ligand-receptor interactions (Fig. 4.6A and B). The cross-linking of the rgTNF-R2 resulted in an additional band of about 75 KDa, which was the expected size of a dimerized TNF-R2 (Fig. 4.6C and D). Cross-linking of co-incubated rgTNF-R2 and rgTNFα-1 or rgTNFα2 resulted in the appearance of an additional band just above 130 KDa, indicative of one ligand

dimer interacting with one dimer of rgTNF-R2 (Fig. 4.6C and D). An additional faint band indicative of multimerization was observed when rgTNFα2 was cross-linked (Fig. 4.6D). This is most likely an artifact of the cross-linking procedure and not a representation of a dominant state of the protein under natural conditions.

In order to confirm the observed receptor-ligand interactions, I examined whether the addition of rgTNF-R1 and rgTNF-R2 to monocyte cultures would affect the rgTNFα1 and rgTNFα2 priming of monocytes for respiratory burst when triggered by a phorbol ester (PMA). Goldfish monocytes were primed with medium or rgIFNγ (100 ng/mL), rgTNFα1 (100 ng/mL), rgTNFα2 (100 ng/mL), rgTNF-R1 (100 ng/mL), rgTNF-R2 (100 ng/mL), a combination of rgIFNγ, rgTNFα1 or rgTNFα2 (100ng/mL each), and rgTNF-R1 or rgTNF-R2 (1, 100, or 10000 ng/mL). The rgIFNγ served as a positive control and medium treated-PMA stimulated cells as a negative control. Significant increases in monocyte ROI production over medium-PMA ($P<0.05$) were observed when the cells were primed with rgTNFα1, rgTNFα2 or rgIFNγ (Fig. 4.7A and B). The addition of 100 ng/mL but not 1 ng/mL of rgTNF-R1 significantly abrogated ($P<0.05$) rgTNFα1 or rgTNFα2 primed ROI responses (Fig. 4.7A). However, the rgTNFα1- and rgTNFα2-primed responses were not completely abolished and were found to be significantly elevated ($P<0.05$) above PMA controls (Fig. 4.7A). The application of higher rgTNF-R1 concentrations did not further dampen the observed ROI responses (data not shown). The addition of either 1 or 100 ng/mL treatments of rgTNF-R2 significantly ($P<0.05$) diminished the rgTNFα1 primed

ROI responses to levels similar to those of PMA alone (Fig 4.7B). Concentration-dependent but not significant (P>0.05) decreases in the rgTNFα2 primed ROI responses were observed when monocyte cultures were co-treated with either 1 or 100 ng/mL of rgTNF-R2 (Fig. 4.7B). The addition of higher amounts of rgTNF-R2 did not result in further decreases of the rgTNFα1 and rgTNFα2-primed ROI responses compared to those induced by the addition of 100 ng/mL of the recombinant receptor only to macrophage cultures. Treatment of cells with either rgTNF-R1 or rgTNF-R2 was not observed to significantly abrogate the rgIFNγ primed ROI responses (P>0.05) at any concentration of either receptor (Fig. 4.7A&B).

3. DISCUSSION

I report on the molecular characterization of goldfish TNF-R1 and TNF-R2. The goldfish TNF-R1 sequence shared the highest homology with the zebrafish TNF-R1 as well as with the zebrafish ovarian TNF receptor, both encoding very similar proteins. The goldfish as well as the other fish TNF-R1 proteins had only three THDs compare to the four seen in higher vertebrate TNF-R1s. This could be the reason why despite the apparent structural similarities of teleost TNF-R1s to their mammalian counterparts, phylogenetically they group closer to Fas molecules. This observation is particularly interesting because in higher vertebrates the second and third domains are thought to mediate ligand binding (1) whereas the fourth domain is thought to aid in ligand-receptor dissociation following receptor-ligand complex internalization (23). The

sequence alignment of the fish TNF-R1 protein sequences revealed the presence of an additional pair of conserved cysteine repeats in the extracellular regions of fish TNF-R1, suggesting an evolutionary deletion of the fourth THD in teleosts or alternatively the addition of the fourth THD in higher vertebrates. It is possible that a different mechanism for TNF-R1-ligand complex dissociation may be operational in teleosts. The goldfish TNF-R2 shared the highest sequence homology with a predicted zebrafish TNF-R2 and to a lesser extent with halibut TNF-R2 sequence. The goldfish TNF-R2 has four THDs, 3 predicted TRAF2 binding sites that are presumably used for signal propagation. Phylogenetic analysis placed the goldfish TNF-R2 closest to the zebrafish predicted TNF-R2 and further from the halibut TNF-R2, with these fish TNF-R2 receptors branching separately from the higher vertebrate TNF-R2s.

It has been reported that in mammals TNF-R1 is present on most cells, while the TNF-R2 is restricted to endothelial cells and cells of the immune system (5, 35). In contrast, goldfish TNF-R2 had significantly higher gene expression than TNF-R1 in all tissues examined as well as being more robustly expressed in immune cell populations. Similarly, flounder TNF-R1 and TNF-R2 were also reported to display a broad pattern of gene expression across tissues, although the expression of flounder TNF-R2 did not exceed that of TNF-R1 (26). The expression of goldfish TNF-R2 was similar in splenocytes, PBLs, granulocytes and monocytes, but significantly lower in mature macrophages. This observation may be explained when one considers the pro-inflammatory/immune roles

attributed to the mammalian TNF-R2 and are consistent with the observed elevated mRNA levels of TNF-R2 in goldfish spleen, kidney, and gill tissues.

In this study I also examined the changes in transcript levels of both goldfish TNF receptors following addition of different goldfish recombinant cytokines to macrophage cultures. Treatment of macrophages with rgTNFα-2 resulted in modest bust significant decrease TNF-R1 mRNA levels and a prominent increase in TNF-R2 mRNA. Similar results were reported when human fibroblasts were treated with recombinant human TNFα, that also caused a decrease in TNF-R1 mRNA and translated protein levels and a marked increase in both the message and protein levels of the TNF-R2 (39). As observed here with rgIFNγ, higher vertebrate cell treatment with IFNγ also caused increases in mRNA levels of both TNF-R1 and TNF-R2 (33, 37). It is well established that in higher vertebrates TGFβ is an antagonist of TNFα via several mechanisms including the inhibition of TNF receptor signaling (18, 30) as well as the down-regulation of TNFα gene expression (24). It is therefore not surprising that rgTGFβ decreased the expression of both goldfish TNF-R1 and TNF-R2 in goldfish macrophages.

In mammals the TNFα ligand binds to TNF-R1 and TNF-R2 as a trimer, with the receptors in turn trimerizing upon ligation. My *in vitro* binding results indicated that both recombinant goldfish TNFα1 and TNFα2 and recombinant extracellular domains of goldfish TNF-R1 and TNF-R2 predominantly formed dimers and not trimers. That these ligand-receptor interactions may have functional significance was confirmed by the results of experiments showing that

goldfish TNF-R1 and TNF-R2 down-regulated the rgTNFα1 or rgTNFα2-primed monocyte ROI responses. This is the first report of TNF receptor-ligand interaction in a teleost species and the first report of the dimerization of a fish TNF-R1 or TNF-R2. Additionally, my observation that goldfish rgTNFα1 and rgTNFα2 formed dimers confirms a previous report that the recombinant seabream TNFα predominantly exists in a dimeric form (10).

Although higher vertebrate TNFα is functionally similar to the teleost TNFs (10, 13, 28), the specifics of the protein interactions within the teleost TNF family are different. The interactions between the neurotrophin (NTFs) family of proteins and their receptors are similar to those of teleost TNF ligands and their receptors. Neurotrophins signal through two different classes of receptors, the trk family of receptor tyrosine kinases and p75 neurotrophin receptor (p75/NTR), a member of the TNF receptor family. The NTF signaling through trk tyrosine kinase results in cell survival while the binding of NTF to p75/NTR has been shown to result in both cell survival as well as cell death (29). The p75/NTR receptor shares majority of the hallmarks of TNF-R1, including four cysteinee rich domains as well as a death domain (2). Interestingly, both the NTR and the p75/NTR form and interact as dimers (6, 12). Similar to NTR/p75NTR receptor interactions, evolutionary selection in teleosts may have led to a TNFα/TNF-receptor system that functions through dimerization. Crystal structure analysis of the human soluble unligated TNF-R1 revealed that the receptor existed in two distinct dimerized states, with one state potentially capable of excluding ligand through changes in the fourth THD (which is absent in fish TNF-R1 proteins)

while the other dimer state had potential capabilities of interacting with the ligand (22, 23). From an evolutionary perspective, this may suggestive of a remnant TNF system where interactions occurred through dimerized and not trimerized protein states.

The results of this study demonstrate that TNF receptors of bony fish share both evolutionarily conserved as well as diverged aspects to their biology. Because of the importance of these receptors in the immune system, an enhanced understanding of their functions in lower vertebrates such as teleosts will aid in elucidating the evolutionary paths that have resulted in the higher vertebrate immune systems.

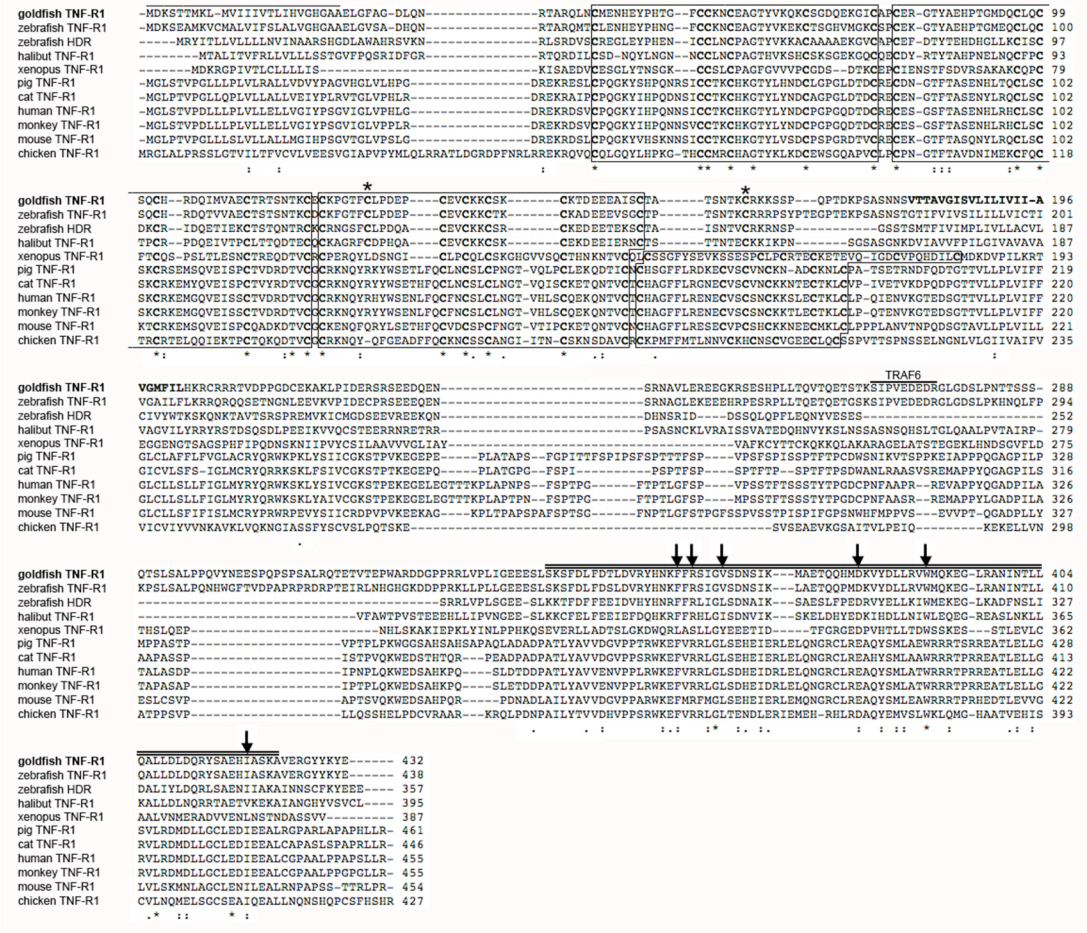

Figure 4.1. Protein alignment of goldfish TNF-R1 with other known fish and higher vertebrate TNF-R1 sequences. The TNF homology domains (THDs) are boxed for all proteins. The goldfish predicted signal sequence is indicated with an overhead line while the transmembrane domain and the conserved cysteines are in bold. The predicted goldfish TRAF6 docking site is indicated with "TRAF6" above it. The predicted goldfish death domain has a double line above it and the six conserved / semi-conserved residues required for signaling, are indicated by arrows. The conserved cysteines unique to teleosts are indicated by (*) overhead. Fully conserved residues are indicated by an asterisk (*) below, partially conserved and semi-conserved substitutions are represented by ":" and ".", respectively.

Figure 4.2. Protein alignment of goldfish TNF-R2 with other known fish and higher vertebrate TNF-R2 protein sequences. The THDs are boxed for all proteins. The goldfish predicted signal sequence is indicated with an overhead line while the trans-membrane domain and the conserved cysteines are in bold. The predicted goldfish TRAF2 docking site is indicated with "TRAF2" above double lines. Fully conserved residues are indicated by an asterisk (*) below, partially conserved and semi-conserved substitutions are represented by ":" and ".", respectively.

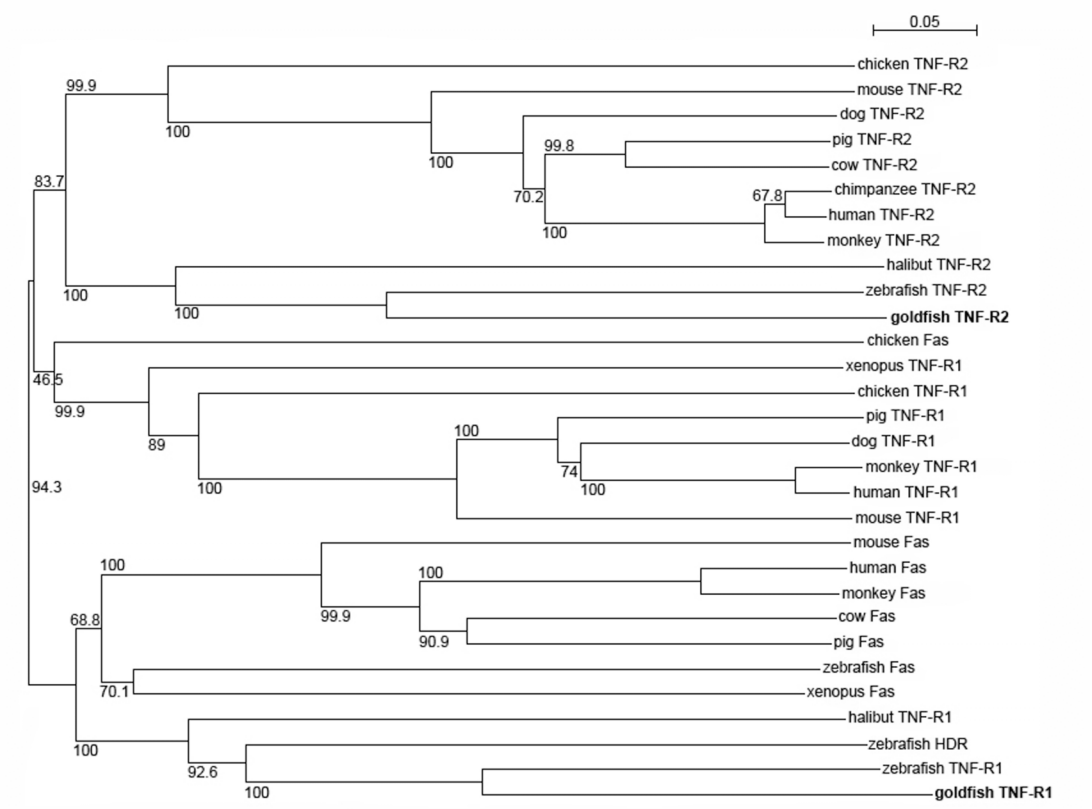

Figure 4.3. Phylogenetic analysis of goldfish TNF-R1 and TNF-R2 in relation to TNF-R1, TNF-R2 and Fas proteins of other fish and higher vertebrate species. The analysis was conducted using the neighbor joining method and was bootstrapped 10000 times and expressed as percent values.

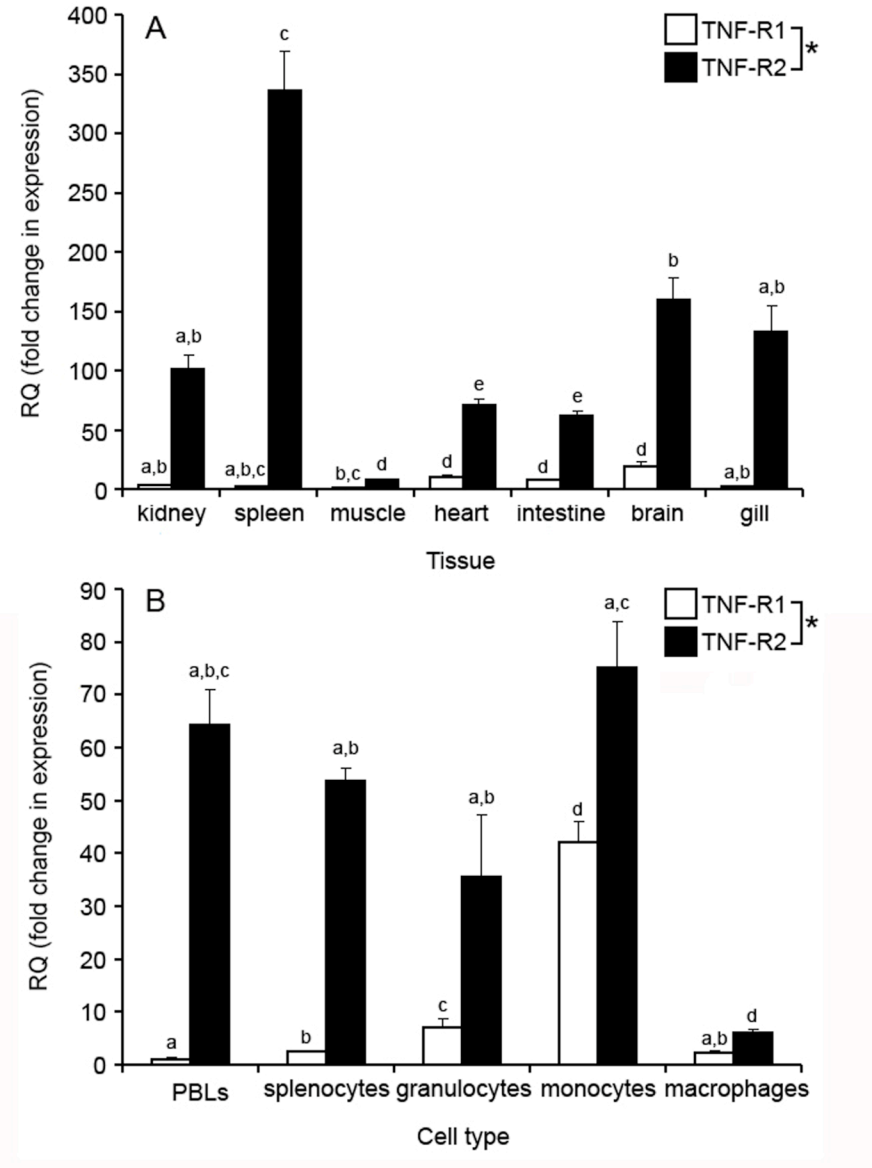

Figure 4.4. Quantitative expression analysis of TNF-R1 and TNF-R2 in goldfish tissues and immune cell populations. (A) Goldfish TNF-R1 and TNF-R2 tissue expression analysis. The goldfish TNF-R1 and TNF-R2 expression is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Analyses of the relative tissue expression data are for tissues from five fish (n=5). All results were normalized against the muscle TNF-R1 mRNA level. **(B)** Goldfish TNF-R1 and TNF-R2 expression in different immune cell populations. Cells cultures were established from 5 fish (n=5) and the expression normalized against that of TNF-R1 mRNA level in PBLs. Statistical analysis was performed using one-way ANOVA. Different letters above each bar indicate significantly different ($P < 0.05$), the same letter indicate no statistical difference between groups. (+) beside lines indicates statistical significance ($P<0.05$) between experimental groups indicated by the lines.

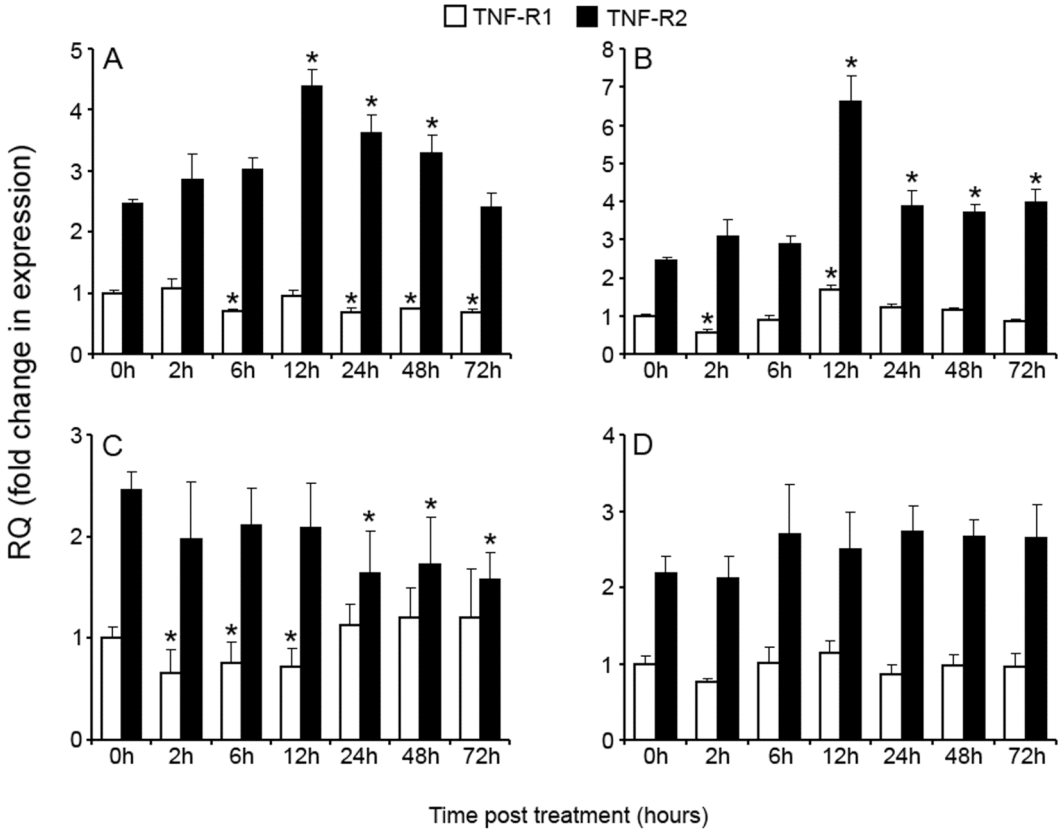

Figure 4.5. Quantitative expression analysis of goldfish TNF-R1 and TNF-R2 in cytokine-stimulated macrophages. Cells were treated with 100 ng/mL of either **(A)** rgTNFα2, **(B)** rgIFNγ, **(C)** rgTGFβ or **(D)** or medium. The expression of goldfish TNF-R1 and TNF-R2 was examined relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for TNF-R1 mRNA level at the 0 hour time point. The results are mean ± SEM of primary macrophage cultures established from five individual fish (*n*=5). (*) indicates significantly different ($P < 0.05$) from the 0 hour time point.

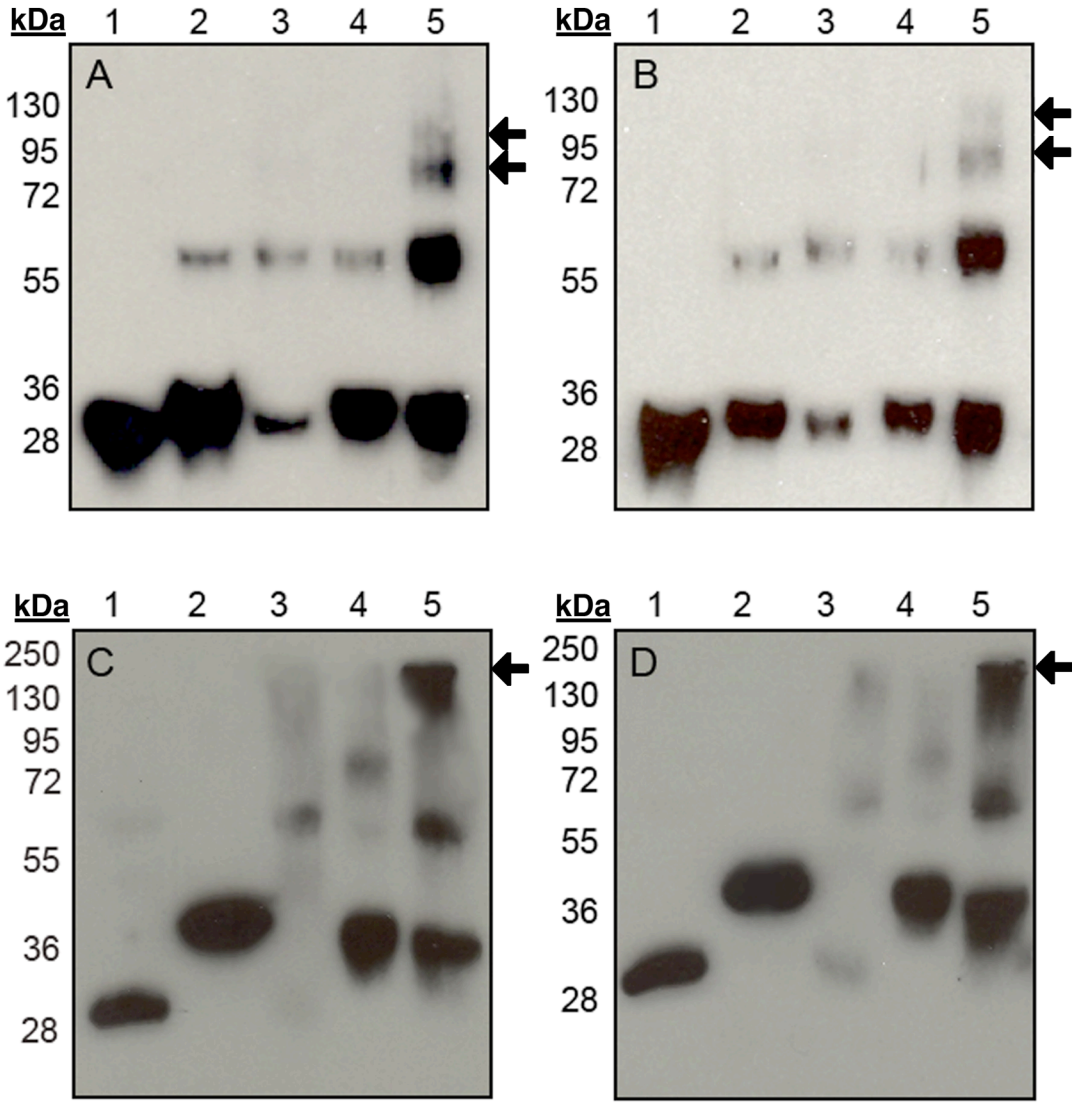

Figure 4.6. Western blot analysis of rgTNF-R1 and rgTNF-R2 interaction with rgTNFα1 and rgTNFα2. For all binding studies, 2.5 μg of rgTNFα1, rgTNFα2, rgTNF-R1 or rgTNF-R2 were incubated individually or in ligand-receptor combinations. Following the initial conjugation period, the ligands, receptors and ligand-receptor combinations were cross-linked using disuccinimidyl suberate (DSS), resolved using reducing SDS-PAGE and visualized by Western blotting against poly histidine tags on the recombinants proteins. **(A)** Lane: 1. rgTNFα1; 2. rgTNF-R1; 3. rgTNFα1 + DSS; 4. rgTNF-R1 + DSS; 5. rgTNFα1 and rgTNF-R1 + DSS. **(B)** Lane: 1. rgTNFα2; 2. rgTNF-R1; 3. rgTNFα2 + DSS; 4. rgTNF-R1 + DSS; 5. rgTNFα2 and rgTNF-R1 + DSS. **(C)** Lane: 1. rgTNFα1; 2. rgTNF-R2; 3. rgTNFα1 + DSS; 4. rgTNF-R2 + DSS; 5. rgTNFα1 and rgTNF-R2 + DSS. **(D)** Lane: 1. rgTNFα2; 2. rgTNF-R2; 3. rgTNFα2 + DSS; 4. rgTNF-R2 + DSS; 5. rgTNFα2 and rgTNF-R2 + DSS.

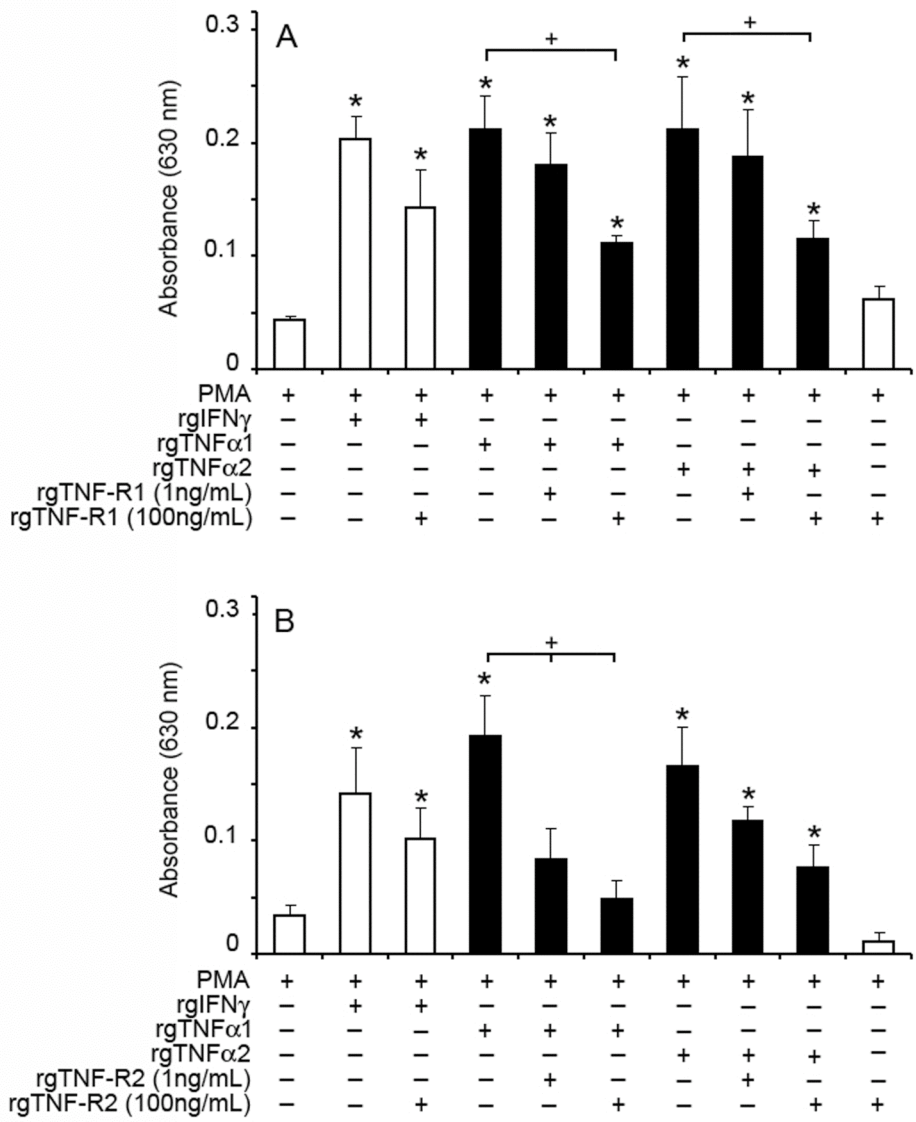

Figure 4.7. The rgTNF-R1 (A) and rgTNF-R2 (B) abrogate the rgTNFα-1 and rgTNFα-2 mediated priming of monocyte respiratory burst responses. Goldfish monocyte cultures were primed with medium, rgIFNγ (100ng/mL), rgTNFα1 (100 ng/mL), rgTNFα2 (100 ng/mL), rgTNF-R1 (100 ng/mL), rgTNF-R2 (100 ng/mL) or a combination of rgIFNγ, rgTNFα1 or rgTNFα2 (100 ng/mL each) and rgTNF-R1 or rgTNF-R2 (1, 100, or 10000 ng/mL). The rgIFNγ served as a positive control and medium treated-PMA treated cells (PMA) as a negative control. The absorbance values of medium treated controls (no PMA) were subtracted from treatment values to factor in background NBT reduction. Results are mean ± SEM of relative reactive oxygen intermediate production by macrophages established from individual fish (n=5). (*) indicates statistically different ($P < 0.05$) compared to PMA only treated cells. (+) above the line denotes significantly different ($P < 0.05$) between experimental groups.

4. REFERENCES

1. **Banner, D. W., A. D'Arcy, W. Janes, R. Gentz, H. J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer.** 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. Cell **73:**431-445.
2. **Barbacid, M.** 1995. Structural and functional properties of the TRK family of neurotrophin receptors. Annals of the New York Academy of Sciences **766:**442-458.
3. **Bobe, J., and F. W. Goetz.** 2001. Molecular cloning and expression of a TNF receptor and two TNF ligands in the fish ovary. Comp Biochem Physiol B Biochem Mol Biol **129:**475-481.
4. **Brockhaus, M., H. J. Schoenfeld, E. J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher.** 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci U S A **87:**3127-3131.
5. **Carpentier, I., B. Coornaert, and R. Beyaert.** 2004. Function and regulation of tumor necrosis factor type 2. Curr Med Chem **11:**2205-2212.
6. **Chapman, B. S., and I. D. Kuntz.** 1995. Modeled structure of the 75-kDa neurotrophin receptor. Protein Sci **4:**1696-1707.
7. **Chen, G., and D. V. Goeddel.** 2002. TNF-R1 signaling: a beautiful pathway. Science **296:**1634-1635.
8. **Dembic, Z., H. Loetscher, U. Gubler, Y. C. Pan, H. W. Lahm, R. Gentz, M. Brockhaus, and W. Lesslauer.** 1990. Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. Cytokine **2:**231-237.
9. **Dranoff, G.** 2004. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer **4:**11-22.
10. **Garcia-Castillo, J., E. Chaves-Pozo, P. Olivares, P. Pelegrin, J. Meseguer, and V. Mulero.** 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci **61:**1331-1340.
11. **Garcia-Castillo, J., P. Pelegrin, V. Mulero, and J. Meseguer.** 2002. Molecular cloning and expression analysis of tumor necrosis factor alpha from a marine fish reveal its constitutive expression and ubiquitous nature. Immunogenetics **54:**200-207.
12. **Gong, Y., P. Cao, H. J. Yu, and T. Jiang.** 2008. Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. Nature **454:**789-793.
13. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Dev Comp Immunol **32:**532-543.
14. **Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, and P. Scheurich.** 1995. The transmembrane form of tumor necrosis factor is the

prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell **83:**793-802.

15. **Hirono, I., B. H. Nam, T. Kurobe, and T. Aoki.** 2000. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder Paralychthys olivaceus. J Immunol **165:**4423-4427.
16. **Kim, E. Y., J. J. Priatel, S. J. Teh, and H. S. Teh.** 2006. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. J Immunol **176:**1026-1035.
17. **Kim, E. Y., and H. S. Teh.** 2004. Critical role of TNF receptor type-2 (p75) as a costimulator for IL-2 induction and T cell survival: a functional link to CD28. J Immunol **173:**4500-4509.
18. **Kloth, J. N., G. J. Fleuren, J. Oosting, R. X. de Menezes, P. H. Eilers, G. G. Kenter, and A. Gorter.** 2005. Substantial changes in gene expression of Wnt, MAPK and TNFalpha pathways induced by TGF-beta1 in cervical cancer cell lines. Carcinogenesis **26:**1493-1502.
19. **Laing, K. J., T. Wang, J. Zou, J. Holland, S. Hong, N. Bols, I. Hirono, T. Aoki, and C. J. Secombes.** 2001. Cloning and expression analysis of rainbow trout Oncorhynchus mykiss tumour necrosis factor-alpha. Eur J Biochem **268:**1315-1322.
20. **Loetscher, H., E. J. Schlaeger, H. W. Lahm, Y. C. Pan, W. Lesslauer, and M. Brockhaus.** 1990. Purification and partial amino acid sequence analysis of two distinct tumor necrosis factor receptors from HL60 cells. J Biol Chem **265:**20131-20138.
21. **Mukhopadhyay, A., J. Suttles, R. D. Stout, and B. B. Aggarwal.** 2001. Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. J Biol Chem **276:**31906-31912.
22. **Naismith, J. H., T. Q. Devine, B. J. Brandhuber, and S. R. Sprang.** 1995. Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor. J Biol Chem **270:**13303-13307.
23. **Naismith, J. H., T. Q. Devine, T. Kohno, and S. R. Sprang.** 1996. Structures of the extracellular domain of the type I tumor necrosis factor receptor. Structure **4:**1251-1262.
24. **Ng, Y. Y., C. C. Hou, W. Wang, X. R. Huang, and H. Y. Lan.** 2005. Blockade of NFkappaB activation and renal inflammation by ultrasound-mediated gene transfer of Smad7 in rat remnant kidney. Kidney Int Suppl**:**S83-91.
25. **Ordas, M. C., M. M. Costa, F. J. Roca, G. Lopez-Castejon, V. Mulero, J. Meseguer, A. Figueras, and B. Novoa.** 2007. Turbot TNFalpha gene: molecular characterization and biological activity of the recombinant protein. Molecular immunology **44:**389-400.
26. **Park, C. I., T. Kurobe, I. Hirono, and T. Aoki.** 2003. Cloning and characterization of cDNAs for two distinct tumor necrosis factor receptor superfamily genes from Japanese flounder Paralichthys olivaceus. Dev Comp Immunol **27:**365-375.

27. **Praveen, K., D. L. Evans, and L. Jaso-Friedmann.** 2006. Constitutive expression of tumor necrosis factor-alpha in cytotoxic cells of teleosts and its role in regulation of cell-mediated cytotoxicity. Mol Immunol **43:**279-291.
28. **Saeij, J. P., R. J. Stet, B. J. de Vries, W. B. van Muiswinkel, and G. F. Wiegertjes.** 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev Comp Immunol **27:**29-41.
29. **Skaper, S. D.** 2008. The biology of neurotrophins, signalling pathways, and functional peptide mimetics of neurotrophins and their receptors. CNS & neurological disorders drug targets **7:**46-62.
30. **Soucek, K., J. Pachernik, L. Kubala, J. Vondracek, J. Hofmanova, and A. Kozubik.** 2006. Transforming growth factor-beta1 inhibits all-trans retinoic acid-induced apoptosis. Leuk Res **30:**607-623.
31. **Tartaglia, L. A., T. M. Ayres, G. H. Wong, and D. V. Goeddel.** 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell **74:**845-853.
32. **Theiss, A. L., J. G. Simmons, C. Jobin, and P. K. Lund.** 2005. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. J Biol Chem **280:**36099-36109.
33. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1986. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. J Immunol **136:**2441-2444.
34. **Turner, S. J., N. L. La Gruta, J. Stambas, G. Diaz, and P. C. Doherty.** 2004. Differential tumor necrosis factor receptor 2-mediated editing of virus-specific CD8+ effector T cells. Proc Natl Acad Sci U S A **101:**3545-3550.
35. **Wajant, H., K. Pfizenmaier, and P. Scheurich.** 2003. Tumor necrosis factor signaling. Cell Death Differ **10:**45-65.
36. **Wallach, D., E. E. Varfolomeev, N. L. Malinin, Y. V. Goltsev, A. V. Kovalenko, and M. P. Boldin.** 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol **17:**331-367.
37. **Wang, F., B. T. Schwarz, W. V. Graham, Y. Wang, L. Su, D. R. Clayburgh, C. Abraham, and J. R. Turner.** 2006. IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. Gastroenterology **131:**1153-1163.
38. **Weiss, T., M. Grell, K. Siemienski, F. Muhlenbeck, H. Durkop, K. Pfizenmaier, P. Scheurich, and H. Wajant.** 1998. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. J Immunol **161:**3136-3142.
39. **Winzen, R., D. Wallach, O. Kemper, K. Resch, and H. Holtmann.** 1993. Selective up-regulation of the 75-kDa tumor necrosis factor (TNF) receptor and its mRNA by TNF and IL-1. J Immunol **150:**4346-4353.

40. **Xiao, J., Z. C. Zhou, C. Chen, W. L. Huo, Z. X. Yin, S. P. Weng, S. M. Chan, X. Q. Yu, and J. G. He.** 2007. Tumor necrosis factor-alpha gene from mandarin fish, Siniperca chuatsi: molecular cloning, cytotoxicity analysis and expression profile. Mol Immunol **44:**3615-3622.
41. **Zhao, X., M. Mohaupt, J. Jiang, S. Liu, B. Li, and Z. Qin.** 2007. Tumor necrosis factor receptor 2-mediated tumor suppression is nitric oxide dependent and involves angiostasis. Cancer Res **67:**4443-4450.
42. **Zou, J., C. J. Secombes, S. Long, N. Miller, L. W. Clem, and V. G. Chinchar.** 2003. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (Ictalurus punctatus). Dev Comp Immunol **27:**845-858.

CHAPTER V:

MOLECULAR CHARACTERIZATION, GENE EXPRESSION AND FUNCTIONAL ANALYSIS OF GOLDFISH (*Carassius auratus* L.) INTERFERON GAMMA[3]

1. INTRODUCTION

Interferon gamma (IFNγ) is a pleiotropic, pro-inflammatory and antiviral cytokine that is produced primarily by activated Th1 T helper cells (23), cytotoxic T lymphocytes of the Th1 phenotype (34) and natural killer (NK) cells (28). This cytokine was first identified in PHA-activated mammalian lymphocyte supernatants and shown to have unique antiviral properties (43). In addition to orchestrating antiviral responses (17, 38), IFNγ regulates the production of an array of cytokines that promote a Th1 phenotype (23) and skewing of T cells toward a Th1 modality (41, 45). Interferon gamma is a central participant in host resistance to various infections (1, 3, 38, 39), demonstrated using IFNγ knockout mice infected with *Leishmania major* (42), *Listeria monocytogenes* (15) and *Mycobacteria* (9).

Recently, IFNγ has been identified in several bony fish species including zebrafish (16), Japanese pufferfish (50), trout (49), Atlantic salmon (32) and catfish (21), with some teleosts having multiple isoforms of the gene. The teleost IFNγs are not as homologous among fishes when compared to the mammalian

[3] A version of this chapter has been published: Grayfer, L., and M. Belosevic. 2009. Molecular characterization, expression and functional analysis of goldfish (*Carassius auratus* L.) interferon gamma. Developmental and Comparative Immunology 33:235-246.

IFNγs, but as in mammals, the fish IFNγ genes are syntenically linked to interleukin-22 and interleukin-26 genes (16, 50). To date there have been limited expression and functional analyses of fish IFNγs. Zou *et al.* (17) have demonstrated that in trout the expression of IFNγ was upregulated in the spleen and kidney following injection of fish with polyriboinosinic:polyribocytidylic acid (PolyI:C). In zebrafish and catfish, the two isoforms of IFNγ are differentially expressed across tissues (16, 21). Furthermore, Zou *et al.* (17) reported that PHA and Poly I:C upregulated expression of IFNγ in trout head-kidney leukocytes and that recombinant trout IFNγ induced reactive oxygen production in trout macrophages and upregulated gene expression of several IFN-related genes in the RTS-11 macrophage cell line (49).

In this chapter, I report on the cloning and comprehensive gene expression and functional analysis of the goldfish IFNγ. Goldfish IFNγ was highly expressed in the spleen and at much lower levels in other tissues examined. The gene expression of IFNγ in different goldfish immune cell populations was upregulated following treatment with PolyI:C, PHA, TNFα and during mixed leukocyte reaction (MLR). A recombinant form of goldfish IFNγ (rgIFNγ) was produced and shown to prime goldfish monocytes and granulocytes for enhanced respiratory burst response. Treatment of goldfish monocytes and macrophages with rgIFNγ increased the phagocytic and nitric oxide responses of these cells, respectively. Treatment of goldfish macrophages with rgIFNγ also induced increased the gene expression of several proinflammatory genes including TNFα1, TNFα2, IL-β-1, IL-β-2, IL-12 p35, IL-12 p40, RSAD-2, CXCL-8, CCL-

1, iNOS A & B, IFNγ, and decreased the expression of TLR-3, while not altering the expression of TGFβ.

2. RESULTS

2.1. Goldfish IFNγ *in silico* analysis

The complete open reading frame and the untranslated regions (UTRs) of the goldfish IFNγ cDNA transcript was obtained (Fig. 5.1). BLASTx analysis of the goldfish IFNγ sequence indicated highest homology to a zebrafish IFNγ sequence. Analysis of the nucleotide sequence revealed the presence of a classical polyadenylation (AATAAA) sequence in the 3' UTR. Additionally present in the 3' UTR were 6 RNA instability regions (ATTTA) characteristic of IFNγ transcripts in different organisms (Fig. 5.1). Also present in the C-terminal third of the protein was the IFNγ signature sequence ([I/V]-QX-[K/Q]-A-X^2-E-[L/F]-X^2-[I/V]) (Fig. 5.1). The goldfish IFNγ also contained a putative nuclear localization signal composed of four contiguous basic amino acids (RRRR) that have been shown to be required for nuclear translocation and cytokine activity (49) (Fig. 5.1). The phylogenetic analysis of IFNγ sequences of different vertebrates indicated that the goldfish IFNγ grouped closely with zebrafish IFNγ and catfish IFNγ (data not shown).

2.2. Analysis of IFNγ gene expression in goldfish tissues

Quantitative gene expression analysis of goldfish IFNγ in tissues of healthy fish revealed that the highest mRNA levels of this cytokine were in the

spleen (Fig. 5.2A). The IFNγ was also expressed in the brain, gill, kidney, heart, intestine and muscle (Fig. 5.2A).

2.3. Analysis of IFNγ gene expression in non-stimulated goldfish immune cell populations

Quantitative gene expression of IFNγ was determined in different goldfish cell populations. The cell populations examined included peripheral blood leukocytes (PBL), kidney-derived granulocytes, kidney-derived leukocytes, splenocytes as well as FACS-sorted kidney-derived monocytes and macrophages. Surprisingly, expression of IFNγ was lower in kidney-derived leukocytes compared to that in other cell populations with the exception of FACS-sorted macrophages (Fig. 5.2B). The IFNγ mRNA levels in sorted monocytes and kidney-derived granulocytes were similar. The goldfish IFNγ mRNA levels were the highest in PBL and splenocytes (Fig. 5.2B).

2.4. Analysis of IFNγ gene expression in stimulated goldfish immune cell populations

Since I cloned goldfish IFNγ from kidney leukocyte cDNA and because others have demonstrated increased IFNγ gene expression in kidney leukocytes following stimulation (49), I investigated the temporal changes of the goldfish IFNγ mRNA levels in kidney leukocytes treated with PHA, PolyI:C or after induction of mixed leukocyte reactions (MLR). I observed that all of these reagents/conditions induced an increased gene expression of IFNγ at 2 hours post

treatment, where the IFNγ mRNA levels in the MLR activated cells were significantly higher than those in other two treatment groups ($P<0.05$) (Fig. 5.3A). By 6 hours post treatment, the IFNγ mRNA levels in MLR activated cells decreased precipitously and remained significantly lower than those in the other two treatment groups until the end of the observation period (72 hours). Kidney leukocytes treated with PHA for 6 hours or longer had consistently higher IFNγ mRNA levels than the other two treatment groups (Fig. 5.3A) while those treated with Poly I:C had IFNγ mRNA levels significantly elevated from baseline (0 hour) at 2, 12 and 72 hours after treatment ($P< 0.05$, one-way ANOVA) (Fig. 5.3A).

I also examined the mRNA levels in different goldfish immune cell populations after treatment with rgTNFα2. Four hours after addition of 100 ng/mL of rgTNFα2 to kidney leukocytes, splenocytes and PBL cell suspensions, I observed a significant up-regulation of IFNγ gene expression in all rgTNFα2-treated cell populations compared to that of non-treated cell populations ($P<0.05$, one-way ANOVA) (Fig. 5.3B). In contrast, the IFNγ gene expression did not change in monocytes, macrophages and granulocytes treated with 100 ng/mL of rgTNFα2 (data not shown).

2.5. Recombinant goldfish IFNγ induces respiratory burst response of goldfish monocytes and granulocytes

I produced recombinant (rgIFNγ) in *E. coli* and evaluated its ability to activate primary goldfish monocytes and granulocytes by measuring the

respiratory burst response of the treated cell populations. Monocyte enriched cultures triggered with PMA, exhibited a significant ($P< 0.05$) concentration-dependent increase in the production of reactive oxygen intermediates when first primed with increasing amounts of rgIFNγ (10 to 1000 ng/mL) when compared to PMA only stimulated cells (Fig. 5.4A). Although priming of cells with 1 ng/mL of rgIFNγ did not induce the respiratory burst in monocytes, a combined treatment of monocytes with 1 ng/mL of rgIFNγ and 10 ng/mL rgTNFα2 induced a significantly higher response than that of PMA only treated controls ($P<0.05$) (Fig. 5.4A).

Similar results were obtained when goldfish kidney granulocytes were primed with rgIFNγ (Fig. 5.4B). Granulocyte responses were significantly higher ($P<0.05$) than in medium treated-PMA stimulated cells for all rgIFNγ concentrations tested (1,10,100,1000 ng/mL). Similar to monocytes, a combination of 1 ng/mL rgIFNγ and 10 ng/mL rgTNFα2 also elicited a higher response than either treatment alone, albeit not significantly so (Fig. 5.4B). When treated with 10 or 100 ng/mL of rgIFNγ in conjunction with 10 ng/mL of rgTNFα2, goldfish granulocytes produced lower ROI levels (significant for the 100 ng/mL rgIFNγ and 10 ng/mL rgTNFα2 treatment, $P<0.05$) than those observed after addition of 10 or 100 ng/mL rgIFNγ alone, respectively. These results are similar to those observed for monocytes (Fig. 5.4A&B).

2.6. Recombinant goldfish IFNγ enhanced goldfish monocyte phagocytosis

The ability of the recombinant IFNγ to enhance the goldfish monocyte phagocytic response was investigated using a flow-cytometry-based phagocytosis assay described previously (11). This assay was optimized to examine cell subpopulations that are highly phagocytic as indicated by ingestion of 3 or more fluorescent latex beads. Goldfish monocytes were treated with medium, MAF, rgTNFα2 (10, 100 ng/mL), rgIFNγ (1,10,100,1000 ng/mL) or a combination of rgIFNγ and rgTNFα2 (1,10,100,1000 ng/mL and 10 ng/mL, respectively). Macrophage activating factors (MAF) and 100 ng

/mL of rgTNFα2 were positive controls. As shown in Figure 5.5, treatment of PKM with rgIFNγ enhanced the phagocytic activity of the cells when higher doses of rgIFNγ were used (100 ng/mL and 1,000 ng/mL). Combined treatments of cells with 10 ng/mL rgIFNγ and 10ng/mL rgTNFα resulted in a further increase in the phagocytic responses compared to those when individual cytokines were added to the cultures (Fig. 5.5).

2.7. Recombinant goldfish IFNγ induces goldfish macrophage nitric oxide responses

Recombinant goldfish IFNγ was tested for its ability to induce a nitric oxide response in goldfish macrophages. Cell cultures from individual fish were either maintained in medium (control) or treated with heat killed *Aeromonas salmonicida* or rgTNFα2 (positive controls), and different amounts of rgIFNγ (1 to 1000 ng/mL) alone or in conjunction with a 10 ng/mL of rTNFα2. The

goldfish rgIFNγ induced a significant nitric oxide response of macrophages in a dose-dependent manner (Fig. 5.6). A combined treatment with 100 ng/mL of rgIFNγ and 10ng/mL of rgTNFα2 induced a nitric oxide response of macrophages that was significantly higher when compared to those induced by treatment with either cytokine alone (Fig. 5.6).

2.8. Expression analysis of proinflammatory genes of goldfish macrophages treated with recombinant goldfish IFNγ

Apart from directly controlling proinflammatory responses, IFNγ can also modulate the inflammatory response by inducing the gene expression of other proinflammatory genes of mammalian macrophages. For this reason, I examined whether rgIFNγ modulated gene expression of goldfish macrophages. A number of macrophage proinflammatory genes were examined including TNFα1 and TNFα2; IL-1β1 and IL-1β2; IL-12 p35 and IL-12 p40; CXCL-8 and CCL-1; RSAD-2; iNOS isoforms A and B; TLR-3; TGFβ and IFNγ at various times after treatment (Fig. 5.7 and Fig. 5.8). A 100 ng/mL concentration of of rgIFNγ was selected to activate macrophages in expression experiments because this was the lowest concentration that significantly induced all antimicrobial responses measured.

The mRNA for goldfish TNFα1 increased significantly 6 hours and that of TNFα2, 12 hours after treatment with rgIFNγ (Fig. 5.7A). The gene expression of IL-1β1 was up-regulated at 12, 24 and 48 hours and that of IL-1β2 at 6, 12 and 24 hours after treatment with rgIFNγ (Fig. 5.7B). Similar to higher vertebrates,

rgIFNγ induced an increase mRNA levels of IL-12 subunits p35 and p40, where IL-12 p35 expression significantly increased at 6 hours and that of IL-12 p40 at 12 hours after treatment (Fig. 5.7C).

Recent reports showed that mammalian macrophages under certain circumstances are capable of expressing IFNγ mRNA (8, 10). Consequently I examined whether rgIFNγ treatment of goldfish macrophages affected its own mRNA level. Indeed, addition of rgIFNγ to goldfish macrophage cultures resulted in upregulation of IFNγ gene expression as early as 2 hours, and the highest mRNA levels were measured 12 hours after treatment (Fig. 5.7D).

It is well established that IFNγ by itself does not induce chemotactic response of inflammatory cells (4) and in some instances it may actually inhibit this process (14, 26). We examined the possible effect of goldfish rgIFNγ on gene expression of two goldfish chemokines, CXCL-8 (= IL-8) and CCL-1 (Fig. 5.9A,B). The transcript levels for both chemokines increased in macrophages treated with rgIFNγ ($P< 0.05$). Treatment of goldfish macrophages with rgIFNγ also induced the expression of the IFN inducible protein RSAD-2 (Fig. 5.8C).

Since rgIFNγ induced nitric oxide response of goldfish macrophages (Fig. 5.6) I examined the gene expression of two iNOS isoforms after treatment with rgIFNγ. As shown in Figure 5.8E, increased expression of iNOS A was observed as early as 6 hours after treatment and the message returned to control level by 72 hours. In contrast, iNOS isoform B was significantly upregulated starting at 48 hours after treatment of macrophages with rgIFNγ.

My work (Fig. 5.3) as well as that of others (17) showed that Poly I:C induced the gene expression of teleost IFNγ, and for this reason I tested whether rgIFNγ treatment affected the mRNA level of the Poly I:C receptor, Toll-like receptor 3 (TLR-3). Interestingly, the gene expression of TLR3 gradually declined after rgIFNγ treatment such that at 48 and 72 hours after treatment mRNA levels were significantly lower than those observed at 0 hour time point (Fig. 5.8D).

Given the fact that IFNγ is generally known as the "macrophage activating cytokine", I examined whether rgIFNγ treatment affected the expression of TGFβ, which is generally known as the "macrophage deactivating cytokine". As shown in Figure 5.8F, the addition of rgIFNγ to macrophage cultures did not significantly alter TGFβ mRNA levels throughout the observation period.

3. DISCUSSION

In this chapter, I report on the cloning and molecular characterization of goldfish IFNγ, and comprehensive gene expression and functional analyses of the molecule. The goldfish IFNγ sequence shared the highest homology with the zebrafish IFNγ and had all the hallmark features present in previously identified IFNγs, including the IFNγ signature sequence and nuclear localization signal [15.19]. Phylogenetic analysis revealed that goldfish IFNγ grouped closely with other teleost IFNγs, in particular with zebrafish IFNγ and catfish IFNγ (splice variants a & b), and to a lesser extent with salmonid and pufferfish IFNγs and zebrafish IFNγrelated (rel) and catfish IFNγrel (data not shown). This close

phylogenetic relationship of the goldfish IFNγ to the zebrafish IFNγ and the catfish IFNγ suggests that two isoforms of IFNγ may also exist in goldfish. This argument is strengthened by a recent report where two isoforms of IFNγ have been reported in carp (28), a close cyprinid relative of the goldfish. Subsequent to the work presented in this chapter, I was successful at identifying the goldfish IFNγrel cDNA.

Quantitative gene expression analysis of goldfish IFNγ indicated high mRNA levels in the spleen. The IFNγ mRNA levels in the other tissues examined (brain, gill, kidney, intestine and muscle) were significantly lower than those in the spleen. The tissue gene expression profiles of fish IFNγ isoforms have been reported to be variable between different fish species. For example, semi-quantitative gene expression analysis revealed that catfish, IFNγrel was broadly expressed in different tissues whereas catfish IFNγ was expressed only in head kidney and spleen (21). In contrast, the expression of zebrafish and trout IFNγ isoforms in different tissues was evident only after treatment with activating agents (16, 49).

In this study, I also measured IFNγ mRNA levels in different cell populations obtained from normal fish, including splenocytes, PBL, kidney leukocytes, primary kidney granulocytes and primary monocytes and macrophages of the goldfish. The highest IFNγ gene expression was observed in PBL and splenocytes and to a lesser extent in granulocytes, macrophages and monocytes. In mammals, the primary producers of IFNγ are T helper 1 lymphocytes and NK cells following activation with various agents (1-3). IFNγ

expression in catfish PBL was upregulated following allogenetic stimulation (21). In trout, increased levels of IFNγ mRNA were observed in head kidney leukocytes after treatment of the cells with PHA or Poly I:C (49). My results confirm the above observation in that treatment of goldfish kidney leukocytes with PHA and in mixed leukocyte reactions, induced an upregulation of goldfish IFNγ gene expression. The induction of IFNγ mRNA in kidney leukocytes by Poly I:C was variable and was influenced by duration of treatment. Furthermore, treatment of kidney leukocytes, PBL and spleenocytes with rgTNFα-2 significantly enhanced IFNγ gene expression in these three primary cell populations. This is the first observation that fish TNFα can induce higher IFNγ mRNA levels in teleosts.

Nathan *et al.* (25) identified IFNγ in mammals and showed that it induced potent antimicrobial responses in cells of the myeloid lineage. Subsequently, it was shown that IFNγ activated macrophages for production of reactive oxygen (5) and nitrogen intermediates (20) and enhanced phagocytic activity (35). In addition, IFNγ was found to be an essential cytokine for host resistance to a variety of obligate intracellular pathogens, including the protozoan *Leishania major* (2, 3). Zou *et al.* (17) previously demonstrated that a recombinant trout IFNγ primed adherent trout-kidney leukocyte populations for an enhanced respiratory burst response. Recombinant goldfish IFNγ primed primary kidney monocytes and granulocytes for enhanced production of reactive oxygen intermediates once the cells were triggered with phorbol ester, PMA. The rgIFNγ also induced a dose-dependent nitric oxide response and enhanced phagocytic

response of goldfish macrophages and monocytes, respectively. This is the first report that examined the influence of teleost IFNγ on a defined immune cell population of fish.

Mammalian IFNγ and TNFα, induce a plethora of responses, many of which are additive as well as redundant (12). Recently, I have characterized goldfish TNFα2 and generated a recombinant protein (22). In addition to using rgTNFα2 as a positive control reagent in the functional assays, we investigated whether there was an additive relationship between rgIFNγ and rgTNFα2. My results clearly indicate that goldfish rgIFNγ and rgTNFα2 cooperate to elicit enhanced nitric oxide and phagocytic responses of macrophages and monocytes, respectively, than individual cytokines alone. Furthermore, when these cytokines were combined in priming the monocyte, enhanced respiratory burst responses were observed. These results indicate that as in mammals, teleost IFNγ and TNFα to induce optimal antimicrobial responses of monocytes/macrophages and to our knowledge represent the first observation of cooperation among proinflammatory cytokines in teleosts.

I also observed a decrease in the production of reactive oxygen intermediates in both monocytes and granulocytes when cell cultures were treated with higher concentrations of rgIFNγ in the presence of 10 ng/mL of rgTNFα2. This decrease in the respiratory burst response was similar to those observed in mammalian systems where higher concentrations of IFNγ and TNFα have been shown to down-regulate the gene expression of p47phox, the component of a

functional NADPH oxidase, the enzyme required for the respiratory burst response (5).

I measured mRNA levels of a number of different proinflammatory genes of goldfish macrophages after treatment with rgIFNγ. In addition to upregulating its own mRNA level in activated macrophages, goldfish IFNγ increased the expression of cytokines TNFα1, TNFα2, IL1β1, IL1β2, IL-12 p35, IL-12 p40, interferon inducible/anti-viral protein RSAD-2, chemokines CXCL-8 and CCL-1, and the enzyme that participates in the production of nitric oxide, iNOS isoforms A and B. The upregulation of TNFα (18), IL-1β (37) and IL-12 (19, 44) in macrophages in response to IFNγ has been reported for mammalian systems but not teleosts.

In general, Type I interferons (e.g. IFNα) are known for their antiviral properties, whereas Type II interferons (e.g. IFNγ) are known for induction of antimicrobial responses in inflammatory cells. However, in mammals, IFNγ has also been shown to influence cellular antiviral mechanisms (36, 48) and induction of gene expression of IFN-inducible proteins (17, 38, 46, 47). Since the expression of IFNγ was upregulated in goldfish macrophages treated with Poly I:C (a compound that resembles dsRNA normally present after viral replication) I examined whether rgIFNγ influenced the gene expression of RSAD-2, an IFN-inducible protein whose mammalian counterparts have been shown to inhibit cytomegalovirus infection (7). As shown in this study, treatment of goldfish macrophages with rgIFNγ also induced RSAD-2 expression, that has recently

been shown to be upregulated in trout spleen following injection of fish with Poly I:C (31).

Although IFNγ by itself does not induce chemotactic response of neutrophils and monocytes (4) it certainly participates in this important inflammatory process by enhancing the expression of various chemokines (6, 22, 29). Interestingly, mammalian IFNγ has also been shown to abrogate the chemotactic response of both eosinophils (25) as well as monocytes (24) in a concentration-dependent manner, suggesting a complex immunomodulatory role of this cytokine in inflammatory response. My results indicate that treatment of goldfish macrophages with rgIFNγ enhanced the expression of CXCL-8 and CCL-1, that have been shown to induce chemotactic responses in mammalian granulocytes (24) and monocytes (33), respectively.

In mammals, IFNγ is primarily produced by activated T lymphocytes (T helper 1 cells) and NK cells. However, there are also reports in the literature that macrophages can be induced to produce IFNγ. For example, IFNγ itself has been demonstrated to induce its own expression in mouse monocytes and macrophages (8, 10). My results also indicate that treatment of goldfish macrophages with rgIFNγ upregulated the IFNγ mRNA in these cells, suggesting a positive feedback loop that may be important for the amplification of the antimicrobial responses of teleost macrophages.

Transforming growth factor-beta and IFNγ have been documented to have numerous antagonistic roles (27, 30, 40). Recently, I reported that recombinant goldfish TGFβ down-regulated antimicrobial responses of macrophages activated

by rgTNFα2 (13). For this reason, I examined whether treatment of goldfish macrophages with rgIFNγ affected the expression of TGFβ, and found that rgIFNγ did not significantly influence TGFβ mRNA levels in macrophages.

The results of this study demonstrate a specific role for goldfish IFNγ in regulation of antimicrobial responses of macrophages and granulocytes and a general role for this cytokine in mediation of teleost inflammatory responses.

Figure 5.1. Goldfish IFNγ nucleotide and predicted protein sequences. The start codon (ATG) is indicated with a line above, and the stop codon is indicated by an asterisk (*) below. The predicted signal sequence is boxed and the IFNγ signature sequence is double underlined. The polyadenylation signal (**AATAAA**) is bolded and the RNA instability motifs are underlined (ATTTA). The NLS (**RRRR**) is bolded and is in larger font.

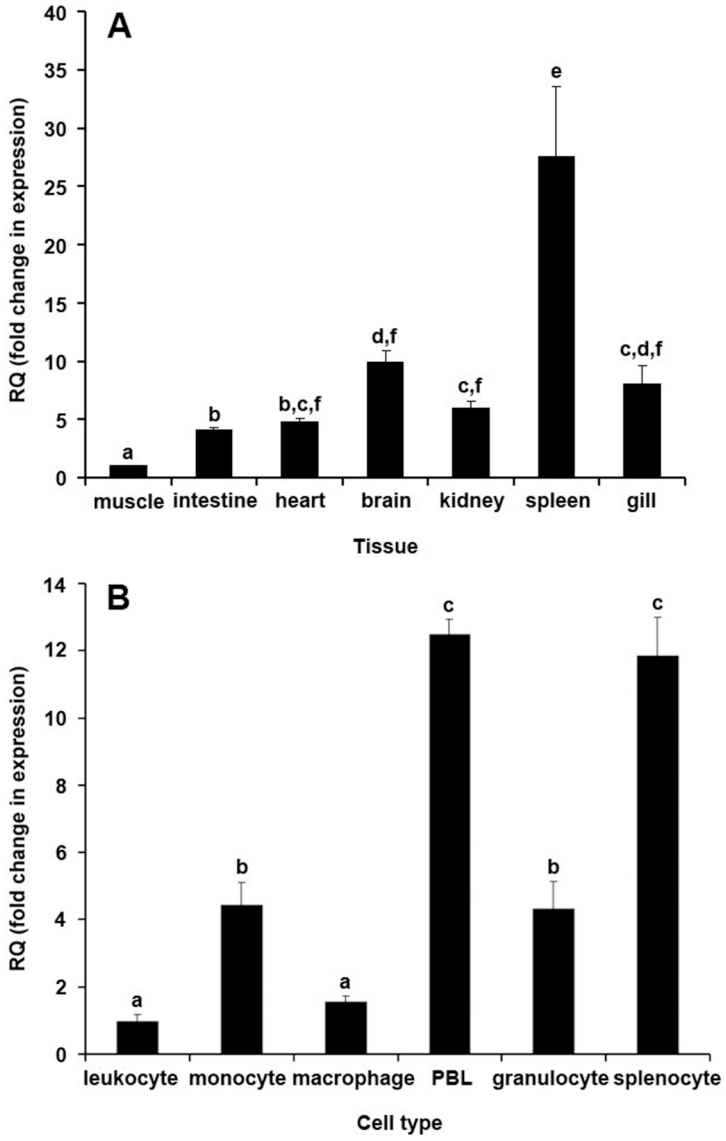

Figure 5.2. Quantitative IFNγ gene expression analysis in goldfish tissues and immune cell populations. (A) Goldfish IFNγ tissue expression analysis. The expression of goldfish IFNγ is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Analyses of the relative tissue expression data are for tissues from five fish (*n*=5). All results were normalized against the muscle IFNγ expression levels. **(B)** Goldfish IFNγ expression in different immune cell populations. Cells cultures were established from 5 fish (*n*=5) and the expression normalized against that of resting kidney leukocytes. Statistical analysis was performed using one-way ANOVA. Different letters above each bar denotes significantly different ($P < 0.05$), the same letter no statistical difference between groups.

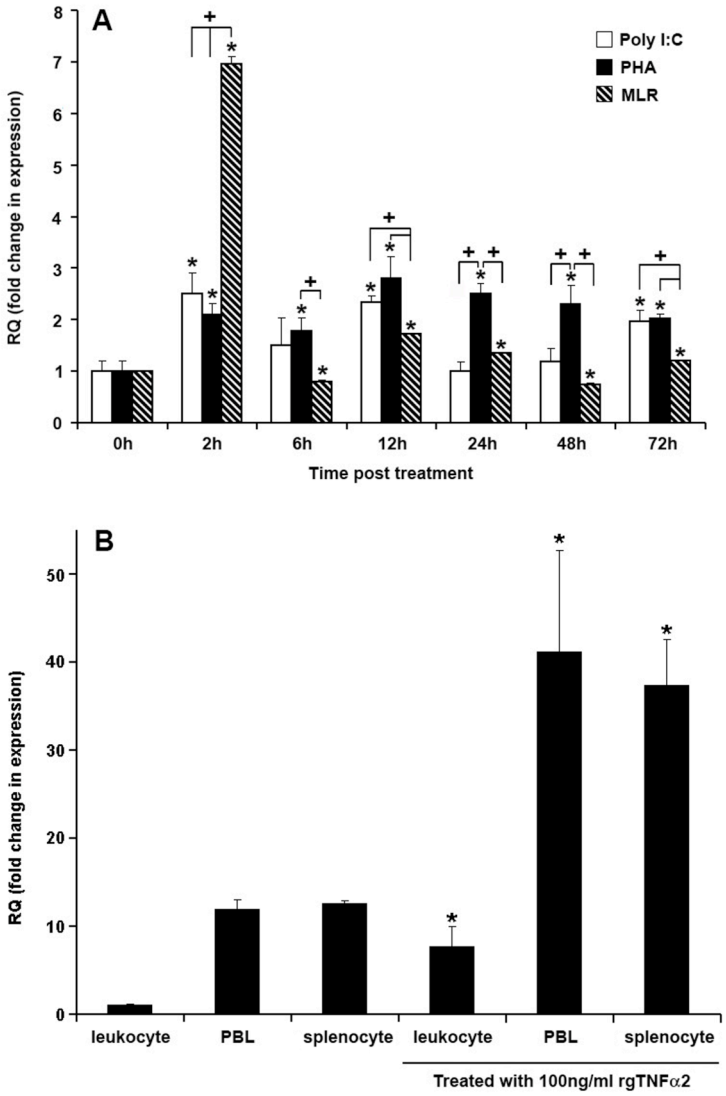

Figure 5.3. Quantitative IFNγ gene expression analysis in goldfish immune cell populations. (A) Goldfish IFNγ expression analysis in kidney-derived leukocytes stimulated with PHA, Poly I:C or MLR. The relative expression of IFNγ relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). Cell cultures established from 5 individual fish (*n*=5). The MLR expression analysis was performed using pooled cells and measured in triplicate for each time point. The expression data were normalized against those for the respective 0 hour time points. **(B)** Goldfish IFNγ expression analysis in immune populations treated with rgTNFα2. Total RNA was isolated from goldfish kidney leukocytes, PBL, kidney-derived granulocytes and splenocytes either immediately after cell isolation or 4 hours after treatment with 100 ng/mL of rgTNFα2. Expression analysis was performed as indicated above. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from respective 0 hour time points (A) or primary kidney leukocytes (B). (+) above the line denotes significantly different ($P< 0.05$) between experimental data bars identified by the lines.

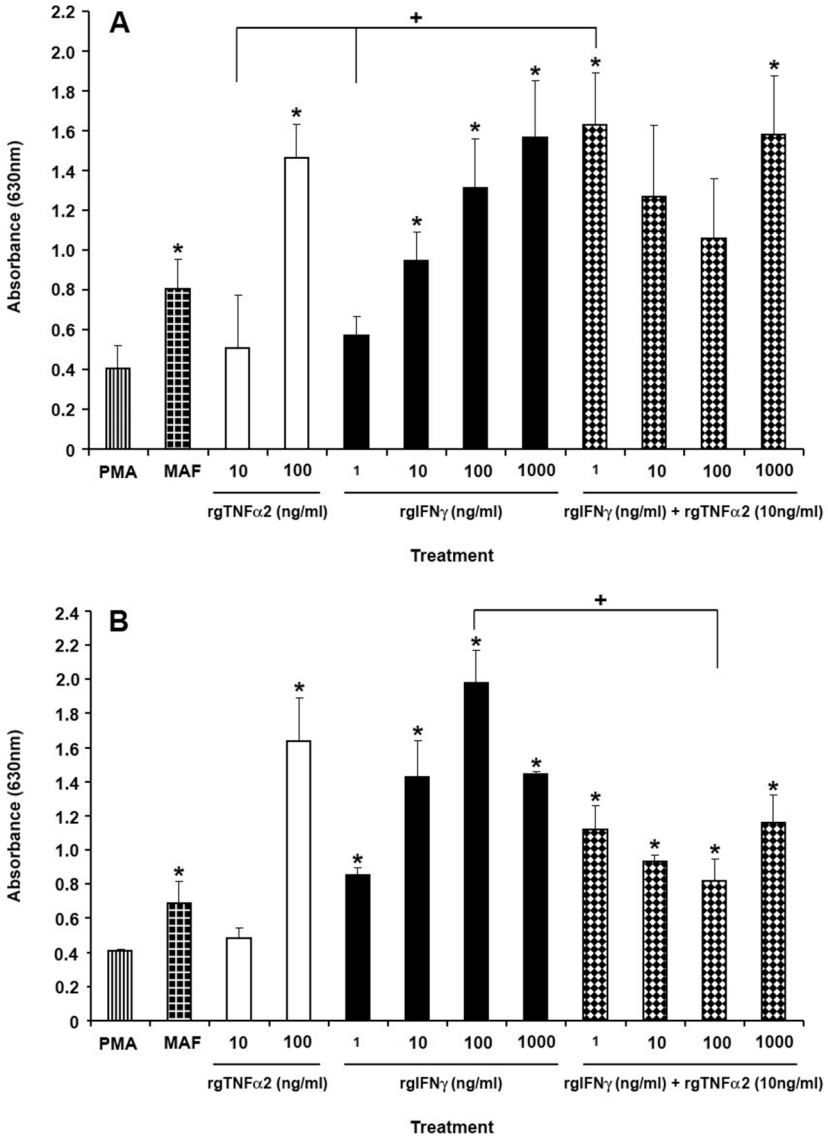

Figure 5.4. Recombinant goldfish IFNγ (rgIFNγ) primes goldfish monocytes and granulocytes for enhanced respiratory burst responses. The absorbance values of medium treated controls (no PMA) were subtracted from treatment values to factor in background NBT reduction. **(A)** Mean ± SEM of relative reactive oxygen intermediate production by monocytes (*n*=5). **(B)** Mean ± SEM of relative reactive oxygen intermediate production by granulocytes. (*) denotes statistically different ($P < 0.05$) compared to PMA only treated cells. (+) above the line denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

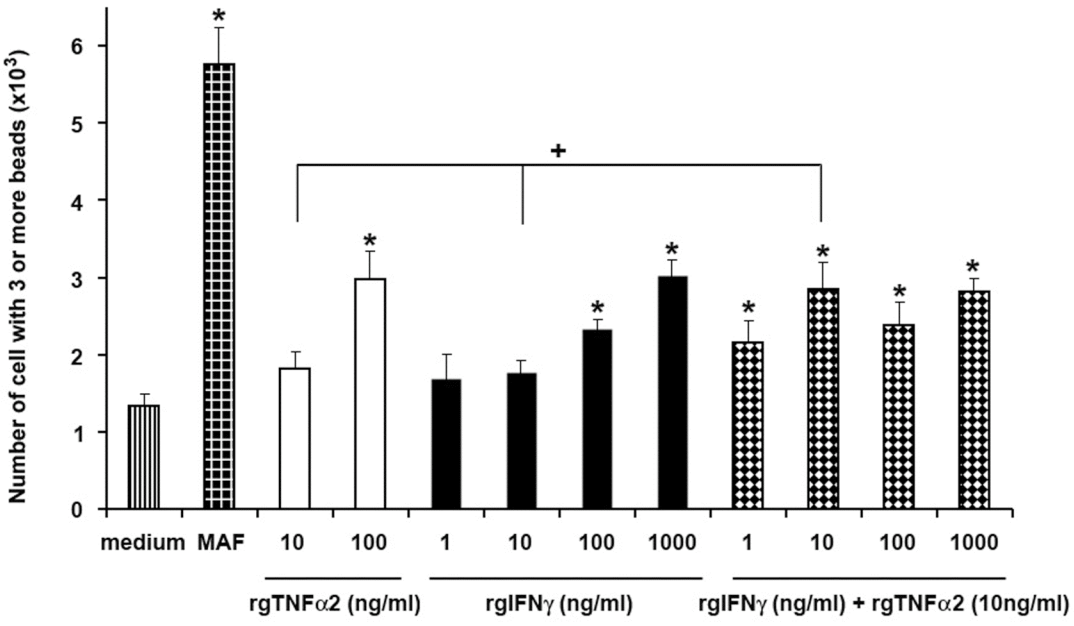

Figure 5.5. Recombinant goldfish IFNγ enhances phagocytic responses of goldfish monocytes. Results are means ± SEM of phagocytic response determined for monocytes cultures established from 5 individual fish (*n*=5). Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) above the line denotes statistical significance between experimental data bars identified by the lines.

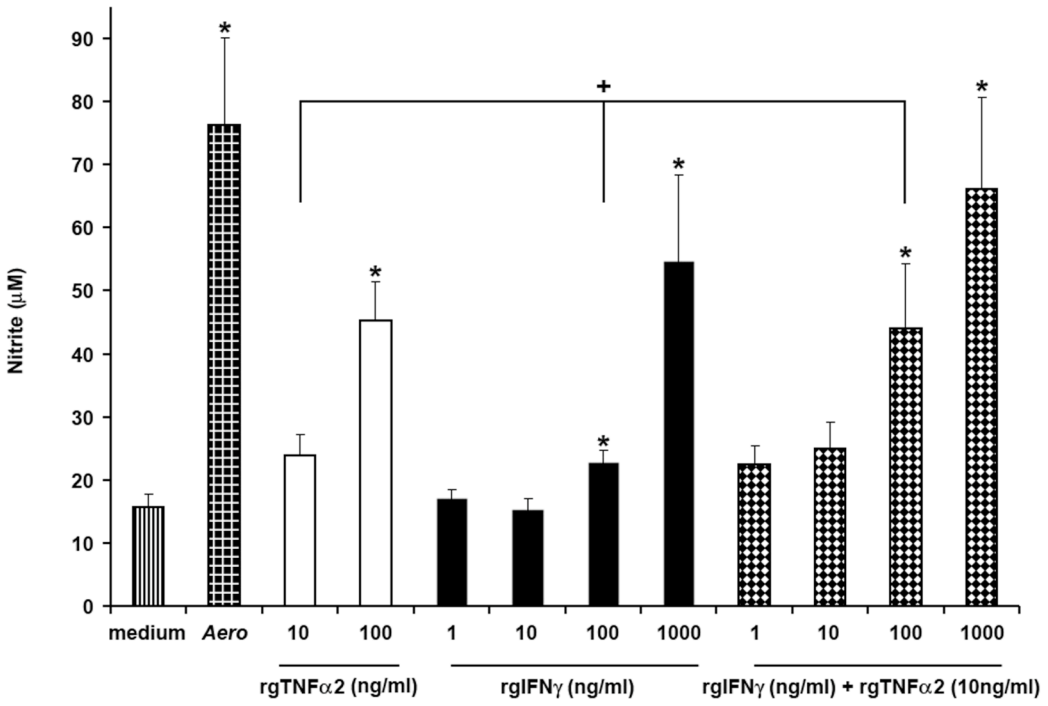

Figure 5.6. Recombinant goldfish IFNγ enhances nitric oxide responses of goldfish macrophages. Nitric oxide production was determined using the Griess reaction and nitrite concentration was determined using a nitrite standard curve. The results are Mean ± SEM nitrite determined for macrophage cultures established from 5 individual fish (n=5). Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) above the line denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

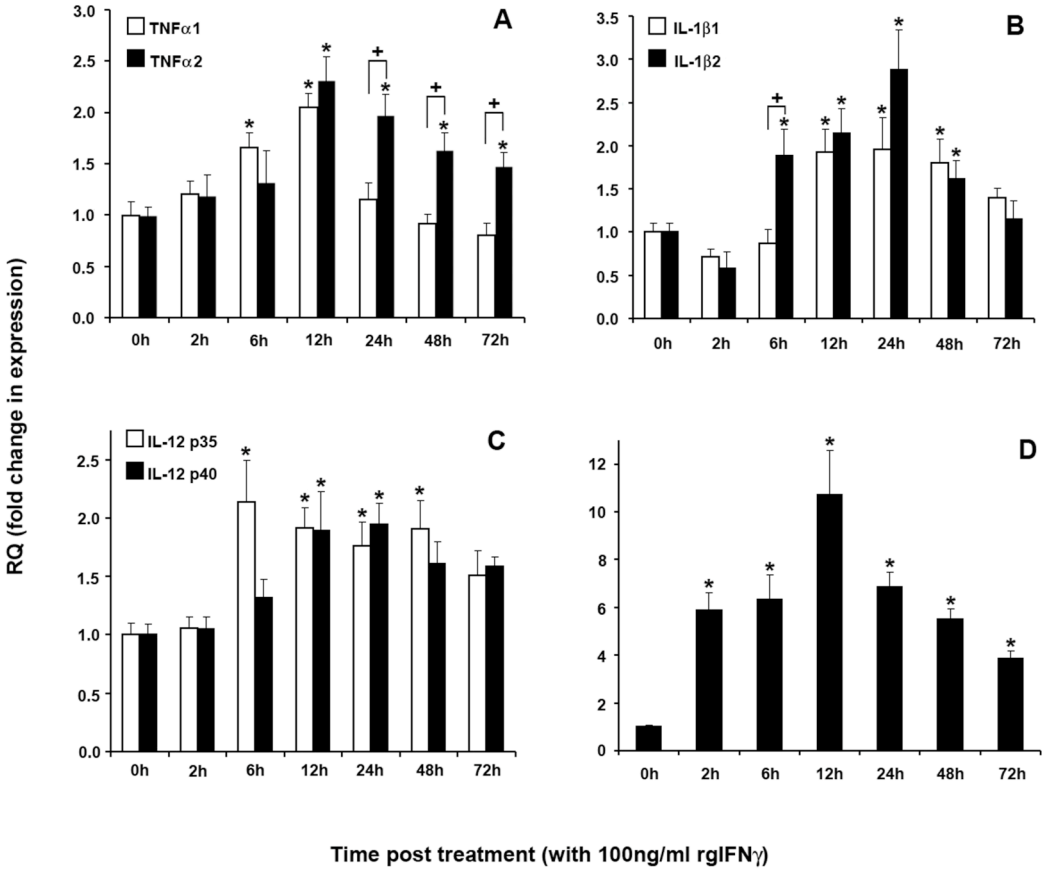

Figure 5.7. Quantitative analysis of proinflammatory cytokine gene expression in rgIFNγ–stimulated goldfish macrophages. The reported expression was relative to EF-1α. **(A)** TNFα1, TNFα2; **(B)** IL-1β1, IL-1β2; **(C)** IL-12-p35, IL-12-p40; and (**D)** IFNγ. The expression data were normalized against those of 0 hour time point for each of the genes. The results are Mean ± SEM of macrophage cultures established from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different from the respective 0 hour time point. (+) above the line denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

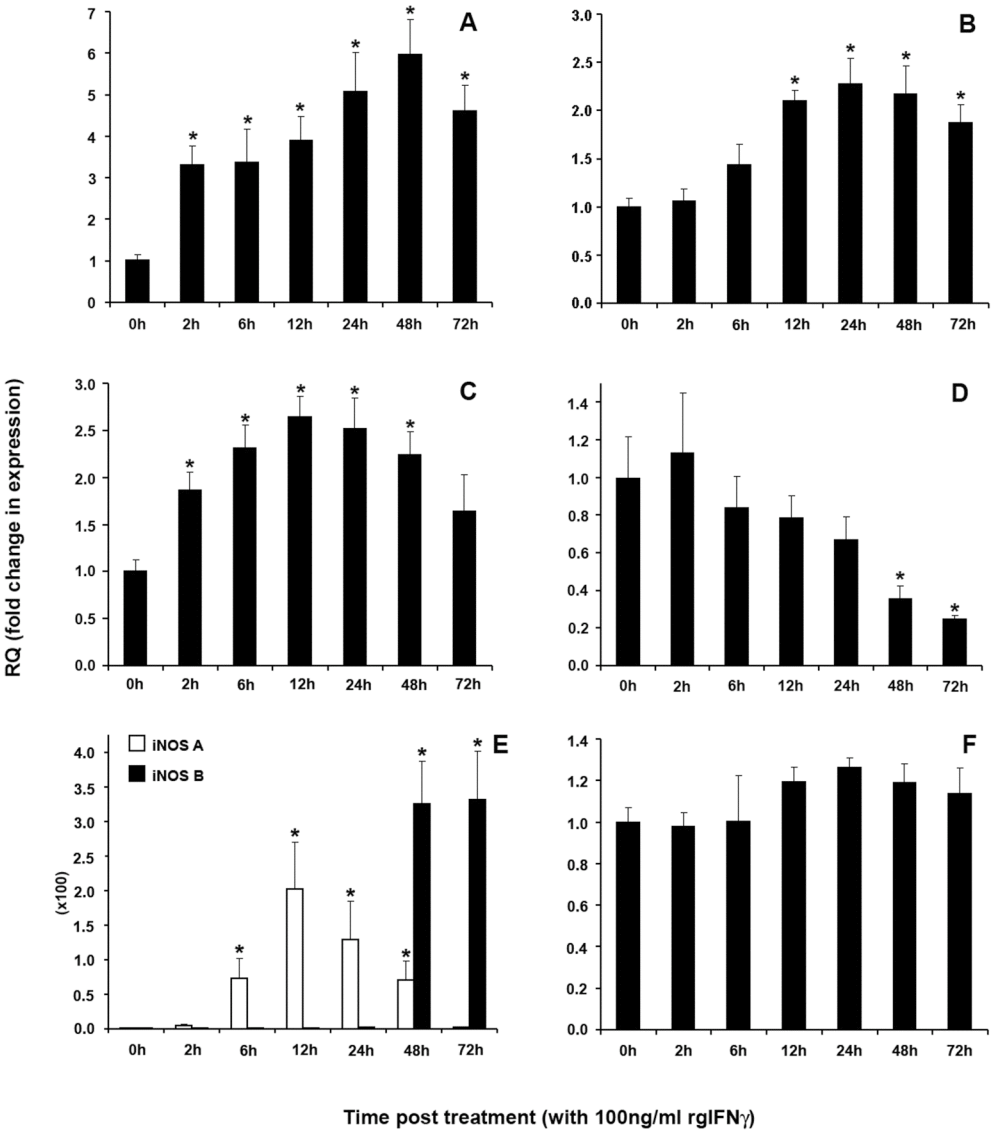

Figure 5.8. Quantitative analysis of immune gene expression in rgIFNγ-stimulated goldfish macrophages. The expression was relative to EF-1α. **(A)** CXCL-8; **(B)** CCL-1; **(C)** RSAD-2; **(D)** TLR-3; **(E)** iNOS isoforms A and B; and **(F)** TGFβ. The expression data were normalized against those of 0 hour time point for each of the genes. The results are Mean ± SEM of macrophage cultures established from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective 0 hour time point. (+) above the line denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

4. REFERENCES

1. **Belosevic, M., C. E. Davis, M. S. Meltzer, and C. A. Nacy.** 1988. Regulation of activated macrophage antimicrobial activities. Identification of lymphokines that cooperate with IFN-gamma for induction of resistance to infection. J Immunol **141:**890-896.
2. **Belosevic, M., D. S. Finbloom, M. S. Meltzer, and C. A. Nacy.** 1990. IL-2. A cofactor for induction of activated macrophage resistance to infection. J Immunol **145:**831-839.
3. **Belosevic, M., D. S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. J Immunol **143:**266-274.
4. **Canono, B. P., M. H. Middleton, and P. A. Campbell.** 1989. Recombinant mouse interferon-gamma is not chemotactic for macrophages or neutrophils. J Interferon Res **9:**79-86.
5. **Cassatella, M. A., F. Bazzoni, R. M. Flynn, S. Dusi, G. Trinchieri, and F. Rossi.** 1990. Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. J Biol Chem **265:**20241-20246.
6. **Chang, H. C., F. Hsu, G. J. Freeman, J. D. Griffin, and E. L. Reinherz.** 1989. Cloning and expression of a gamma-interferon-inducible gene in monocytes: a new member of a cytokine gene family. Int Immunol **1:**388-397.
7. **Chin, K. C., and P. Cresswell.** 2001. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci U S A **98:**15125.15130.
8. **Cockfield, S. M., V. Ramassar, J. Noujaim, P. H. van der Meide, and P. F. Halloran.** 1993. Regulation of IFN-gamma expression in vivo. IFN-gamma up-regulates expression of its mRNA in normal and lipopolysaccharide-stimulated mice. J Immunol **150:**717-725.
9. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med **178:**2243-2247.
10. **Di Marzio, P., P. Puddu, L. Conti, F. Belardelli, and S. Gessani.** 1994. Interferon gamma upregulates its own gene expression in mouse peritoneal macrophages. J Exp Med **179:**1731-1736.
11. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Dev Comp Immunol **32:**532-543.
12. **Gupta, J. W., M. Kubin, L. Hartman, M. Cassatella, and G. Trinchieri.** 1992. Induction of expression of genes encoding components of the respiratory burst oxidase during differentiation of human myeloid

cell lines induced by tumor necrosis factor and gamma-interferon. Cancer Res **52:**2530-2537.

13. **Haddad, G., P. C. Hanington, E. C. Wilson, L. Grayfer, and M. Belosevic.** 2008. Molecular and functional characterization of goldfish (Carassius auratus L.) transforming growth factor beta. Dev Comp Immunol **32:**654-663.
14. **Hu, Y., X. Hu, L. Boumsell, and L. B. Ivashkiv.** 2008. IFN-gamma and STAT1 arrest monocyte migration and modulate RAC/CDC42 pathways. J Immunol **180:**8057-8065.
15. **Huang, S. H., W., Althage, A., Hemmi, S., Bluethmann, H., Kamijo, R., Vilcek, J., Zinkernagel, RM., Aguet, M. .** 1993. Immune response in mice that lack the interferon-γ receptor. Science **259**.
16. **Igawa, D., M. Sakai, and R. Savan.** 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Mol Immunol **43:**999-1009.
17. **Kerr, I. M., Stark., G.R.** 1992. The antiviral effects of the interferons and their inhibition. J. Interferon Res. **12:**237– 240
18. **Koerner, T. J., D. O. Adams, and T. A. Hamilton.** 1987. Regulation of tumor necrosis factor (TNF) expression: interferon-gamma enhances the accumulation of mRNA for TNF induced by lipopolysaccharide in murine peritoneal macrophages. Cell Immunol **109:**437-443.
19. **Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri.** 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J Exp Med **183:**147-157.
20. **Martin, E., C. Nathan, and Q. W. Xie.** 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J Exp Med **180:**977-984.
21. **Milev-Milovanovic, I., S. Long, M. Wilson, E. Bengten, N. W. Miller, and V. G. Chinchar.** 2006. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics **58:**70-80.
22. **Minty, A., P. Chalon, J. C. Guillemot, M. Kaghad, P. Liauzun, M. Magazin, B. Miloux, C. Minty, P. Ramond, N. Vita, and et al.** 1993. Molecular cloning of the MCP-3 chemokine gene and regulation of its expression. Eur Cytokine Netw **4:**99-110.
23. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol **7:**145.173.
24. **Murphy, P. M.** 1997. Neutrophil receptors for interleukin-8 and related CXC chemokines. Semin Hematol **34:**311-318.
25. **Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin.** 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med **158:**670-689.
26. **Park, C. S., E. N. Choi, J. S. Kim, Y. S. Choi, T. Y. Rhim, H. S. Chang, and I. Y. Chung.** 2005. Interferon-gamma inhibits in vitro

mobilization of eosinophils by interleukin-5. Int Arch Allergy Immunol **136:**295.302.

27. **Park, I. K., J. J. Letterio, and J. D. Gorham.** 2007. TGF-beta 1 inhibition of IFN-gamma-induced signaling and Th1 gene expression in CD4+ T cells is Smad3 independent but MAP kinase dependent. Mol Immunol **44:**3283-3290.
28. **Perussia, B.** 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. Curr. Opin. Immunol. **3:**49– 55
29. **Proost, P., A. Wuyts, and J. Van Damme.** 1996. Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. J Leukoc Biol **59:**67-74.
30. **Reardon, C., and D. M. McKay.** 2007. TGF-beta suppresses IFN-gamma-STAT1-dependent gene transcription by enhancing STAT1-PIAS1 interactions in epithelia but not monocytes/macrophages. J Immunol **178:**4284-4295.
31. **Rise, M. L., J. Hall, M. Rise, T. Hori, A. K. Gamperl, J. Kimball, S. Hubert, S. Bowman, and S. C. Johnson.** 2008. Functional genomic analysis of the response of Atlantic cod (Gadus morhua) spleen to the viral mimic polyriboinosinic polyribocytidylic acid (pIC). Dev Comp Immunol **32:**916-931.
32. **Robertsen, B.** 2006. The interferon system of teleost fish. Fish Shellfish Immunol **20:**172-191.
33. **Roos, R. S., M. Loetscher, D. F. Legler, I. Clark-Lewis, M. Baggiolini, and B. Moser.** 1997. Identification of CCR8, the receptor for the human CC chemokine I-309. J Biol Chem **272:**17251-17254.
34. **Sad S, M. R., Mosmann TR.** 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or T2 cytokines. Immunity **2:**271– 279.
35. **Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedersky, B. S. Finkle, and M. A. Palladino, Jr.** 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J Immunol **135:**2069-2073.
36. **Shikata, A., T. Sugimoto, H. Hosoi, Y. Sotozono, T. Shikata, T. Sawada, and L. F. Parada.** 1994. Increased expression of trk proto-oncogene by gamma-interferon in human neuroblastoma cell lines. Jpn J Cancer Res **85:**122-126.
37. **Sone, S. E. O., K Mizuno, S Yano, Y Nishioka, T Haku, A Nii, and T Ogura.** 1994. Production of IL-1 and its receptor antagonist is regulated differently by IFN-gamma and IL-4 in human monocytes and alveolar macrophages. Eur Respir J **7:**657-663.
38. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Virus Res **38**.
39. **Stevenson, M. M., M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba.** 1990. Role of endogenous gamma interferon in host response to infection with blood-stage Plasmodium chabaudi AS. Infect Immun **58:**3225.3232.

40. **Takaki, H., Y. Minoda, K. Koga, G. Takaesu, A. Yoshimura, and T. Kobayashi.** 2006. TGF-beta1 suppresses IFN-gamma-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation. Genes Cells **11:**871-882.
41. **Trinchieri, G., and P. Scott.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. Res Immunol **146:**423-431.
42. **Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley.** 1994. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. J Exp Med **179:**1367-1371.
43. **Wheelock, E. F.** 1965. nterferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. Science **149:**310– 311.
44. **Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida.** 1994. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. Biochem Biophys Res Commun **198:**857-861.
45. **Young, H. A., Hardy, K.J.** 1995. Role of interferon-γ in immune cell regulation. J. Leuk. Biol. **58:**373– 381.
46. **Zeng, X., T. A. Moore, M. W. Newstead, J. C. Deng, N. W. Lukacs, and T. J. Standiford.** 2005. IP-10 mediates selective mononuclear cell accumulation and activation in response to intrapulmonary transgenic expression and during adenovirus-induced pulmonary inflammation. J Interferon Cytokine Res **25:**103-112.
47. **Zeremski, M., M. Markatou, Q. B. Brown, G. Dorante, S. Cunningham-Rundles, and A. H. Talal.** 2007. Interferon gamma-inducible protein 10: a predictive marker of successful treatment response in hepatitis C virus/HIV-coinfected patients. J Acquir Immune Defic Syndr **45:**262-268.
48. **Ziegler, S. F., C. B. Wilson, and R. M. Perlmutter.** 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene (hck) after macrophage activation. J Exp Med **168:**1801-1810.
49. **Zou, J., A. Carrington, B. Collet, J. M. Dijkstra, Y. Yoshiura, N. Bols, and C. Secombes.** 2005. Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. J Immunol **175:**2484-2494.
50. **Zou, J., Y. Yoshiura, J. M. Dijkstra, M. Sakai, M. Ototake, and C. Secombes.** 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish Shellfish Immunol **17:**403-409.

CHAPTER VI: MOLECULAR CHARACTERIZATION OF NOVEL INTERFERON GAMMA RECEPTOR 1 ISOFORMS IN ZEBRAFISH (*Danio rerio*) AND GOLDFISH (*Carassius auratus* L.)[4]

1. Introduction

Interferon gamma (IFNγ) is a highly pleotropic pro-inflammatory and anti-viral cytokine produced primarily by activated Th1 phenotype CD4+ cells (29), CD8+ cells (33) and natural killer (NK) cells (31). Aside from its initial identification (42) and subsequent characterization for its unique anti-viral roles (24, 36), IFNγ has been associated with the facilitation of a Th1-induced cell mediated immunity (39, 43). Additionally, this cytokine has been demonstrated to being central to host defenses against numerous infectious diseases (3, 4, 36, 37). This was further demonstrated using IFNγ gene knock-out mice that were unable to control infections with *Leishmania major* (41), *Listeria monocytogenes* (19) and *Mycobacteria* (6).

The biological effects mediated by IFNγ are initiated by the formation of anti-parallel homodimers (7) and subsequent interaction with a cognate receptor complex composed of interferon gamma receptors 1 and 2 (IFNGR1 and IFNGR2), also referred to as alpha and beta chains, respectively. IFNγ dimers directly interact with two molecules of IFNGR1 (5, 11, 40) and this interaction is

---

[4] A version of this chapter has been published: Grayfer, L., and M. Belosevic. 2009. Molecular characterization of novel interferon gamma receptor 1 isoforms in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus* L.). Molecular Immunology 46:3050-3059.

further stabilized by two molecules of the IFNGR2 (27), which facilitate receptor complex stability by interacting with IFNGR1 but not with the ligand. The formation of this active complex consisting of IFNγ:IFNGR1:IFNGR2 results in downstream signaling events through the JAK-STAT signaling pathway where, upon complex ligation. IFNGR1 is responsible for the recruitment and subsequent activation of JAK1 (20, 23) and STAT1 (10, 17) and IFNGR2 of JAK2 (1, 25, 34).

Homologues of the IFNγ have been identified in a number of bony fish (teleosts) including zebrafish (21), Japanese pufferfish (45), trout (44), Atlantic salmon (32), catfish (28), carp (38) and goldfish (14). Of these fish species, zebrafish, catfish, carp and goldfish appear to possess two isoforms of IFNγ, which differ markedly within each species in sequence identity and expression patterns (21, 28, 38). These were initially coined as IFNγ1 and IFNγ2, but since have been renamed as IFNγ related (IFNγrel) and IFNγ, respectively.

In contrast to the increasing pool of published data pertaining to IFNγ ligand biology, relatively little is known about the IFNγ receptors of fish. The annotated sequence for the zebrafish IFNGR1 can be found in the gene database. There has been a recent report on the identification of IFNGR1 and IFNGR2 in trout (12). The trout IFNGR1 and 2 shared many of the characteristics of mammalian counterparts, were widely expressed across tissues, and were differentially expressed in a trout cell line (12). Aside from this solitary report, to my knowledge there have been no other studies on the characterization of teleost IFNGRs.

In this chapter, I report on the identification of a novel isoform of the zebrafish (*Danio rerio*) IFNGR1 and the cloning of corresponding IFNGR1-1 and IFNGR1-2 isoforms in the goldfish (*Carassius auratus* L.). Predicted peptide analysis revealed that all known fish IFNGR1 species (including the ones described here) have potential JAK1 and STAT1 docking sites. Phylogenetic analysis grouped the teleost IFNGR1 proteins separately from those of higher vertebrates, with zebrafish and goldfish IFNGR1-2 protein sequences demonstrating a closer relationship to the recently identified trout IFNGR1. Comprehensive Q-PCR analysis revealed that IFNGR1-1 and IFNGR1-2 constitutive expression was different between zebrafish and goldfish. Whereas the goldfish IFNGR1-1 exhibited substantially greater gene expression in all tissues examined compared to IFNGR1-2, the constitutive gene expression of the two zebrafish IFNGR1 isoforms was similar in all tissues examined. Q-PCR analysis of goldfish immune cell populations revealed that IFNGR1-1 was more robustly expressed in all cell types compared to the IFNGR1-2. Incubation of goldfish macrophages with recombinant goldfish IFNγ (rgIFNγ) up-regulated gene expression of both IFNGR1-1 and IFNGR1-2, while treatment of cells with rgTNFα2 only increased the expression of IFNGR1-1. Treatment of goldfish macrophages with rgTGFβ caused modest increases in the mRNA levels of both IFNGR1-1 and IFNGR1-2 after prolonged treatment, while non-treated cells did not exhibit significant changes in mRNA levels of the two receptor isoforms. Recombinant extracellular domains of goldfish receptor isoforms (rgIFNGR1-1 and rgIFNGR1-2) were produced. Binding studies indicated that rgIFNGR1-1

bound to rgIFNγrel but not rgIFNγ, whereas the rgIFNGR1-2 preferentially bound to rgIFNγ and not to rgIFNγrel.

2. RESULTS

2.1. *In silico* analysis of zebrafish and goldfish IFNGR1-1 and IFNGR1-2

Synteny analysis of the zebrafish genome revealed the presence of a second isoform of an IFNGR1 on chromosome 13 (denoted here as IFNGR1-2) in addition to the previously identified zebrafish IFNGR1 isoform (denoted here as IFNGR1-1) present on chromosome 1 (Fig. 6.1). Comparisons of the human chromosome 6 with the zebrafish chromosome 1 indicated that the human genes syntenic to the human IFNGR1 and coding for peroxiredoxin 2 (PRDX2) and LTV1 homologue (*S. cerevisiae*) appear to have conserved localization relative to the zebrafish IFNGR1-1 on the zebrafish chromosome 1 (Fig. 6.1). Conversely, other genes syntenic to the IFNGR1 gene on the human chromosome 6 including TNF alpha-induced protein 3 (TNFAIP3), PERP TP53 apoptosis effector (PERP) and oligodendrocyte transcription factor 3 (OLIG3), are syntenic to the zebrafish IFNGR1-2 gene on the zebrafish chromosome 13 (Fig. 6.1).

RACE-PCR was employed to identify the goldfish IFNGR1-1 and IFNGR1-2 cDNA sequences corresponding to the zebrafish IFNGR1-1 and IFNGR1-2 sequences. The goldfish IFNGR1-1 displayed substantially longer 3' and 5' untranslated regions (UTRs) than those observed in the goldfish IFNGR1-2 (Fig. 6.2). The goldfish IFNGR1-1 3' UTR contained a typical polyadenylation signal (AATAAA) while goldfish IFNGR1-2 had a less common polyadenylation

signal (CATTAAA) in its 3' UTR (Fig. 6.2). While the goldfish IFNGR1-1 had 5 RNA instability motifs characteristic of Type II IFN transcripts (ATTTA, 1 in the 5' and 4 in the 3' UTRs), the goldfish IFNGR1-2 contained only a single instability motif in its 3' UTR (Fig. 6.2).

Protein alignments of the zebrafish and goldfish IFNGR1-1 and IFNGR1-2 with other known vertebrate IFNGR1 sequences indicated that the IFNGR1 amino acid sequence conservation across species was minimal (Fig. 6.3). With the exception of the zebrafish IFNGR1-1, all IFNGR1 protein sequences contained predicted signal peptide sequences in their respective N termini (Fig. 6.3). The extracellular regions of all sequences contained several, conserved cysteine repeats, presumably employed for protein conformation (Fig. 6.3). All of the examined sequences also had single trans-membrane domains in relatively similar positions across the residue sequences (Fig. 6.3). Additionally, the intracellular regions of all of the examined IFNGR1 sequences had putative JAK1 and STAT1 docking sites, with the zebrafish IFNGR1-2 and both of the goldfish isoforms having two possible JAK1 binding sites (Fig. 6.3).

Phylogenetic analysis of the zebrafish and goldfish IFNGR1 isoforms as well as other known vertebrate IFNGR1 and IFNGR2, confirmed the close relationship of the respective zebrafish and goldfish IFNGR1 isoforms (Fig. 6.4). The trout IFNGR1 grouped closest to the zebrafish and goldfish IFNGR1-2 (Fig. 6.4). All of the fish IFNGR1 sequences grouped separate from but closer to the other vertebrate IFNGR1 and further still from IFNGR2 sequences (Fig. 6.4).

2.2. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in zebrafish tissues

Quantitative gene expression analysis of the zebrafish IFNGR1-1 and IFNGR1-2 in healthy fish tissues was performed (Fig. 6.5). The two IFNGR1 isoforms exhibited generally similar expression patterns with the highest transcript levels for both receptor isoforms observed in muscle, followed closely by spleen and brain (Figure 6.5). Lower IFNGR1-1 and IFNGR1-2 mRNA levels were measured in zebrafish gill, kidney, heart and intestine tissues (Fig. 6.5). The gene expression of the zebrafish IFNGR1-1 was significantly higher than that of IFNGR1-2 in all of the tissues examined with the exception of muscle (Fig. 6.5).

2.3. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in goldfish tissues

In contrast to zebrafish receptor expression patterns, the goldfish IFNGR1-1 exhibited substantially greater magnitudes of gene expression (several logs difference) across all goldfish tissues examined, as compared to IFNGR1-2 (Fig. 6.6). The transcript levels for IFNGR1-1were highest in the spleen, moderate in the kidney, brain and gill tissues and lowest in the muscle, intestine and heart (Fig. 6.6). Conversely, the gene expression of IFNGR1-2 was greatest in the brain, moderate in most other tissues examined and lowest in the muscle tissues (Fig. 6.6A).

2.4. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in goldfish immune cells

The gene expression of IFNGR1-1 and IFNGR1-2 was investigated in different goldfish immune cell populations (Fig. 6.6B). The cell populations examined included peripheral blood leukocytes (PBL), kidney-derived granulocytes, splenocytes as well as FACS-sorted goldfish monocytes and macrophages. In comparison to the tissue expression patterns described above, the differences between the transcript levels of IFNGR1-1 and IFNGR1-2 across goldfish immune populations were not as drastic (Fig. 6.6B). However, as observed across goldfish tissues, the mRNA levels of IFNGR1-1 were also greater than those of IFNGR1-2 in all cell populations examined (Fig. 6.6B). The gene expression of IFNGR1-1 was highest in monocytes, relatively similar in PBLs, splenocytes and granulocytes and lower in mature macrophages (Fig. 6.6B). In contrast, IFNGR1-2 gene expression was only slightly greater in monocytes as compared to the other cell types (Fig. 6.6B).

2.5. Analysis of IFNGR1-1 and IFNGR1-2 gene expression in activated goldfish macrophages

In order to examine possible cytokine-induced changes in goldfish macrophage IFNGR1-1 and IFNGR1-2 expression, mature macrophage cultures (6 to 8 days of cultivation) were either left untreated or stimulated with 100 ng/mL of rgIFNγ, rgTGFβ or rgTNFα2. The addition of rgIFNγ to macrophage cultures resulted in significant increases in the expression of both IFNGR1-1 and

IFNGR1-2 at 12 and 24 h after treatment (Fig. 6.7A). Treatment of macrophages with rgTGFβ induced a modest increase in the gene expression of IFNGR1-1 and IFNGR1-2 at later times following stimulation, where the mRNA levels of both receptor isoforms were significantly elevated at 24 and 72 h after treatment (Fig. 6.7B). In contrast, treatment with rgTNFα2 induced higher expression of IFNGR1-1 at 6 and 12 h after treatment while not altering the mRNA levels of IFNGR1-2 (Fig. 6.7C). Although slight variability in gene expression of both IFNGR1 isoforms was observed in non-treated cells, no significant changes in IFNGR1-1 or IFNGR1-2 mRNA levels were evident during a 72 h time period (Fig. 6.7D).

2.6. *In vitro* analysis of rgIFNγrel and rgIFNγ interaction with rgIFNGR1-1 and rgIFNGR1-2

To determine whether goldfish IFNGR1-1 and/or IFNGR1-2 interacted with their potential ligands, rgIFNγrel and rgIFNγ, the extracellular domains of both receptors and the active forms of the two ligands were produced as recombinant proteins. When cross-linked with DSS, the rgIFNγrel and rgIFNγ both displayed band shifts indicative of dimerization (Fig. 6.8A, B and Fig. 6.8C, D, respectively). Following the cross-linking of the rgIFNGR1-1 or the rgIFNGR1-2, no bands could be visualized by methods employed here (Fig. 6.8A, C and Fig. 6.8B, D, respectively). When I co-incubated rgIFNγrel and rgIFNGR1-1 and then cross-linked with DSS, an additional band was observed corresponding to the predicted size of a ligand dimer-receptor dimer complex

(Fig. 6.8A). Cross-linking of co-incubated rgIFNγrel and rgIFNGR1-2 did not yield any detectable additional bands (Fig. 6.8B). Cross-linking of co-incubated rgIFNγ with rgIFNGR1-2, but not with rgIFNGR1-1 also resulted in a band shift indicative of a ligand dimer-receptor dimer interaction (Fig. 6.8D and Fig. 6.8C, respectively)

3. DISCUSSION

Unlike mammals, that have a single IFNGR1 isoform, I report that zebrafish and goldfish each possess two distinct IFNGR1 isoforms. My results indicate that these IFNGR1-1 and IFNGR1-2 isoforms preferentially bound to corresponding ligands, IFNγrel and IFNγ, respectively, suggesting that the type II interferon system of these fish species is distinct from those of higher vertebrates.

Gene synteny comparisons of the human and the zebrafish IFNGR1 loci indicated that several genes neighboring the human IFNGR1 gene on chromosome 6 (PDX2, OLIG3, TNFAIP3, PERP, LTV1) were segregated to either zebrafish chromosome 1 with IFNGR1-1 (PRDX2, LTV1) or to chromosome 13 with the IFNGR1-2 (TNFAIP3, PERP, OLIG3). Since the conservation of syntenic gene organization across evolution suggests a biological relationship between the neighboring genes, the fact that the zebrafish IFNGR1 isoforms have been segregated to distinct chromosomes with some but not other neighboring genes implies that while all other characterized metazoans mediate the type II IFN signaling through a single receptor, in zebrafish and goldfish the type II IFN biological outcomes might be partitioned between two distinct

IFNGR1 receptors. It is also possible that signaling through one IFNGR1 isoform in teleosts induces traditional pro-inflammatory roles associated with IFNγ whereas signaling through the second IFNGR1 isoform may induce novel and yet to be identified, distinct biological function(s) for type II IFNs.

This is the first report on the characterization of bony fish IFNGR1-1 and IFNGR1-2. The goldfish IFNGR1-1 and IFNGR1-2 were shared high identity with zebrafish IFNGR1-1 and IFNGR1-2, respectively. The 5' and 3' UTRs of IFNGR1-1 were substantially longer than those of IFNGR1-2. Present on the 3' UTRs of both goldfish isoforms as well as the 5' UTR of goldfish IFNGR1-1, were AU-rich RNA instability motifs, otherwise referred to as AREs (35, 46). The IFNGR1-1 transcript had substantially more AREs than IFNGR1-2. The degradation through AREs is a highly regulated process, with transcripts containing AREs elements displaying greatly increased stabilization during times of cell stress (13), stimulation with growth factors (26), and following neoplastic transformation (18). Furthermore, it has been demonstrated that under some circumstances, factors that normally facilitate AREs based RNA degradation, confer increased stabilization of their target transcripts (8, 30). This suggests a complex system for regulation of the teleost IFNGR1-1 and IFNGR1-2 transcript stability and that surface expression of these receptors may be subject to checkpoint regulation at multiple levels in the cell.

Phylogenetic analysis of known vertebrate IFNGR1s and IFNGR2s indicated that, as predicted, the respective IFNGR1-1 and IFNGR1-2 of zebrafish and goldfish were highly related. The trout IFNGR1 displayed greater

phylogenetic similarity to the zebrafish and goldfish IFNGR1-2. It is possible that a second, yet undefined isoform of IFNGR1 may be present in trout. However, it is known that zebrafish and goldfish have two isoforms of the IFNγ ligand, while trout has only one IFNγ isoform, suggesting that trout may also have a single IFNGR1 isoform.

The fish IFNGR1 protein sequences contain the predicted JAK1 and STAT1 binding sites, which are crucial for the activity of the mammalian IFNR1 (9, 10, 16). In mammals these docking sites correspond to LPKS and YDKPH sequences respectively, with the Y, D and H residues (**YD**KP**H**) crucial for the STAT1 activity (10, 16, 17) and the P residue (L**P**KS) dominantly involved in the JAK1 recruitment (17, 23). The zebrafish and goldfish IFNGR1-2, the goldfish IFNGR1-1 and the trout IFNR1 appeared to have 2 possible JAK1 sites that were in similar regions of respective proteins to the mammalian LPKS sequences. All of these contained the essential P residue but differed in the surrounding sequence compositions. Similarly, all of the fish IFNR1 isoforms displayed variations of the YDKPH sequence with conserved Y, D, and H residues and in very similar locations on respective protein sequences to the mammalian counterpart. These observations suggest that the JAK1-STAT1 signaling of the IFNGR1 has been conserved in teleosts.

Expression analysis of the zebrafish tissues demonstrated that IFNGR1-1 and IFNGR1-2 gene expression patterns were relatively similar, albeit with IFNGR1-1 displaying higher expression in most tissues. Conversely, the goldfish IFNGR1-1 expression was substantially higher (~1000 times higher) than that of

IFNGR1-2 for all tissues examined, with the highest IFNGR1-1 transcript levels in the spleen, followed by brain, kidney, gill and intestine. In contrast, the IFNGR1-2 gene expression was greatest in the brain followed by spleen, heart and the gill. Based on the above observations, it would appear that transcriptional regulation of each goldfish IFNGR1 isoform is distinct from the other and that different regulation mechanisms for respective IFNGR1 gene isoforms are in place for these two fish species. Furthermore, the drastic expression differences between the two goldfish isoforms may be indicative of the yet undefined functional differences conferred by respective IFNGR1 isoforms.

Amongst the goldfish immune cell populations, monocytes had the highest mRNA levels of both IFNGR1 isoforms, which was not surprising given the known immunomodulatory effects of IFNγ on monocytes/macrophages of vertebrates. Interestingly, the treatment of goldfish macrophages with rgIFNγ induced increased gene expression of both IFNGR1-1 and IFNGR1-2, while the treatment of macrophages with rgTNFα2 resulted in the up-regulation of the IFNGR1-1 isoform only. This supports my assertion that goldfish IFNGR1-1 and IFNGR1-2 isoforms may have distinct cellular regulatory mechanisms and possibly non-overlapping roles. The later observation is in contrast to the recent report that showed that trout recombinant IFNγ down-regulated the gene expression of the trout IFNGR1 (12). This discrepancy may be attributed to differences in cellular mechanisms of the IFNGR1 gene regulation between these two fish species. Alternatively, these differences might stem from

the fact that while the trout expression studies involved rainbow a trout gonadal cell line (RTG-2), the work described here utilized primary macrophage cultures.

My *in vitro* binding studies demonstrated that rgIFNγrel bound to the rgIFNGR1-1 but not rgIFNGR1-2. Similarly, I observed that under the described experimental conditions, rgIFNγ bound to rgIFNGR1-2 but not rgIFNGR1-1. It is also possible that each ligand may have higher affinity for the respective receptor, but still retained a reduced ability to interact with alternative receptor isoform beyond detection levels permitted by the Western blot. Cross-linking of equal concentrations of the individual ligands resulted in faint rgIFNγrel dimer bands but prominent rgIFNγ dimer band formation. Because the detection method employed here relied on antibody recognition of the poly-His tags on respective recombinant proteins, it is possible that cross-linking of the dimers may have imposed a protein conformation of rgIFNγrel that may have reduced access to the His tags. Similar reasoning could be applied to the observation that when cross-linked, both rgIFNGR1-1 and rgIFNGR1-2 failed to display any banding pattern. However, it is well documented that the mammalian IFNGR1 proteins do not undergo homotypic interactions and instead remain a distance apart during ligand interactions (5, 7, 40).

It is important to note that in addition to the expression profile differences of IFNγrel and IFNγ in respective teleost species, the peptide sequences of these cytokine isoforms demonstrate a great deal of diversity (Fig. 7.8). Although the fish IFNγrel and IFNγ contain slightly altered versions of IFNγ signature sequence ([I/V]-QX-[K/Q]-A-X^2-E-[L/F]-X^2-[I/V]), the sizes and amino acid

sequences of respective fish isoforms are substantially different. Additionally, only the IFNγ but not IFNγrel fish isoforms, have the nuclear localization signals that are thought to be essential for the mammalian and trout IFNγ activity (2, 22). Indeed, as outlined in chapter VII, the recombinant goldfish IFNγrel and IFNγ appear to possess distinct capabilities to elicit monocte/macrophage antimicrobial mechanisms (15). From an evolutionary perspective it seems less likely that a second cytokine receptor isoform would be retained rather than deleted following a gene duplication event, unless the signaling through that receptor conferred distinct biological function(s). Together, these observations indicate that cyprinids (zebrafish and goldfish) have evolved novel mechanisms of orchestrating the type II IFN-inducible responses, distinct from all other IFNγ systems characterized across vertebrate species.

The results presented in this chapter demonstrate that the type II interferon system of bony fish has substantially diverged from that of mammals. Because of the importance of this system in innate and adaptive immune responses in higher vertebrates, a more thorough understanding of the type II interferon system of lower vertebrates such as bony fish may shed light on the evolution of this crucial host defense pathway.

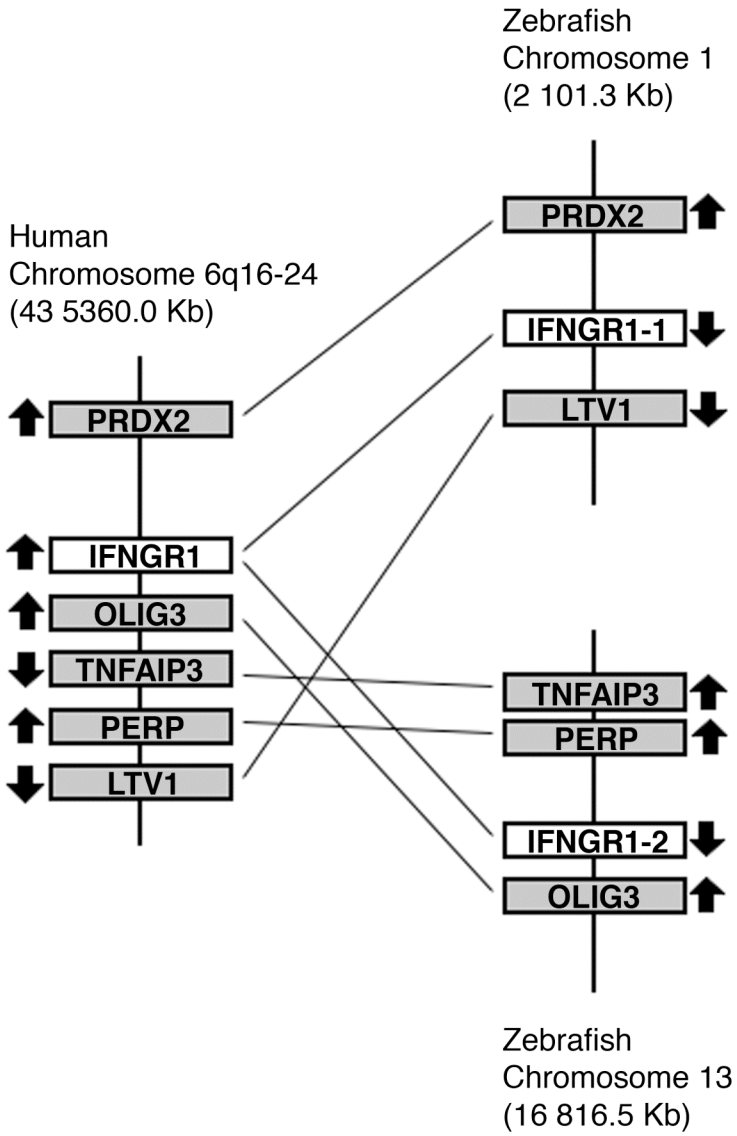

Figure 6.1. Synteny analysis of human and zebrafish IFNGR1 gene loci. The abbreviated genes are: interferon gamma receptor 1(IFNGR1); peroxiredoxin 2 (PRDX2); LTV1 homolog (*S. cerevisiae*) (LTV1); TNF alpha-induced protein 3 (TNFAIP3), PERP, TP53 apoptosis effector (PERP); oligodendrocyte transcription factor 3 (OLIG3). Arrows indicate the orientation of respective genes.

Figure 6.2. The untranslated region cDNA sequences of goldfish IFNGR1-1 and IFNGR1-2. The open reading frames are denoted by boxes. The polyadenlylation signals (*AATAAA*:IFNGR1-1 and *CATAAA*: IFNGR1) are in italic larger font. The RNA instability motifs (ATTTA) are underlined.

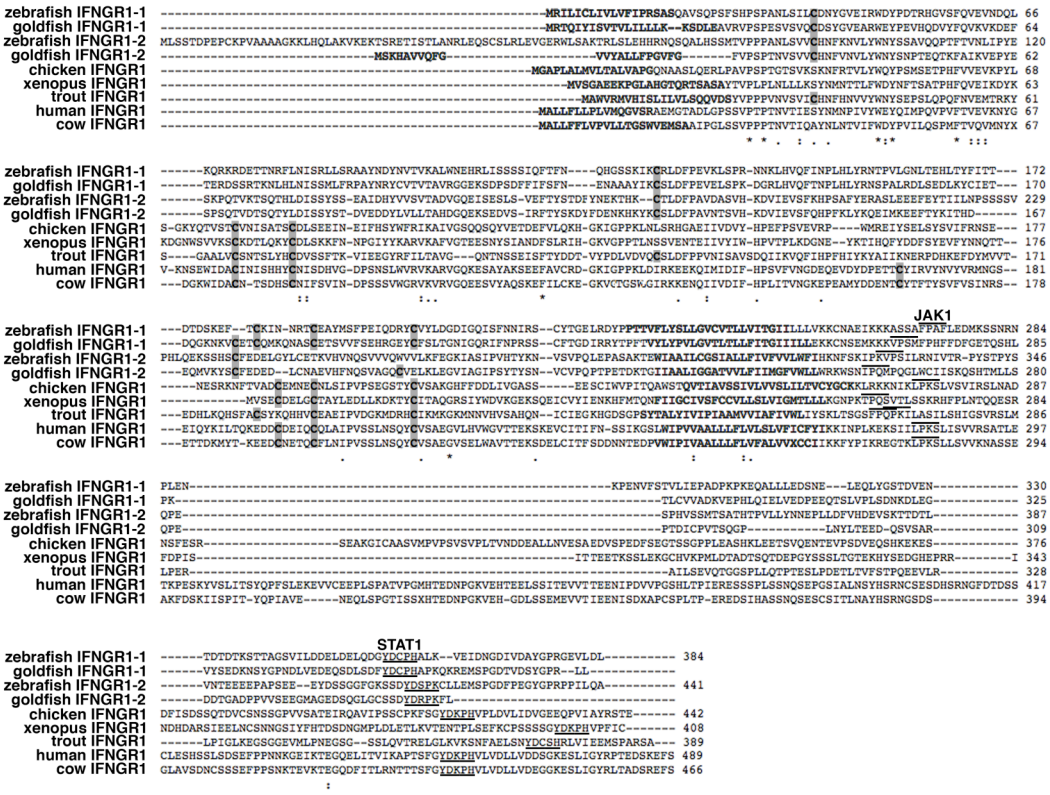

Figure 6.3. Protein sequence alignment of zebrafish and goldfish IFNGR1-1 and IFNGR1-2, trout and higher vertebrate IFNGR1s. Predicted signal sequences and transmembrane domains are in bold. The conserved cysteine residues are highlighted. Potential JAK1 binding sites are denoted with overhead lines and potential STAT1 binding sites are denoted by underhand lines. Fully conserved residues are indicated by an asterisk (*) below, partially conserved and semi-conserved substitutions are represented by ":" and ".", respectively.

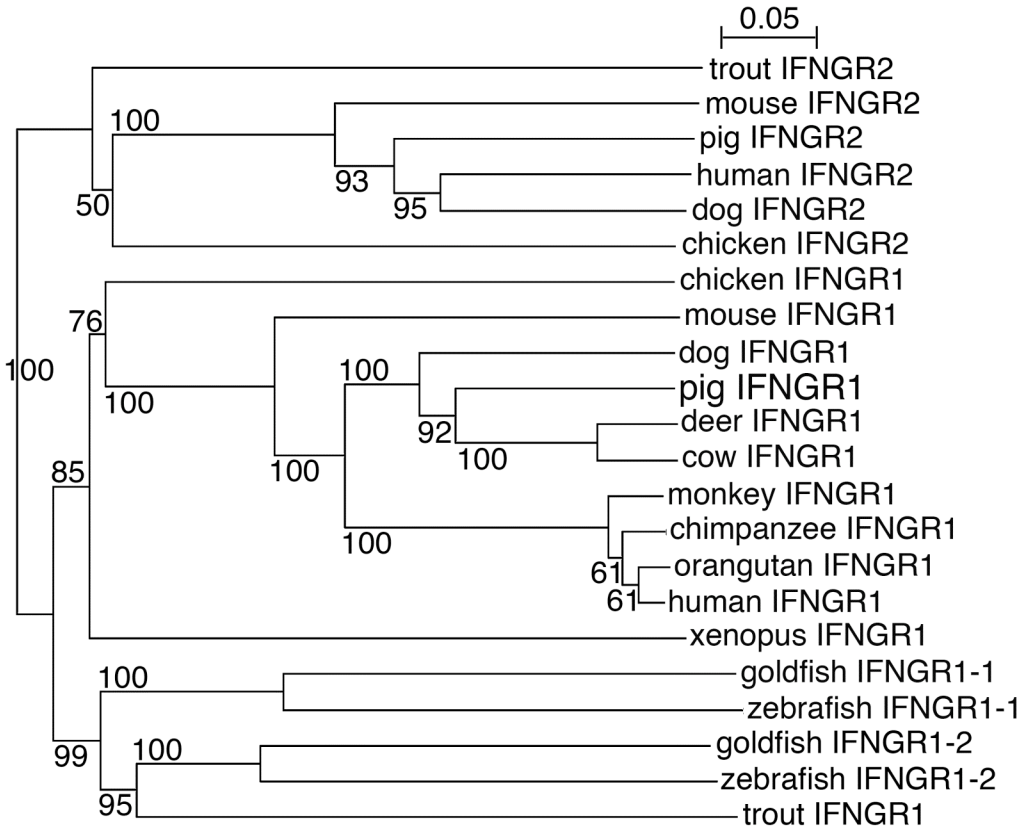

Figure 6.4. Phylogenetic analysis of zebrafish and goldfish IFNGR1-1 and IFNGR1-2 isoforms and IFNGR1 and IFNGR2 of other vertebrates. The analysis was conducted using the neighbor joining method and was bootstrapped 10,000 times and expressed as percent values.

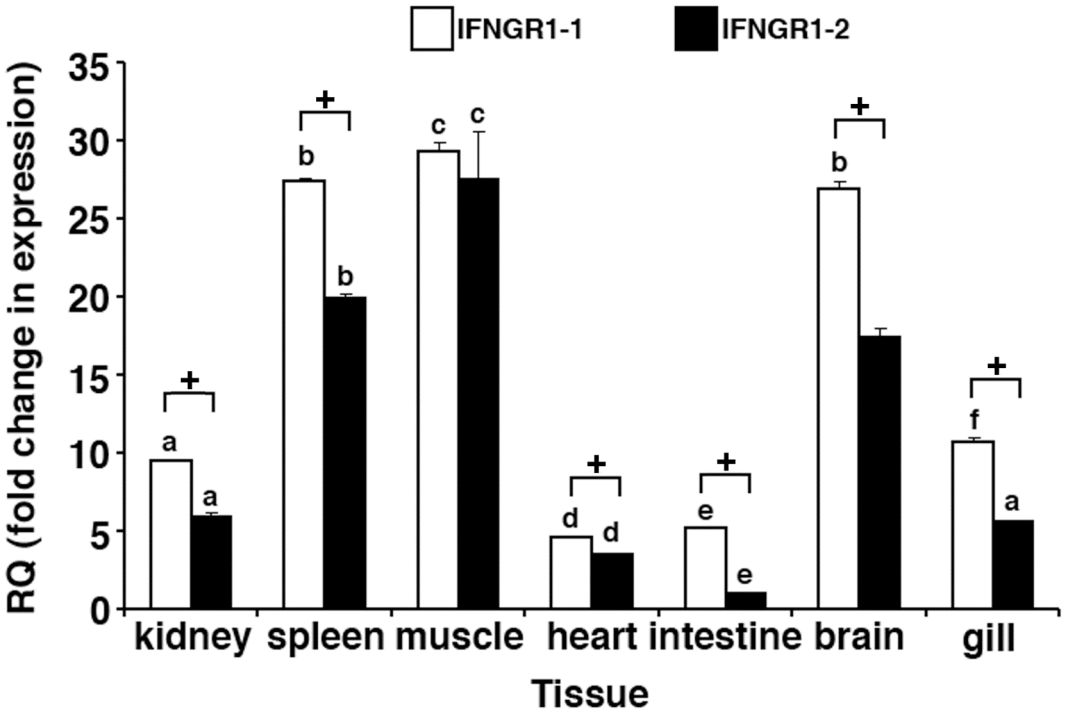

Figure 6.5. Quantitative analysis of IFNGR1-1 and IFNGR1-2 gene expression in zebrafish tissues. The zebrafish IFNGR1-1 and IFNGR1-2 expression is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Analyses of the relative tissue expression data are for pooled tissues from 6 individual fish, performed in triplicate. All results were normalized against the intestine IFNGR1-2 mRNA levels. Statistical analysis was performed using one-way ANOVA. Different letters above individual bars indicate significantly different ($P < 0.05$), the same letters indicate no statistical difference between groups. The asterisks (+) above lines indicate statistical differences ($P < 0.05$) between data bars indicated by the lines

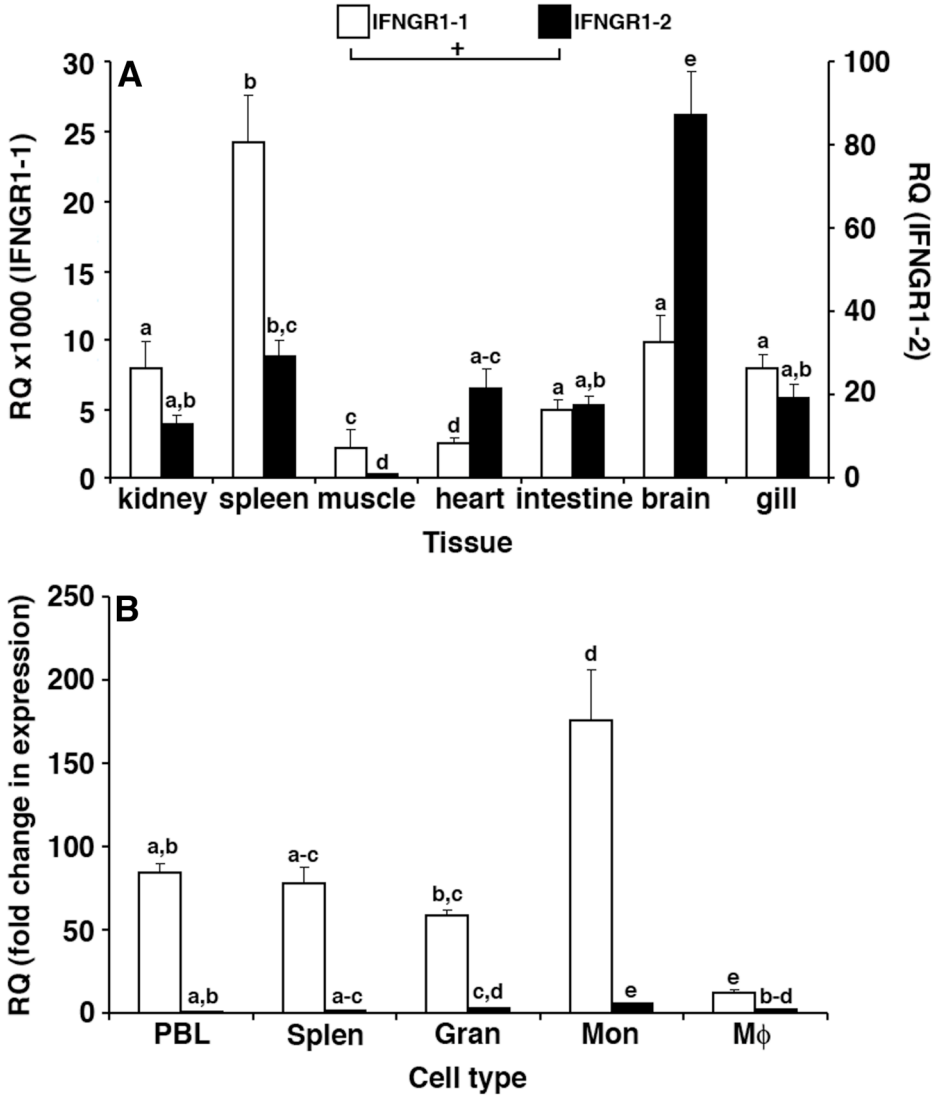

Figure 6.6. Quantitative analysis of IFNGR1-1 and IFNGR1-2 gene expression goldfish in tissues and immune cell populations. (A) Goldfish IFNGR1-1 and IFNGR1-2 tissue expression analysis. The goldfish IFNGR1-1 and IFNGR1-2 expression is relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Analyses of the relative tissue expression data are for tissues from five fish (*n*=5). All results were normalized against the muscle IFNGR1-2 mRNA levels. **(B)** Goldfish IFNGR1-1 and IFNGR1-2 expression in different immune cell populations. Cell cultures were established from 5 fish (*n*=5) and the expression normalized against that of IFNGR1-2 mRNA levels in PBLs. Cell populations examined are: peripheral blood leukocytes (PBLs), splenocytes (Splen), kidney-derived granulocytes (Gran), monocytes (Mon) and mature macrophages (Mϕ). Statistical analysis was performed using one-way ANOVA. Different letters above each bar indicates significantly different ($P < 0.05$), the same letter indicates no statistical difference between groups. (+) above lines denotes a significant difference between groups indicated by the lines.

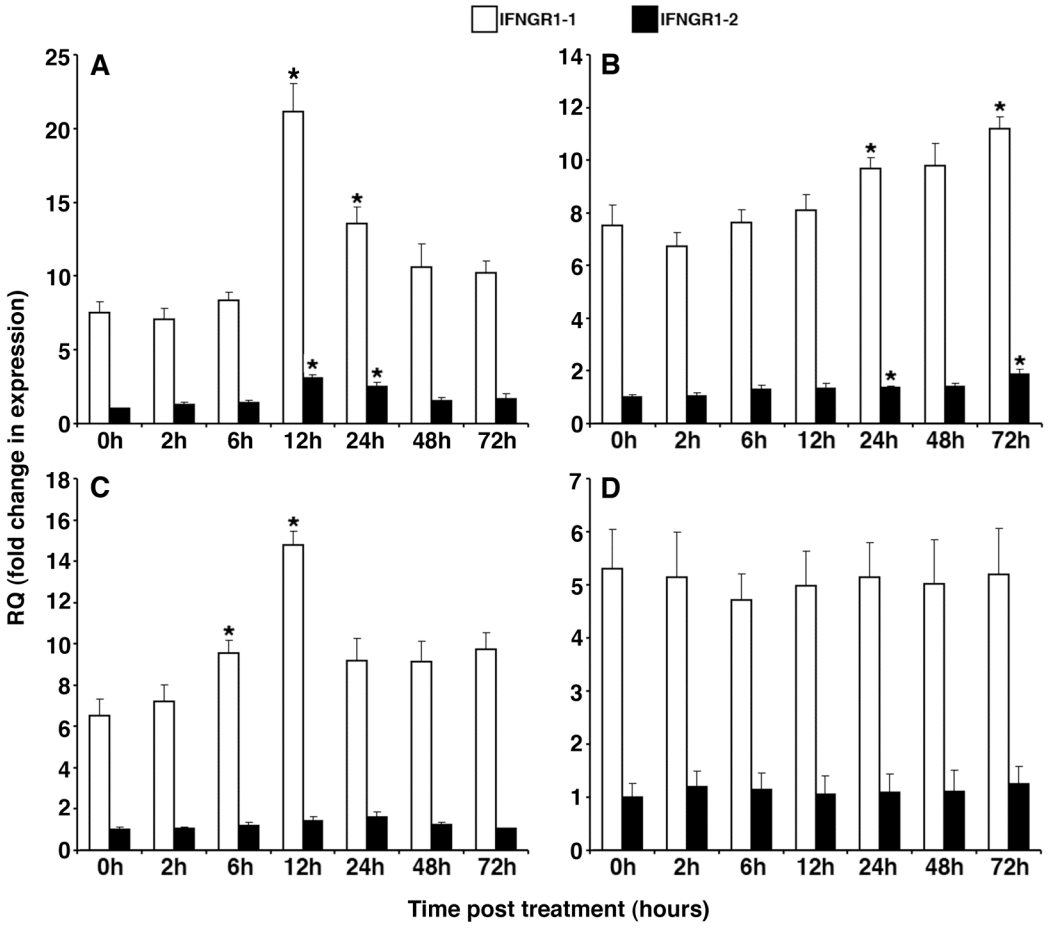

Figure 6.7. Quantitative analysis of IFNGR1-1 and IFNGR1-2 gene expression in cytokine-stimulated goldfish macrophages. Goldfish macrophages were treated with 100 ng/mL of **(A)** rgIFNγ, **(B)** rgTGFβ, **(C)** rgTNFα2 or **(D)** medium. The expression of goldfish IFNGR1-1 and IFNGR1-2 was examined relative to the endogenous control gene, elongation factor 1 alpha (EF-1α). The expression data were normalized against those observed for IFNGR1-2 mRNA levels at the 0 hour time point. The results are mean ± SEM of primary macrophage cultures established from five individual fish (*n*=5). (*) indicates significantly different ($P < 0.05$) from the 0 hour time point.

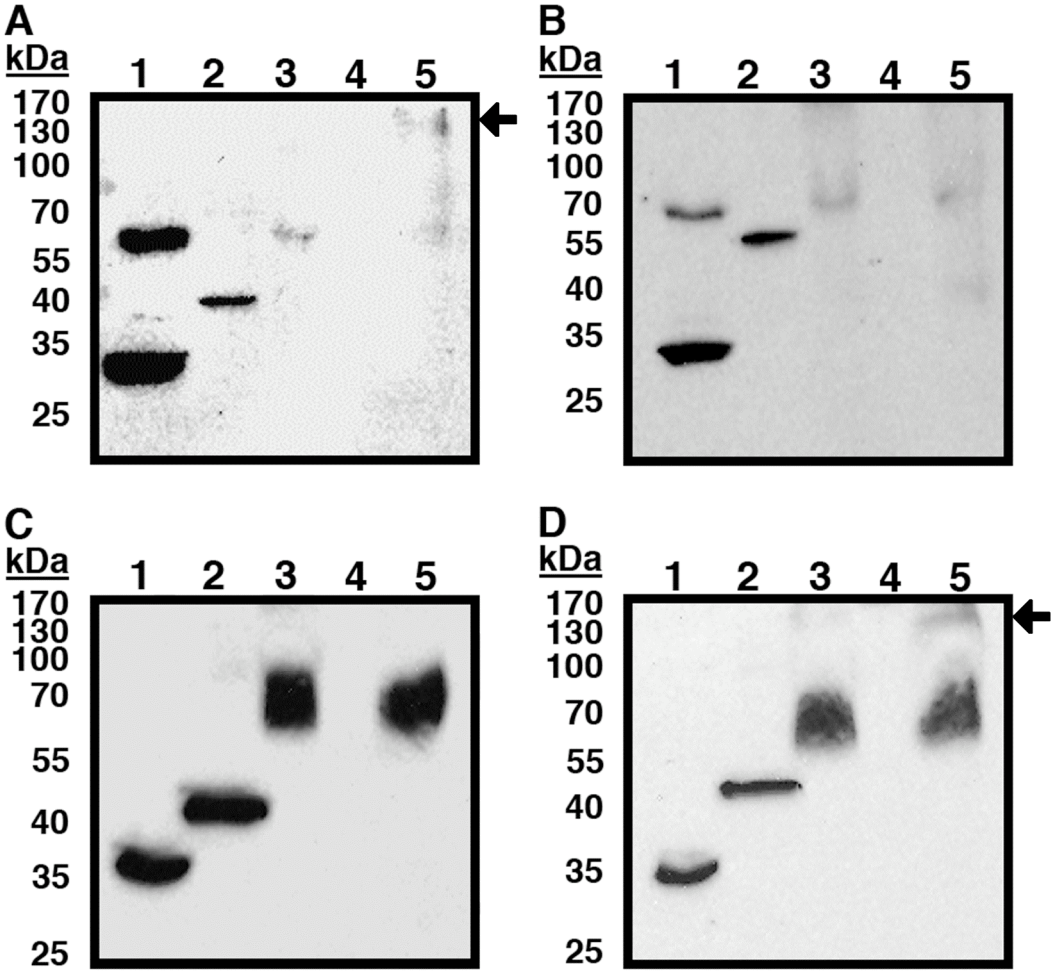

Figure 6.8. Western blot analysis of rgIFNGR1-1 and rgIFNGR1-2 interaction with rgIFNγrel and rgIFNγ. For all binding studies, 2.5 μg of each recombinant protein was incubated in conjugation buffer (20 mM Hepes) for half an hour. The rgIFNγ, rgIFNγrel, rgIFNGR1-1 and rgIFNGR1-2 were incubated individually or in ligand-receptor combinations. Following the initial conjugation period, the ligands, receptors and ligand-receptor combinations were cross-linked using disuccinimidyl suberate (DSS, Therom Scientific). All studies were done using a final concentration of 1 mM DSS. The reactions were then resolved using reducing SDS-PAGE and western blot against poly histidine tags. **(A)** Lane: 1. rgIFNγrel; 2. rgIFNGR1-1; 3. rgIFNγrel + DSS; 4. rgIFNGR1-1 + DSS; 5. rgIFNγrel and rgIFNGR1-1 + DSS. **(B)** Lane: 1. rgIFNγrel; 2. rgIFNGR1-2; 3. rgIFNγrel + DSS; 4. rgIFNGR1-2 + DSS; 5. rgIFNγrel and rgIFNGR1-2 + DSS. **(C)** Lane: 1. rgIFNγ; 2. rgIFNGR1-1; 3. rgIFNγ + DSS; 4. rgIFNGR1-1 + DSS; 5. rgIFNγ and rgIFNGR1-1 + DSS. **(D)** Lane: 1. rgIFNγ; 2. rgIFNGR1-2; 3. rgIFNγ + DSS; 4. rgIFNGR1-2 + DSS; 5. rgIFNγ and rgIFNGR1-2 + DSS.

4. REFERENCES

1. **Bach, E. A., J. W. Tanner, S. Marsters, A. Ashkenazi, M. Aguet, A. S. Shaw, and R. D. Schreiber.** 1996. Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. Molecular and cellular biology **16:**3214-3221.
2. **Bader, T., and J. Weitzerbin.** 1994. Nuclear accumulation of interferon gamma. Proceedings of the National Academy of Sciences of the United States of America **91:**11831-11835.
3. **Belosevic, M., C. E. Davis, M. S. Meltzer, and C. A. Nacy.** 1988. Regulation of activated macrophage antimicrobial activities. Identification of lymphokines that cooperate with IFN-gamma for induction of resistance to infection. J Immunol **141:**890-896.
4. **Belosevic, M., D. S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. J Immunol **143:**266.274.
5. **Chene, C., M. Fountoulakis, H. Dobeli, B. D'Arcy, F. Winkler, and A. D'Arcy.** 1995. Crystallization of the complex of human IFN-gamma and the extracellular domain of the IFN-gamma receptor. Proteins **23:**591-594.
6. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. The Journal of experimental medicine **178:**2243-2247.
7. **Ealick, S. E., W. J. Cook, S. Vijay-Kumar, M. Carson, T. L. Nagabhushan, P. P. Trotta, and C. E. Bugg.** 1991. Three-dimensional structure of recombinant human interferon-gamma. Science (New York, N.Y **252:**698-702.
8. **Fan, X. C., and J. A. Steitz.** 1998. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. The EMBO journal **17:**3448-3460.
9. **Farrar, M. A., J. D. Campbell, and R. D. Schreiber.** 1992. Identification of a functionally important sequence in the C terminus of the interferon-gamma receptor. Proceedings of the National Academy of Sciences of the United States of America **89:**11706.11710.
10. **Farrar, M. A., J. Fernandez-Luna, and R. D. Schreiber.** 1991. Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. The Journal of biological chemistry **266:**19626.19635.
11. **Fountoulakis, M., M. Zulauf, A. Lustig, and G. Garotta.** 1992. Stoichiometry of interaction between interferon gamma and its receptor. Eur J Biochem **208:**781-787.
12. **Gao, Q., P. Nie, K. D. Thompson, A. Adams, T. Wang, C. J. Secombes, and J. Zou.** 2009. The search for the IFN-g receptor in fish: Functional and expression analysis of putative binding and signalling

chains in rainbow trout Oncorhynchus mykiss. Developmental and comparative immunology **doi:10.1016/j.dci.2009.03.001**.

13. **Gorospe, M., X. Wang, and N. J. Holbrook.** 1998. p53-dependent elevation of p21Waf1 expression by UV light is mediated through mRNA stabilization and involves a vanadate-sensitive regulatory system. Molecular and cellular biology **18:**1400-1407.
14. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization, expression and functional analysis of goldfish (Carassius auratus L.) interferon gamma. Developmental and comparative immunology **33:**235-246.
15. **Grayfer, L., E. G. Garcia, and M. Belosevic.** 2010. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (Carassius auratus L.). The Journal of biological chemistry **285:**23537-23547.
16. **Greenlund, A. C., M. A. Farrar, B. L. Viviano, and R. D. Schreiber.** 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). The EMBO journal **13:**1591-1600.
17. **Greenlund, A. C., M. O. Morales, B. L. Viviano, H. Yan, J. Krolewski, and R. D. Schreiber.** 1995. Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. Immunity **2:**677-687.
18. **Hirsch, H. H., A. P. Nair, V. Backenstoss, and C. Moroni.** 1995. Interleukin-3 mRNA stabilization by a trans-acting mechanism in autocrine tumors lacking interleukin-3 gene rearrangements. The Journal of biological chemistry **270:**20629-20635.
19. **Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet.** 1993. Immune response in mice that lack the interferon-gamma receptor. Science (New York, N.Y **259:**1742-1745.
20. **Igarashi, K., G. Garotta, L. Ozmen, A. Ziemiecki, A. F. Wilks, A. G. Harpur, A. C. Larner, and D. S. Finbloom.** 1994. Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. The Journal of biological chemistry **269:**14333-14336.
21. **Igawa, D., M. Sakai, and R. Savan.** 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Molecular immunology **43:**999-1009.
22. **Johnson, H. M., B. A. Torres, M. M. Green, B. E. Szente, K. I. Siler, J. Larkin, 3rd, and P. S. Subramaniam.** 1998. Hypothesis: ligand/receptor-assisted nuclear translocation of STATs. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y **218:**149-155.
23. **Kaplan, D. H., A. C. Greenlund, J. W. Tanner, A. S. Shaw, and R. D. Schreiber.** 1996. Identification of an interferon-gamma receptor alpha

chain sequence required for JAK-1 binding. The Journal of biological chemistry **271:**9-12.

24. **Kerr, I. M., and G. R. Stark.** 1992. The antiviral effects of the interferons and their inhibition. Journal of interferon research **12:**237-240.
25. **Kotenko, S. V., L. S. Izotova, B. P. Pollack, T. M. Mariano, R. J. Donnelly, G. Muthukumaran, J. R. Cook, G. Garotta, O. Silvennoinen, J. N. Ihle, and et al.** 1995. Interaction between the components of the interferon gamma receptor complex. The Journal of biological chemistry **270:**20915-20921.
26. **Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson.** 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science (New York, N.Y **244:**339-343.
27. **Marsters, S. A., D. Pennica, E. Bach, R. D. Schreiber, and A. Ashkenazi.** 1995. Interferon gamma signals via a high-affinity multisubunit receptor complex that contains two types of polypeptide chain. Proceedings of the National Academy of Sciences of the United States of America **92:**5401-5405.
28. **Milev-Milovanovic, I., S. Long, M. Wilson, E. Bengten, N. W. Miller, and V. G. Chinchar.** 2006. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics **58:**70-80.
29. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology **7:**145-173.
30. **Peng, S. S., C. Y. Chen, N. Xu, and A. B. Shyu.** 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. The EMBO journal **17:**3461-3470.
31. **Perussia, B.** 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. Current opinion in immunology **3:**49-55.
32. **Robertsen, B.** 2006. The interferon system of teleost fish. Fish & shellfish immunology **20:**172-191.
33. **Sad, S., R. Marcotte, and T. R. Mosmann.** 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity **2:**271-279.
34. **Sakatsume, M., K. Igarashi, K. D. Winestock, G. Garotta, A. C. Larner, and D. S. Finbloom.** 1995. The Jak kinases differentially associate with the alpha and beta (accessory factor) chains of the interferon gamma receptor to form a functional receptor unit capable of activating STAT transcription factors. The Journal of biological chemistry **270:**17528-17534.
35. **Savant-Bhonsale, S., and D. W. Cleveland.** 1992. Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a > 20S degradation complex. Genes & development **6:**1927-1939.
36. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Advances in virus research **38:**147-200.

37. **Stevenson, M. M., M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba.** 1990. Role of endogenous gamma interferon in host response to infection with blood-stage Plasmodium chabaudi AS. Infection and immunity **58:**3225-3232.
38. **Stolte, E. H., H. F. Savelkoul, G. Wiegertjes, G. Flik, and B. M. Lidy Verburg-van Kemenade.** 2008. Differential expression of two interferon-gamma genes in common carp (Cyprinus carpio L.). Developmental and comparative immunology **32:**1467-1481.
39. **Trinchieri, G., and P. Scott.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. Research in immunology **146:**423-431.
40. **Walter, M. R., W. T. Windsor, T. L. Nagabhushan, D. J. Lundell, C. A. Lunn, P. J. Zauodny, and S. K. Narula.** 1995. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. Nature **376:**230-235.
41. **Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley.** 1994. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. The Journal of experimental medicine **179:**1367-1371.
42. **Wheelock, E. F.** 1965. Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. Science (New York, N.Y **149:**310-311.
43. **Young, H. A., and K. J. Hardy.** 1995. Role of interferon-gamma in immune cell regulation. Journal of leukocyte biology **58:**373-381.
44. **Zou, J., A. Carrington, B. Collet, J. M. Dijkstra, Y. Yoshiura, N. Bols, and C. Secombes.** 2005. Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. J Immunol **175:**2484-2494.
45. **Zou, J., Y. Yoshiura, J. M. Dijkstra, M. Sakai, M. Ototake, and C. Secombes.** 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish & shellfish immunology **17:**403-409.
46. **Zubiaga, A. M., J. G. Belasco, and M. E. Greenberg.** 1995. The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. Molecular and cellular biology **15:**2219-2230.

CHAPTER VII: COMPARISON OF MACROPHAGE ANTIMICROBIAL RESPONSES INDUCED BY TYPE II INTERFERONS OF THE GOLDFISH (*Carassius auratus* L.)[5]

1. INTRODUCTION

Interferon gamma (IFNγ) is a highly pleiotropic pro-inflammatory and anti-viral cytokine produced primarily by activated Th1 phenotype CD4+ cells (38) CD8+ cells (45) and natural killer (NK) cells (43). In addition to its weak antiviral activity (29, 49, 60), IFNγ is a central cytokine regulator of host defence against obligate and facultative intracellular pathogens (2, 3, 49, 50). For example, IFNγ gene knock-out mice are unable to control infections with *Leishmania major* (59), *Listeria monocytogenes* (26) and *Mycobacteria* (13), indicating that IFNγ is pivotal for the regulation of macrophage antimicrobial responses (2, 6, 8, 17, 32).

Homologues of the IFNγ have been identified in a number of bony fish (teleosts) including zebrafish (27), Japanese pufferfish (66), trout (64), Atlantic salmon (44), catfish (37), common carp (51), and goldfish (19). Of these fish species, zebrafish, catfish, common carp and goldfish have two isoforms of IFNγ, which differ markedly within each species in both sequence homology and

---

[5] A version of this chapter has been published: Grayfer, L., E. G. Garcia, and M. Belosevic. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (*Carassius auratus* L.). The Journal of Biological Chemistry 285:23537-23547.

expression in different tissues (27, 37, 51). Both isoforms contain IFNγ signature motifs (27, 37) and were initially named IFNγ1 and IFNγ2. IFNγ2 is structurally similar to mammalian IFNγ, while IFNγ1 is shorter and does not contain C-terminal cationic residues characteristic of a nuclear localization signal (NLS) and required for the IFNγ activity (1, 64). Consequently, the fish IFNγ1 and IFNγ2 are now referred to as IFNγ related (IFNγrel) and IFNγ, respectively.

IFNγrel was expressed in LPS-stimulated common carp leukocytes enriched for B-cells (51). In grass carp, increased IFNγrel mRNA levels were observed in immune organs following infection with reovirus and stimulation with peptidoglycan, LPS and poly I:C (11). However, there is conflicting evidence as to possible roles of the fish type II IFNs *in vivo* (31, 48) and the functional roles of the IFNγrel have not bee explored.

The mammalian IFNγ mediates its biological effects by ligating interferon gamma receptor 1 (IFNGR1) which then associates with IFNGR2, forming a signaling complex. Receptor complex assembly leads to activation of Janus kinases (Jak) 1 and 2, associated with the receptor chains 1 and 2, respectively (28). These phospho-tyrosine kinases then phosphorylate the IFNGR1-associated Stat1 (14) and to a lesser extent Stat2 (54) transcription factors. The activation of a plethora of other genes then ensues through homodimeric Stat1, heterodimeric Stat1:Stat2 as well as through the transcription factor complexes ISGF3 and Stat1-p48, composed of Stat1:Stat2:IRF-9 and Stat1:Stat1:IRF-9, respectively (7, 33, 54). The above transcription factors orchestrate gene regulation through recognition of IFNγ-activated sequences (GAS) in the promoter regions of target

genes (55). Within the first 30 min of IFNγ signalling, an upregulation in the expression of several interferon regulatory factors (IRFs) occurs, which then modulate subsequent waves of gene expression in the IFNγ signalling cascade (62). Several, but not all of the genes in the IFNγ signalling pathway have been cloned (12, 25, 44) and an IFNγ-specific trout reporter cell line has been established (10). However, the consensus sequences of the GAS elements of the fish IFNγ-responsive promoters are less specific than their mammalian counterparts (9) and the precise mechanisms by which the fish IFNγ and IFNγrel signal are not known.

This report represents the first comprehensive functional characterisation and direct comparison of the fish (rg)IFNγrel and rgIFNγ. My findings indicate that rgIFNγrel and rgIFNγ possess distinct capacities to mediate specific pro-inflammatory responses of goldfish myeloid cells. The regulation of macrophage antimicrobial functions by two functionally segregated type II interferons in certain bony fish species is distinct from the single Type II IFN system present in all other vertebrates examined.

2. RESULTS

2.1. Analysis of IFNγrel and IFNγ gene expression in goldfish tissues and immune cell populations

The gene expression analysis of goldfish IFNγ and IFNγrel revealed that the highest mRNA levels for both cytokines were in the spleen compared to other tissues and the lowest mRNA levels were in the muscle (Fig. 7.1A). However,

significantly higher mRNA levels of IFNγ compared to IFNγrel were observed in most tissues (Fig. 7.1A).

The gene expression of IFNγ was significantly greater than that of IFNγrel in all goldfish immune cell population except granulocytes (Fig. 7.1B). The highest mRNA levels of both IFNγ and IFNγrel were observed in the PBLs and splenocytes (Fig. 7.1B). Lower mRNA levels were measured in monocytes and granulocytes, while the expression of both IFNγ and IFNγrel was lowest in mature macrophages (Fig. 7.1B).

2.2. Analysis of immune gene expression in monocytes treated with rgIFNγrel and rgIFNγ

To examine the immune gene expression in monocytes, cells were treated with medium, rgIFNγrel, rgIFNγ, or with a combination of both cytokines and the mRNA levels of select immune genes measured using quantitative PCR. The following genes were examined: IFNGR1-1 and IFNGR1-2, components of the NADPH oxidase pathway, IL-1β isoforms -1 and -2, TNFα isoforms -1 and -2, the chemokines CXCL-8 and CCL-1, TGFβ and ceruloplasmin.

The gene expression of the NADPH oxidase components, after treatment of monocytes with rgIFNγrel or rgIFNγ was variable. For example, the expression of $p47^{phox}$ was significantly higher than that in medium-treated cells (Fig. 7.2A). The treatment of monocytes with rgIFNγrel alone or in combination with rgIFNγ caused a significant down-regulation in the gene expression of $p40^{phox}$, whereas treatment with rgIFNγ alone had no effect on the expression of this

gene (Fig. 7.2A). Combined, but not individual treatments of monocytes with rgIFNγrel and rgIFNγ also resulted in decreased gene expression of $p67^{phox}$ (Fig. 7.2A). The expression of $gp91^{phox}$, a gene that encodes a membrane bound NADPH pathway component, was significantly elevated by all treatments while the gene expression of $p22^{phox}$ did not change (Fig. 7.2B).

An increase in the gene expression of both IL-1β isoforms was observed after treatment with individual or combined rgIFNγrel and rgIFNγ (Fig. 7.2C). However, cells treated with rgIFNγrel alone or in combination with rgIFNγ had significantly higher IL-1β1 mRNA levels than those treated with rgIFNγ alone (Fig. 7.2C).

Monocytes treated with rgIFNγrel and IFNγ had elevated TNFα1 and TNFα2 mRNA levels (Fig. 7.2D). Similar to the IL-1β gene expression, the mRNA levels of both TNF isoforms were substantially higher in cells treated with rgIFNγrel alone and in combination with rgIFNγ, compared to cells treated with rgIFNγ alone (Fig. 7.2D).

Although the treatment of monocytes with rgIFNγrel or rgIFNγ induced an up-regulation in the CXCL-8 mRNA levels, the rgIFNγrel stimulation induced significantly greater magnitude of increase in the gene expression of CXCL-8 compared to that induced by rgIFNγ (Fig. 7.2E). Interestingly, the combined treatment of monocytes with both rgIFNγrel and rgIFNγ down-regulated the gene expression of CXCL-8 compared to that induced by rgIFNγrel alone (Fig. 7.2E). In contrast, the expression of CCL-1 in monocytes was not affected after treatment with either cytokine (Fig. 7.2E).

Monocytes treated with either cytokine alone or in combination exhibited significantly elevated mRNA levels of IFNGR1-1 but not IFNGR1-2 (Fig. 7.2F).

When cells were treated with either rgIFNγrel or rgIFNγ, no significant changes were observed in the gene expression of the goldfish TGFβ gene (Fig. 7.2G). In contrast, monocytes treated with both rgIFNγrel and rgIFNγ exhibited significantly lower TGFβ mRNA levels compared to medium-treated cells (Fig. 7.2G).

In mammals, IFNγ stimulation of myeloid cells results in increased expression and production of the acute phase protein, ceruloplasmin. In order to address whether this upregulation is also a feature of goldfish monocytes treated with rgIFNγrel and/or rgIFNγ, we cloned goldfish ceruloplasmin cDNA and design Q-PCR primers against it. To our surprise, treatment of monocytes with rgIFNγ alone or in combination with rgIFNγrel did not affect the gene expression of ceruloplasmin (Fig. 7.2F). In contrast, the rgIFNγrel stimulations resulted in significant up-regulation in goldfish monocyte ceruloplasmin mRNA (Fig. 7.2F). The gene expression of select immune genes following exposure of mature macrophages to rgIFNγrel and rgIFNγ was similar to that observed for monocytes (data not shown).

2.3. The rgIFNγrel and rgIFNγ confer distinct monocyte-ROI priming potentials

I previously reported that goldfish kidney derived cultures enriched in monocytes exhibited significant ROI production and phagocytosis after treatment

with rgIFNγ (20). In contrast, activated mature macrophages conferred robust nitric oxide responses but drastically reduced ability to produce ROI (20).

To determine whether rgIFNγrel primed goldfish monocytes for ROI production, I incubated cells with medium, rgIFNγ, rgIFNγrel, rgTNFα2, a combination of rgIFNγrel + rgIFNγ, or rgIFNγrel + rgTNFα2. Surprisingly, when goldfish monocytes were treated with rgIFNγrel overnight (16 hours), I did not observe significantly enhanced ROI production (Fig. 7.3A). Furthermore, when monocytes were primed overnight with either rgIFNγ or rgTNFα2 in conjunction with rgIFNγrel, the PMA-triggered ROI production by monocytes was substantially reduced compared to the ROI production of cells treated with either rgIFNγ or rgTNFα2 alone (Fig. 7.3A). This down-regulation of the priming response and ultimately ROI production was evident when as little as 1 ng/mL of rgIFNγrel was added to the monocyte cultures (Fig. 7.3B). To elucidate whether rgIFNγrel was the cause of the decrease in rgIFNγ or rgTNFα2 mediated ROI production, 5 μg/mL of α-rgIFNγrel affinity-purified rabbit IgG was added to monocyte cultures while priming with rgIFNγ + rgIFNγrel or rgTNFα2 + rgIFNγrel (Fig. 7.3C). The addition of α-rgIFNγrel antibody partially restored the rgIFNγ or rgTNFα2 mediated ROI production (Fig. 7.3B). I did not observe further restoration of ROI production when higher concentrations of α-rgIFNγrel IgG were added to the cultures (data not shown).

To address whether rgIFNγrel had the capacity to down-regulate the priming for ROI production at shorter incubation times, I treated monocytes with rgIFNγrel alone or in combination with rgIFNγ for 1 or 9 hours (Fig. 7.3D). To

my surprise, monocytes treated with as little as 1 ng/mL of rgIFNγrel alone for 1 hour, primed the cells for significant ROI production (Fig. 7.3D). The combined treatments of monocytes with rgIFNγrel and rgIFNγ for 1 hour resulted in ROI production similar to that induced by treatment of cells with rgIFNγrel alone (Fig 7.3D). As expected, the rgIFNγrel-mediated priming for ROI production was significantly reduced after addition of 5 μg/mL of α-rgIFNγrel IgG to the monocyte cultures. (Fig. 7.3D). The addition of higher amounts of rgIFNγrel did not result in further increases in ROI production (data not shown).

When monocytes were treated longer (9 hour) with rgIFNγrel, a significant decrease in the production of ROI was observed (Fig. 7.3D). In contrast, prolonged treatment of monocytes with rgIFNγ for 9 hours resulted in a further increase of ROI production, compared to that by cells treated for 1 hour. (Fig. 7.3D). Interestingly, a nine-hour incubation of monocytes with a combination of rgIFNγ and rgIFNγrel caused a decreased ROI response compared to that induced by rgIFNγ alone (Fig. 7.3D).

2.4. Recombinant goldfish IFNγrel induces higher phagocytosis than rgIFNγ

I previously reported that rgIFNγ enhanced the phagocytosis of fluorescent latex beads by monocytes (19). To determine whether rgIFNγrel activated monocytes to ingest fluorescent latex beads, cells were treated with either medium alone, rgIFNγ (100 ng/mL), rgIFNγrel (1 or 100 ng/mL) or a combination of both cytokines. The capacity of monocytes to engulf fluorescent

latex beads was determined using flow cytometry. As seen in FACS plots of monocyte cultures from a representative fish, treatment of cells with 100 ng/mL of rgIFNγ induced a modest increase in the uptake of latex beads (Fig 7.4A). In contrast, monocytes obtained from the same fish and treated with 100 ng/mL of rgIFNγrel exhibited substantially higher phagocytic activity compared to those treated with rgIFNγ (Fig. 7.4A).

The flow cytometry-based phagocytosis assay allows for analysis of discrete populations of monocytes that have ingested 1, 2, 3 or more beads. I previously reported that enhanced phagocytic activity of activated monocytes was related to the uptake of 3 or more beads (19). Treatment of monocytes with 100 ng/mL rgIFNγ or rgIFNγrel resulted in a significant increase in phagocytosis of activated monocytes (Fig. 7.4B), where rgIFNγrel induced significantly higher phagocytosis compared to rgIFNγ (Fig. 7.4B). The addition of α-rgIFNγrel IgG to the monocyte cultures partially decreased the phagocytic activity of monocytes induced by rgIFNγrel (Fig. 7.4B).

2.5. Recombinant goldfish IFNγrel induces iNOS gene expression and nitric oxide response of goldfish macrophages

I previously reported that activated mature goldfish macrophages exhibit significant nitric oxide responses after treatment with pro-inflammatory cytokines (19, 20). To compare the ability of rgIFNγrel and rgIFNγ to induce nitric oxide response, 6 to 8 day old macrophage cultures were treated with these two cytokines either individually or in combination and the gene expression of iNOS

isoforms A and B measured by Q-PCR (Fig. 7.5A). An increase in the gene expression of both iNOSA and iNOSB was observed when macrophages were treated with either rgIFNγ or rgIFNγrel, however, mRNA levels of iNOSA and iNOSB were significantly higher after rgIFNγrel stimulation (Fig 7.5A). Interestingly, treatment of macrophages with rgIFNγ + rgIFNγrel caused a substantial down-regulation of the gene expression of both iNOS isoforms (Fig. 7.5A).

I then examined the ability of goldfish macrophages to produce nitrite after treatment with rgIFNγrel and/or rgIFNγ using Griess reaction assay. The treatment of macrophage cultures with rgIFNγ induced significantly elevated nitrite production, compared to medium-treated cells (Fig. 7.5B). The addition of rgIFNγrel to macrophage cultures resulted in significantly higher nitrite production compared to that induced by rgIFNγ (Fig. 7.5B). The addition of α-rgIFNγrel IgG partially decreased the nitrite production of macrophages (Fig. 7.5B).

2.6. Analysis of cellular association of rgIFNγrel and rgIFNγ

Monocytes were treated for 0, 15, 30 or 90 minutes with either rgIFNγrel or rgIFNγ and the cellular association of the two proteins determined using Western blot. Analysis of the whole cell lysates revealed that more of rgIFNγrel was associated with the cells compared to rgIFNγ. Increased association of rgIFNγrel was evident as early as 15 minutes and persisted during the observation period (90 minutes) (Fig. 7.6A). In contrast, most of rgIFNγ was present in whole

cell lysates at 30 minutes (Fig. 7.6A). The incubation of monocytes with rgIFNγ + rgIFNγrel for 30 minutes did not alter the association of either cytokine with the cells (Fig. 7.6B). Since monocytes were incubated with equal amounts of either rgIFNγ or rgIFNγrel (Fig. 7.6C), it appears that more rgIFNγrel was associated with the cells (Fig. 7.6A).

2.7. Analysis rgIFNγrel- and rgIFNγ-mediated Stat1-(Y)- phosphorylation and nuclear accumulation

Western blot analyses of rgIFNγrel- and rgIFNγ-treated monocytes using an α-phospho-(Y)-Stat1 antibody were performed (Fig. 7.6 D,F). When cells were treated with rgIFNγrel, Stat1 tyrosine phosphorylation was evident at 30 and 90 minutes after stimulation (Fig. 7.6D). In contrast, rgIFNγ treatment resulted in substantial Stat1-(Y)-phosphorylation as early as 15 and 30 minutes after stimulation, which was then substantially reduced by 90 minutes (Fig. 7.6D).

To determine whether rgIFNγrel or rgIFNγ stimulation resulted in nuclear accumulation of phospho-(Y)-Stat1, nuclei were isolated from monocyte at 0, 15, 30 or 90 minutes after treatment with either rgIFNγrel or rgIFNγ (Fig. 7.6E). Nuclear accumulation of phospho-(Y)-Stat1 was evident 30 and 90 minutes after treatment with rgIFNγ (Fig. 7.6E). In contrast, no phospho-(Y)-Stat1 was detected in the nuclei isolated from rgIFNγrel treated cells (Fig. 7.6E).

2.8. Gene expression analysis of interferon regulatory factors in monocytes treated with rgIFNγrel and rgIFNγ

In mammals, the first wave of IFNγ-induced gene activation, including those that encode interferon regulatory factors (IRFs) occurs 15-30 minutes after treatment of cells with IFNγ (62). These IRFs then help to regulate the next wave of gene transcription in the IFNγ signalling cascade. To compare the signalling pathways of rgIFNγrel and rgIFNγ, we measured the gene expression of IRFs after stimulation with the two cytokines. In addition to goldfish IRF-1 and IRF-7 sequences, available in the NCBI database, I cloned the goldfish IRF-2, IRF-5, IRF-8 and IRF-9 and designed specific Q-PCR primers (Table 2.6). Monocytes were treated with 100 ng/mL of rgIFNγrel or rgIFNγ for 0, 15, 30 and 90 minutes and the gene expression levels of different IRFs determined (Fig. 7.7A-F).

Treatment of monocytes with rgIFNγ caused increased gene expression of IRF-1, IRF-2, IRF-8 and IRF-9, while treatment with rgIFNγrel caused increased expression of IRF-2 and IRF-9 and decreased gene expression (at 15 minutes) of IRF-7 (Fig. 7.7A-F). No changes in expression of IRF-5 were observed after treatment of monocytes with either cytokine (Fig. 7.7C).

3. DISCUSSION

Unlike mammals, bony fish possess two Type II interferons, IFNγ and IFNγrel, whose pro-inflammatory functions have not been fully characterized. I previously reported that rgIFNγ primed monocytes for ROI production, enhances monocyte phagocytosis and nitrite production by mature macrophages (19). In this Chapter I report that rgIFNγrel elicited a robust, but relatively short-lived priming of monocytes for ROI production, and subsequently renders the cells

unresponsive to the priming potentials of other pro-inflammatory cytokines (rgIFNγ and rgTNFα2). To my knowledge this is the first report of a type II interferon directly down-regulating monocyte/macrophage antimicrobial functions.

My results indicate differences in the signalling pathways utilized by the bony fish IFNγ and IFNγrel. In mammals it has been documented that Stat1 activation and concomitant IRF-1 production after IFNγ stimulation, determine the differentiation and fate of the activated cells (4, 5). My findings indicate that while rgIFNγ induced both Stat1 nuclear translocation and increased IRF-1 gene expression, rgIFNγrel did not mediate Stat1 nuclear translocation and did not affect IRF-1 gene expression. Indeed the two cytokines induced very unique profiles of functional responses in the goldfish monocytes and macrophages. For example, rgIFNγ exhibited long-lasting priming effects for monocyte ROI production, contrary to rgIFNγrel, whose ROI priming effects were short-lived and were followed by a down-regulation in the monocyte priming for this response. When compared to rgIFNγ, rgIFNγrel induced significantly greater magnitudes of phagocytosis, increased iNOS gene expression and nitrite production in monocytes and macrophages, respectively. Schroder *et al.* (47) reported that in general ROI responses are better suited to deal with phagocytosed extracellular pathogens and that nitric oxide responses evolved for more efficient destruction of obligate intracellular pathogens. We previously reported that fish macrophages mounted sequential antimicrobial responses following stimulation with macrophage activating factors (MAF) contained in mitogen-induced cell

supernatants (40). The ROI response was selectively deprogrammed once maximal induction had occurred without affecting the nitric oxide response of activated macrophages. The ability of the host to selectively deactivate ROI production may play an important role in host defence, because the regulation of the duration and intensity of the ROI response would minimize tissue damage at an inflammatory site, in an otherwise futile attempt to eliminate ROI resistant pathogens. It is likely that MAF contain both IFNγrel and IFNγ. Consequently, the differences in the induction of antimicrobial responses of monocytes/macrophages by rgIFNγrel and rgIFNγ may have evolved to regulate the intensity and the duration of specific antimicrobial functions during an inflammatory response.

Treatment of monocytes with rgIFNγrel caused a significant decrease in the p67^{phox} mRNA while the addition of rgIFNγrel + rgIFNγ induced significant decreases of both p67^{phox} and p40^{phox} mRNA. The p67^{phox} domain of NADPH oxidase is essential for electron transfer through flavocytochrome b centers (22, 42) and p40^{phox} participates in the activation of NADPH oxidase (24, 53, 57). Humans suffering from chronic granulomotous disease have dysfunctional p67^{phox} and exhibit a concomitant decrease in p40^{phox} expression (58, 61). It is possible that the observed down-regulation of monocyte ROI production by rgIFNγrel may be at least partially due to the transcriptional decreases of p67^{phox} and p40^{phox}.

Monocytes and macrophages treated with rgIFNγrel and/or rgIFNγ exhibited distinct gene expression of select immune genes. The mammalian IFNγ has been documented to up-regulate the gene expression of NADPH oxidase

components p67 ^{phox} (8, 41) and gp91 ^{phox} (21). In contrast, goldfish rgIFNγ and/or rgIFNγrel were found to up-regulate only gp91^{phox} and p47 ^{phox}. It is possible that the mechanisms that regulate NADPH oxidase activation may be different between mammals and fish.

I previously reported that goldfish monocytes derived from cultures of kidney leukocytes exhibited significant ROI production and phagocytosis after treatment with rgIFNγ (20). In contrast, activated mature goldfish macrophage cultures possess robust nitric oxide potentials but drastically reduced abilities to produce ROI (19, 20). My results indicate that iNOS A and B gene expression was significantly reduced after stimulation of goldfish macrophages with rgIFNγ and rgIFNγrel, when compared to rgIFNγrel alone, supporting distinct biological roles for the two cytokines in activation of antimicrobial functions of macrophages.

Human IFNγ has been shown to increase the expression of the gene encoding the acute phase protein, ceruloplasmin, and also differentially affect the translation of this protein (34, 35, 46). The elevated ceruloplasmin transcript levels correlated with increases in protein level shortly after activation (35), however, significant inhibition of translation of ceruloplasmin was reported at later times after IFNγ treatment (34, 46). Interestingly, rgIFNγrel, which is structurally less related to the mammalian IFNγ, significantly increased the expression of goldfish ceruloplasmin, while the more related rgIFNγ did not. Unfortunately, goldfish recombinant ceruloplasmin and α-goldfish ceruloplasmin

antibodies are currently not available, preventing the examination of the relationship between fish type II IFNs and ceruloplasmin at the protein level.

Within the first 30 minutes of treatment of mammalian cells with IFNγ, the expression of specific IRF transcription factors is upregulatedand and these IRFs participate in further signalling events (62). In this study, I examined the gene expression of goldfish IRFs after treatment of monocytes with IFNγ or IFNγrel. The expression of IRF-1 is dependent on Stat1 activation (16, 36) while the expression of IRF-8 is strictly induced by IFNγ but not by Type I IFNs (15, 39). My results indicate that like the mammalian IFNγ, the goldfish rgIFNγ also induced phospho-Stat1 nuclear translocation and upregulation in IRF-1 and IRF-8 gene expression. In contrast, IFNγrel did not induce phospho-Stat1 nuclear translocation or IRF-1 and IRF-8 gene upregulation, suggesting that it this cytokine signals through distinct signalling pathways. Interestingly IRF-2, which primarily serves as a transcriptional repressor of IRF-1 and ISGF3 (23), was upregulated in fish monocytes treated with either rgIFNγrel or rgIFNγ. It is possible that consistent down-regulation of goldfish IRFs at 90 minutes post-stimulation may be due to increased IRF-2 protein levels.

The changes in gene expression of both IRF-5 (56) and IRF-7 (63) are believed to be controlled by Type I but not Type II IFNs. This is consistent with my observations that the expression of the goldfish IRF-5 and IRF-7 is not upregulated in monocyte stimulated with either rgIFNγ or rgIFNγrel.

In addition to transcriptional regulation as a homodimer, IFNγ-activated Stat1 also regulates gene expression by forming the interferon signalling gene

factor (ISGF3) complex composed of Stat1, Stat2 and IRF-9 (33, 54) as well as a different complex composed of a Stat1 homodimer and IRF-9 (7). My results indicate that both rgIFNγ and rgIFNγrel induced an upregulation in the expression of goldfish IRF-9.

In chapter VI, I demonstrated that zebrafish and goldfish have two distinct IFNGR1 genes (18). Most vertebrate species have a single IFNGR1 gene, and it is reasonable to speculate that these distinct fish genes arose from a gene duplication of a single ancestral gene. As such it is important to emphasize that the two zebrafish IFNGR1 isoforms have not been evolutionarily retained on a single chromosome, but instead reside on distinct chromosomes, each with some but not all homologues of genes that are syntenic to the single mammalian IFNGR1 gene (18). Since gene synteny is suggestive of biological relationships between respective genes, the lack of synteny between the zebrafish IFNGR1 genes, support the hypothesis that the genes encoding these receptors have evolved to mediate distinct biological functions.

A recent report using morpholino knockdowns in zebrafish embryos showed that zebrafish IFNγrel appears to be essential for clearance of *E.coli* (48). The knockdown of both IFNγrel and IFNγ had a more drastic effect on embryo mortality during the course of the infection compared to that caused by the knockdown of either cytokine alone (48). It should be noted that the injection of zebrafish with recombinant IFNγ failed to protect fish against viral and bacterial infections possibly due to high rate of clearance of the recombinant protein in the injected animals (31).

In chapter VI, I demonstrated that the rgIFNγrel (~rgIFNγ1) and rgIFNγ (~rgIFNγ2) each bound to one but not the other IFNGR1 isoform (18). *In silico* analyses revealed that the two zebrafish and the two goldfish IFNGR1 isoforms had putative and evolutionarily conserved docking sites for both Jak1 and Stat1 (18). In the present study, both rgIFNγrel and rgIFNγ induced Stat1 tyrosine phosphorylation, suggesting a role for Stat1 in their signalling pathways. It should be noted that nuclear translocation of phospho-Stat1 was observed only after monocyte stimulation with rgIFNγ but not with rgIFNγrel and that only goldfish IFNγ has the nuclear localization signal sequence (NLS).

The leading model for mammalian IFNγ signalling, as proposed by Subramaniam *et al.* (52), suggests that following ligation of IFNγ to its receptor complex, Stat1 is delivered into the nucleus via the IFNγ NLS in a complex consisting of Stat1:IFNGR1:IFNγ. This NLS is made up of a positively charged stretch of residues at the C-terminal end of the protein (Fig. 7.8). While treatment of monocytes with either rgIFNγ or rgIFNγrel resulted in the presence of tyrosine phosphorylated Stat1 in the whole cell lysates, phospho-(Y)-Stat1 was observed in the nuclei of IFNγ but not IFNγrel-treated cells. I used the α-phospho-(Y)-Stat1 antibody because, as determined by protein alignments, the epitope recognized by this antibody has been evolutionarily conserved. In general this is not the case with other fish Stat proteins, since they do not have a high sequence identity with the mammalian Stats. My attempts to immunodetect goldfish Stat2 and Stat3 using antibodies raised against mammalian proteins were not successful. The findings of this study and our previous work (19) indicate that

goldfish and mammalian IFNγ are structurally more related and may signal through similar pathways. In contrast, IFNγrel which induces a plethora of significant biological effects in goldfish monocytes and macrophages, does not appear to signal through Stat1, because phospho-(Y)-Stat1 was not detected in nuclei of IFNγrel-stimulated monocytes. A more thorough investigation of signalling mechanisms used by the fish Type II IFNs and in particular IFNγrel will follow the generation of fish specific reagents.

Recently, Type I IFNs of teleosts have been identified and grouped into two groups based on structural similarities, with one group found in all teleost and the other present only in relatively primitive fish species (65). As with the fish Type II IFNs, these also appear to possess functionally distinct properties (31, 65). Using morpholino knockdowns in the context of embryo reactivity to a zebrafish type I IFN, Levrad *et al.* (30) identified potential candidates for IFNAR1 and IFNAR2. However similar studies have not been performed with other recently identified Type I IFNs. It is possible that like Type II fish interferons, teleost Type I IFNs may mediate biological events through distinct receptor/ligand complexes.

The findings presented in this chapter suggest the presence of a functional segregation in the induction of monocyte and macrophage antimicrobial functions by type II interferons of bony fish. This is different from the single Type II IFN systems present in all other vertebrates examined thus far. Given the importance of innate immunity in host defence of bony fish, it is perhaps not surprising that they have evolved a more elaborate cytokine-regulation system of macrophage

antimicrobial responses. However, the precise evolutionary as well as practical advantage for a more segregated Type II interferon system in bony fish remains to be fully elucidated.

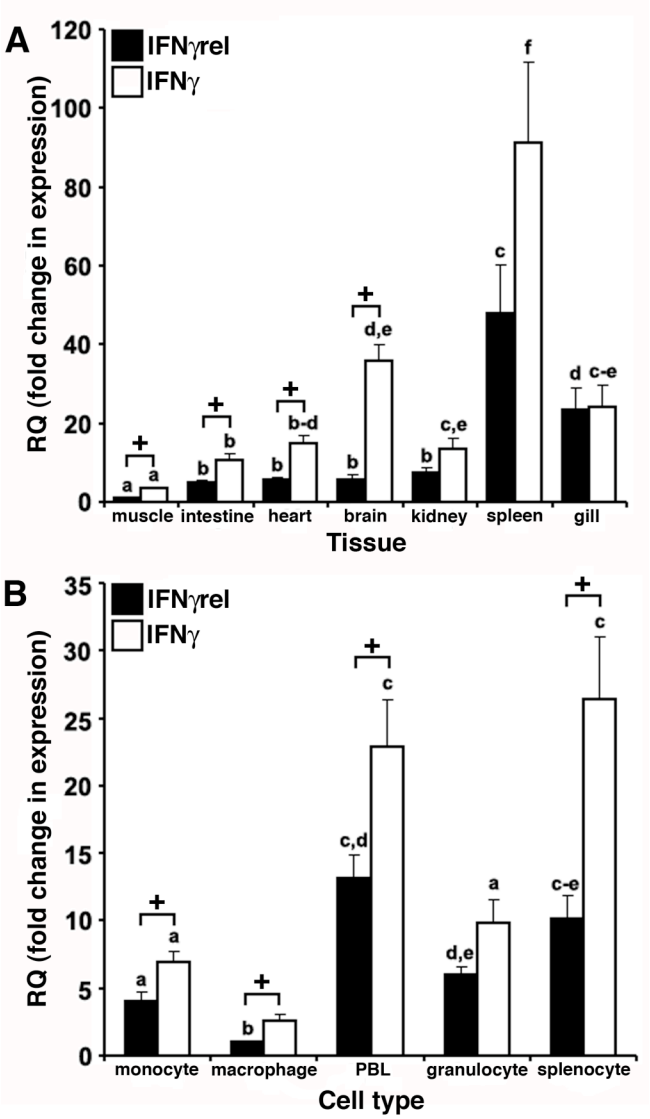

Figure 7.1. Quantitative expression analysis of IFNγ and IFNγrel in goldfish tissues and immune cell populations. **(A)** Goldfish IFNγrel tissue expression analysis. The expression of goldfish IFNγrel was assessed relative to endogenous control gene, elongation factor 1 alpha (EF-1a). Analyses of the relative tissue expression data are for tissues from five fish (*n*=5). All results were normalized against the muscle IFNγrel expression levels. **(B)** Goldfish IFNγrel expression in different immune cell populations. Immune cells populations were derived from four fish (*n*=4) and the expression normalized against that of FACS-sorted macrophages. Direct comparisons of IFNγ and IFNγrel expression was achieved by performing ddCT analysis using lowest expression as the standard for the expression of both cytokines. The RQ values were normalized against the lowest observed tissue or cell expression (IFNγrel, muscle and monocytes respectively). Statistical analysis was performed using one-way ANOVA. Different letters above each bar denote significant differences ($P < 0.05$), the same letter indicate no statistical difference between groups. (+) above lines denote statistical differences ($P < 0.05$) between indicated experimental bars.

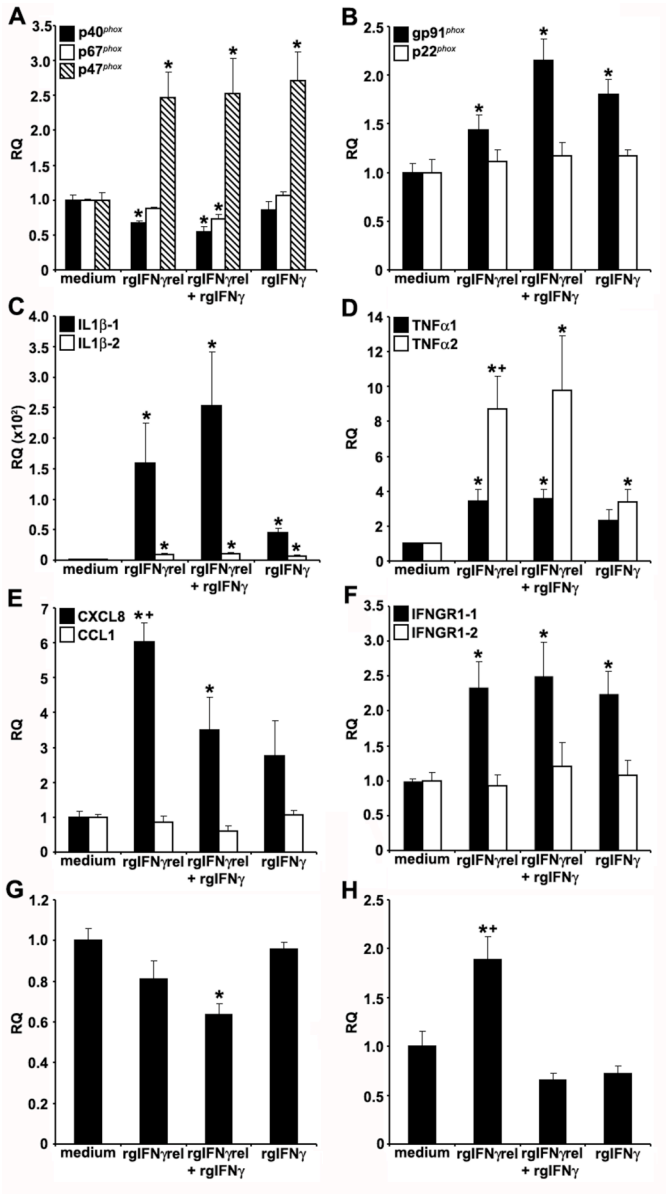

Figure 7.2. Quantitative gene expression analysis of goldfish immune genes in monocytes stimulated with rgIFNγrel, rgIFNγ or a combination of both cytokines. The reported expression was relative to EF-1α. The genes examined included: **(A)** $p40^{phox}$, $p47^{phox}$, $p67^{phox}$; **(B)** $gp91^{phox}$, $p22^{phox}$; **(C)** IL-1β1, IL-1β2; **(D)** TNFα1, TNFα2; **(E)** CXCL8, CCL1; **(F)** IFNGR1-1, IFNGR1-2; **(G)** TGFβ; **(E)** ceruloplasmin. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are mean ± SEM RQ values for monocytes obtained from cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls and (+) denotes significantly different ($P < 0.05$) from respective rgIFNγ treated cells.

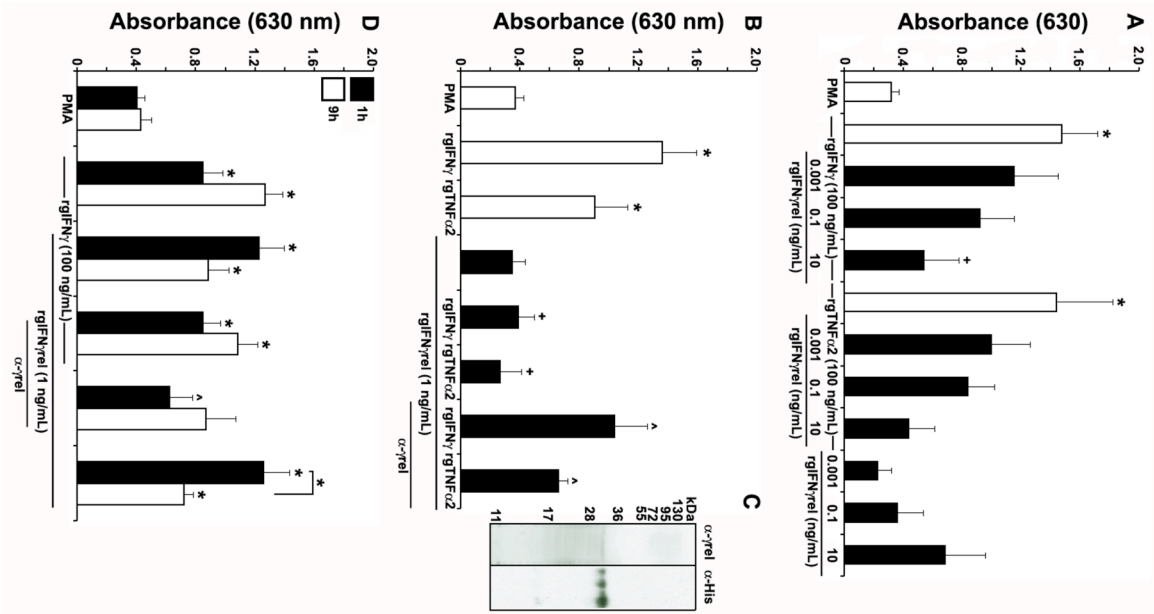

Figure 7.3. Recombinant goldfish IFNγrel temporally regulates the priming of the monocyte reactive oxygen production. (A) rgIFNγrel reduces rgIFNγ- and rgTNFα2- primed ROI. Cells were treated with medium, rgTNα2 (100 ng/mL), rgIFNγ (100 ng/mL), rgIFNγrel (0.001, 0.1, 10 ng/mL) or a combination of rgTNFα2 or rgIFNγ and rgIFNγrel. **(B)** α-rgIFNγrel Ab restores rgIFNγrel-mediated ROI down-regulation. Cells were treated with medium, rgTNα2 (100 ng/mL), rgIFNγ (100 ng/mL), rgIFNγrel (1 ng/mL) or a combination of rgTNα2 or rgIFNγ and rgIFNγrel (1 ng/mL), alone or in conjuction with α-rgIFNγrel polyclonal IgG (5 μg/ml). **(C)** Western blot detection of rgIFNγrel with α-rgIFNγrel IgG and α-His IgG. **(D)** rgIFNγrel elicits short-lived priming of monocyte ROI. Cells were treated with medium, rgIFNγ (100 ng/mL), rgIFNγrel (1 ng/mL) or a combination of rgIFNγ and rgIFNγrel, alone or in conjuction with α-rgIFNγrel IgG (5 μg/mL). All experiments used monocyte cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from medium controls. (+) denotes significantly different from respective treatments (rgIFNγ or rgTNFα2) without rγIFNγrel ($P < 0.05$). (^) denotes significantly different from respective treatments without Ab application.

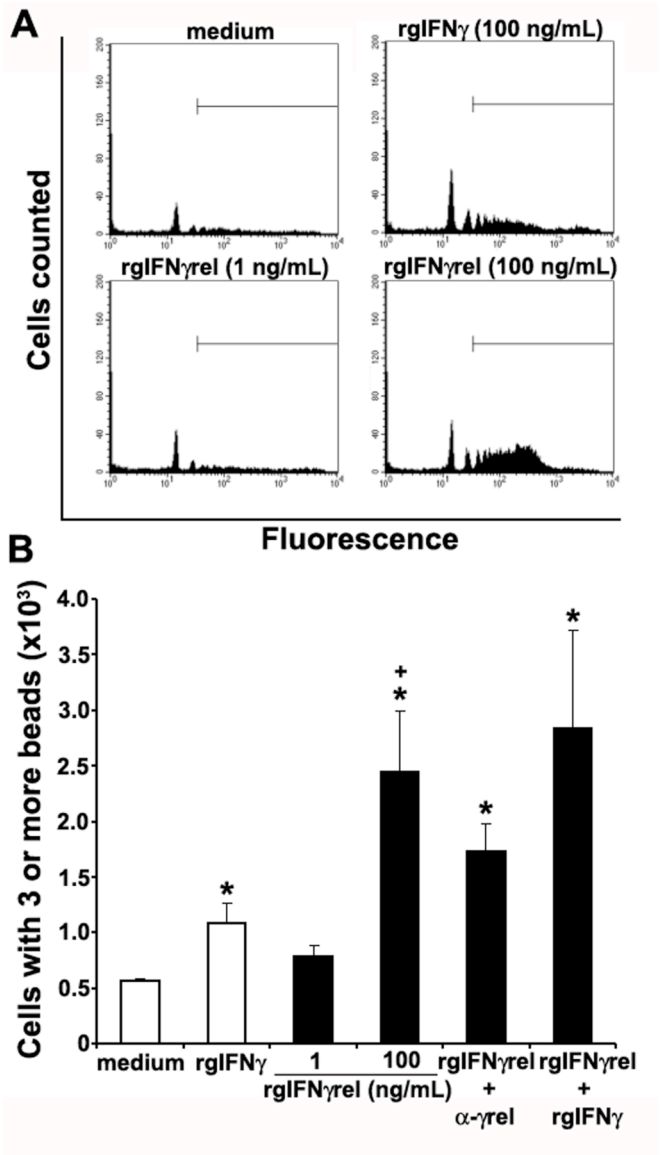

Figure 7.4. Recombinant goldfish IFNγrel induces higher monocyte phagocytic responses compared to rgIFNγ. Goldfish monocyte cultures were treated with medium, rgIFNγ (100ng/mL), or rgIFNγrel (1, 100 ng/mL) and phagocytosis assessed by FACS. **(A)** Representative phagocytosis histogram plots of cells from an individual fish treated with medium, rgIFNγ or rgIFNγrel. **(B)** Mean ± SEM phagocytic response of monocytes obtained from cultures established from individual fish (*n*=5) that have ingested 3 or more beads following treatment with medium, rgIFNγ (100 ng/mL), rgIFNγrel (1, 100 ng/mL), a combination of rgIFNγ (100 ng/mL) and rgIFNγrel (100 ng/mL), or rgIFNγrel (100 ng/mL) in conjuction with α-rgIFNγrel IgG (5 μg/mL). Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium control. (+) denotes statistically significant ($P < 0.05$) from rgIFNγ induced phagocytosis values.

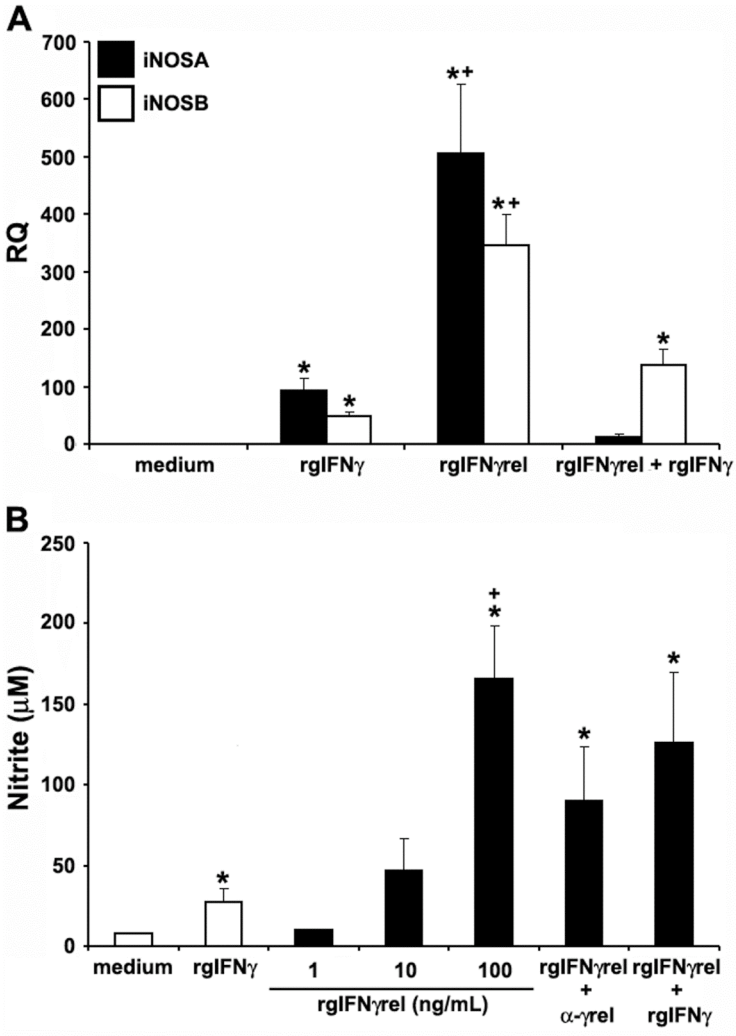

Figure 7.5. Recombinant goldfish IFNγrel induces higher macrophage iNOS gene expression and nitric oxide production compared to rgIFNγ. (A) Q-PCR analysis of gene expression of iNOS isoforms A and B in goldfish macrophages treated with medium, rgIFNγrel (100 ng/mL), rgIFNγ (100 ng/mL) or both recombinant cytokines. Q-PCR was performed using the delta CT method against the endogenous control, elongation factor 1 alpha (EF-1α). The results are mean ± SEM RQ values for macrophage cultures established from five individual fish (*n*=5) and normalized against the RQ values from medium treated cells. **(B)** Nitrite production by cytokine stimulated goldfish macrophages. Macrophage cultureswere established from individual fish (*n*=5) and were treated with medium, rgIFNγ (100 ng/mL), rgIFNγrel (1, 10, 100 ng/mL) or a combination of rgIFNγ and rgIFNγrel, or rgIFNγrel (100 ng/mL) in conjunction with α-rgIFNγrel IgG (5 μg/mL). Nitrite production was determined using the Griess reaction. The results are mean ± SEM μM nitrite. Statistical analysis was done using one-way ANOVA. (*) denotes statistically different ($P < 0.05$) from medium controls. (+) denotes significant difference ($P < 0.05$) form rgIFNγ treated cells.

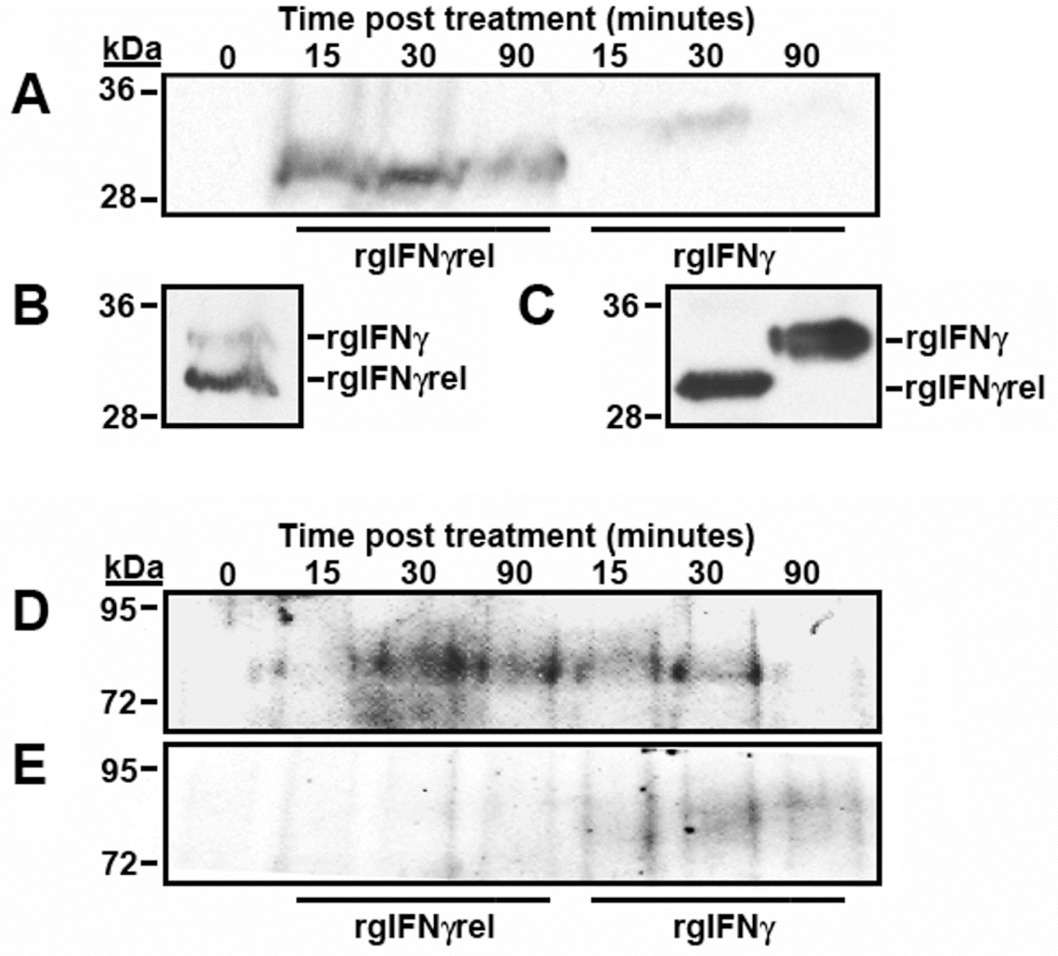

Figure 7.6. Analysis of rgIFNγrel and rgIFNγ cellular association, Stat1 tyrosine phosphorylation and phospho-(Y)-Stat1 nuclear accumulation in monocytes treated with rgIFNγrel or rgIFNγ. Five million monocytes were incubated with either medium alone, 5 mg of rgIFNγrel or 5 mg of rgIFNγ for 0, 15, 30 or 90 minutes. Whole cell lysates **(A)** were assayed by Western blot using a-polyHis antibody. Cells were also co-incubated with 5 mg of rgIFNγrel and 5 mg of rgIFNγ for half an hour **(B)**. The relative amounts of rgIFNγrel and rgIFNγ added to cells can be seen in **(C)**. Five million monocytes were incubated with medium, 100 ng/mL of rgIFNγrel or 100 ng/mL of rgIFNγ for 0, 15, 30 or 90 minutes. Whole cell lysates **(D)** or isolated nuclei **(E)** were assessed by Western blot with an α-phospho-(Tyr)-Stat1 antibody.

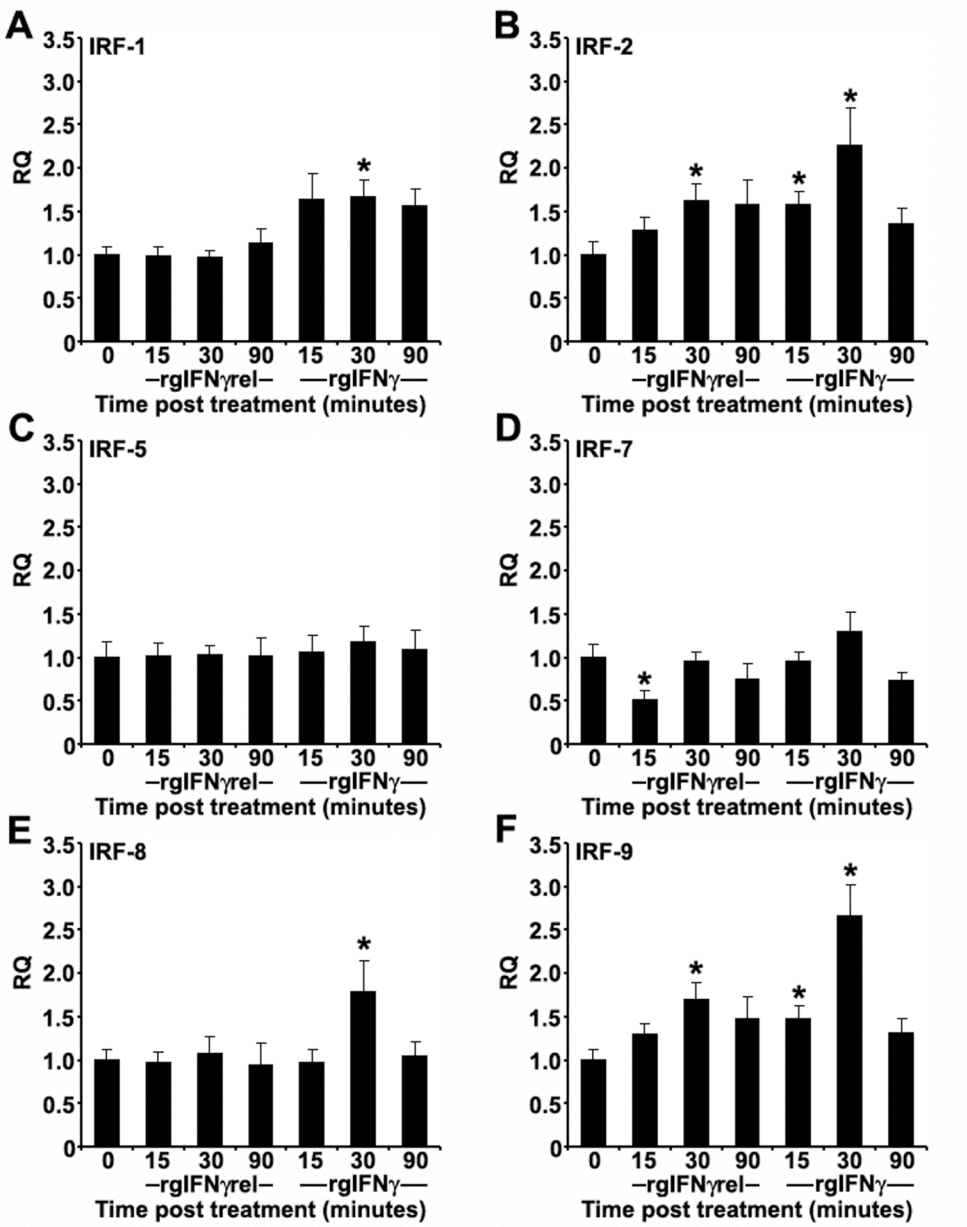

Figure 7.7. Quantitative gene expression analysis of goldfish interferon responsive factors (IRFs) in monocytes treated with medium, 100 ng/mL of rgIFNγrel or 100 ng/mL of rgIFNγ for 0, 15, 30, or 90 minutes. The reported expression was relative to EF-1α. The genes examined included: **(A)** IRF-1; **(B)** IRF-2; **(C)** IRF-5; **(D)** IRF-7; **(E)** IRF-8; **(F)** IRF-9. The expression data were normalized against expression of respective IRFs at 0 minute time point. The results are mean ± SEM RQ values for monocytes obtained from cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective 0 time point control.

Figure 7.8. Protein sequence alignments of IFNγrel (A) and IFNγ (B). Sequence alignments were performed using ClustalW. The signal sequences are in bold face, the IFNγ signature sequences are highlighted in grey and indicated overhead and the nuclear localization signals (**NLS**) are bold face with the "NLS" overhead. Fully conserved residues are indicated by an asterisk (*) below, partially conserved and semi-conserved substitutions are represented by ":" and ".", respectively.

4. REFERENCES

1. **Bader, T., and J. Weitzerbin.** 1994. Nuclear accumulation of interferon gamma. Proc Natl Acad Sci U S A **91:**11831-11835.
2. **Belosevic, M., C. E. Davis, M. S. Meltzer, and C. A. Nacy.** 1988. Regulation of activated macrophage antimicrobial activities. Identification of lymphokines that cooperate with IFN-gamma for induction of resistance to infection. J Immunol **141:**890-896.
3. **Belosevic, M., D. S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy.** 1989. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major. J Immunol **143:**266-274.
4. **Bernabei, P., A. Allione, L. Rigamonti, M. Bosticardo, G. Losana, I. Borghi, G. Forni, and F. Novelli.** 2001. Regulation of interferon-gamma receptor (INF-gammaR) chains: a peculiar way to rule the life and death of human lymphocytes. European cytokine network **12:**6-14.
5. **Bernabei, P., E. M. Coccia, L. Rigamonti, M. Bosticardo, G. Forni, S. Pestka, C. D. Krause, A. Battistini, and F. Novelli.** 2001. Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. Journal of leukocyte biology **70:**950-960.
6. **Berton, G., L. Zeni, M. A. Cassatella, and F. Rossi.** 1986. Gamma interferon is able to enhance the oxidative metabolism of human neutrophils. Biochem Biophys Res Commun **138:**1276-1282.
7. **Bluyssen, H. A., R. Muzaffar, R. J. Vlieststra, A. C. van der Made, S. Leung, G. R. Stark, I. M. Kerr, J. Trapman, and D. E. Levy.** 1995. Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. Proceedings of the National Academy of Sciences of the United States of America **92:**5645-5649.
8. **Cassatella, M. A., F. Bazzoni, R. M. Flynn, S. Dusi, G. Trinchieri, and F. Rossi.** 1990. Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. J Biol Chem **265:**20241-20246.
9. **Castro, R., S. A. Martin, S. Bird, J. Lamas, and C. J. Secombes.** 2008. Characterisation of gamma-interferon responsive promoters in fish. Molecular immunology **45:**3454-3462.
10. **Castro, R., S. A. Martin, J. Zou, and C. J. Secombes.** Establishment of an IFN-gamma specific reporter cell line in fish. Fish & shellfish immunology **28:**312-319.
11. **Chen, W. Q., Q. Q. Xu, M. X. Chang, J. Zou, C. J. Secombes, K. M. Peng, and P. Nie.** 2009. Molecular characterization and expression analysis of the IFN-gamma related gene (IFN-gammarel) in grass carp Ctenopharyngodon idella. Vet Immunol Immunopathol.

12. **Collet, B., G. Ganne, S. Bird, and C. M. Collins.** 2009. Isolation and expression profile of a gene encoding for the Signal Transducer and Activator of Transcription STAT2 in Atlantic salmon (Salmo salar). Developmental and comparative immunology **33:**821-829.
13. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med **178:**2243-2247.
14. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-1421.
15. **Driggers, P. H., D. L. Ennist, S. L. Gleason, W. H. Mak, M. S. Marks, B. Z. Levi, J. R. Flanagan, E. Appella, and K. Ozato.** 1990. An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. Proceedings of the National Academy of Sciences of the United States of America **87:**3743-3747.
16. **Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy.** 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell **84:**443-450.
17. **Fertsch, D., and S. N. Vogel.** 1984. Recombinant interferons increase macrophage Fc receptor capacity. J Immunol **132:**2436-2439.
18. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of novel interferon gamma receptor 1 isoforms in zebrafish (Danio rerio) and goldfish (Carassius auratus L.). Mol Immunol **46:**3050-3059.
19. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization, expression and functional analysis of goldfish (Carassius auratus L.) interferon gamma. Dev Comp Immunol **33:**235-246.
20. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Dev Comp Immunol **32:**532-543.
21. **Gupta, J. W., M. Kubin, L. Hartman, M. Cassatella, and G. Trinchieri.** 1992. Induction of expression of genes encoding components of the respiratory burst oxidase during differentiation of human myeloid cell lines induced by tumor necrosis factor and gamma-interferon. Cancer Res **52:**2530-2537.
22. **Han, C. H., J. L. Freeman, T. Lee, S. A. Motalebi, and J. D. Lambeth.** 1998. Regulation of the neutrophil respiratory burst oxidase. Identification of an activation domain in p67(phox). J Biol Chem **273:**16663-16668.
23. **Harada, H., E. Takahashi, S. Itoh, K. Harada, T. A. Hori, and T. Taniguchi.** 1994. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for a gene network in the interferon system. Molecular and cellular biology **14:**1500-1509.
24. **He, R., M. Nanamori, H. Sang, H. Yin, M. C. Dinauer, and R. D. Ye.** 2004. Reconstitution of chemotactic peptide-induced nicotinamide

adenine dinucleotide phosphate (reduced) oxidase activation in transgenic COS-phox cells. J Immunol **173:**7462-7470.

25. **Holland, J. W., S. Bird, B. Williamson, C. Woudstra, A. Mustafa, T. Wang, J. Zou, S. C. Blaney, B. Collet, and C. J. Secombes.** 2008. Molecular characterization of IRF3 and IRF7 in rainbow trout, Oncorhynchus mykiss: functional analysis and transcriptional modulation. Molecular immunology **46:**269-285.
26. **Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet.** 1993. Immune response in mice that lack the interferon-gamma receptor. Science **259:**1742-1745.
27. **Igawa, D., M. Sakai, and R. Savan.** 2006. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. Mol Immunol **43:**999-1009.
28. **Ihle, J. N., and I. M. Kerr.** 1995. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet **11:**69-74.
29. **Kerr, I. M., and G. R. Stark.** 1992. The antiviral effects of the interferons and their inhibition. J Interferon Res **12:**237.240.
30. **Levraud, J. P., P. Boudinot, I. Colin, A. Benmansour, N. Peyrieras, P. Herbomel, and G. Lutfalla.** 2007. Identification of the zebrafish IFN receptor: implications for the origin of the vertebrate IFN system. J Immunol **178:**4385-4394.
31. **Lopez-Munoz, A., F. J. Roca, J. Meseguer, and V. Mulero.** 2009. New insights into the evolution of IFNs: zebrafish group II IFNs induce a rapid and transient expression of IFN-dependent genes and display powerful antiviral activities. J Immunol **182:**3440-3449.
32. **Martin, E., C. Nathan, and Q. W. Xie.** 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J Exp Med **180:**977.984.
33. **Matsumoto, M., N. Tanaka, H. Harada, T. Kimura, T. Yokochi, M. Kitagawa, C. Schindler, and T. Taniguchi.** 1999. Activation of the transcription factor ISGF3 by interferon-gamma. Biological chemistry **380:**699-703.
34. **Mazumder, B., and P. L. Fox.** 1999. Delayed translational silencing of ceruloplasmin transcript in gamma interferon-activated U937 monocytic cells: role of the 3' untranslated region. Mol Cell Biol **19:**6898-6905.
35. **Mazumder, B., C. K. Mukhopadhyay, A. Prok, M. K. Cathcart, and P. L. Fox.** 1997. Induction of ceruloplasmin synthesis by IFN-gamma in human monocytic cells. J Immunol **159:**1938-1944.
36. **Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber.** 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell **84:**431-442.

37. **Milev-Milovanovic, I., S. Long, M. Wilson, E. Bengten, N. W. Miller, and V. G. Chinchar.** 2006. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics **58:**70-80.
38. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol **7:**145-173.
39. **Nelson, N., Y. Kanno, C. Hong, C. Contursi, T. Fujita, B. J. Fowlkes, E. O'Connell, J. Hu-Li, W. E. Paul, D. Jankovic, A. F. Sher, J. E. Coligan, A. Thornton, E. Appella, Y. Yang, and K. Ozato.** 1996. Expression of IFN regulatory factor family proteins in lymphocytes. Induction of Stat-1 and IFN consensus sequence binding protein expression by T cell activation. J Immunol **156:**3711-3720.
40. **Neumann, N. F., and M. Belosevic.** 1996. Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). Dev Comp Immunol **20:**427.439.
41. **Newburger, P. E., R. A. Ezekowitz, C. Whitney, J. Wright, and S. H. Orkin.** 1988. Induction of phagocyte cytochrome b heavy chain gene expression by interferon gamma. Proc Natl Acad Sci U S A **85:**5215-5219.
42. **Nisimoto, Y., S. Motalebi, C. H. Han, and J. D. Lambeth.** 1999. The p67(phox) activation domain regulates electron flow from NADPH to flavin in flavocytochrome b(558). J Biol Chem **274:**22999-23005.
43. **Perussia, B.** 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. Curr Opin Immunol **3:**49-55.
44. **Robertsen, B.** 2006. The interferon system of teleost fish. Fish Shellfish Immunol **20:**172-191.
45. **Sad, S., R. Marcotte, and T. R. Mosmann.** 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity **2:**271-279.
46. **Sampath, P., B. Mazumder, V. Seshadri, and P. L. Fox.** 2003. Transcript-selective translational silencing by gamma interferon is directed by a novel structural element in the ceruloplasmin mRNA 3' untranslated region. Mol Cell Biol **23:**1509-1519.
47. **Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume.** 2004. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of leukocyte biology **75:**163-189.
48. **Sieger, D., C. Stein, D. Neifer, A. M. van der Sar, and M. Leptin.** 2009. The role of gamma interferon in innate immunity in the zebrafish embryo. Dis Model Mech **2:**571-581.
49. **Staeheli, P.** 1990. Interferon-induced proteins and the antiviral state. Adv Virus Res **38:**147.200.
50. **Stevenson, M. M., M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba.** 1990. Role of endogenous gamma interferon in host response to infection with blood-stage Plasmodium chabaudi AS. Infect Immun **58:**3225-3232.

51. **Stolte, E. H., H. F. Savelkoul, G. Wiegertjes, G. Flik, and B. M. Lidy Verburg-van Kemenade.** 2008. Differential expression of two interferon-gamma genes in common carp (Cyprinus carpio L.). Dev Comp Immunol **32:**1467.1481.
52. **Subramaniam, P. S., B. A. Torres, and H. M. Johnson.** 2001. So many ligands, so few transcription factors: a new paradigm for signaling through the STAT transcription factors. Cytokine **15:**175-187.
53. **Suh, C. I., N. D. Stull, X. J. Li, W. Tian, M. O. Price, S. Grinstein, M. B. Yaffe, S. Atkinson, and M. C. Dinauer.** 2006. The phosphoinositide-binding protein p40phox activates the NADPH oxidase during FcgammaIIA receptor-induced phagocytosis. J Exp Med **203:**1915-1925.
54. **Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi.** 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. Science **288:**2357.2360.
55. **Takaoka, A., and H. Yanai.** 2006. Interferon signalling network in innate defence. Cellular microbiology **8:**907.922.
56. **Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka.** 2001. IRF family of transcription factors as regulators of host defense. Annual review of immunology **19:**623-655.
57. **Tian, W., X. J. Li, N. D. Stull, W. Ming, C. I. Suh, S. A. Bissonnette, M. B. Yaffe, S. Grinstein, S. J. Atkinson, and M. C. Dinauer.** 2008. Fc{gamma}R-stimulated activation of the NADPH oxidase: phosphoinositide-binding protein p40phox regulates NADPH oxidase activity after enzyme assembly on the phagosome. Blood **112:**3867.3877.
58. **Tsunawaki, S., H. Mizunari, M. Nagata, O. Tatsuzawa, and T. Kuratsuji.** 1994. A novel cytosolic component, p40phox, of respiratory burst oxidase associates with p67phox and is absent in patients with chronic granulomatous disease who lack p67phox. Biochem Biophys Res Commun **199:**1378-1387.
59. **Wang, Z. E., S. L. Reiner, S. Zheng, D. K. Dalton, and R. M. Locksley.** 1994. CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major. J Exp Med **179:**1367.1371.
60. **Wheelock, E. F.** 1965. Interferon-Like Virus-Inhibitor Induced in Human Leukocytes by Phytohemagglutinin. Science **149:**310-311.
61. **Wientjes, F. B., J. J. Hsuan, N. F. Totty, and A. W. Segal.** 1993. p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem J **296 (Pt 3):**557.561.
62. **Young, H. A., and K. J. Hardy.** 1995. Role of interferon-gamma in immune cell regulation. Journal of leukocyte biology **58:**373-381.
63. **Zhang, L., and J. S. Pagano.** 1997. IRF-7, a new interferon regulatory factor associated with Epstein-Barr virus latency. Molecular and cellular biology **17:**5748-5757.

64. **Zou, J., A. Carrington, B. Collet, J. M. Dijkstra, Y. Yoshiura, N. Bols, and C. Secombes.** 2005. Identification and bioactivities of IFN-gamma in rainbow trout Oncorhynchus mykiss: the first Th1-type cytokine characterized functionally in fish. J Immunol **175:**2484-2494.

65. **Zou, J., C. Tafalla, J. Truckle, and C. J. Secombes.** 2007. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. J Immunol **179:**3859-3871.

66. **Zou, J., Y. Yoshiura, J. M. Dijkstra, M. Sakai, M. Ototake, and C. Secombes.** 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish & shellfish immunology **17:**403-409.

CHAPTER VIII: CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF GOLDFISH (*Carassius auratus* L.) INTERLEUKIN-10[6]

1. INTRODUCTION

Interleukin-10 (IL-10) is a central anti-inflammatory cytokine that was first identified in supernatants of Con-A-stimulated T cells based on its ability to inhibit the synthesis of pro-inflammatory cytokines (9). Aside from T cells, the production of IL-10 has been noted in other cell types including B cells, eosinophils, epithelial cells, keratinocytes, mesangial cells, monocytes/macrophages, NK cells and tumor cells (8). Although IL-10 was initially shown to inhibit T and NK cell function, it is now clear that the primary targets of IL-10 are cells of the monocyte/macrophage lineage and that the inhibition of lymphocyte function by IL-10 is an indirect bystander effect (3, 7, 10, 11). In addition to inhibiting monocyte/macrophage cytokine production in mammals, IL-10 has also been shown to down-regulate the production of reactive oxygen (ROI) and to a much lower extent, reactive nitrogen (NO) intermediates (3, 17). The primary mechanism behind the down-regulation of these processes has been elegantly linked to the IL-10-mediated inhibition of TNFα synthesis (17).

Interleukin-10 mediates its biological effects as a homodimer (25, 31) structurally similar to IFNγ (32, 34) through an interaction with a receptor

---

[6] A version of this chapter has been published: Grayfer, L., J. Hodgkinson, S.J. Hitchen, and M. Belosevic. 2010. Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin-10. Molecular Immunology 48:563-571.

complex composed of ligand binding (16, 27, 28), and accessory (15, 24) receptor subunits (IL10R1 and IL10R2, respectively). IL-10 signals via a Jak-Stat signalling pathway, with the signal transducer of activation 3 (Stat3), transcription factor recruited to and activated (through phosphorylation) by the ligated IL-10R complex (33). Stat3 activation is indispensable to the anti-inflammatory effects mediated by IL-10 (21, 26) and its activation results in a rapid transcriptional up-regulation of suppressor of cytokine signaling-3 (SOCS-3) (1) which is believed to be one of the primary mechanisms by which IL-10 mediates its anti-inflammatory effects in the mammalian systems (2, 20).

Interleukin-10 has been identified in several bony fish species including puffer fish (36), carp (22), trout (13) zebrafish (35), seabass (18) and cod (23). In the initial report of IL-10 in puffer fish, the single copy of the IL-10 gene exhibited very low tissue gene expression (36). The tissue expression of the IL-10 gene in carp, trout and zebrafish was reported to be the greatest in the head kidney, spleen and gill tissues and was subject to up-regulation following administration of LPS (13, 22, 35). The injection of cod with formaline-killed *Vibrio anguillarum* and polyI:C resulted in the up-regulation of spleen IL-10 gene expression (23). The sea bass spleen and kidney tissues exhibited increased IL-10 mRNA levels following immune stimulation with UV-killed *Photobacterium damselae ssp. Piscicida* (18). Furthermore, the tissue gene expression of Atlantic salmon IL-10 increased in fish infected with pancreatic necrosis virus (12), while carp intraepithelial lymphocytes fluctuated in their expression of IL-10 following soybean meal-induced enteritis (30). Despite the growing evidence of the fish IL-

10 being involved in immune regulation, to date there have been no reports focusing on the functional characterization of this cytokine in bony fish.

In this chapter, I describe the first comprehensive functional characterization of a bony fish interleukin-10. I cloned and expressed goldfish IL-10 (rgIL-10) using a prokaryotic expression system. Phylogenetic analysis confirmed a close relationship of goldfish IL-10 with those of carp and zebrafish, respectively. The tissue gene expression of goldfish IL-10 mRNA was found to be the highest in the spleen, peripheral blood leukocytes (PBL) and granulocytes. The rgIL-10 substantially reduced the gene expression of TNFα1, TNFα2, IL-1β1, IL-10, CXCL-8, and NADPH oxidase component, p47phox in monocytes activated with heat-killed *Aeromonas salmonicida.* The rgIL-10 also down-regulated the IFNγ gene expression in *A. salmonicida*-activated splenocytes. Furthermore, pre-treatment of monocyets with rgIL-10 resulted in substantial reduction in the their ability to up-regulate ROI in response to *A. salmonicida* or rgIFNγ. Western blot analysis indicated that rgIL-10 was capable of multimerization, cellular interaction and the induction of phosphorylation and nuclear translocation of Stat3. Furthermore, rgIL-10 induced rapid and robust increases in mRNA levels of the goldfish monocyte SOCS-3.

2. RESULTS

2.1. *In silico* analysis of the goldfish IL-10

The complete open reading frame and the untranslated (UTR) regions of the goldfish IL-10 cDNA transcript were obtained (Fig. 8.1). Sequence analysis of

the UTR regions revealed the presence of a typical polyadenylation sequence (AATAAA) in the 3' UTR (Fig. 8.1). The 5' and 3' UTRs also contained RNA instability regions (ATTTA): one in the 5' and 5 in the 3' regions (Fig. 8.1). As denoted in Figure 8.1, the predicted goldfish IL-10 protein sequence contained a signal peptide (underlined), 4 cysteine residues thought to be essential for cytokine structure as well as 6 predicted helical domains. Also present in the predicted goldfish IL-10 protein sequence was the IL-10 family signature (G-X2-KA-X2-[D,E]-X-D[ILV]-[FLY]-[FILMV]-X2-[ILMV][EKQR] with the exception of an M in the [FLY] position (Fig. 8.1).

Phylogenetic analysis of the goldfish IL-10 demonstrated closest relationship to the carp IL-10, followed by IL-10 of zebrafish and then trout (Fig. 8.2). The IL-10 proteins of the above fish species branched independently to the IL-10s of the other fish species including seabass, fugu (puffer fish), tetraodon and cod (Fig. 8.2). All known fish IL-10 proteins in turn branched independently from the IL-10 protein sequences of higher vertebrates (Fig. 8.2). Goldfish IFNγ and IFNγrel were used as out-groups (Fig. 8.2).

2.2. Gene expression analysis of IL-10 in goldfish tissues and immune cell populations

Assessment of IL-10 gene expression in the tissues of healthy goldfish revealed highest IL-10 mRNA transcript levels in goldfish spleens, followed by relatively similar gene expression in kidney, brain and gill tissues (Fig. 8.3A).

Lower gene expression levels were observed in the intestines and hearts, with goldfish muscle tissues exhibiting the lowest IL-10 transcript levels (Fig. 8.3A).

Splenocytes, granulocytes, PBL and monocytes had similar IL-10 mRNA transcript levels, while mature macrophages expressed substantially less of the IL-10 transcript (Fig. 8.3B). Cell stimulation with rgTNFα2 caused a significant down-regulation of the IL-10 mRNA levels in granulocyte and monocyte populations (Fig. 8.3B) while not affecting gene expression of IL-10 in the other cell populations examined (data not shown).

2.3. Recombinant goldfish IL-10 down-regulates immune gene expression in *A. salmonicida*-activated monocytes

To assess the ability of the rgIL-10 to down-regulate monocyte immune gene expression, cells were pre-treated with 500 ng/mL of the recombinant cytokine and then stimulated with heat killed *A. salmonicida* (2 μg/mL) (Fig. 8.4). The immune genes examined were: TNFα1 and TNFα2; IL-1β1 and IL-1β2; IL-10; TGFβ; CCL1; CXCL8; $p40^{phox}$; $p47^{phox}$; $p67^{phox}$; $p22^{phox}$ and $gp91^{phox}$. The pre-treatment of monocytes with rgIL-10 significantly abrogated the up-regulation of TNFα1 and TNFα2, IL-10, CXCL-8 and $p47^{phox}$ gene expression induced by *A. salmonicida* (Fig. 8.3A,C,D,E, respectively). Although not significantly, rgIL-10 pre-treatments also caused lower *Aeromonas*-induced IL-1β1 gene up-regulation compared to cells treated with *A. salmonicida* alone (Fig. 8.4B). The rgIL-10 pre-treatment of non-stimulated cells (no *Aeromonas*) did not cause gene expression changes (Fig. 8.4A-F).

Splenocytes pre-treated with rgIL-10 had significantly diminished up-regulation of IFNγ following *A. salmonicida* stimulation, compared to splenocytes pre-treated with medium prior to the bacterial stimulation (Fig. 8.5). The splenocyte IFNγrel mRNA levels were not altered by any of the treatments (Fig. 8.5).

2.4. Recombinant goldfish IL-10 down-regulates the ROI priming effects of heat-killed A. *salmonicida* and rgIFNγ

Given that rgIL-10 abrogated the induction of NADPH component $p47^{phox}$ (Fig. 8.6), I assessed whether treatment of monocytes with rgIL-10 resulted in decreased ability of this cell population to mount an ROI response. Cells were pre-incubated with 10, 100 or 1000 ng/mL of rgIL-10 or medium alone and subsequently primed with either heat-killed *Aeromonas salmonicida* (2 μg/mL) or rgIFNγ (100 ng/mL). ROI production was induced with the phorbol ester, PMA. Pre-treatment of cells with 10 to 1000 ng/mL of rgIL-10 substantially abrogated the *A. salmonicida* and rgIFNγ mediated enhancement of ROI production (Fig. 8.6A,B). Statistically significant ($P<0.05$) down-regulation of rgIFNγ-mediated ROI production was observed when cells were pre-treated with 1000 ng/mL of rgIL-10 (Fig. 8.6B). Interestingly, none of the examined rgIL-10 concentrations had a significant effect on the ROI production induced by PMA (Fig. 8.6A,B). Longer rgIL-10 pre-incubation times and longer assay incubation times did not result in further ROI reduction (data not shown).

2.5. Cross-linking analysis of rgIL-10

I performed *in vitro* cross-linking studies to determine whether the rgIL-10 was capable of multimerization (Fig 8.7A), which has been reported for mammalian IL-10. The rgIL-10 was incubated in the absence or in the presence of the cross-linker DSS, resolved by SDS-PAGE and visualized by Western blot against the polyHis tag on the recombinant protein. While the non-cross-linked rgIL-10 resolved as a monomer (Fig 8.7A, lane 1), the cross-linked rgIL-10 appeared to be a dimer and to a lesser extent, a trimer (Fig. 8.7A, lane 2).

2.6. Analysis of cellular association of rgIL-10

Goldfish monocytes were incubated with 5 μg of rgIL-10 (Fig. 8.7B) for 0, 15, 30 or 90 minutes and then assessed by Western blot (Fig. 8.7C). The cellular association of rgIL-10 appeared to increase with increased incubation time for all time points tested (Fig. 8.7C).

2.7. Analysis of rgIL-10-mediated Stat3-(Y)-phosphorylation and nuclear translocation

The biological effects of the mammalian IL-10 result from receptor ligation and subsequent tyrosine phosphorylation and nuclear translocation of the signal transducer of activation-3 (Stat3) transcription factor (33). In order to assess the ability of rgIL-10 to mediate these affect in goldfish monocytes, I treated cells for 0, 15, 30 or 90 minutes with 500 ng/mL of rgIL-10, resolved the whole cell lysates or isolated nuclei of these cells on SDS-gels and assessed Stat-

3-Y-phosphorylation by western blotting (Fig. 8.7D). A band of about 90 kDa (approximate size of Stat3) was detected in the cell lysates of cells treated for 15 minutes and 30 minutes (a weaker signal) and in the nuclei of cell treated for 15 minutes with rgIL-10 (Fig. 8.7D). An additional, fainter band of approximately 100 kDa was also detected in the cell lysates of monocytes treated for 15 and 30 minutes and an in isolated nuclei of monocytes treated for 15 minutes with the rgIL-10 (Fig. 8.7D).

2.8. The rgIL-10 up-regulates the gene expression of SOCS-3 in goldfish monocytes

Since one of the central mechanisms by which the mammalian IL-10 mediates its inhibitory effects is through increasing the gene expression of suppressor of cytokine signalling-3 (SOCS-3), I next assessed whether the goldfish rgIL-10 operates through the similar means. To examine this, I cloned the goldfish SOCS-3 cDNA and designed Q-PCR primers to measure gene expression changes of this gene following rgIL-10 stimulation. The goldfish SOCS-3 mRNA transcript levels were significantly elevated in monocytes treated for 2, 6 or 12 hours with 500 ng/mL of rgIL-10 (Fig. 8.8).

3. DISCUSSION

In this chapter, I report on the cloning, molecular analysis and comprehensive functional characterization of the goldfish interleukin-10. The goldfish IL-10 predicted peptide possessed the characteristic signature residues

that are highly conserved among IL-10 molecules of different species. Additionally present in the 5' and 3' prime UTRs were mRNA instability motifs which have been reported to regulate post-transcriptional regulation of the mammalian IL-10 (19). Interestingly, while these instability regions (ATTTA) are only present on the 3' UTRs of the mammalian (19) and other known fish IL-10 (22, 35, 36) sequences, the goldfish IL-10 possesses an additional instability motif in it's 5' UTR. This is perhaps a manifestation of having to tightly regulate the production of this anti-inflammatory cytokine in a species that possesses multiple isoforms of key pro-inflammatory mediators such as TNFα, IL-1β and IFNγ.

Similar to other reports on fish IL-10 (22, 35, 37), the goldfish IL-10 exhibited the highest mRNA levels in the spleen, kidney and gill tissues. Among the immune populations examined, PBLs, splenocytes, granulocytes and monocytes expressed similar IL-10 mRNA levels, while mature macrophages had substantially lower levels of this transcript. More recently, it has been demonstrated that the IL-10 gene can be regulated by the transcription factors SP1 and SP3, which are constitutively active in numerous cell types (4, 29). Likely, the goldfish immune cell gene expression of IL-10 is to some extent constitutive but the subsequent stability of the IL-10 transcripts is subject to autocrine and paracrine stimuli as well as the general state of the given cell. Supporting this hypothesis is my observation that the IL-10 mRNA levels decreased following rgTNFα2 stimulation of monocytes and granulocytes, but not other cell types examined. This observation corroborates the reported presence of a

TNFα–responsive element in the promoter region of the puffer fish IL-10 gene (36), and suggest that the regulation of IL-10 production is both complex and cell-type dependent.

Goldfish monocytes pre-treated with rgIL-10 prior to stimulation with heat-killed *Aeromonas salmonicida* exhibited substantially lower mRNA levels of TNFα1, TNFα2, IL-1β1, CXCL-8 and IL-10, compared to cells that were not pre-treated with rgIL-10. The rgIL-10 pre-treatment of isolated splenocytes also down-regulated the *Aeromonas*-induced gene expression of IFNγ in these cells. These observation are consistent with those reported for the mammalian IL-10, which has been shown to inhibit the production of TNFα, IL-1β , CXCL-8 and IL-10 by activated monocytes/macrophages (3, 6, 10, 11, 14) and inhibited the production of IFNγ by activated lymphocytes (5). Together these observations indicate that one of the central roles for IL-10, as a cytokine synthesis inhibitor, may be evolutionarily conserved.

The functional assessment of the mammalian recombinant IL-10 (3) revealed that treatment of peritoneal macrophages with IL-10 at concentrations 50-100 fold greater than those required for the inhibition of TNFα synthesis, abrogated the PMA-triggered reactive oxygen production and to a much lesser extent the IFNγ-triggered NO production. Oswald *et al.* (1992) and Flesch et al. (1994) demonstrated that the deactivation of macrophages by IL-10 was not direct and that the observed down-regulation of macrophage antimicrobial responses was due to IL-10 down-regulation of TNFα production. Here I report that aside from inhibiting the expression of TNFα1 and TNFα2 in activated monocytes, the

goldfish recombinant IL-10 also abrogated the *Aeromonas*-induced and IFNγ-induced monocyte ROI production. Interestingly, I also observed a significant IL-10-induced decrease in the gene expression of NADPH oxidase component, $p47^{phox}$ in activated monocytes. Presumably, this transcriptional down-regulation was a contributing factor in the observed decrease of ROI production.

Biochemical and crystal structure studies of the mammalian recombinant IL-10 revealed that this cytokine forms and functions as a homodimer (25, 31). My *in vitro* cross-linking studies suggest that the goldfish recombinant IL-10 also adopts primarily a homodimeric state.

The goldfish IL-10 signals through the phosphorylation and nuclear translocation of Stat3, similar to the mammalian IL-10 (21, 26, 33). While the cellular association of rgIL-10 persisted for up to 90 min, both cellular and nuclear phospho-Y-Stat3 induced by rgIL-10 was more transient, peaking 15 min after treatment. This observation suggests that rgIL-10 receptor association becomes uncoupled from the downstream signalling events at later treatment points. Supporting this is my observation that rgIL-10 also rapidly induced the gene expression of SOCS-3, which would negatively regulate the signal from the IL-10 receptor. Indeed rgIL-10 significantly reduced the gene expression of the IL-10 gene in *A. salmonicida*-activated monocytes. Thus, goldfish IL-10 signalling is tightly regulated, with rapid induction of SOCS-3, concomitantly serving as both effector and negative feedback regulation mechanisms.

The anti-phospho-Y-Stat3 Western blot analysis of whole cell lysates and isolated nuclei from cells treated with rgIL-10 revealed the presence of an

additional band of slightly higher molecular weight than that predicted for Stat3. I performed protein alignments of the mammalian and zebrafish Stat3 and found that the epitopes recognized by the commercial reagent employed here were entirely conserved. Given that the goldfish Stat3 sequence is not known and since goldfish have had additional genome duplication events compared to zebrafish, I speculate that that the additional band may be an isoform of goldfish Stat3. Unfortunately, the commercially available anti-N-terminus Stat3 antibody that recognizes human, mouse and chicken Stat3 did not recognize the goldfish protein, preventing further examination of goldfish Stat3.

My findings indicate that the function of interleukin-10 as a central regulator of the anti-inflammatory response has been conserved in bony fish. Further *in vivo* studies using recombinant fish IL-10 may pave the way for therapeutic applications in controlling excessive inflammation in fish.

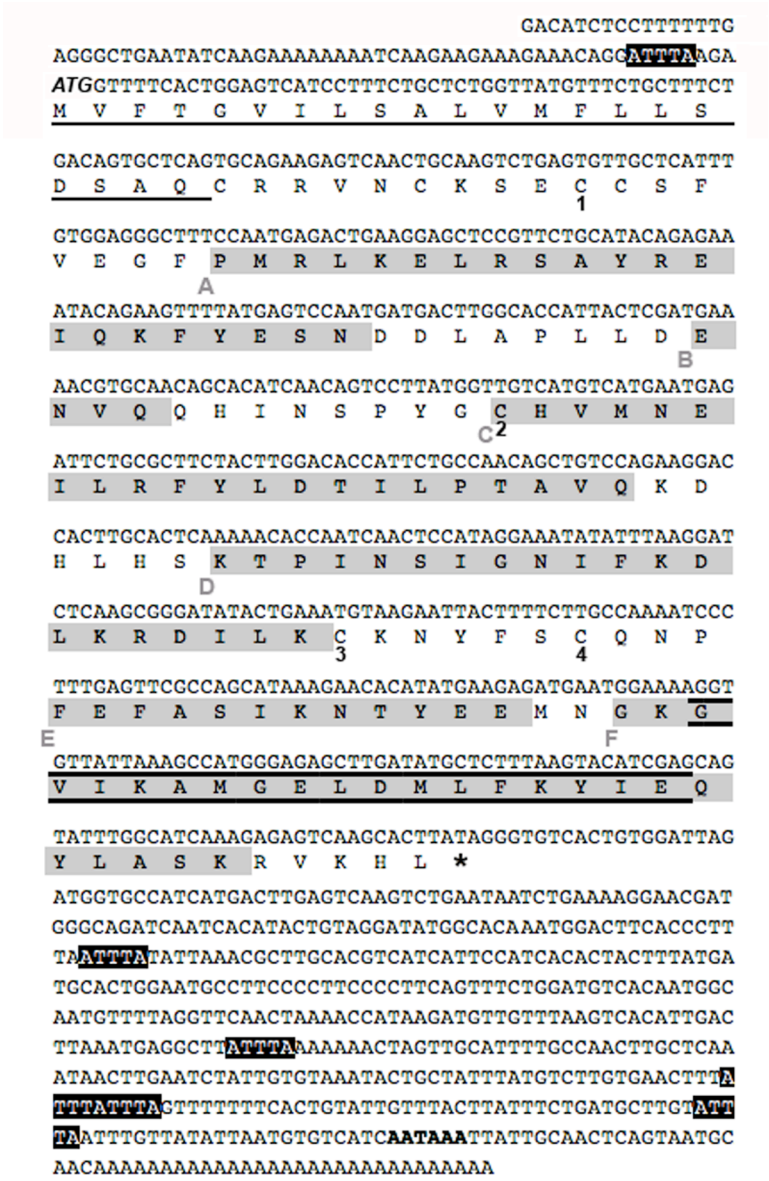

Figure 8.1. Goldfish interleukin-10 cDNA and predicted peptide sequences. The start codon (***ATG***) is in bold italics and the stop codon is indicated with (*). The signal peptide is underlined. The helical domains are depicted in grey boxes and numbered (A-F). The structurally essential cysteine residues are numbered (1-4). The IL-10 signature sequence is indicated by the under- and over-head lines. The mRNA instability motifs (ATTTA) are in black boxes and the polyadenylation signal (**AATAAA**) is in bold.

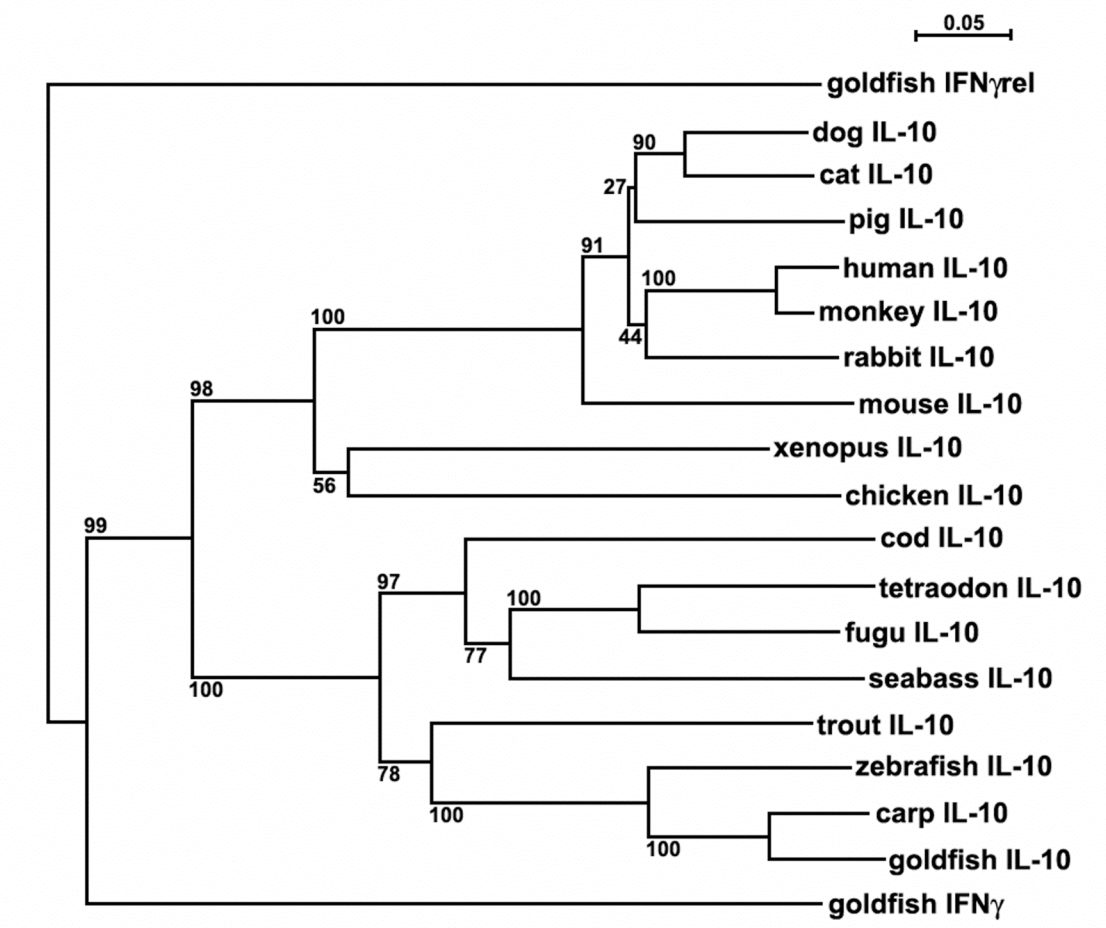

Figure 8.2. Phylogenetic analysis of the goldfish IL-10 in relation to other fish and vertebrate IL-10 proteins. The analysis was conducted using the neighbour joining method and resulting tree was bootstrapped 10,000 times and expressed as percent values. The goldfish IFNγ and IFNγrel were used as out-groups.

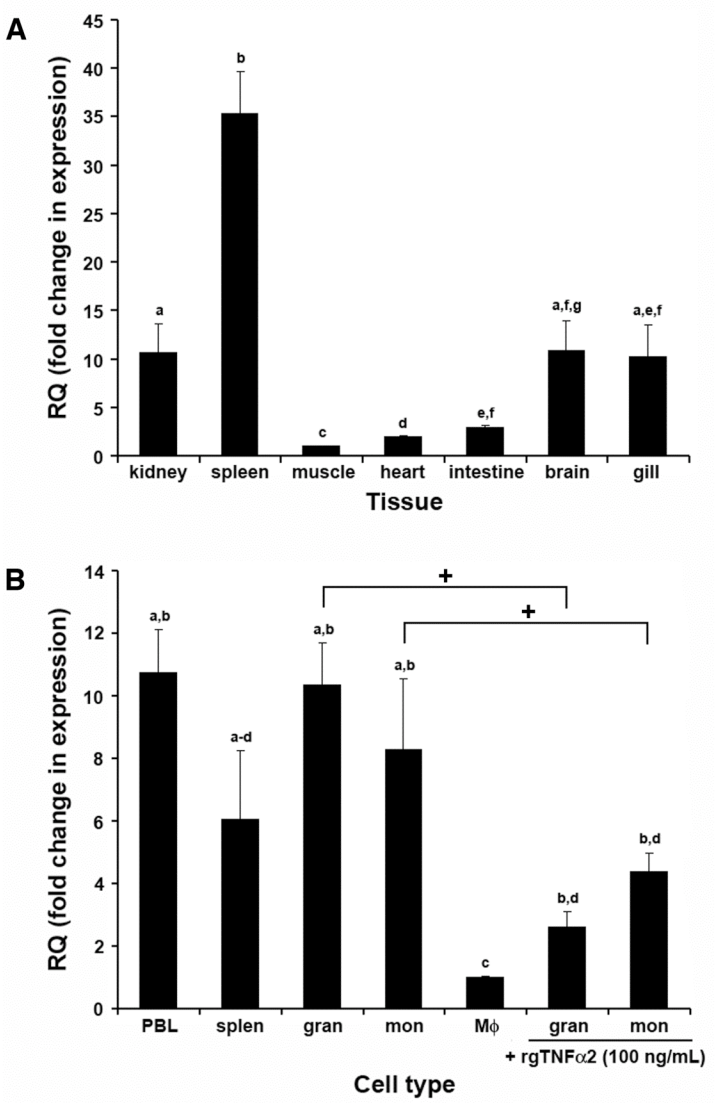

Figure 8.3. Quantitative gene expression analysis of IL-10 in goldfish tissues and immune cell populations. **(A)** Goldfish IL-10 tissue expression analysis. The tissues examined were: kidney, spleen, muscle, heart, intestine, brain and gill. The expression of goldfish IL-10 was assessed relative to endogenous control gene, elongation factor 1 alpha (EF-1α). Analyses of the relative tissue expression data are for tissues from five fish (*n*=5). All results were normalized against the muscle IL-10 expression levels. **(B)** Goldfish IL-10 expression in resting and rgTNFα2-activated immune cell populations. The cells examined were: monocytes (mon), macrophages (Mϕ), peripheral blood leukocytes (PBL), granulocytes (gran) and splenocytes (spleen). All cell populations were derived from 4 fish (*n*=4) and the expression normalized against that of FACS-sorted macrophages. Statistical analysis was performed using one-way ANOVA. Different letters above each bar denote significant differences ($P < 0.05$), the same letter indicate no statistical difference between groups and (+) above lines denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

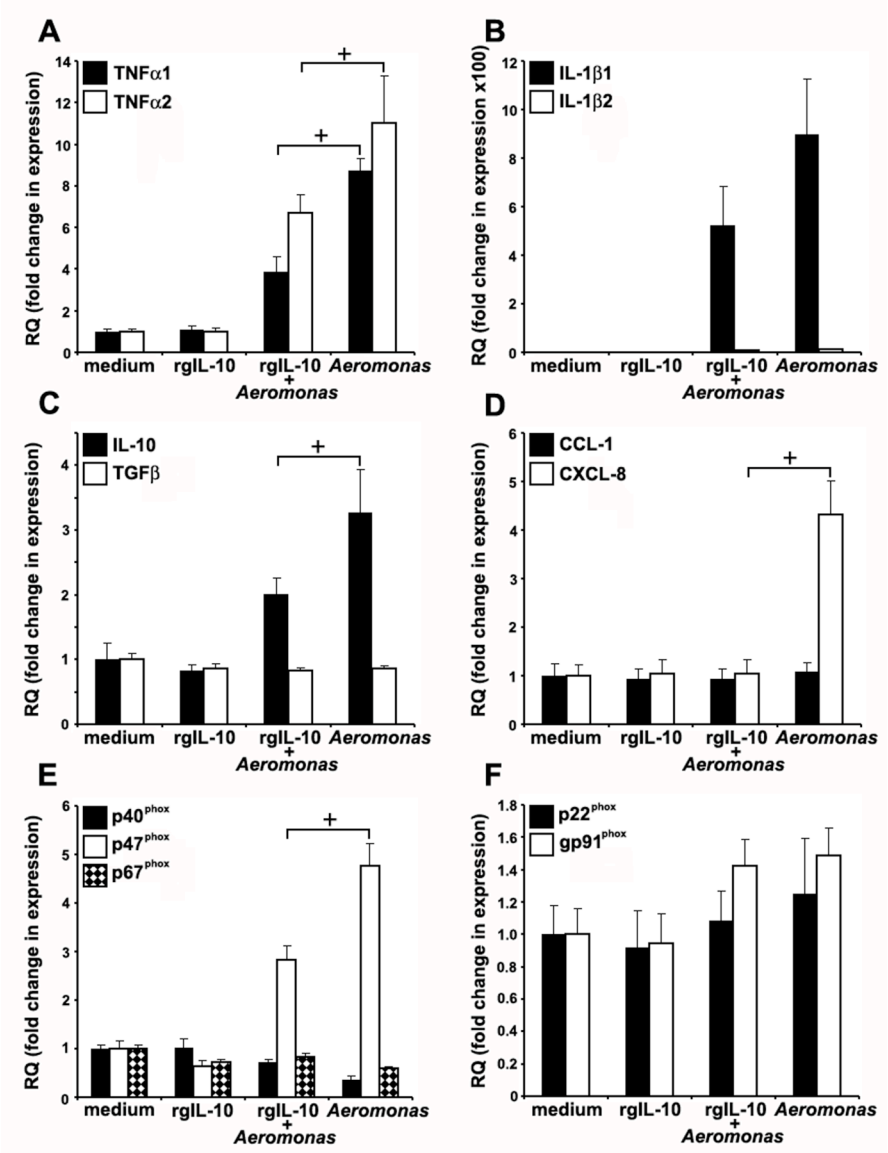

Figure 8.4. Quantitative expression analysis of goldfish immune genes in *Aeromonas salmonicida* -activated monocytes pre-treated with rgIL-10. Monocytes were pre-treated for 2h with medium alone or rgIL-10 (500 ng/mL) before being stimulated with heat-killed *A. salmonicida* for an additional 6h. The reported expression was relative to EF-1α. The genes examined included: **(A)** TNFα1, TNFα2; **(B)** IL-1β-1, IL-1β-2; **(C)** IL-10, TGFβ; **(D)** CCL1,CXCL8; **(E)** $p40^{phox}$, $p47^{phox}$, $p67^{phox}$; **(F)** $gp91^{phox}$, $p22^{phox}$. The expression data were normalized against those observed in medium treated cells for each gene. The results are means ± SEM of RQ values derived from monocyte cultures established from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls and (+) above lines denotes significantly different ($P < 0.05$) between experimental data bars identified by the lines.

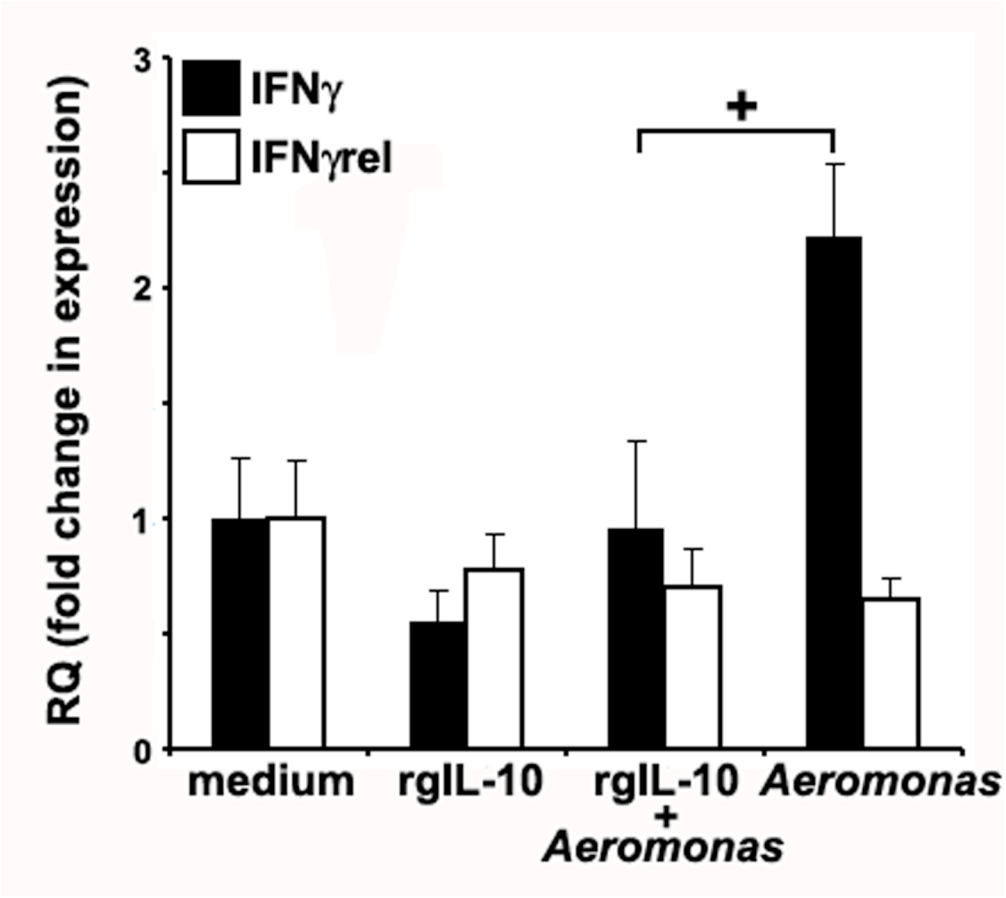

Figure 8.5. Quantitative gene expression analysis of goldfish IFNγ and IFNγrel in *Aeromonas salmonicida* -activated splenocytes pre-treated with rgIL-10. Splenocytes were pre-treated for 2h with medium alone or rgIL-10 (500 ng/mL) before being stimulated with heat-killed *A. salmonicida* for an additional 6h. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived from splenocyte cultures established from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls and (+) above lines denotes significantly different ($P < 0.05$) between experimental groups identified by the lines.

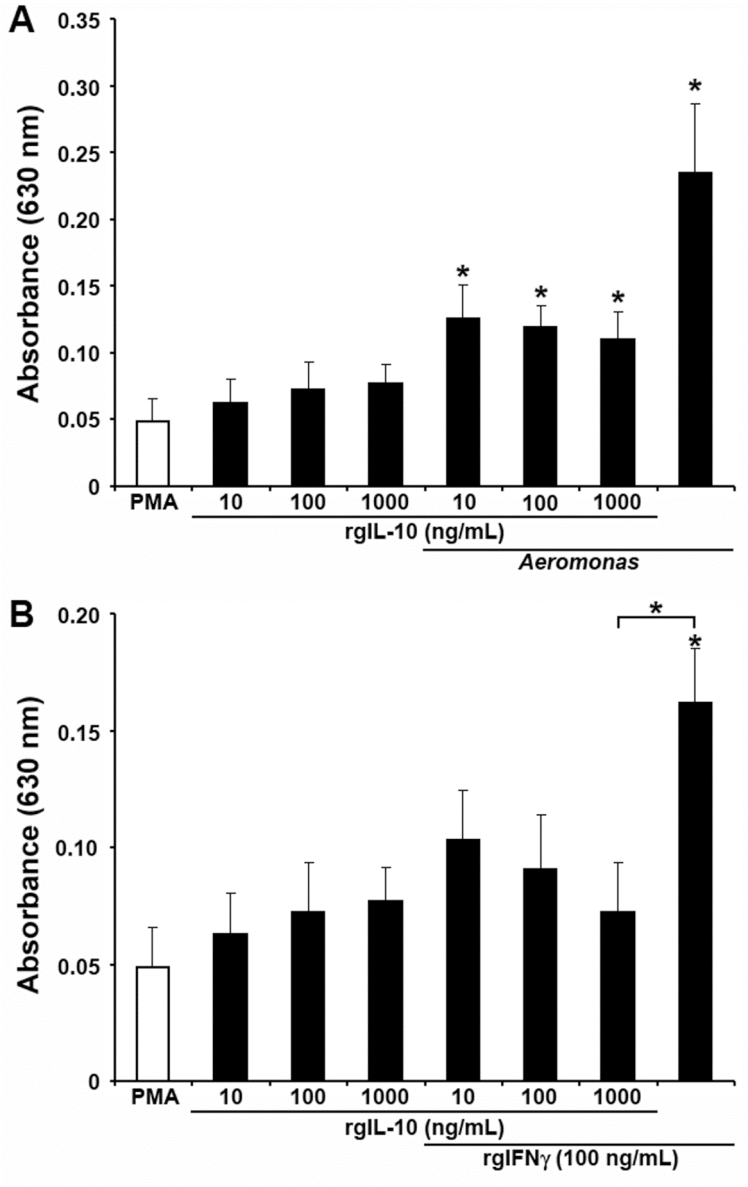

Figure 8.6. Pre-treatment of goldfish monocytes with rgIL-10 abrogates the ROI priming effects of *Aeromonas salmonicida* and rgIFNγ. Monocyte cultures were pre-treated for 2h with medium only or rgIL-10 (10, 100 or 1000 ng/mL) and stimulated for an additional 6h with **(A)** heat-killed *A. salmonicida* or **(B)** rgIFNγ (100ng/mL). The ROI response was then induced with PMA (100 ng/mL). All experiments employed cells from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls and (+) above lines denotes significantly different ($P < 0.05$) between experimental groups identified by the lines.

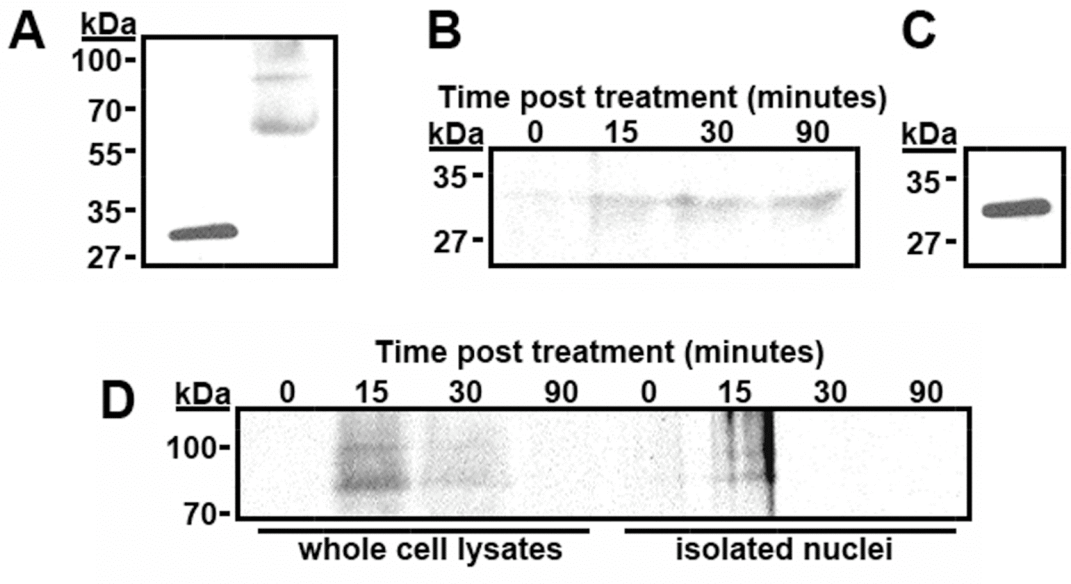

Figure 8.7. Analysis of multimerization, cellular interaction and signaling mechanisms employed by rgIL-10. **(A)** rgIL-10 (2.5 μg) was incubated in the absence (lane 1) or presence (lane 2) of 1mM cross-linker, DSS and the reactions were analyzed by western blotting with an α-polyHis Ab. **(B)** Five million cells from three-day-old cultures were incubated with either medium alone, or 5 μg of rgIL-10 for 0, 15, 30 or 90 minutes. Whole cell lysates were then assayed by western blotting with an α-polyHis Ab. The relative amounts of rgIL-10 (5 μg) added to cells can be seen in **(C)**. **(D)** Five million cells from three-day-old cultures were incubated with either medium alone or rgIL-10 (500 ng/mL) for 0, 15, 30 or 90 minutes. Whole cell lysates or isolated nuclei were assessed by Western blot using α-phospho-(Y)-Stat3 Ab.

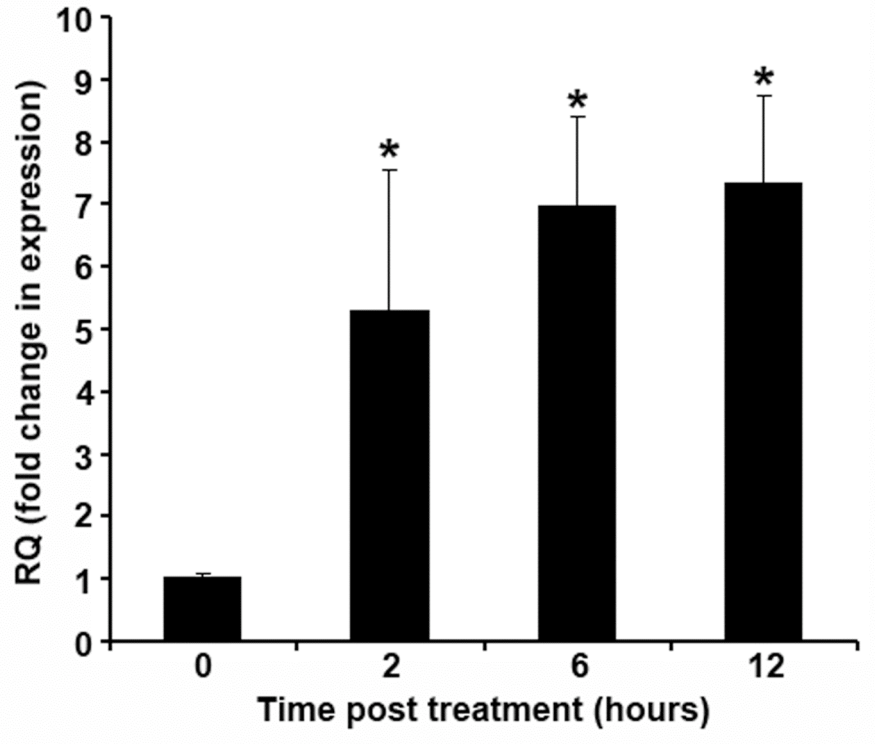

Figure 8.8. Quantitative gene expression analysis of goldfish suppressor of cytokine signalling-3 (SOCS3) in monocytes stimulated with 500 ng/mL of rgIL-10 for 0, 2, 6, or 12 hours. The reported expression was relative to EF-1α. The expression data were normalized against SOCS-3 expression at 0 hour time point. The results are means ± SEM of RQ values derived from monocyte cultures established from five individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from the respective 0 time point control.

4. REFERENCES

1. **Auernhammer, C. J., C. Bousquet, and S. Melmed.** 1999. Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. Proc Natl Acad Sci U S A **96:**6964-6969.
2. **Berlato, C., M. A. Cassatella, I. Kinjyo, L. Gatto, A. Yoshimura, and F. Bazzoni.** 2002. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol **168:**6404-6411.
3. **Bogdan, C., Y. Vodovotz, and C. Nathan.** 1991. Macrophage deactivation by interleukin 10. The Journal of experimental medicine **174:**1549-1555.
4. **Brightbill, H. D., S. E. Plevy, R. L. Modlin, and S. T. Smale.** 2000. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. J Immunol **164:**1940-1951.
5. **D'Andrea, A., M. Aste-Amezaga, N. M. Valiante, X. Ma, M. Kubin, and G. Trinchieri.** 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. The Journal of experimental medicine **178:**1041-1048.
6. **de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries.** 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. The Journal of experimental medicine **174:**1209-1220.
7. **Ding, L., and E. M. Shevach.** 1992. IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. J Immunol **148:**3133-3139.
8. **Ding Y, F. S., Zamarin D, Bromberg JS.** 2003. Interleukin-10. In: Thomson AW, Lotze MT, editors. The cytokine handbook. California: Academic Press 603-625.
9. **Fiorentino, D. F., M. W. Bond, and T. R. Mosmann.** 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. The Journal of experimental medicine **170:**2081-2095.
10. **Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra.** 1991. IL-10 inhibits cytokine production by activated macrophages. J Immunol **147:**3815-3822.
11. **Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra.** 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol **146:**3444-3451.
12. **Ingerslev, H. C., A. Ronneseth, E. F. Pettersen, and H. I. Wergeland.** 2009. Differential expression of immune genes in Atlantic salmon (Salmo salar L.) challenged intraperitoneally or by cohabitation with IPNV. Scand J Immunol **69:**90-98.

13. **Inoue, Y., S. Kamota, K. Ito, Y. Yoshiura, M. Ototake, T. Moritomo, and T. Nakanishi.** 2005. Molecular cloning and expression analysis of rainbow trout (Oncorhynchus mykiss) interleukin-10 cDNAs. Fish Shellfish Immunol **18:**335-344.
14. **Kopydlowski, K. M., C. A. Salkowski, M. J. Cody, N. van Rooijen, J. Major, T. A. Hamilton, and S. N. Vogel.** 1999. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. J Immunol **163:**1537-1544.
15. **Kotenko, S. V., C. D. Krause, L. S. Izotova, B. P. Pollack, W. Wu, and S. Pestka.** 1997. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. Embo J **16:**5894-5903.
16. **Liu, Y., S. H. Wei, A. S. Ho, R. de Waal Malefyt, and K. W. Moore.** 1994. Expression cloning and characterization of a human IL-10 receptor. J Immunol **152:**1821-1829.
17. **Oswald, I. P., T. A. Wynn, A. Sher, and S. L. James.** 1992. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. Proc Natl Acad Sci U S A **89:**8676-8680.
18. **Pinto, R. D., D. S. Nascimento, M. I. Reis, A. do Vale, and N. M. Dos Santos.** 2007. Molecular characterization, 3D modelling and expression analysis of sea bass (Dicentrarchus labrax L.) interleukin-10. Mol Immunol **44:**2056-2065.
19. **Powell, M. J., S. A. Thompson, Y. Tone, H. Waldmann, and M. Tone.** 2000. Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. J Immunol **165:**292-296.
20. **Qin, H., C. A. Wilson, K. L. Roberts, B. J. Baker, X. Zhao, and E. N. Benveniste.** 2006. IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. J Immunol **177:**7761-7771.
21. **Riley, J. K., K. Takeda, S. Akira, and R. D. Schreiber.** 1999. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. J Biol Chem **274:**16513-16521.
22. **Savan, R., D. Igawa, and M. Sakai.** 2003. Cloning, characterization and expression analysis of interleukin-10 from the common carp, Cyprinus carpio L. Eur J Biochem **270:**4647-4654.
23. **Seppola, M., A. N. Larsen, K. Steiro, B. Robertsen, and I. Jensen.** 2008. Characterisation and expression analysis of the interleukin genes, IL-1beta, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). Mol Immunol **45:**887-897.
24. **Spencer, S. D., F. Di Marco, J. Hooley, S. Pitts-Meek, M. Bauer, A. M. Ryan, B. Sordat, V. C. Gibbs, and M. Aguet.** 1998. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. The Journal of experimental medicine **187:**571-578.

25. **Syto, R., N. J. Murgolo, E. H. Braswell, P. Mui, E. Huang, and W. T. Windsor.** 1998. Structural and biological stability of the human interleukin 10 homodimer. Biochemistry **37:**16943-16951.
26. **Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Forster, and S. Akira.** 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity **10:**39-49.
27. **Tan, J. C., S. Braun, H. Rong, R. DiGiacomo, E. Dolphin, S. Baldwin, S. K. Narula, P. J. Zavodny, and C. C. Chou.** 1995. Characterization of recombinant extracellular domain of human interleukin-10 receptor. J Biol Chem **270:**12906-12911.
28. **Tan, J. C., S. R. Indelicato, S. K. Narula, P. J. Zavodny, and C. C. Chou.** 1993. Characterization of interleukin-10 receptors on human and mouse cells. J Biol Chem **268:**21053-21059.
29. **Tone, M., M. J. Powell, Y. Tone, S. A. Thompson, and H. Waldmann.** 2000. IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. J Immunol **165:**286-291.
30. **Uran, P. A., A. A. Goncalves, J. J. Taverne-Thiele, J. W. Schrama, J. A. Verreth, and J. H. Rombout.** 2008. Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.). Fish Shellfish Immunol **25:**751-760.
31. **Walter, M. R., and T. L. Nagabhushan.** 1995. Crystal structure of interleukin 10 reveals an interferon gamma-like fold. Biochemistry **34:**12118-12125.
32. **Walter, M. R., W. T. Windsor, T. L. Nagabhushan, D. J. Lundell, C. A. Lunn, P. J. Zauodny, and S. K. Narula.** 1995. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. Nature **376:**230-235.
33. **Weber-Nordt, R. M., J. K. Riley, A. C. Greenlund, K. W. Moore, J. E. Darnell, and R. D. Schreiber.** 1996. Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. J Biol Chem **271:**27954-27961.
34. **Zdanov, A., C. Schalk-Hihi, A. Gustchina, M. Tsang, J. Weatherbee, and A. Wlodawer.** 1995. Crystal structure of interleukin-10 reveals the functional dimer with an unexpected topological similarity to interferon gamma. Structure **3:**591-601.
35. **Zhang, D. C., Y. Q. Shao, Y. Q. Huang, and S. G. Jiang.** 2005. Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (Danio rerion). J Biochem Mol Biol **38:**571-576.
36. **Zou, J., M. S. Clark, and C. J. Secombes.** 2003. Characterisation, expression and promoter analysis of an interleukin 10 homologue in the puffer fish, Fugu rubripes. Immunogenetics **55:**325-335.
37. **Zou, J., Y. Yoshiura, J. M. Dijkstra, M. Sakai, M. Ototake, and C. Secombes.** 2004. Identification of an interferon gamma homologue in Fugu, Takifugu rubripes. Fish Shellfish Immunol **17:**403-409.

CHAPTER IX:

ANALYSIS OF THE ANTIMICROBIAL RESPONSES OF PRIMARY PHAGOCYTES OF THE GOLDFISH (*Carassius auratus* L.) AGAINST *Mycobacterium marinum*[7]

1. INTRODUCTION

Pathogenic *Mycobacterium* spp. reside primarily in phagocytic cells and are the causative agents of serious vertebrate diseases including tuberculosis (*M. tuberculosis*) and leprosy (*M. leprae*). Despite the growing need to understand the interaction of this pathogen with its host cells, research has been hampered by very slow replication of these microorganisms and a lack of suitable *in vitro* model systems to adequately recapitulate host-pathogen interface of the infection in primary immune cell populations. Research efforts have focused on utilizing surrogate model systems (different species of faster-growing *Mycobacterium* spp.) to study the host-pathogen interface of the ethiological agent of tuberculosis (54, 57). The use of the fish/amphibian pathogen *Mycobacterium marinum* has been reported since the analysis of 16S rRNA of various mycobacterial species showed that *M. marinum* is most closely related to the *M. tuberculosis* complex, with 99.4% sequence homology (59). Experimental infections of fish with *M. marinum* have revealed very similar pathologies and disease progression to those seen in *M. tuberculosis* infections of humans (10, 21). These include bacterial invasion of host phagocytes, granuloma formation, bacterial dissemination,

[7] A version of this chapter has been accepted for publication: Grayfer, L., J. Hodgkinson, and M. Belosevic. 2011. Developmental and Comparative Immunologcvbny. doi:10.1016/j.dci.2011.04.007

emaciation and eventual death of the infected hosts (11, 15). In contrast to *M. tuberculosis*, the use of *M. marinum* has additional advantages since it is only a level 2 pathogen and it has a relatively short (4 h) doubling time making it ideal for studies on the mechanisms of host-pathogen interactions. Furthermore, while it is difficult to establish specific mutant strains of *M. tuberculosis* due to its long doubling time (50, 52), homologous recombination-based mutagenesis of *M. marinum* has proven to be relatively effective (55, 56), yielding new insight into the roles of specific mycobacteial genes and gene products. For example, signature-tagged mutagenesis of *M. marinum* prior to infection of goldfish, has allowed for the identification of numerous putative genes crucial for infectivity and pathogenesis (62), while research using the zebrafish embryo-infection model systems has revealed that, unlike previously believed, granuloma formation is actually the direct result of the bacteria dissemination strategy (15, 36).

Although pathogenic mycobacteria reside in phagocytes (21), these host cells are not entirely permissive to bacterial invasion and intracellular survival. It has been elegantly demonstrated that although *M. marinum* can infiltrate zebrafish phagocytes, replicate and disseminate within them, embryos that are depleted of phagocytes have higher bacterial burdens even at early times of the infection (11). To survive and thrive in the intracellular environment of the phagocyte, *Mycobacterium spp.* must subvert numerous microbicidal mechanisms in order to make the intra-phagosomal environment hospitable for bacterial growth [reviewed in references (22, 24)]. These phagocyte barriers include phagosome acidification, phago-lysosome fusion and oxidative bursts, aimed at eliminating

the intracellular pathogen. In order to circumvent these barriers, it has been reported that mycobacteria escape the phagosome entirely and reside in the host cell cytosol (26, 64). The production of pro-inflammatory cytokines by phagocytes is crucial in tipping the balance between intracellular survival of bacteria or their eventual elimination by antimicrobial armamentarium of activated macrophages (25, 53). Not surprisingly, mycobacteria have been shown to manipulate cytokine production by activated phagocytes in order to promote their own survival (2, 20).

Our group has developed and functionally characterized a goldfish kidney-derived primary monocyte/macrophage (PKM) culturing system (27-32, 42-44). In this chapter I examine the specific roles of the respective inflammatory and immune mediators identified and characterized in the previous chapters of this thesis (Chapters III-VIII) in the context of *Mycobacterium marinum* infections of goldfish monocytes and macrophages. I believe that the goldfish PKM system coupled with the previously described *in vivo* goldfish infection model (63) will be an invaluable tool for investigating the mechanisms of immunity and pathogenesis of mycobacteria-host interactions.

2. RESULTS

2.1. *M. marinum* down-regulates goldfish monocyte cytokine-primed ROI

The kidney of bony fish (teleosts) is the main hematopoietic organ, analogous to the mammalian bone marrow. It has been previously demonstrated that *in vitro* derived goldfish primary kidney macrophage cultures (PKM)

comprise of at least three distinct populations: myeloid progenitor cells, monocytes and mature macrophages (42, 44). Unlike mammalian bone-marrow-derived macrophages that require the addition of exogenous growth factors (M-CSF and/or GM-CSF), bony fish macrophages grow spontaneously and differentiate *in vitro* because they produce copious amounts of endogenous growth factors (primarily M-CSF) (42-47). Furthermore, these monocyte and macrophage subpopulations exhibit distinct capabilities to mount specific antimicrobial functions. For example, monocytes are the primary producers of ROI while the production of RNI is restricted to mature macrophages (29, 42, 44). In this study, I employed this *in vitro* culture system to study the interactions between the distinct monocyte- or macrophage-enriched PKM cultures and the fish pathogen, *M. marinum* (ATCC 927).

From several recombinant cytokines characterized in the context of the PKM culture system, the recombinant goldfish (rg) interferon gamma (rgIFNγ) and tumor necrosis factor alpha (isoform 2, rgTNFα2) proved to be the most effective in priming the production of ROI by goldfish monocytes (29, 30, 32). I first examined whether the exposure of monocytes to *M marinum* affected their ability to prime ROI. Monocyte cultures exposed to 10^5, 10^6 or 10^7 colony-forming units (cfu/mL) of *M. marinum* for 5 hours before priming with rgIFNγ, exhibited significant decrease in the production of ROI (Fig. 9.1A). Non-stimulated cells infected with 10^4 cfu/mL had increased (not significantly) ROI response, while higher bacterial numbers (10^5, 10^7 cfu/mL) induced ROI responses similar to or significantly lower (10^6 cfu/mL) compared to those of

medium-treated controls (Fig. 9.1A,C). Monocytes pre-treated with rgIFNγ prior to infection with *M. marinum* also exhibited diminished production of ROI (Fig. 9.1C). The 10^6 cfu/mL dose of *M. marinum* consistently induced greater decrease in the ROI response compared to the 10^7 cfu/mL dose (Fig. 9.1A,C).

The heat-killed *M. marinum* also down-regulated the monocyte rgIFNγ-primed ROI response regardless of whether the recombinant cytokine was added to cultures before or after stimulation with heat-killed mycobacteria (Fig. 9.1B,D). Similar results were obtained when rgTNFα2 was used to prime monocyte ROI production (Fig. 9.2). None of the *M. marinum* doses used had an effect on monocyte viability for the duration of the experiments (Table 9.1).

2.2. *M. marinum* induces changes in gene expression of monocytes

To examine the possible mechanisms by which *M. marinum* was down-regulating the monocyte ROI production, monocytes were exposed to viable or heat-killed *M. marinum*, respectively, and the gene expression for various goldfish NADPH oxidase components determined using quantitative PCR (Fig. 9.3A-E). Compared to non-treated controls, monocyte cultures incubated with 10^6 cfu/mL of live *M. marinum* exhibited significantly decreased expression of $p22^{phox}$ and $p47^{phox}$ (Fig. 9.3A,C) while the expression of $p67^{phox}$ was significantly elevated in these cells (Fig. 9.3D). Monocytes incubated with 10^7 cfu/mL of *M. marinum* displayed significantly decreased transcript levels of $p22^{phox}$, $p67^{phox}$ and $gp91^{phox}$ as well as decreased (not significant) mRNA levels of $p40^{phox}$ and $p47^{phox}$ (Fig. 9.3). Monocytes incubated with 10^7 cfu/mL of heat-

killed *M. marinum* displayed significantly decreased gene expression of $p22^{phox}$ (Fig. 9.3A) while the mRNA levels of the other NADPH components remained unchanged (Fig. 9.3). In addition, live but not the heat-killed *M. marinum* induced significantly higher gene expression of the natural resistance-associated macrophage protein (NRAMP) (Fig. 9.3F) while the expression of idoleamine 2,3 dioxygenase (IDO), the enzyme that catalyses the degradation of tryptophan, remained unchanged (data not shown).

To examine whether *M. marinum* elicited anti-inflammatory mechanisms in goldfish monocytes, I measured the mRNA levels of IL-10, TGFβ1 and suppressor of cytokine signaling-3 (SOCS-3) (Fig. 9.4A-C). Treatments 10^7 cfu/mL of viable or heat-killed *M. marinum*, significantly increased the gene expression of IL-10, while the exposure of monocytes to 10^6 cfu/mL of live but not heat-killed bacteria elicited modest but significantly elevated gene expression of IL-10 (Fig. 9.4A). The gene expression of monocyte TGFβ1 was significantly upregulated after exposure to10^6 cfu/mL of live or heat killed bacteria, and decreased after the addition of 10^7 cfu/mL of viable bacteria to the cultures (Fig. 9.4B). The addition of 10^7 cfu/mL of live and heat-killed *M. marinum* to monocyte cultures also caused significantly elevated mRNA levels of monocyte SOCS-3 gene (Fig. 9.4C).

Subsequently, I examined the effects of *M. mariunum* on goldfish monocyte pro-inflammatory gene expression. Monocyte cultures were exposed to *M. marinum* as above and assayed by Q-PCR for the expression of goldfish TNFα (isoforms 1 and 2); IL-1β (isoforms 1 and 2); IFNγ and IFNγrel (a Type II

interferon present in certain bony fish) and the chemokines CXCL-8 and CCL-1 (Fig. 9.5A-H). Exposure of monocytes to 10^7 cfu/mL of viable *M. marinum* induced significantly increased monocyte mRNA levels of TNFα1 and TNFα2, IL-1β1 (but not IL-1β2), IFNγ and CXCL-8 (Fig. 9.5A-C, E,G). The exposure of monocytes to 10^6 cfu/mL of live or heat-killed bacteria and 10^7 cfu/mL of live but not heat-killed bacteria, also induced significantly higher gene expression of IFNγrel (Fig. 9.5F). The 10^6 cfu/mL of live bacteria decreased the gene expression of both IL-1β isoforms, while the heat-killed 10^6 cfu/mL treatment caused IL-1β1 (but not IL-1β2) expression (Fig. 9.5C,D). The 10^6 cfu/mL of live but not heat-killed *M. marinum* also induced modest but significant increase in monocyte CCL-1 chemokine gene expression (Fig. 9.5H).

The gene expression of monocyte TNFα receptors (TNFR1 and TNFR2) and IFNγ receptors (IFNGR1-1 and IFNGR1-2) was investigated following exposure of monocytes to live or heat-killed *M. marinum* (Fig. 9.6). The infection of monocytes with 10^6 cfu/mL of live bacteria induced a decrease in the expression of TNFR1, while the addition of 10^7 cfu/mL of heat killed but not live *M. marinum* caused a significant increase in the mRNA levels of this gene (Fig. 9.6A). The mRNA levels of IFNGR1-1 and IFNGR1-2 were both significantly elevated in monocytes infected with 10^6 cfu/mL of live (but not heat-killed) bacteria, whereas monocytes exposed to 10^7 cfu/mL of the live (but not heat-killed) exhibited significantly reduced mRNA levels of both receptors (Fig. 9.6C,D). The monocyte gene expression of TNFR2 was not affected by any of the treatments (Fig. 9.6B).

2.3. *M. marinum* down-regulates goldfish macrophage nitric oxide responses

As the goldfish PKM cultures age, the mature macrophages predominate in the cultures with a concomitant gain in the ability of these cells to produce RNI (42, 44). Of the several pro-inflammatory fish cytokines at my disposal, rgIFNγrel and rgTNFα2 have proven to be the most potent inducers of the production of RNI by mature goldfish macrophages (30, 32). Accordingly, I examined the effects of *M. marinum* infection on rgIFNγrel- and rgTNFα2-induced RNI production by mature macrophages (Fig. 9.7 and Fig. 9.8). When macrophages were stimulated with rgIFNγrel before or after infections with *M. marinum*, I observed significant reduction in the production of RNI (Fig. 9.7A,C). In contrast, the treatment of macrophage cultures with heat-killed *M. marinum* did not affect the rgIFNγrel-induced RNI production (Fig. 9.7B,D and Fig. 9.8B,D). Interestingly, while macrophages exposed to live *M. marinum* alone did not produce RNI (Fig. 9.7A,C), those treated with higher numbers of the heat-killed bacteria exhibited a modest RNI production (Fig. 9.7B,D and Fig. 9.8B,D).

In contrast to rgIFNγrel-induced NO responses, macrophages pre-treated with rgTNFα2 prior to bacterial infection, did not exhibit a reduction in nitrite production (Fig. 9.8A,C). Heat-killed *M. marinum* failed to significantly alter the rgTNFα2-induced RNI production, whether it was added before or after the exposure of macrophage cultures (Fig. 9.8B,D).

To confirm *M. marinum* effects on NO responses of macrophages, I quantified the gene expression of the two isoforms of the iNOS gene that encode the enzyme that catalyzes the conversion of argenine to citruline and eventual nitric oxide production by macrophages. (Fig. 9.9A,B). The exposure of macrophages to live bacteria elicited higher mRNA levels of iNOS A and B (Fig. 9.9A,B) while the treatment of macrophage cultures with heat-killed bacteria only up-regulated the gene expression of iNOS B (Fig. 9.9B).

2.4. *M. marinum* changes immune gene expression in mature macrophages

The assessment of the gene expression of different antimicrobial genes showed that mRNA levels of the NRAMP were not affected by various exposures of live or heat-killed *M. marinum* (Fig. 9.10D). However, macrophages exposed to *M. marinum* had significantly higher mRNA levels of tryptophan degradation enzyme, 2,3 indoleamine dioxygenase (IDO) (Fig. 9.10C). Furthermore, similar cytokine and cytokine receptor gene expression patterns to those seen in *M. marinum* exposed monocytes, were also observed in mature macrophages (Fig. 9.10 - 9.12). Briefly, live *M. marinum* increased the macrophage expression of IL-10, TGFβ1 and SOCS-3 (Fig. 9.10); TNFα1 and TNFα2, IL-1β2, IFNγ, IFNγrel, p35 and p40 subunits of IL-12, CCL-1 (Fig. 9.11); TNFR2 and the IFNGR1-1 and IFNGR1-2 (Fig. 9.12).

2.5. Cytokine activation of goldfish phagocytes decreases the survival of intracellular *M. marinum*

I examined the effects of recombinant cytokine stimulation of goldfish monocyte and macrophage cultures on the viability of intracellular *M. marinum*. Monocytes (Fig. 9.13A,B) or mature macrophages (Fig. 9.13C,D) activated with rgTNFα2, rgIFNγ or rgIFNγrel and infected with 10^5 cfu/mL of *M. marinum,* regardless of the order of cytokine stimulation and/or bacterial infection, exhibited reduced bacterial survival compared to medium-treated controls. Monocytes and mature macrophages treated with rgIL-10 after following the addition of *M. marinum* to the cultures caused a modest increase (not significant) in bacterial survival (Fig. 9.13B,D).

3. DISCUSSION

To date, the majority of *in vitro* mycobacterial infection studies have relied on murine macrophage model systems to infer on immune outcomes of mycobacterial infections of human cells (16, 23, 60). This is not an ideal model system to study host-pathogen interactions since mice appear to be naturally resistant to mycobacterial infections, while susceptible mouse strains do not exhibit mycobacterial disease progression characteristic of human disease (48, 49). Human cell lines have been employed for mycobacterial infection studies, however these cells are not fully differentiated since in mammalian systems the final macrophage differentiation occurs following interaction with tissue microenvironments. To circumvent this problem, researchers have resorted to

artificially differentiating the human cell lines using phorbol esters (37, 61) or LPS (1, 39), which inevitably resulted in activation of these cells, further confounding studies of this nature. Furthermore, due to the long doubling time of the *M. tuberculosis and M. leprae* that naturally infect humans, researchers have examined the interaction of the mammalian cells and the faster growing species such as *M. marinum*. However, the natural hosts of *M. marinum* are fish and amphibians, it is not surprising that while *M. marinum* is able to infect mammalian cell lines at 37 °C, the pathogen is only able to replicate in these cells at lower temperatures (54). In fact it has been proposed that one of the reasons why *M. marinum* infections manifest as systemic tuberculosis-like disease in fish and amphibians (9, 18), while being restricted to primarily cutaneous infections in mammals, may be due to the lower temperature requirement for this species to grow (10). Undoubtedly, altering cell culture conditions to facilitate bacterial growth does not provide for an ideal *in vitro* infection model system. In this chapter I described the use of a well-characterized primary goldfish macrophage culture system as an *in vitro* mycobacteria-natural host cell infection model for comprehensive analyses of *M. marinum*-host cell interface. Unlike mammalian macrophages that require the addition of exogenous growth factors (M-CSF and/or GM-CSF) to survive in culture, bony fish macrophages grow spontaneously and fully differentiate *in vitro* because they produce copious amounts of endogenous growth factors (primarily M-CSF) (42-47). Furthermore, these monocyte and macrophage subpopulations have distinct abilities to mount specific antimicrobial functions. For example, the monocytes are the primary

producers of ROI, while the production of RNI is restricted to mature macrophages (29, 42, 44). To my knowledge, this is the first report that comprehensively characterized the natural host cell-*M. marinum* interactions.

Upon activation, mononuclear phagocytes express NADPH oxidase and iNOS, enzymes responsible for the production of ROI and RNI, respectively. These reactive radicals are capable of damaging numerous bacterial components such as nucleic acids, proteins, lipids and carbohydrates. There are discrepancies in the literature as to the susceptibility of mycobacterial species to killing by these reactive radicals. For example, it has been reported that the occurrence of mycobacterial disease is much greater in patients suffering from chronic granulomatous disease (5) while mice with NADPH oxidase deficiency are relatively resistant to *M. tuberculosis* (13, 34). My results showed that *M. marinum* down-regulated the goldfish monocyte recombinant cytokine-induced ROI production regardless of whether cells were cytokine-stimulated before or after to bacterial challenge. Although the mechanisms by which *Mycobacterium* spp. down-regulate the production of reactive radicals are poorly understood, these pathogens are well known for counteracting the phagocyte oxidative bursts [reviewed in reference (19)]. Mycobacteria possess numerous strategies for eliminating reactive radicals including a catalase-peroxidase (46), two superoxide dismutases (67), as well as mycothiol (45), an alternative of glutathione shared by all *Actinobacteria*. Indeed, I also observed that viable *M. marinum* significantly down-regulated the gene expression of several NADPH oxidase components, providing further evidence about the possible mechanism (s) used by these

pathogens to suppress the ROI responses. Interestingly, I observed that heat-killed *M. marinum* also down-regulated the cytokine-induced ROI, suggesting that this immune evasion strategy may not require bacterial viability. It is becoming more apparent that mycobacterial cell wall components such as the 19 kDa lipoprotein, facilitate immune evasion by redirecting the immune response through distinct TLR signaling pathways from those desired for proper pathogen elimination (47). Presumably such bacterial strategies could account for the negative effects of heat-killed *M. marinum* on the monocyte ROI production.

The efficacy mycobacteria killing by phagocyte RNI has also been subject to some debate (41). Some lines of evidence suggest that RNI play a direct role in the clearance of mycobacteria (17), while other reports suggest that phagocytes rely on non-oxidative mechanisms to eliminate these pathogens (35). I observed that *M. marinum* abrogated the goldfish cytokine-induced macrophage production of RNI, when the cells were challenged with the pathogen prior to cytokine-stimulation. However, unlike the down-regulation of monocyte ROI responses, only viable *M. marinum* were capable of down-regulating the NO response, while the heat-killed bacteria actually induced a dose-dependent production of nitrite by macrophages. This suggests that the *M. marinum* evasion of the NO response are at least in part non-overlapping and are likely mediated by different mechanisms. Mycobacterial cell wall components have been well documented to elicit phagocyte iNOS gene expression and RNI production (3, 8), suggesting that *M. marinum* may actively counteract this antimicrobial function of macrophages. While both live and heat-killed *M. marinum* induced comparable macrophage

iNOS isoform A expression, the expression of iNOS isoform B was more substantially up-regulated by the viable bacteria. Since the respective involvement of these two isoforms in goldfish macrophage RNI production has not been fully elucidated, it is difficult to speculate what influence the differences in the iNOS isoform gene expression have on this antimicrobial function of fish mature macrophages. However, since the viable *M. marinum* did not induce lower iNOS expression levels compared to the heat-killed pathogen, it is likely the *M. marinum* manipulation of macrophage RNI production occurs down-stream of iNOS gene expression.

Unlike most vertebrates that have a single type II IFN, certain fish species such as goldfish possess two type II IFNs, IFNγ and IFNγrel. As described in chapters V and VII, I have observed that while rgIFNγ induces modest macrophage RNI production, rgIFNγrel induces a substantially greater NO response of activated macrophages (29, 30). *M. marinum* infecton of macrophages down-regulated the rgIFNγrel and rgTNFα2-induced RNI production when cells were challenged with the pathogen before cytokine stimulation. Interestingly, while *M. marinum* also down-regulated RNI production of cells pre-treated with rgIFNγrel, macrophages stimulated with rgTNFα2 prior to bacterial challenge retained full ability to generate NO response. This may suggest that *M. marinum* down-regulation strategy targets a pathway of NO activation not utilized or otherwise circumvented by rgTNFα2.

My gene expression studies indicate that *M. marinum* is modulating the expression of monocyte and macrophage TNFα and IFNγ receptors. It is possible

that *M. marinum* may be actively altering the host cell responsiveness to the pro-inflammatory cytokines by modulating receptor gene expression on the surface of macrophages through a mechanism that remains to be elucidated.

Mycobacterial cell wall components are recognized by the pattern recognition receptors and this recognition results in induction of numerous pro-inflammatory and other immune genes (8, 58, 68). Changes in the expression of some of these genes appears to be part of the bacterial evasion strategy, while the expression of other genes is thought to be part of the normal host cell immune response to the infection. As is the case in mycobacterial infections of human and mouse macrophages (68), I also observed that *M. marinum* infection induced increases in goldfish monocyte and macrophage expression of the anti-inflammatory mediators, IL-10 and TGFβ (51, 68). In chapter VIII, I reported that a recombinant goldfish IL-10 substantially increased the expression of the goldfish suppressor of cytokine signaling-3 gene (31), the product of which has been well established to down-regulate the signaling from immune receptors in mammalian systems. More recently, mycobacterial species have been shown to induce the expression of SOCS-3 in mammalian cells and it was postulated that this could constitute a mycobacterial immune evasion strategy (40). In my study, *M. marinum* also induced increased mRNA levels of SOCS-3 in goldfish monocytes and macrophages. Whether these changes in gene expression are the result of the IL-10 production, or are directly mediated by the pathogen, remains to be determined. Presumably the *M. marinum* induced expression of these

immunosuppressive genes aids in its immune evasion, manifested in the down-regulation of ROI and RNI production.

M. marinum also induced the expression of several key goldfish pro-inflammatory genes including isoforms of TNFα and IL-1β, IFNγ, IFNγrel, CXCL-8 and CCL-1 in both monocytes and macrophages. The mammalian counterparts of these pro-inflammatory genes have been implicated in mycobacterial clearance, so it is reasonable to speculate that these expression changes are indicative of the monocyte and macrophage immune responses to *M. marinum* recognition and/or infection. Indeed the importance of pro-inflammatory cytokines such as TNFα in fish mycobacterial elimination has already been demonstrated (12). I believe that the use of the natural host model systems for *M. marinum* such as fish and fish-derived primary macrophage cultures will yield a greater understanding of the exact roles and mechanisms of action of these respective cytokines in mycobacterial infections.

The viable *M. marinum* induced monocyte but not macrophages up-regulation of the expression of the NRAMP gene. NRAMP has been reported to be important for the clearance of intracellular pathogens by macrophages [reviewed in references (6)]. *M. marinum*-induced NRAMP expression has previously been shown in striped bass leukocytes (4). Similarly, viable but not heat-killed *M. marinum* induced substantially greater increase in the mRNA levels of indoleamine 2,3 dioxygenase gene in mature macrophages. This enzyme catalyzes key reactions during tryptophan degradation and deprivation of tryptophan has been identified as an important strategy for enhanced immunity

against *M. avium* (33). While NRAMP functions upon pathogens enclosed in phagosomes, IDO mediates its effects in the milieu surrounding the effector cells. We have previously observed that majority of stimulus-induced phagocytosis is mediated by goldfish monocytes while the mature macrophages gain the ability to undergo antimicrobial functions such reactive nitrogen production (42, 43). This might explain why the NRAMP up-regulation was seen in monocytes while IDO gene expression increased in mature macrophages.

My results indicate that *M. marinum* viability is reduced in goldfish monocyte and macrophage cultures treated with rgTNFα2, rgIFNγ or rgIFNγrel before or after exposure to the pathogen. I also observed that when higher numbers of *M. marinum* were added to the cultures, the decreased viability effect was abrogated suggesting that there may be a finite capacity of cultured cells to clear mycobacteria. Interestingly, with increased mycobacterial burden, I observed an enhanced down-regulation of the production of monocyte ROI and macrophage RNI that was related to the restoration of bacterial viability in monocyte and macrophage cultures exposed to higher numbers of mycobacteria. This suggests that goldfish monocytes and macrophages likely rely, at least in part, on ROI and RNI responses for mycobacterial clearance. Indeed it has been observed in mammals that the TNFα- and IFNγ-induce destruction of infiltrating mycobacteria in mammalian systems occurs through both oxidative (23) and non-oxidative mechanisms (35).

Mycobacterial species are also notorious for circumventing host cell phagosome maturation and acidification (38, 65), and the ability of mycobacteria

to do so is abrogated when macrophages are activated with cytokines such as IFNγ (7). This appears to be the case for goldfish cytokine-stimulated macrophages, since the reduction in the number of intracellular organisms by activated macrophages has been shown to be related to the ability of mycobacteria and other pathogens to survive at low pH (66).

During mycobacterial infections, myeloid cells serve as a central line of defense as well as the primary sites of infection and lines of dissemination of mycobactria. The use of zebrafish and goldfish as an *in vivo* natural host model system for *M. marinum*, has already yielded a substantial amount of new insight into the biology the host-pathogen interactions in mycobacterial infections (12, 14, 15, 63). The goldfish infections with *M.marinum* have been proposed as an *in vivo* model system for the study of mycobacterial disease in a natural host (63). I believe that greater understanding of the immune mechanisms and events involved in mycobacterial infiltration, evasion and elimination by phagocytes will come from utilizing natural host-derived primary cell cultures such as the fish monocyte/macrophage system characterized in this study.

Table 9.1. Viability of goldfish monocytes infected with *M. marinum*[1]

M. marinum	**0 cfu/mL**	**10^4 cfu/mL**	**10^5 cfu/mL**	**10^6 cfu/mL**	**10^7 cfu/mL**
viable	2.83 ± 0.39	3.17 ± 0.36	2.98 ± 0.48	2.65 ± 0.45	3.13 ± 0.50
heat-killed	3.05 ± 0.38	2.67 ± 0.09	2.79 ± 0.52	3.06 ± 0.47	2.65 ± 0.39

[1]3 x10^5 goldfish monocytes were incubated with medium alone, 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum* for 12 hours. Following incubation, monocytes were enumerated. Above cell counts are means ± SEM (x10^5) of cells derived from three individual fish (*n*=3).

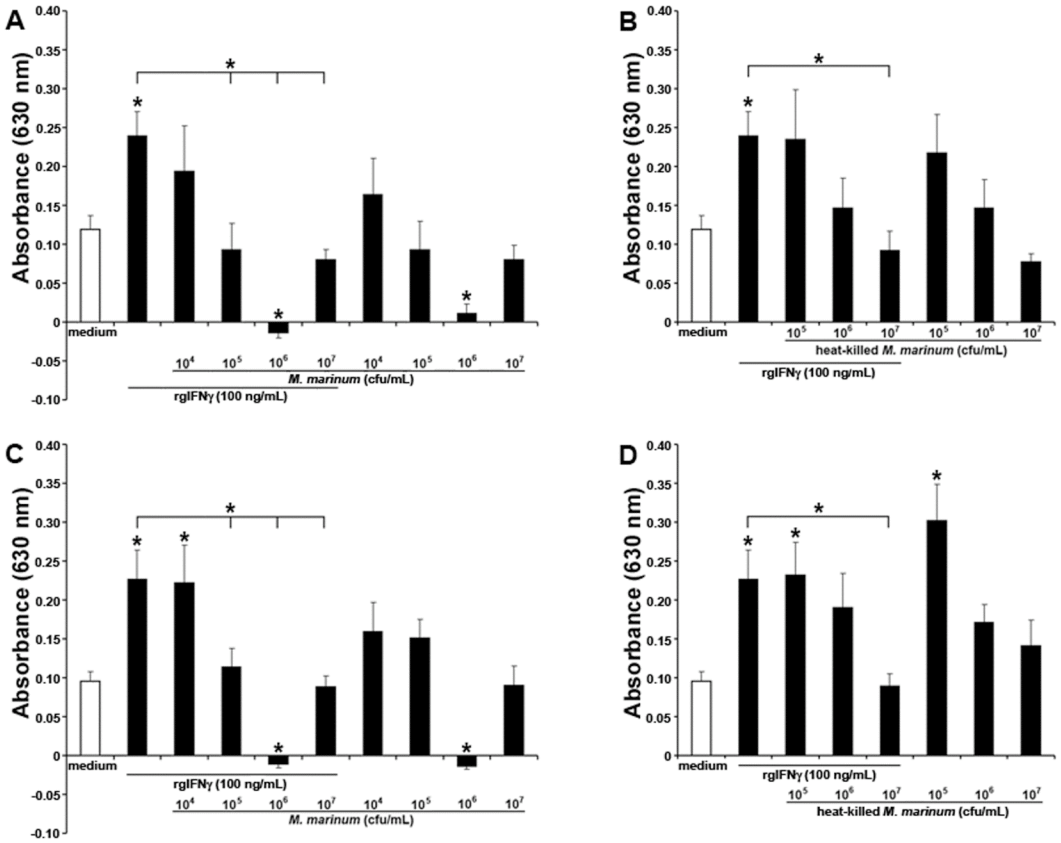

Figure 9.1. ***Mycobacterium marinum*** **down-regulates rgIFNγ-induced monocyte ROI.** Monocyte cultures were pre-incubated for 5 hours with medium only, 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(A)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed *M. marinum* **(B)**, subsequently treated with rgIFNγ. Alternatively, cell were pre-treated for 5 hours with rgIFNγ, before being incubated with 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(C)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed **(D)** *M. marinum*. The ROI response was then induced with PMA (100 ng/mL). Monocytes were obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were considered significant at $P < 0.05$. (*) indicates significantly different ($P < 0.05$) from the PMA only treated controls and (*) above lines denotes significant difference ($P<0.05$) between different experimental groups indicated by the lines.

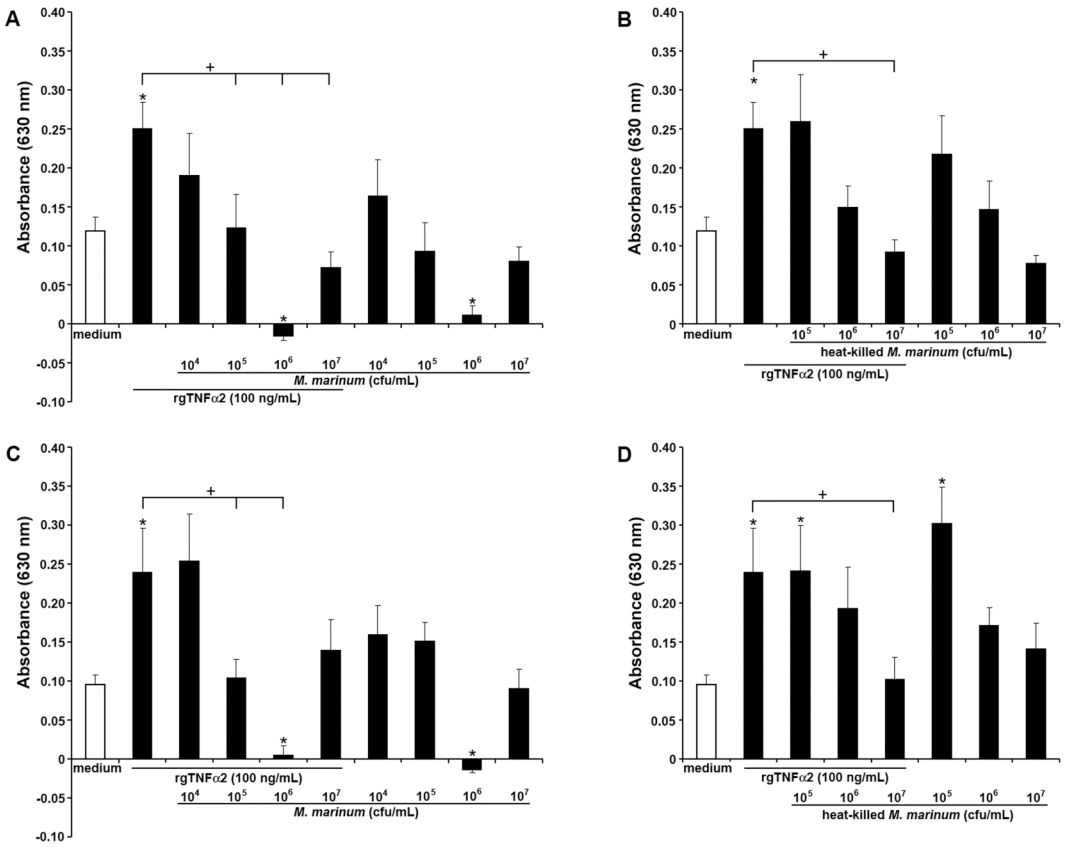

Figure 9.2. ***Mycobacterium marinum*** **down-regulates rgTNFα2-induced monocyte ROI.** Monocyte cultures were pre-incubated for 5 hours with medium only, 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(A)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed *M. marinum* **(B)**, subsequently treated with 100 ng/mL of rgTNFα2 and incubated for an additional 12 hours. Alternatively cell were pre-treated for 5 hours with 100 ng/mL of rgTNFa2 before being incubated with 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(C)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed **(D)** *M. marinum*. The ROI response was then induced with PMA (100 ng/mL). Monocytes were obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) indicates significantly different ($P < 0.05$) from the PMA only treated controls and (+) above lines denotes significant difference ($P<0.05$) between experimental groups indicated by the lines.

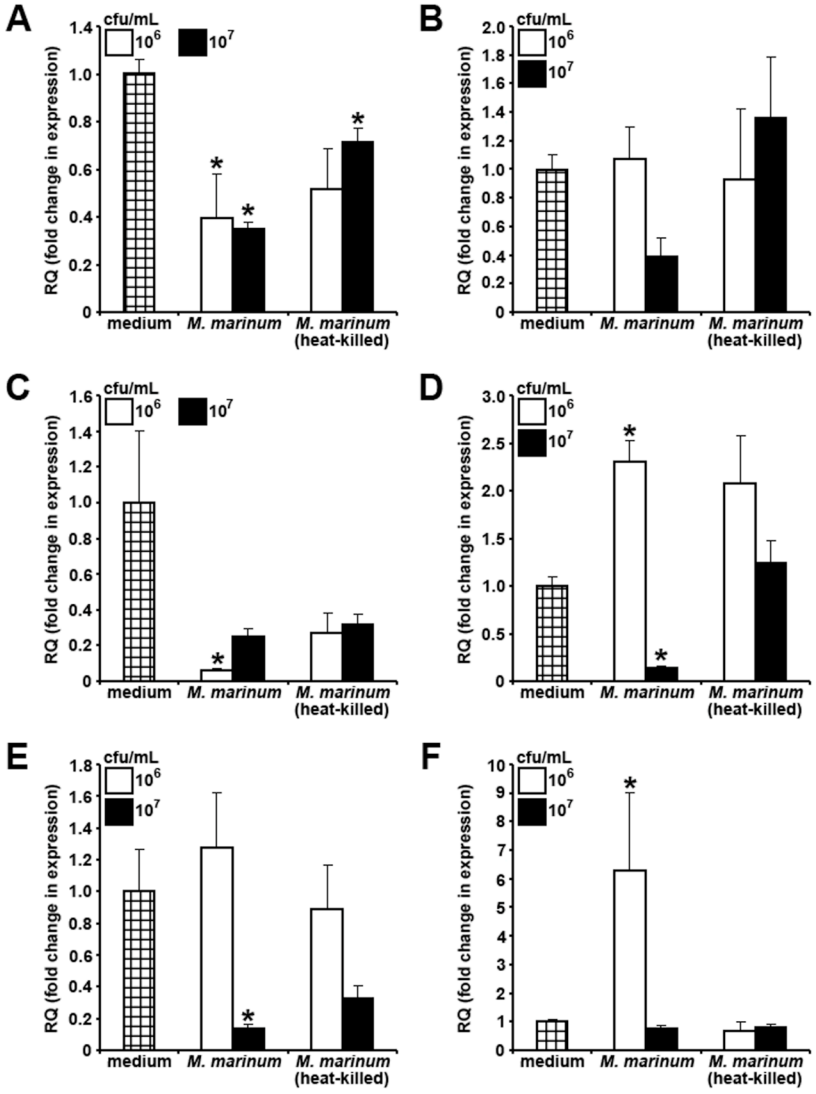

Figure 9.3. ***Mycobacterium marinum*** **infections alter the expression of genes encoding goldfish monocyte NADPH oxidase components and NRAMP.** Monocytes were incubated with viable or heat-killed *M. marinum*. The genes examined included: **(A)** $p22^{phox}$; **(B)** $p40^{phox}$; **(C)** $p47^{phox}$; **(D)** $p67^{phox}$; **(E)** $gp91^{phox}$; **(F)** NRAMP. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using monocytes obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). (*) indicates significantly different ($P < 0.05$) from the respective medium treated controls.

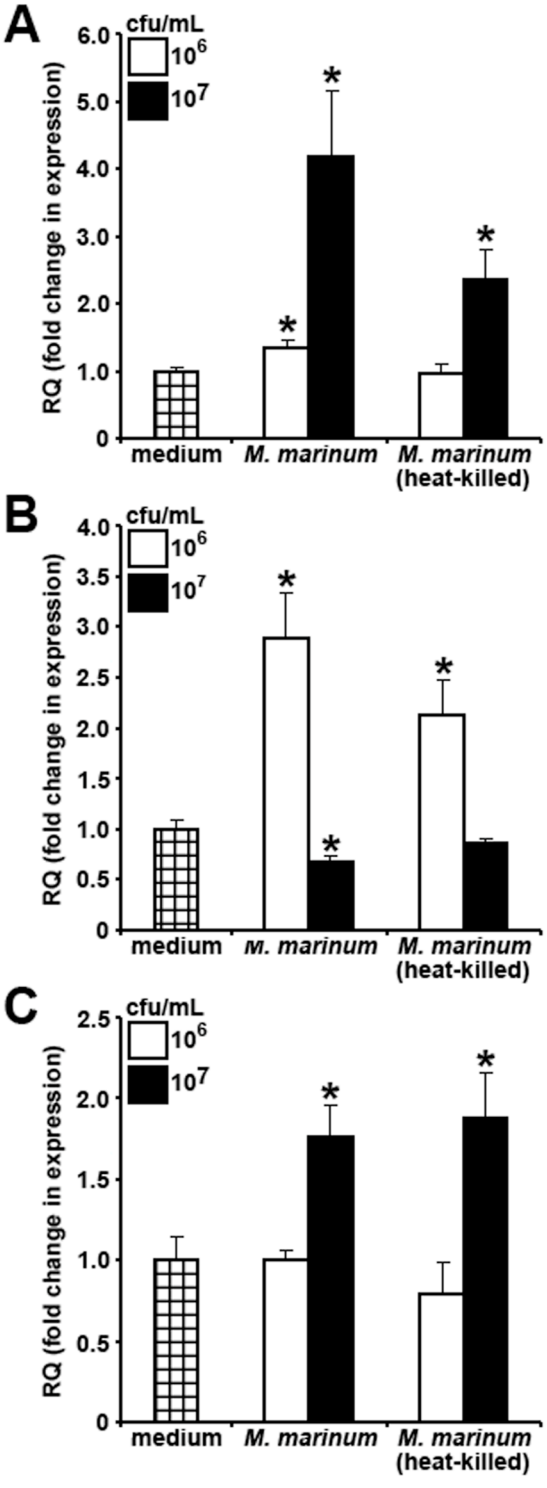

Figure 9.4. ***Mycobacterium marinum*** **infections alter the gene expression of goldfish monocyte immunosuppressive genes.** Monocytes were incubated with viable or heat-killed *M. marinum*. The genes examined included: **(A)** IL-10, **(B)** TGFβ1, **(C)** SOCS-3. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using monocytes obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls.

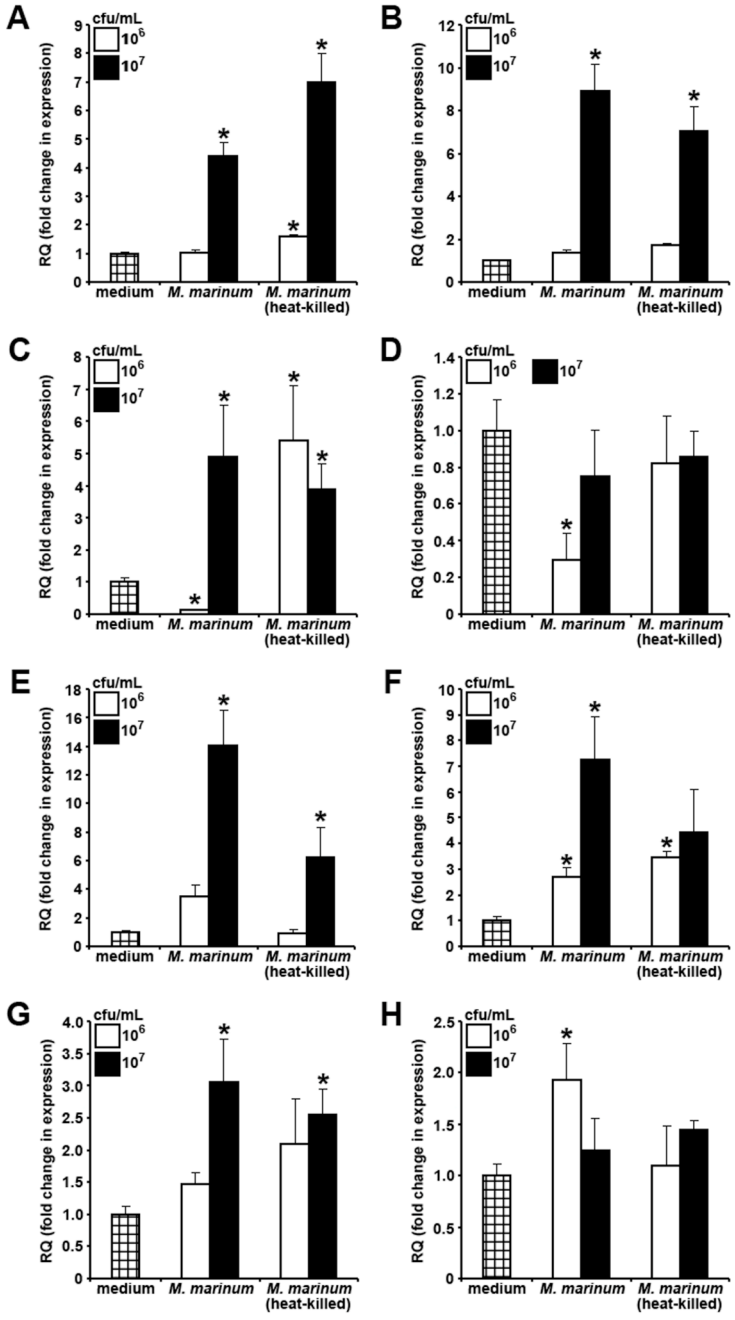

Figure 9.5. ***Mycobacterium marinum*** **infections alter the gene expression of goldfish monocyte pro-inflammatory genes.** Monocytes were incubated with viable or heat-killed *M. marinum*. The genes examined included: **(A)** TNFα1; **(B)** TNFα2 **(C)** IL-1β1; **(D)** IL-1β2; **(E)** IFNγ; **(F)** IFNγrel; **(G)** CXCL-8; **(H)** CCL-1. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using monocytes obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls.

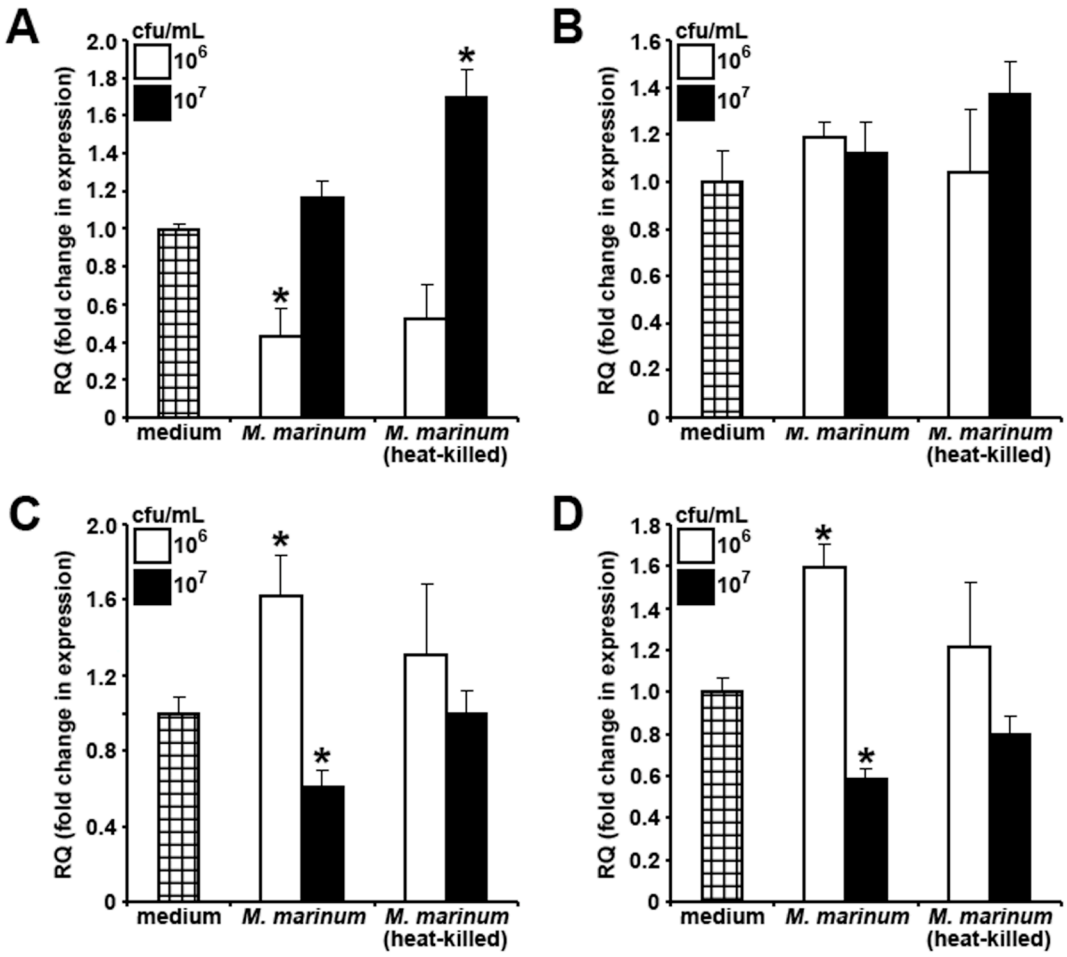

Figure 9.6. ***Mycobacterium marinum*** **infections alter the gene expression of goldfish monocyte pro-inflammatory cytokine receptor genes.** Monocytes were incubated with viable or heat-killed *M. marinum*. The genes examined included: **(A)** TNFR1; **(B)** TNFR2; **(C)** IFNGR1-1; **(D)** IFNGR1-2. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using monocytes obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). (*) indicates significantly different ($P < 0.05$) from the respective medium treated controls.

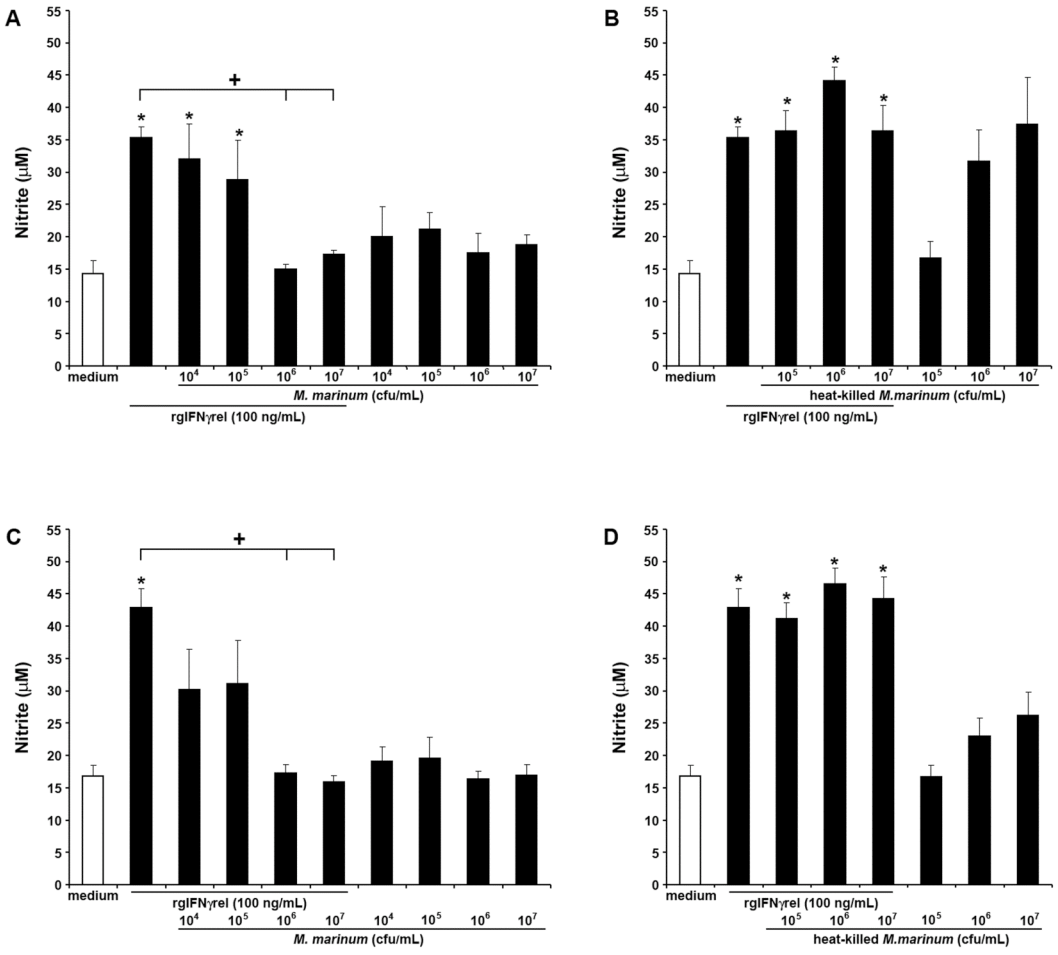

Figure 9.7. ***Mycobacterium marinum*** **suppresses goldfish macrophage rgIFNγrel-induced RNI.** Macrophage cultures were pre-incubated for 5 hours with medium only, or 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(A)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed *M. marinum* **(B)**, and subsequently treated with rgIFNγrel. Alternatively, cell were pre-treated for 5 hours with rgIFNγrel, before addition of 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(C)** or heat-killed **(D)** *M. marinum*. Nitrite production was determined by Griess reaction and nitrite concentrations determined using a nitrite standard curve. Macrophages were obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) indicates significantly different ($P < 0.05$) from medium only treated controls and (+) above lines denotes significant difference ($P<0.05$) between experimental groups indicated by the lines.

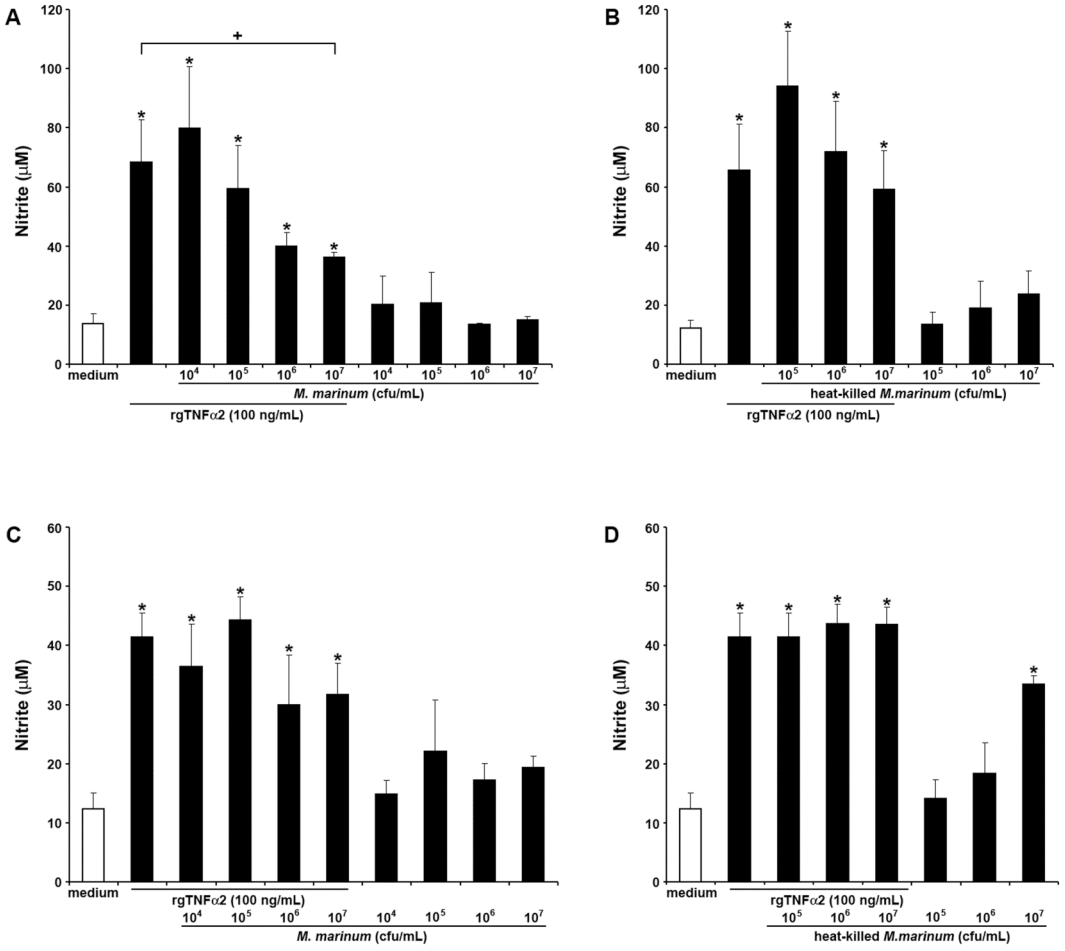

Figure 9.8. ***Mycobacterium marinum*** **infection suppresses rgTNFα2-induced RNI of pre-infected but not pre-rgTNFα2 stimulated macrophages.** Macrophage cultures were pre-incubated for 5 hours with medium only or 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(A)** or 10^5, 10^6 or 10^7 cfu/mL of heat-killed *M. marinum* **(B)**, and subsequently treated with rgTNFa2. Alternatively, cell were pre-treated for 5 hours with rgTNFa2, before addition of 10^4, 10^5, 10^6 or 10^7 cfu/mL of viable **(C)** or heat-killed **(D)** *M. marinum.* Nitrite production was determined by Griess reaction and nitrite concentrations were calculated using a nitrite standard curve. Macrophages were obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) indicates significantly different ($P < 0.05$) from medium only treated controls and (+) above lines denotes significant difference ($P<0.05$) between experimental groups indicated by the lines.

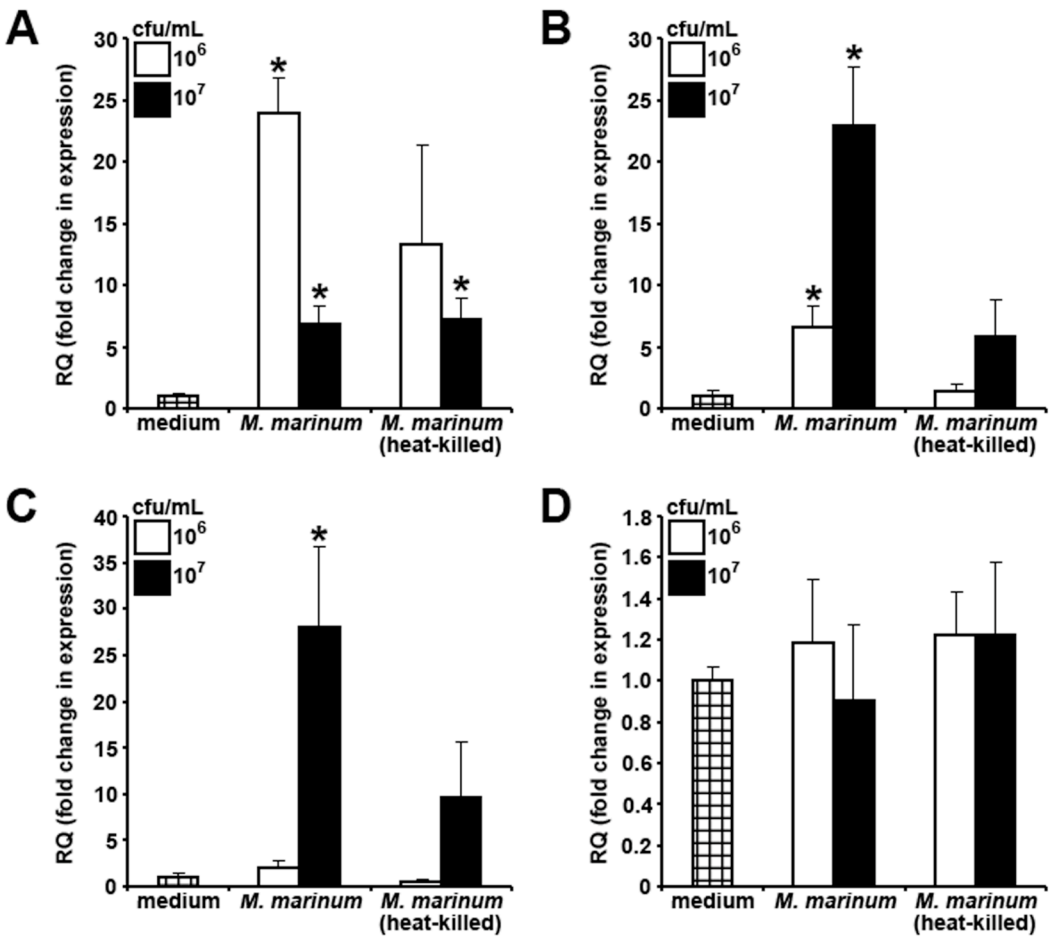

Figure 9.9. ***Mycobacterium marinum*** **infection alters gene expression of iNOS (isoforms A and B), IDO and NRAMP in goldfish macrophages.** Mature macrophage cultures were incubated with viable or heat-killed *M. marinum*. The genes examined included: **(A)** iNOS A; **(B)** iNOS B; **(C)** IDO; **(D**) NRMAP. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using macrophages obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). (*) indicates significantly different ($P < 0.05$) from the respective medium treated controls.

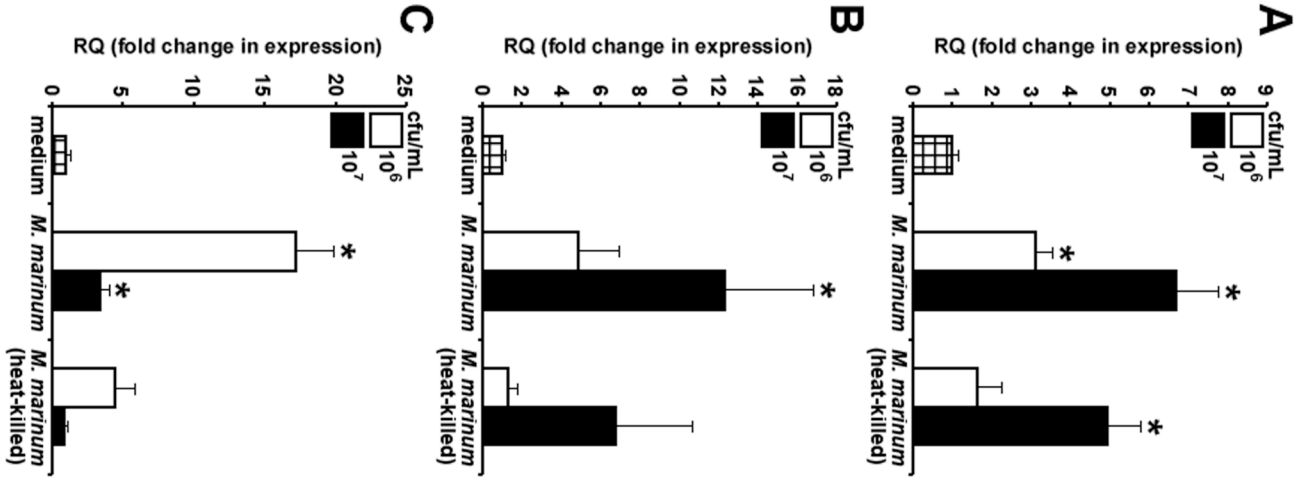

Figure 9.10. Quantitative analysis of *M. marinum*-induced gene expression changes of goldfish macrophage immunosuppressive genes. Mature macrophage cultures were incubated for 12h with 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum*. The genes examined included: **(A)** IL-10; **(B)** TGFβ1; **(C)** SOCS-3. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using macrophages obtained from primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls.

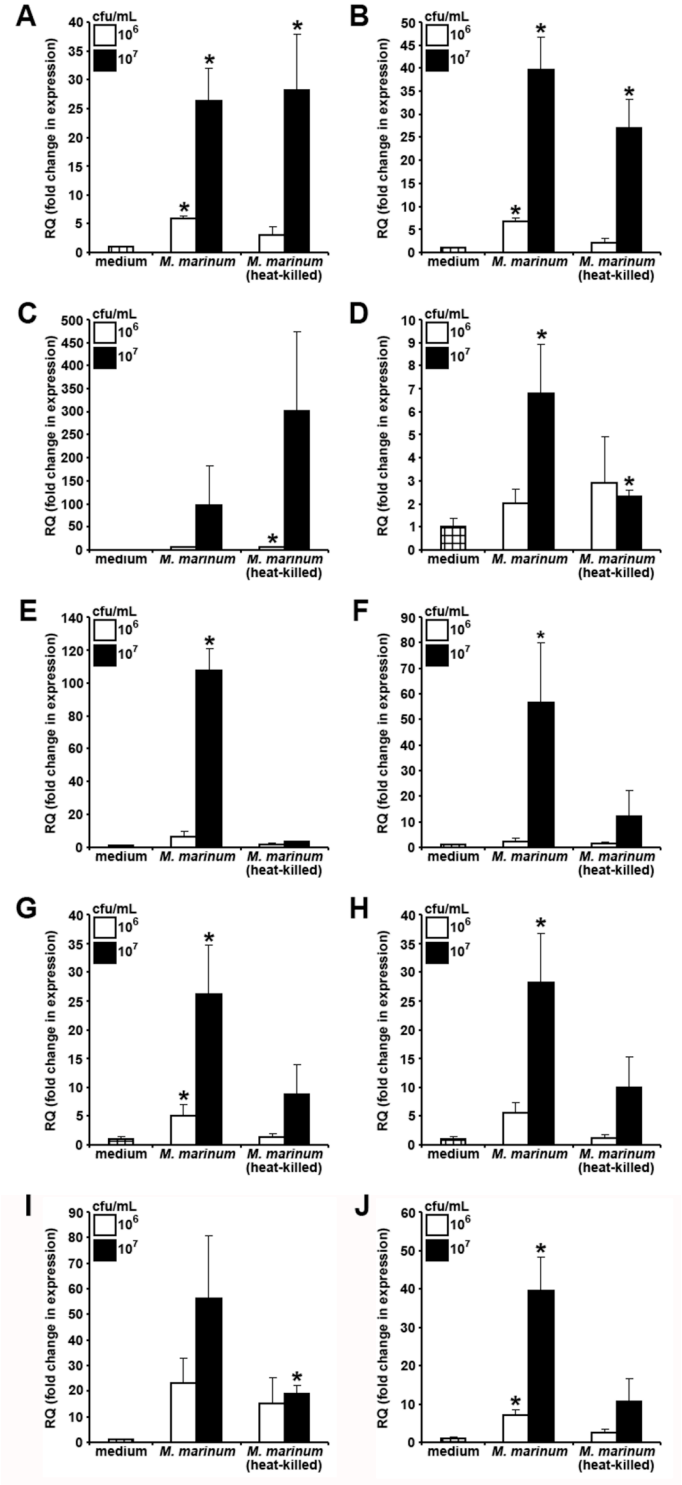

Figure 9.11. Quantitative analysis of *M. marinum*-induced gene expression changes of goldfish macrophage pro-inflammatory genes. Mature macrophage cultures were incubated for 12h with 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum*. The genes examined included: **(A)** TNFα1; **(B)** TNFα2; **(C)** IL-1β-1; **(D)** IL-1β-2; **(E)** IFNγ; **(F)** IFNγrel; **(G)** IL-12 p35; **(H)** IL-12 p40; **(I)** CXCL-8; **(J)** CCL-1. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using macrophages obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). (*) denotes significantly different ($P < 0.05$) from the respective medium treated controls.

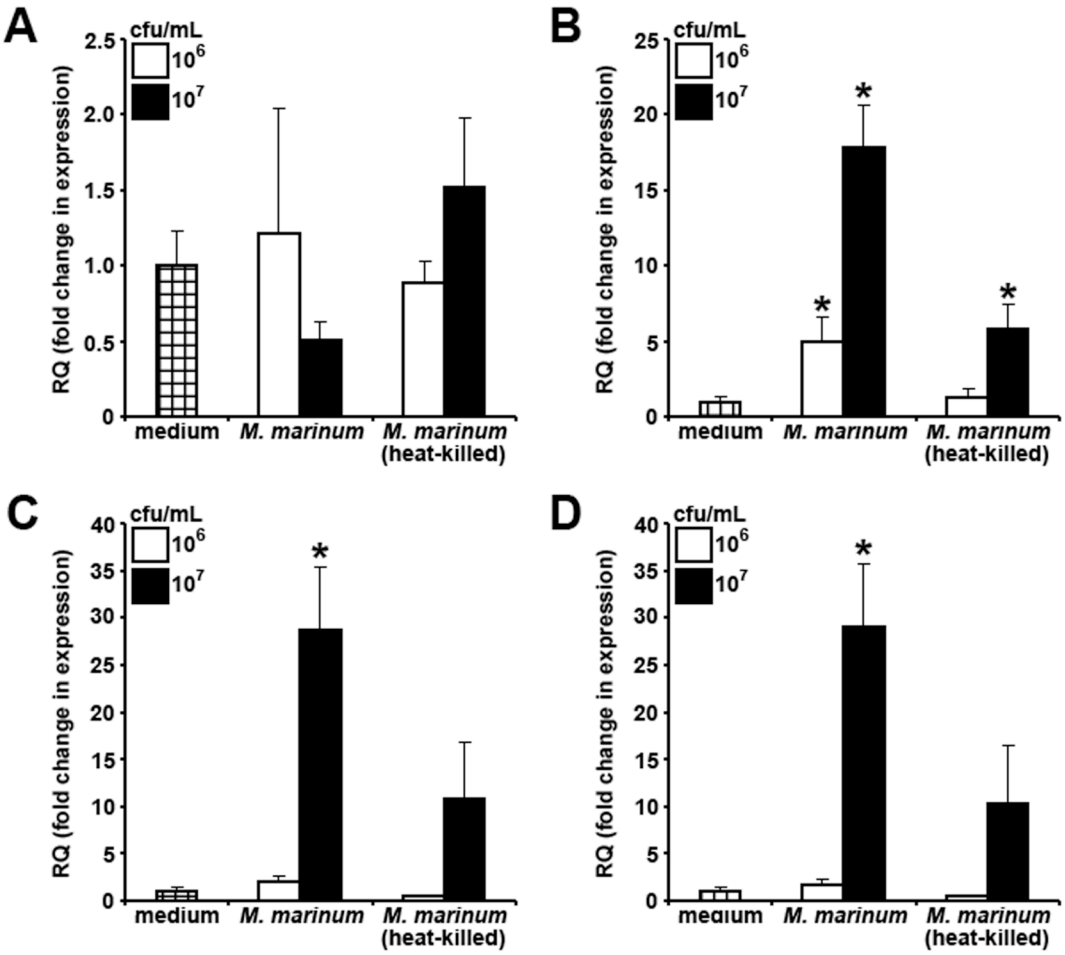

Figure 9.12. Quantitative analysis of *M. marinum*-induced gene expression changes of goldfish macrophage pro-inflammatory cytokine receptor genes. Mature macrophage cultures were incubated for 12h with 10^6 or 10^7 cfu/mL of viable or heat-killed *M. marinum*. The genes examined included: **(A)** TNFR1; **(B)** TNFR2; **(C)** IFNGR1-1; **(D)** IFNGR1-2. The reported expression was relative to EF-1α. The expression data were normalized against those observed in medium treated cells, respectively for each gene. The results are means ± SEM of RQ values derived using macrophages obtained form primary kidney macrophage (PKM) cultures established from individual fish (*n*=5). (*) indicates significantly different ($P < 0.05$) from the respective medium treated controls.

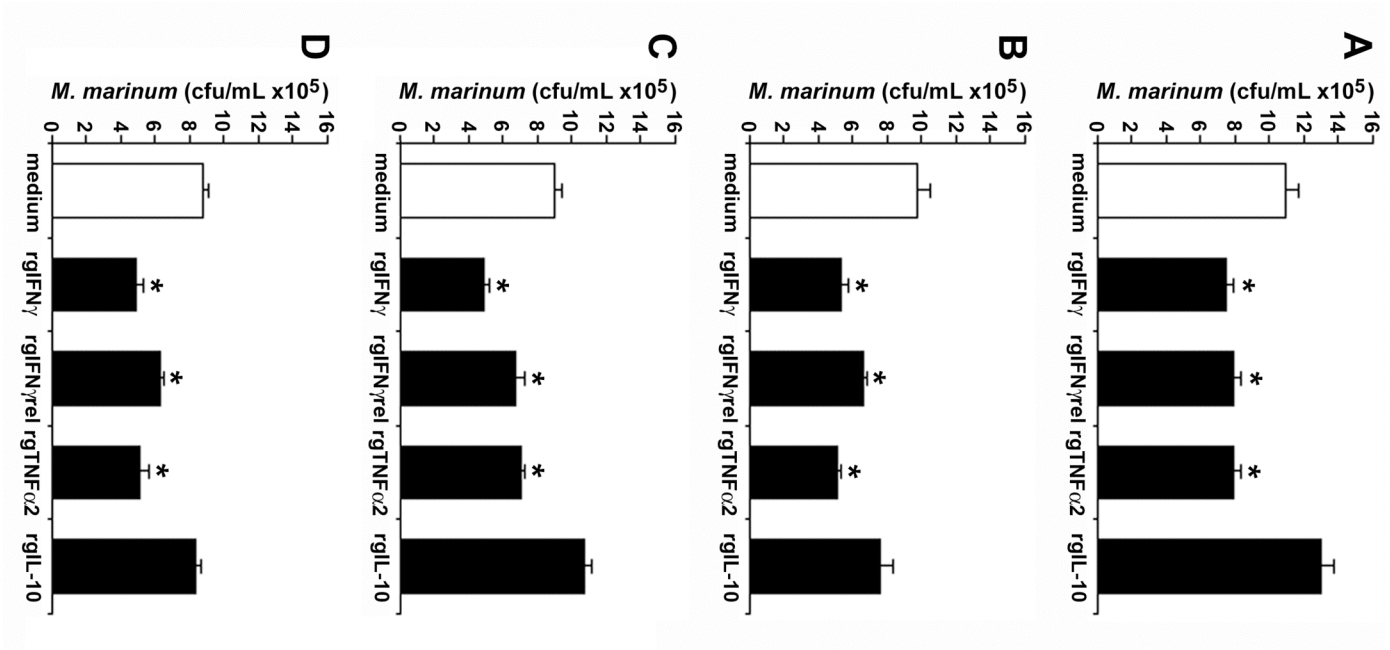

Figure 9.13. Intracellular survival of *M. marinum* in cytokine-activated goldfish phagocytes. Day 3 PKM cultures enriched in monocytes **(A)** and day 6-8 cultures comprising of mature macrophages **(C)** were incubated for 5 hours with 10^5, 10^6 or 10^7 cfu/mL of viable *M. marinum*, stimulated with 100 ng/mL of rgTNFα2, rgIFNγ, rgIFNγrel or 500 ng/mL of rgIL-10. Conversely, monocytes **(B)** and macrophages **(D)** were pre-treated for 5 hours with 100 ng/mL of rgTNFα2, rgIFNγ, rgIFNγrel or 500 ng/mL of rgIL-10 prior to infection with viable *M. marinum.* After incubation (12 hours), cells were washed twice MGFL in order to remove any cell-free bacteria, the medium was removed and 50 µL sterile 0.1% Tween-20 in distilled water was added to lyse the phagocytes. Cells were washed, lysed and the lysates were serially diluted and plated on Middlebrook 7H9 plates to determine cfu/mL. Monocytes or macrophages were obtained from primary kidney macrophage (PKM) cultures established from individual fish (n=5). Statistical analysis was performed using one-way ANOVA and the results were deemed to be significant at $P < 0.05$. (*) denotes significantly different ($P < 0.05$) from medium only treated controls.

4. REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1984. The cell biology of macrophage activation. Annual review of immunology **2:**283-318.
2. **Beltan, E., L. Horgen, and N. Rastogi.** 2000. Secretion of cytokines by human macrophages upon infection by pathogenic and non-pathogenic mycobacteria. Microbial pathogenesis **28:**313-318.
3. **Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin.** 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science (New York, N.Y **285:**732-736.
4. **Burge, E. J., D. T. Gauthier, C. A. Ottinger, and P. A. Van Veld.** 2004. Mycobacterium-inducible Nramp in striped bass (Morone saxatilis). Infection and immunity **72:**1626-1636.
5. **Bustamante, J., G. Aksu, G. Vogt, L. de Beaucoudrey, F. Genel, A. Chapgier, O. Filipe-Santos, J. Feinberg, J. F. Emile, N. Kutukculer, and J. L. Casanova.** 2007. BCG-osis and tuberculosis in a child with chronic granulomatous disease. The Journal of allergy and clinical immunology **120:**32-38.
6. **Canonne-Hergaux, F., S. Gruenheid, G. Govoni, and P. Gros.** 1999. The Nramp1 protein and its role in resistance to infection and macrophage function. Proceedings of the Association of American Physicians **111:**283-289.
7. **Casanova, J. L., and L. Abel.** 2002. Genetic dissection of immunity to mycobacteria: the human model. Annual review of immunology **20:**581-620.
8. **Chan, E. D., K. R. Morris, J. T. Belisle, P. Hill, L. K. Remigio, P. J. Brennan, and D. W. Riches.** 2001. Induction of inducible nitric oxide synthase-NO* by lipoarabinomannan of Mycobacterium tuberculosis is mediated by MEK1-ERK, MKK7-JNK, and NF-kappaB signaling pathways. Infection and immunity **69:**2001-2010.
9. **Clark, H. F., and C. C. Shepard.** 1963. Effect of Environmental Temperatures on Infection with Mycobacterium Marinum (Balnei) of Mice and a Number of Poikilothermic Species. Journal of bacteriology **86:**1057-1069.
10. **Clark, R. B., H. Spector, D. M. Friedman, K. J. Oldrati, C. L. Young, and S. C. Nelson.** 1990. Osteomyelitis and synovitis produced by Mycobacterium marinum in a fisherman. Journal of clinical microbiology **28:**2570-2572.
11. **Clay, H., J. M. Davis, D. Beery, A. Huttenlocher, S. E. Lyons, and L. Ramakrishnan.** 2007. Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. Cell host & microbe **2:**29-39.

12. **Clay, H., H. E. Volkman, and L. Ramakrishnan.** 2008. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. Immunity **29:**283-294.
13. **Cooper, A. M., B. H. Segal, A. A. Frank, S. M. Holland, and I. M. Orme.** 2000. Transient loss of resistance to pulmonary tuberculosis in p47(phox-/-) mice. Infection and immunity **68:**1231-1234.
14. **Davis, J. M., H. Clay, J. L. Lewis, N. Ghori, P. Herbomel, and L. Ramakrishnan.** 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity **17:**693-702.
15. **Davis, J. M., and L. Ramakrishnan.** 2008. "The very pulse of the machine": the tuberculous granuloma in motion. Immunity **28:**146-148.
16. **Denis, M.** 1991. Killing of Mycobacterium tuberculosis within human monocytes: activation by cytokines and calcitriol. Clinical and experimental immunology **84:**200-206.
17. **Douglas, T., D. S. Daniel, B. K. Parida, C. Jagannath, and S. Dhandayuthapani.** 2004. Methionine sulfoxide reductase A (MsrA) deficiency affects the survival of Mycobacterium smegmatis within macrophages. Journal of bacteriology **186:**3590-3598.
18. **Dulin, M. P.** 1979. A review of tuberculosis (mycobacteriosis) in fish. Vet Med Small Anim Clin **74:**731-735.
19. **Ehrt, S., and D. Schnappinger.** 2009. Mycobacterial survival strategies in the phagosome: defence against host stresses. Cellular microbiology **11:**1170-1178.
20. **Falcone, V., E. B. Bassey, A. Toniolo, P. G. Conaldi, and F. M. Collins.** 1994. Differential release of tumor necrosis factor-alpha from murine peritoneal macrophages stimulated with virulent and avirulent species of mycobacteria. FEMS immunology and medical microbiology **8:**225-232.
21. **Fenton, M. J., and M. W. Vermeulen.** 1996. Immunopathology of tuberculosis: roles of macrophages and monocytes. Infection and immunity **64:**683-690.
22. **Flannagan, R. S., G. Cosio, and S. Grinstein.** 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nature reviews **7:**355-366.
23. **Flesch, I. E., J. H. Hess, I. P. Oswald, and S. H. Kaufmann.** 1994. Growth inhibition of Mycobacterium bovis by IFN-gamma stimulated macrophages: regulation by endogenous tumor necrosis factor-alpha and by IL-10. International immunology **6:**693-700.
24. **Flynn, J. L., and J. Chan.** 2003. Immune evasion by Mycobacterium tuberculosis: living with the enemy. Current opinion in immunology **15:**450-455.
25. **Flynn, J. L., and J. Chan.** 2001. Immunology of tuberculosis. Annual review of immunology **19:**93-129.
26. **Gao, L. Y., S. Guo, B. McLaughlin, H. Morisaki, J. N. Engel, and E. J. Brown.** 2004. A mycobacterial virulence gene cluster extending RD1 is

required for cytolysis, bacterial spreading and ESAT-6 secretion. Molecular microbiology **53:**1677-1693.

27. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of novel interferon gamma receptor 1 isoforms in zebrafish (Danio rerio) and goldfish (Carassius auratus L.). Molecular immunology **46:**3050-3059.
28. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization of tumor necrosis factor receptors 1 and 2 of the goldfish (Carassius auratus L.). Molecular immunology **46:**2190-2199.
29. **Grayfer, L., and M. Belosevic.** 2009. Molecular characterization, expression and functional analysis of goldfish (Carassius auratus L.) interferon gamma. Developmental and comparative immunology **33:**235-246.
30. **Grayfer, L., E. G. Garcia, and M. Belosevic.** 2010. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (Carassius auratus L.). The Journal of biological chemistry **285:**23537-23547.
31. **Grayfer, L., J. W. Hodgkinson, S. J. Hitchen, and M. Belosevic.** 2011. Characterization and functional analysis of goldfish (Carassius auratus L.) interleukin-10. Molecular immunology **48:**563-571.
32. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Developmental and comparative immunology **32:**532-543.
33. **Hayashi, T., S. P. Rao, K. Takabayashi, J. H. Van Uden, R. S. Kornbluth, S. M. Baird, M. W. Taylor, D. A. Carson, A. Catanzaro, and E. Raz.** 2001. Enhancement of innate immunity against Mycobacterium avium infection by immunostimulatory DNA is mediated by indoleamine 2,3-dioxygenase. Infection and immunity **69:**6156-6164.
34. **Jung, Y. J., R. LaCourse, L. Ryan, and R. J. North.** 2002. Virulent but not avirulent Mycobacterium tuberculosis can evade the growth inhibitory action of a T helper 1-dependent, nitric oxide Synthase 2-independent defense in mice. The Journal of experimental medicine **196:**991-998.
35. **Kisich, K. O., M. Higgins, G. Diamond, and L. Heifets.** 2002. Tumor necrosis factor alpha stimulates killing of Mycobacterium tuberculosis by human neutrophils. Infection and immunity **70:**4591-4599.
36. **Lesley, R., and L. Ramakrishnan.** 2008. Insights into early mycobacterial pathogenesis from the zebrafish. Current opinion in microbiology **11:**277-283.
37. **Lotem, J., and L. Sachs.** 1982. Regulation of growth and differentiation by phorbol esters and the mechanism of tumor promotion. Carcinogenesis; a comprehensive survey **7:**385-390.
38. **MacMicking, J. D., G. A. Taylor, and J. D. McKinney.** 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science (New York, N.Y **302:**654-659.
39. **Morrison, D. C., and J. A. Rudbach.** 1981. Endotoxin-cell-membrane interactions leading to transmembrane signaling. Contemporary topics in molecular immunology **8:**187-218.

40. **Narayana, Y., and K. N. Balaji.** 2008. NOTCH1 up-regulation and signaling involved in Mycobacterium bovis BCG-induced SOCS3 expression in macrophages. The Journal of biological chemistry **283:**12501-12511.
41. **Nathan, C., and M. U. Shiloh.** 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proceedings of the National Academy of Sciences of the United States of America **97:**8841-8848.
42. **Neumann, N. F., D. R. Barreda, and M. Belosevic.** 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. Fish & shellfish immunology **10:**1-20.
43. **Neumann, N. F., J. L. Stafford, D. Barreda, A. J. Ainsworth, and M. Belosevic.** 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Developmental and comparative immunology **25:**807-825.
44. **Neumann, N. F., J. L. Stafford, and M. Belosevic.** 2000. Biochemical and functional characterisation of macrophage stimulating factors secreted by mitogen-induced goldfish kidney leucocytes. Fish & shellfish immunology **10:**167-186.
45. **Newton, G. L., N. Buchmeier, and R. C. Fahey.** 2008. Biosynthesis and functions of mycothiol, the unique protective thiol of Actinobacteria. Microbiol Mol Biol Rev **72:**471-494.
46. **Ng, V. H., J. S. Cox, A. O. Sousa, J. D. MacMicking, and J. D. McKinney.** 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Molecular microbiology **52:**1291-1302.
47. **Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding.** 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of Mycobacterium tuberculosis. J Immunol **167:**910-918.
48. **Orme, I. M., and A. M. Cooper.** 1999. Cytokine/chemokine cascades in immunity to tuberculosis. Immunology today **20:**307-312.
49. **Orme, I. M., A. D. Roberts, S. K. Furney, and P. S. Skinner.** 1994. Animal and cell-culture models for the study of mycobacterial infections and treatment. Eur J Clin Microbiol Infect Dis **13:**994-999.
50. **Otero, J., W. R. Jacobs, Jr., and M. S. Glickman.** 2003. Efficient allelic exchange and transposon mutagenesis in Mycobacterium avium by specialized transduction. Applied and environmental microbiology **69:**5039-5044.
51. **Othieno, C., C. S. Hirsch, B. D. Hamilton, K. Wilkinson, J. J. Ellner, and Z. Toossi.** 1999. Interaction of Mycobacterium tuberculosis-induced transforming growth factor beta1 and interleukin-10. Infection and immunity **67:**5730-5735.

52. **Pavelka, M. S., Jr., and W. R. Jacobs, Jr.** 1999. Comparison of the construction of unmarked deletion mutations in Mycobacterium smegmatis, Mycobacterium bovis bacillus Calmette-Guerin, and Mycobacterium tuberculosis H37Rv by allelic exchange. Journal of bacteriology **181:**4780-4789.
53. **Raja, A.** 2004. Immunology of tuberculosis. The Indian journal of medical research **120:**213-232.
54. **Ramakrishnan, L., and S. Falkow.** 1994. Mycobacterium marinum persists in cultured mammalian cells in a temperature-restricted fashion. Infection and immunity **62:**3222-3229.
55. **Ramakrishnan, L., N. A. Federspiel, and S. Falkow.** 2000. Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science (New York, N.Y **288:**1436-1439.
56. **Ramakrishnan, L., H. T. Tran, N. A. Federspiel, and S. Falkow.** 1997. A crtB homolog essential for photochromogenicity in Mycobacterium marinum: isolation, characterization, and gene disruption via homologous recombination. Journal of bacteriology **179:**5862-5868.
57. **Ramakrishnan, L., R. H. Valdivia, J. H. McKerrow, and S. Falkow.** 1997. Mycobacterium marinum causes both long-term subclinical infection and acute disease in the leopard frog (Rana pipiens). Infection and immunity **65:**767-773.
58. **Rich, E. A., M. Torres, E. Sada, C. K. Finegan, B. D. Hamilton, and Z. Toossi.** 1997. Mycobacterium tuberculosis (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. Tuber Lung Dis **78:**247-255.
59. **Rogall, T., J. Wolters, T. Flohr, and E. C. Bottger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus Mycobacterium. International journal of systematic bacteriology **40:**323-330.
60. **Rook, G. A., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology **59:**333-338.
61. **Rovera, G., D. Santoli, and C. Damsky.** 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proceedings of the National Academy of Sciences of the United States of America **76:**2779-2783.
62. **Ruley, K. M., J. H. Ansede, C. L. Pritchett, A. M. Talaat, R. Reimschuessel, and M. Trucksis.** 2004. Identification of Mycobacterium marinum virulence genes using signature-tagged mutagenesis and the goldfish model of mycobacterial pathogenesis. FEMS microbiology letters **232:**75-81.

63. **Ruley, K. M., R. Reimschuessel, and M. Trucksis.** 2002. Goldfish as an animal model system for mycobacterial infection. Methods in enzymology **358:**29-39.
64. **Stamm, L. M., J. H. Morisaki, L. Y. Gao, R. L. Jeng, K. L. McDonald, R. Roth, S. Takeshita, J. Heuser, M. D. Welch, and E. J. Brown.** 2003. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. The Journal of experimental medicine **198:**1361-1368.
65. **Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russell.** 1994. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science (New York, N.Y **263:**678-681.
66. **Sung, N., and M. T. Collins.** 2003. Variation in resistance of Mycobacterium paratuberculosis to acid environments as a function of culture medium. Applied and environmental microbiology **69:**6833-6840.
67. **Wu, C. H., J. J. Tsai-Wu, Y. T. Huang, C. Y. Lin, G. G. Lioua, and F. J. Lee.** 1998. Identification and subcellular localization of a novel Cu,Zn superoxide dismutase of Mycobacterium tuberculosis. FEBS letters **439:**192-196.
68. **Zhang, M., J. Gong, Z. Yang, B. Samten, M. D. Cave, and P. F. Barnes.** 1999. Enhanced capacity of a widespread strain of Mycobacterium tuberculosis to grow in human macrophages. The Journal of infectious diseases **179:**1213-1217.

CHAPTER X: GENERAL DISCUSSION

1. OVERVIEW OF FINDINGS

Vertebrate species have evolved effective but complex, multi-component, temporally regulated immune mechanisms to deal with both infiltrating pathogens and repair of damaged tissues. Collectively, these processes are known as inflammation, whose primary role is the resolution of tissue damage, clearance of damaged or dead cells and infiltrating pathogens and ultimately the restoration of homeostasis. Cells of the monocyte/ macrophage lineage are among the first to recognize invading pathogens, and effectively respond to foreign invaders to facilitate the onset and progression of appropriate host defense strategies. Macrophages use a battery of autocrine, paracrine and endocrine regulatory mechanisms, to architect the onset, progression and resolution of inflammation in accordance to the specifics of immune cues and stimuli. Through potent phagocytic and microbicidal pathways, these cells eliminate infiltrating organisms and clear the inflammation site of apoptotic and necrotic cells and debris. During the resolution of inflammation, monocytes/macrophages undergo an alternative polarization from classically activated antimicrobial killer cells to a non-classically activated healing cell phenotypes (2, 34, 69, 71).

Although the inflammatory response is multifaceted, at all stages of its progression and resolution, it is regulated by distinct soluble mediators of inflammation, known as cytokines. The main objective of this doctoral thesis was to assess the roles and efficacies of the inflammatory cytokines in the regulation

of macrophage antimicrobial responses of the ectothermic vertebrate, the goldfish (*Carassius auratus* L.).

The bony fish kidney serves as the main source of hematopoietic pluripotent cell populations, akin to the mammalian bone marrow. In response to soluble molecules released during the onset of infection, these progenitor pools may be recruited into the blood and commit, in accordance with the local stimuli, to particular cell differentiation pathways, such as the myeloid pathway, that gives rise to monocyte/macrophages. The Belosevic group has previously demonstrated that the goldfish kidney derived leukocyte (containing pluripotent cell populations) selectively differentiate into myeloid progenitor cells *in vitro* in absence of exogenous mediators, by producing copious amounts of endogenous growth factors (such as macrophage growth and differentiation factor, M-CSF) (42-47). At distinct phases of *in vitro* development, these primary cultures comprise of at least three distinct populations: myeloid progenitor cells, monocytes and mature macrophages, which have been extensively characterized by morphology, immunohistochemistry and function (37, 39). The less mature cultures comprise primarily of monocytic cells while mature macrophages predominate in older cultures. During the initial characterization of these populations (37, 38) as well as early on during the work described here (Chapter II) (18) it became evident that these specific populations possess distinct capabilities to undergo the hallmark antimicrobial responses attributed to the mammalian inflammatory monocytes and macrophages. Specifically, goldfish monocytes possess greater phagocytic capabilities as well as being more potent

producers of reactive oxygen intermediates, while the goldfish mature macrophages are the main producers of reactive nitrogen intermediates. In accordance with these earlier findings, the investigations presented in this thesis employed less mature monocyte cultures for the study of antimicrobial mechanisms such as phagocytosis and production of reactive oxygen intermediates, while mature macrophage cultures were used to measure the nitric oxide responses.

The majority of studies of lower vertebrate host defense pathways have focused on identifying homologues of the mammalian cytokines, while the functional verification of these moieties was infrequently investigated. Since cytokine regulation is pivotal to nearly all aspects of vertebrate macrophage inflammatory responses, it was imperative to identify and functionally characterize pro- and anti-inflammatory cytokines and their receptors in the goldfish model system. Given that tumor necrosis factor-alpha and interferon-gamma pathways are pivotal to the onset and progression of inflammation, and interleukin-10 is crucial for the inflammatory response, I decided to generate these goldfish cytokines and their receptors as recombinant proteins and assess their capacities to modulate antimicrobial responses of goldfish macrophages. In addition to investigating the abilities of these inflammatory homologues to modulate the antimicrobial responses of the goldfish kidney derived myeloid cells, it was also important to examine their respective roles in a natural host cell-pathogen interactions. For this reason, I developed a comprehensive *in vitro*

system for the examination of goldfish macrophage antimicrobial functions in response to *Mycobacterium marinum*.

The recombinant goldfish TNFα2 was a potent elicitor of hallmark monocyte and macrophage inflammatory mechanisms such as chemotaxis, phagocytosis, ROI and NO (Chapter III). The goldfish TNFα1 and TNFα2 isoforms displayed similar capabilities of priming the goldfish monocyte ROI responses and both were capable of interacting with either rgTNF-R1 or rgTNF-R2 in *in vitro* cross-linking studies. This suggests that the two goldfish TNFα isoforms may possess largely overlapping roles. Surprisingly, both TNF cytokines and their receptors were in dimerized states, and the ligand-receptor interaction occurred as ligand-receptor dimer pairs (Chapter IV). Interestingly, the recombinant seabream TNFα was also reported to predominantly exist in a dimeric form (16). This is unique to teleosts, since the mammalian TNFα and TNFα receptors, are predominantly trimers. Thus, despite being functionally similar to their mammalian counterparts (16, 18, 61), the specifics of the protein interactions and receptor-ligand interactions of the teleost TNFα appear to be different.

Unlike higher vertebrate species, certain bony fish posses not one but two type II IFNs, coined IFNγ and IFNγ related cytokine (IFNγrel). I demonstrated through gene synteny analyses of higher vertebrate IFNγ receptor-1 (IFNGR1) loci with those of zebrafish, that this teleost species has two distinct IFNGR1 genes, each located on different chromosomes in synteny with select gene homologues of those positioned along the mammalian IFNGR1 locus (Chapter

VI). I confirmed the presence of these IFNGR1 isoforms in the goldfish, and through *in vitro* binding studies, I demonstrated that the rgIFNγ and rgIFNγrel, each bound to a specific, cognate IFNGR1 isoform, suggesting a possible functional segregation. In a series of functional comparison studies, I demonstrated that rgIFNγ and rgIFNγrel indeed had distinct propensities to elicit goldfish monocyte and macrophage antimicrobial responses and immune gene expression profiles (Chapter VII). Furthermore, my work suggests that while the teleost IFNγ employed the cell signaling pathways utilized by its mammalian counterpart, the IFNγrel had distinct, as of yet not identified signaling mechanisms. These results indicate that while a single mammalian IFNγ serves as a key cytokine in the regulation of inflammatory and immune processes, certain teleosts have evolutionarily diverged to rely on an additional type II IFN in orchestrating antimicrobial processes. Undoubtedly, these fish species have been subject to very distinct evolutionary pressures, as compared to mammals, the consequences of which are reflected in the presence of this unique type II IFN system. As reagents become available, it will be interesting to examine the downstream signaling mechanism employed by IFNγrel and how these relate to other known cytokine signaling pathways of higher vertebrates.

Interleukin-10 is believed to a primordial immune mediator, ancestral to even IFNγ. Its functional importance is reflected by the fact that the protein sequences of evolutionarily distant species such as fish and man share very high identity. Therefore, it was not surprising that the recombinant goldfish IL-10 exhibited conserved immunosuppressive role attributed to its mammalian

counterpart (Chaptre VIII). Indeed, like the mammalian cytokine, the rgIL-10 ablated the induced inflammatory gene expression of goldfish monocytes as well their production of ROI. Furthermore, it would appear that this goldfish anti-inflammatory mediator also uses similar cell signaling mechanisms employed by the mammalian IL-10.

The roles of the pro- and anti-inflammatory cytokines in antimicrobial and immune responses of the goldfish monocytes and macrophages challenged with the fish pathogen *Mycobacterium marinum* were investigated *in vitro* (Chapter IX). At higher bacterial cell numbers and regardless of viability, *M. marinum* abrogated the recombinant goldfish (rg)TNFα2 and rgIFNγ-primed monocyte ROI. Live but not heat-killed *M. marinum* also ablated rgIFNγrel and rg-TNFα2-elicited macrophage NO. These anti-inflammatory effects could be explained in part by the observation that *M. marinum* also significantly decreased the gene expression of several NADPH oxidase components and inflammatory cytokine receptors while upregulating the expression of immunosuppressive genes IL-10, TGFβ1 and SOCS-3. Interestingly, the bacteria also elicited a clear host cell defense response, since monocytes and mature macrophages up-regulated the mRNA levels of several pro-inflammatory genes in response to bacterial challenges. Furthermore, the treatment of monocytes and macrophages with rgTNFα2, rgIFNγ, or rgIFNγrel reduced the survival of intracellular mycobacteria, again underlining the centrality of these immune mediators in regulation of goldfish antimicrobial functions. Together, these results suggest that a delicate balance exists between the goldfish monocyte/macrophage immune

defense and the evasion strategies of *M. marinum*, which has undoubtedly co-evolved with the fish immune system. Furthermore, it would appear that the goldfish inflammatory cytokines are indispensable in shifting the balance of this host cell-pathogen battle towards pathogen elimination rather than infiltrate survival and propagation.

2. EVOLUTION OF THE METAZOAN INFLAMMATORY RESPONSE

The initial steps in the evolution of the inflammatory response can be linked to events where unicellular eukaryotes that inbitially used phagocytosis for nutrient uptake, adopted this process as a defense strategy against invading pathogens (31, 64). It has been well documented that primordial single celled amoeba has a number of lectin and mannose receptors, akin to mammalian phagocytic receptors, which facilitate engulfment of bacteria by these protozoans. Furthermore, the cellular mechanisms of phagocytosis are similar between the mammalian and single celled phagocytes (57-60). Unicellular eukaryotes evolved from prokaryotes approximately 2000 million years ago (4), while the emergence multicellular metazoans is believed to have occurred some 800-1000 million years ago (46). Therefore, it can be reasoned that the transition from phagocytosis as a trophic mechanism, and use of phagocytosis for host defense occurred between 1000-2000 million years ago.

Specialized phagocyte, geared towards clean-up of apoptotic cells and infiltrating pathogens first arose in sponges, where invertebrate phagocytes have

been referred to as hemocytes, coelomocytes or amoebocytes (7). Sponges have existed for some 550 million years, making them the oldest living group in possession of a rudimentary inflammatory response, complete with pattern recognition receptors, pathogen phagocytosis, complement like components, and macrophage-like cytokine regulation reminiscent of the mammalian mediators of inflammation.

Similar to the mammalian professional phagocytes, sponge hemocytes also express cysteine-rich scavenger receptors (SRCRs) [reviewed in (53)]. In fact, as in mammals, sponges possess both type A and type B SRCRs, comprising of six and eight cysteines, respectively. Interestingly, sponges express at least 3 alternative splice variants of SRCR type A, and the administration of an antibody raised against the sponge SRCR type A, inhibits hemocyte aggregation (5, 46). The sponge SRCR type B is a multi-adhesion protein with high phylogenetic identity to bonafide mammalian macrophage phagocytic receptors (45). Thus, the sponges not only possess functional phagocytes with bactericidal properties but they also have a repertoire of receptors for discriminating the phagocytic targets. Sponge hemocytes also possess a number of lysosomal enzymes, such as cathepsins, reminiscent of those found in mammalian phagolysosomes (24). Furthermore, invertebrate phagocytes are capable of undergoing the production of reactive oxygen (21) and nitrogen intermediates (36, 65, 66), which are hallmark antimicrobial mechanisms of the mammalian phagocytes.

The emergence of pathogen pattern recognition is also believed to be very primordial, predating the arthropod and deuterostome split as exemplified by the

Drosophila toll pathway (26, 27, 30). Indeed a recent genomic analysis of the sea urchin has revealed that these ancient organisms encode 222 putative toll-like receptors, more than 200 NACHT domain-leucine-rich repeat proteins (similar to nucleotide-binding and oligomerization domain (NOD) and NALP proteins of vertebrates), and a broad family of scavenger receptor cysteine-rich proteins [reviewed in (52)]. It remains to be seen, whether these structurally related proteins also confer functions akin to their mammalian homologues.

Invertebrate species also have a complement-like system currently believed to comprise of much fewer proteins and primarily serving the roles of pathogens opsonization (15, 41-43). For example, the horseshoe crab has functional homologues of C3 as well as other complement components (70) and in fact a cDNA transcript encoding a C3-like molecule has been identified in gorgonian coral (12), suggesting that at least this complement component diverged before the protostome and deuterostome separation. Additionally, it is widely believed that the prophenoloxidase activating system of invertebrates is the functional predecessor to the complement pathways seen in vertebrate species [reviewed in (6)]. This primordial defense mechanism involves pathogen recognition and coordination of host defense through a complex cascade involving serine protease activation of the melanin enzyme, which in turn confers fungicidal, bactericidal and/or anti-viral effects [reviewed in (22, 63)].

Despite the broad evolutionary distance between teleosts and mammals, the results of my studies suggest that the fundamental monocyte/macrophage regulatory mechanisms pivotal for onset, progression and resolution of

mammalian inflammation are for the most part present in bony fish. The hallmark pro-inflammatory mediators IFNγ and TNFα have functionally analogous roles to their respective mammalian counterparts, as does the immunosuppressive goldfish IL-10. Furthermore, the work described in this thesis outlines numerous goldfish monocyte/macrophage immune and inflammatory gene expression studies following a range of immune stimuli. The changes in the mRNA transcripts of these genes in response to respective stimuli also strongly suggest that their transcriptional regulation is occurring in similar manner to their mammalian counterparts. Although the products of many of these genes have yet to be functionally assessed, their predicted roles are supported by their expression patterns. Collectively, this suggests that the networks of genes and gene products involved in the regulation of the metazoan inflammatory processes were largely intact 350-400 million years ago, prior to the split of ray finned-fishes and lobe-finned fishes (ancestral to lung fish). In fact, there is growing evidence suggesting that cartilaginous fish, ancestral to the above two clades, possess intact TNFα, IFNγ and IL-10 pathways of immune regulation (20, 62). This would place the origins of these cytokines to almost 450 million years ago.

Indeed the TNF and IL-1 immune signaling pathways are extremely primordial, predating the emergence of vertebrate species. For example, sponges and molluscs appear to possess immune factors with immune activities similar to those of TNFα (19, 48), sea urchins possess seven TNF receptor and four TNF ligand family members, the sea squirts encode two TNF-Rs and two potential ligands, while sea anemones have two TNF receptors and one TNF ligand (54).

Although none of the above have been suggested to being the direct TNF-R1 and/or TNFα homologues, recently a molluscan TNFα homolog has been identified (10).

Sea urchin genome database mining for potential IFNγ failed to yield positive results (62), suggesting that IFNγ may have evolved with the emergence of vertebrate species. Since the prevailing theory suggests that the IFNγ system evolved from gene duplication of an ancestral IL-10 ligand and receptor genes, the above would imply that IL-10 like molecules were present prior to the emergence of the first vertebrate species. However, keeping in mind that the IFNγ gene sequence identities across fish are incredibly low, it is all together possible that an ancestral IFNγ (and circumstantially IL-10) may exist in invertebrates, beyond molecular recognition.

The TNFα, IL-1β and IL-6-like molecules (3), and TNFα and IL-1β signaling pathways (17, 68) have clearly been demonstrated in invertebrate species. Indeed early reports using mammalian cytokines to elicit hallmark inflammatory processes from molluscan hemocytes suggest the presence and functional conservation of endogenous invertebrate counterparts of TNFα, IL-1β and IL-6 (44). Despite this, to my knowledge there have been no direct functional studies of such invertebrate inflammatory mediators. Regardless, the above discoveries indirectly suggest the potential presence of counter-regulatory, immunosuppressive molecules such as IL-10. Until experimentally proven, it will remain debatable whether the ancestral genes from which the classic inflammatory cytokines evolved, initially possessed inflammatory roles or

acquired them with increased organism complexity and new pathogen pressures during evolution. Indeed, it has been proposed that while TGFβ signaling pathways exist in invertebrates, that the anti-inflammatory roles of TGFβ evolved as a result of more recent gene duplication events (23).

It is believed that the inflammatory response of currently existing metazoan species arose form a monophyletic origin. It seems rational, when examining inflammatory processes across these evolutionarily distinct species, that with increased organism complexity there is a concomitant increase in the number of mechanisms that regulate these processes. Understanding the respective inflammatory response strategies across metazoans in a broader context, keeping in mind other potential tradeoffs to developing more elaborate inflammatory strategies, will gain a better understanding of why these species might possesses more or less complex ways of dealing with tissue damage and host defense against infection. In turn, a more fundamental insight into species-specific strategies of defense will yield more reliable approaches to anticipate and deal with immunological outcomes of emerging aquacultural, livestock and human pathogens.

3. THE USE OF FISH MONOCYTE/MACROPHAGE *Mycobacterium marinum* INFECTIONS AS SURROGATE SYSTEMS FOR THE STUDY OF MYCOBACTERIAL DISEASE

As emphasized in chapter IX, the majority of *in vitro* mycobacterium infection studies have relied on murine macrophage model systems (11, 13, 55),

which are far from ideal due to the natural resistance of mice to mycobacterial infections. Using human cell lines requires differentiating these cell types with phorbol esters (29, 56) or LPS (1, 32), by which the cell activation status confounds the interpretation of the results obtained in these experiments. Furthermore, the mycobacterial species that naturally infect humans (chiefly *M. tuberculosis* and *M. leprae*) grow very slowly in culture and are difficult to maintain and manipulate. As a result a surrogate model system has recently been employed to study the host-pathogen association in mycobacterial infections, *M marinum*. This mycobacterial species is relatively fast growing, easily manipulated and naturally infects fish and amphibians (49). *M. marinum* infections of natural host model systems have yielded a substantial amount of new valuable insight into the biology of this pathogen as well as its interactions with the host immune system (9, 28). I believe that the use primary host derived *in vitro* cultures for the study of *M. marinum* immune interactions is the next logical step in gaining a better understanding of the biology of this host-pathogen association. Unfortunately, such research has been hampered by the fact that while zebrafish (the main species used thus far to study *M. marinum*) are genetically ideal for *in vivo* infection studies, the small size of zebrafish makes it very difficult to establish primary cell cultures for *ex vivo* mycobacterium studies. Consequently, a significant portion of my thesis work has focused on the generation of inflammatory reagents and the characterization of the processes involved in immune regulation of goldfish macrophage antimicrobial mechanisms. I believe that I have successfully adopted the goldfish reagents and

monocyte/macrophage culturing system in investigating the *M. marinum*-natural host cell interface. Overall, the results of this thesis and the developed *in vitro* model system provide a stepping-stone for enhanced mycobacterial research.

4. FUTURE DIRECTIONS

4.1 Goldfish tumor necrosis factor alpha

It is well established that the mammalian TNFα mediates its biological roles both as a membrane bound moiety as well as a soluble mediator, following enzymatic cleavage. The mammalian TNFα converting enzyme (TACE) responsible for cleaving the membrane bound TNFα has been identified and thoroughly characterized (25, 33, 47). Similarly, TNFα of bony fish possess the highly conserved TACE cleavage sites (Chapter III) and exhibit membrane bound and soluble forms of this mediator (14). Despite this, distinct biological roles of the membrane and soluble forms of teleost TNFα are currently poorly understood. I believe that the next logical step in the research of the biology of goldfish TNFα would be to elucidate the respective biological roles of the membrane bound form of this fish cytokine. It is likely that membrane and soluble forms of TNFα engage different TNF receptors or engage the same receptors with different affinities. Thus, the membrane-bound and soluble TNFα may confer unique biological outcomes in the same cell types or target distinct immune populations.

To investigate the roles of the membrane-bound TNFα, it would be advantageous to generate specific polyclonal and monoclonal Abs against the recombinant goldfish ligand(s), the membrane-tethered portion of the TNFα as

well as the TNF-R1 and TNF-R2, hopefully some of which would be blocking antibodies. Goldfish monocyte/macrophage production of TNFα could be immunostimulated while chemically inhibiting the TNFα converting enzyme [TACE, using chemical inhibitors such as previously described for mammals (40)], thereby preventing the cleavage and release of the soluble TNFα. The presence of the membrane-bound TNFα could then be confirmed using the Ab specific for the N-terminal, tethering portion of the cytokine (by Western blot, confocal microscopy and/or FACS). The cells would then be methanol fixed to preserve their membrane protein compositions (membrane-bound TNFα) but prevent them from undergoing further changes. The fixed cells (and appropriate controls) would be employed to conduct a battery of studies such those described for the characterization of the rgTNFα in this thesis. The TNF-R1 and TNF-R2 blocking antibodies could be incorporated into these studies in order to deduce which receptor(s) was/were responsible for the observed effects. These results would need to be recapitulated using a reporter system, expressing each of the goldfish TNF-R1 or TNF-R2 proteins individually or in combination, using cells that are not normally expressing these receptors.

The above reagents as well as those described throughout this thesis could be used to investigate the effects of soluble and membrane bound goldfish TNFα on immune gene expression profiles of various goldfish immune populations. Additionally, mammalian reagents could be adopted to analyze whether distinct signaling pathways are employed in the context of each population and stimulus combination.

The anti-TNFα antibodies could also be used to investigate the variables attributing to whether the goldfish TNFα becomes cleaved or otherwise remains cell bound both *in vitro* and *in vivo* at the inflammatory sites. Such studies could incorporate the recombinant cytokines described in this thesis, several of which have been observed to increase the expression of the goldfish TNFα isoforms.

I predict that the soluble and membrane bound goldfish TNFα will elicit distinct antimicrobial responses of goldfish monocytes and macrophages. It is likely that as in mammals (67), the membrane-bound cytokine will induce the apoptosis of the goldfish neutrophil populations. Since the goldfish immune populations express distinct proportions of TNF-R1 and TNF-R2 (Chapter IV), it is probable that stimulation of these respective populations with soluble and/or membrane bound goldfish TNFα will result in unique signal transduction and gene expression profiles in different cell types. Because TNFα is a key inflammatory mediator of mammals and appears to have the same role in bony fish, it will be invaluable to gain a better understanding of its biology in a lower vertebrate, both from comparative and fundamental perspectives.

4.2 Goldfish interferon gamma related cytokine

It is intriguing that while most vertebrates possess a single type II IFN (IFNγ), certain bony fish rely on an additional type II IFN, IFNγ related protein (IFNγrel), to orchestrate inflammatory processes. I demonstrated that, unlike mammals, certain cyprinids also possess not one but two IFNGR1 genes and that the two recombinant goldfish IFNGR1 proteins each bound to rgIFNγ or

rgIFNγrel, respectively (Chapter VI). Furthermore, rgIFNγ and rgIFNγrel had non-overlapping capabilities to elicit goldfish monocyte and macrophage antimicrobial responses and immune gene expression (Chapter VII). While rgIFNγ utilized the cell signaling pathways employed by the mammalian counterpart (including tyrosine phosphorylation/activation of Stat1), the signaling of rgIFNγrel appeared to be different (Chapter VII). Presumably, in the case of rgIFNγrel, the Stat1 tyrosine phosphorylation could be a remnant of a diverging receptor. In mammals, Stat1 may also be activated through Jak-independent serine phosphorylation (8, 35), and this may be the mechanism of rgIFNγrel Stat-1 activation. Unfortunately, the mammalian reagents against the serine-phosphorylated Stat1 did not cross-react with goldfish Stat-1, preventing me form answering this question.

The next logical step in the investigation of the biology of IFNγrel would be to elucidate its signaling pathways. It would appear that the IFNγrel signaling mechanisms diverged from the classical IFNγ signal pathways, thus I believe that identifying these IFNγrel signaling pathways will provide an important perspective on the evolution of inflammatory and immune processes in teleosts. Presumably, IFNγrel signals specifically through IFNGR1-1 and an as of yet unidentified distinct IFNGR2 chain. It is difficult to speculate as to the downstream signaling events elicited by IFNγrel, but as suggested above it is possible that Stat1 serine phosphorylation/activation predominates in this unique system. Alternatively, this cytokine might adopt distinct or novel Stats (there are

fish Stats with no mammalian counterparts), or all together distinct signaling pathways.

In order to elucidate some these mechanisms, goldfish monocytes and macrophages could be incubated with rgIFNγrel, cross-linked for any potential protein interactions with formaldehyde, and immunoprecipitated (IP) against rgIFNγrel-receptor complexes (and hopefully docked signaling molecules). After rgIFNγrel IP, the cross-linking should be reversed and the products resolved (preferably by 2-D SDS-PAGE). The distinct proteins would then be excised from gels and peptide identification of these performed by mass spectrometry.

In a separate series of experiments, goldfish fibroblast cell line CCL-1, could be transfected with an expression vector encoding tagged full length IFNGR1-1 and IFNGR1-2, individually or in combination, stimulated with rgIFNγrel and/or rgIFNγ to facilitate receptor activation and IP against the tagged receptors performed as outlined above for rgIFNγrel. Upon successful identification of a partial protein sequence of a putative rgIFNγrel second receptor chain, reverse genetics could be employed to identify the goldfish full length cDNA transcript for this receptor. The deduced cDNA sequence could then be utilized in IP experiments similar to those described above. Also, the identified receptor sequences could be employed to perform monocyte and macrophage IFNγ receptor(s) RNAi knockdowns to deduce respective receptor roles in the biological outcomes elicited by IFNγ and IFNγrel, respectively.

The generation of the specific monoclonal antibodies to IFNγ and IFNγrel receptor epitopes would also be ideal in order to examine the distribution of these

receptors on different goldfish immune cell populations. The Western blot analysis of rgIFNγ- and rgIFNγrel- stimulated goldfish macrophage lysates and isolated nuclei (as described in Chapter VII) could be done using Abs specific for a range of Stats in various activation states (tyrosine or serine-phosphorlated). For example, as described in Chapter VIII, the anti-mammalian phospho-Y-Stat3 Ab cross-reacted with the goldfish protein. Possibly other mammalian reagents, such as a different anti-phospho-S-Stat1 Ab could be adopted for goldfish studies.

I expect that the above studies will result in the discovery of a novel IFNGR2 receptor isoform. As predicted based on putative docking sites on the IFNGR1 isoforms (Chapter VI), the IP experiments will likely pull down molecules such as Stat1 and Jak1 as well as possibly Jak2 (with IFNGR2 chain). The pull-downs will likely also yield other signaling mediators such as IRFs and SOCS. If Stat1 is not the main signaling molecule employed by IFNγrel, would be difficult to speculate what other signal transducers might be involved in signaling. As it stands, it is quite probable that IFNγrel may act through specific Stat1 serine (instead of tyrosine) phosphorylation and activation.

Since the possession of a second type II IFN in a given species is unique to some teleosts, understanding the regulation mechanisms and cell targets of IFNγ and IFNγrel, respectively, would greatly enhance our understanding of why these teleosts have evolved an additional IFNγ ligand-receptor system. To reiterate, gaining a better understanding of the signaling mechanisms of this unique teleost inflammatory cytokine that likely diverged from a classical

immune signaling pathway, present in all known vertebrates, will lend an inimitable perspective on the evolution of inflammatory and immune processes.

4.3 Using the kidney-derived goldfish monocyte/macrophage cultures to study *M. marinum* infections *in vitro*

It has been extensively demonstrated that unlike *M. tubeculosis*, gene mutagenesis of *M. marinum* is relatively effective (50, 51). Until now a reliable *in vitro* primary culture system for the study of mycobacterium-host cell interactions was not available. In this thesis I have described the comprehensive characterization of key goldfish inflammatory cytokines in the context of an *in vitro* goldfish monocyte and macrophage cultures (Chapters III-VIII). Furthermore, I have demonstrated that these biologically active reagents and the primary monocyte/macrophage culturing system can be adopted for the study of the *M. marinum* infections. Thus, in order to gain a better understanding of the mycobacterial strategies for survival and pathogenesis, I believe that it would be rewarding to perform shotgun mutagenesis of the *M. marinum* genome. This approach would enable the assessment of the abilities of mutant mycobacteria to evade antimicrobial responses and survive in goldfish monocytes and macrophages. I believe that this approach will uncover new mycobacterial genes essential for intracellular survival and evasion of the host antimicrobial responses.

To accomplish this, *M. marinum* whole genome mutagenesis should be performed. Derived bacterial mutants should be used to infect goldfish cells and investigate which of these bacterial clones would confer altered sensitivities to

specific monocyte and/or macrophage antimicrobial mechanisms (ROI, NO, phagocytosis, chemotaxis). In this thesis, I have demonstrated that rgTNFα, rgIFNγ and rgIFNγrel have distinct capabilities to elicit monocyte and macrophage antimicrobial responses. Should the *M. marinum* shotgun mutagenesis approach yield clones with increased sensitivity to specific antimicrobial responses, it would be interesting to examine whether these sensitivities would hold regardless of the recombinant cytokine used to elicit a particular monocyte or macrophage response. For example, if an *M. marinum* mutant has an increased susceptibility to ROI, is it equally hampered following the activation of host cells by either rgTNFα, rgIFNγ or rgIFNγrel. Since *M. marinum* manipulates numerous cytokine pathways in order to ensure its own survival, this approach may result in the identification of novel pathogen evasion pathways and allow us to specifically target these at distinct junctures, in attempts to prevent persistent mycobacterial infections.

Understanding what virulence factors are indispensable to *M. marinum* pathogenicity and intracellular survival will lead to advancements in the maximizing of host defense strategies to control this important infectious disease.

4.4 Using the goldfish *M. marinum* infection model system to study *in vivo* host defense against mycobacterial infections

In order to perform *in vivo* goldfish *M. marinum* infection studies, specific polyclonal antibodies against the goldfish inflammatory cytokines should be generated. An *M. marinum* time course of infection should be performed using

low, intermediate and high doses of the pathogen. Subsequently, relevant fish tissues should be harvested and Q-PCR immune gene expression studies and immunohistochemistry analyses performed. This work would elucidate the involvement of the specific goldfish inflammatory cytokines during the onset, progression and resolution of mycobacterial disease *in vivo*.

Should *M. marinum* mutagenesis yield any promising mutants that are highly susceptible to monocyte/macrophage antimicrobial responses, these can then be used to infect goldfish in order to establish the importance (an hence lack there of) of specific mycobacterial evasion strategies in *in vivo* persistence and mycobacterial virulence. Furthermore, the ablation (partial or complete) of specific cytokines by injection of goldfish with anti-recombinant goldfish cytokine antibodies may allow for the analysis of the importance of specific cytokines in the regulation of the course of infection of *M. marinum*.

I believe that the approach described above would also reveal the importance of the specific *M. marinum* host defense evasion strategies. Furthermore, the results of these studies would help characterize the inflammatory mechanisms employed by the host to deal with the infection and the efficacy by which distinct goldfish inflammatory cytokines contribute to host-pathogen interactions.

5. SUMMARY

The discipline of comparative immunology is a relatively new and expanding area of research with investigations in this field addressing the immune

response strategies of lower vertebrates, with central focus on bony fish. However, majority of this research to date has focused on identifying cDNA sequences of key homologues of mammalian immune mediators and to a lesser extent on the function of these molecules in respective fish species.

In this thesis, I reported on the molecular identification and functional characterization of a number of goldfish cytokines and their receptors. Using these newly characterized goldfish reagents, I believe that I have significantly extended our knowledge about inflammatory and antimicrobial responses of bony fish. Furthermore, I believe that the research contributions described in this dissertation have contributed to our knowledge on the origin and function of the innate immune responses of ectothermic vertebrates.

6. REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1984. The cell biology of macrophage activation. Annual review of immunology **2:**283-318.
2. **Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau, and F. Geissmann.** 2007. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science (New York, N.Y **317:**666-670.
3. **Beck, G., and G. S. Habicht.** 1996. Characterization of an IL-6-like molecule from an echinoderm (Asterias forbesi). Cytokine **8:**507-512.
4. **Bengtson, S. e.** 1994. Early life on earth. . New York: Columbia University Press.
5. **Blumbach, B., Z. Pancer, B. Diehl-Seifert, R. Steffen, J. Munkner, I. Muller, and W. E. Muller.** 1998. The putative sponge aggregation receptor. Isolation and characterization of a molecule composed of scavenger receptor cysteine-rich domains and short consensus repeats. Journal of cell science **111 (Pt 17):**2635-2644.
6. **Cerenius, L., and K. Söderhäll.** 1995. Crustacean immunity and complement: a premature comparison? Am Zool **35**.
7. **Cooper, E. L.** 1976. Evolution of blood cells. Annales d'immunologie **127:**817-825.
8. **Dalpke, A. H., S. Eckerle, M. Frey, and K. Heeg.** 2003. Triggering of Toll-like receptors modulates IFN-gamma signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signaling. European journal of immunology **33:**1776-1787.
9. **Davis, J. M., and L. Ramakrishnan.** 2008. "The very pulse of the machine": the tuberculous granuloma in motion. Immunity **28:**146-148.
10. **De Zoysa, M., S. Jung, and J. Lee.** 2009. First molluscan TNF-alpha homologue of the TNF superfamily in disk abalone: molecular characterization and expression analysis. Fish & shellfish immunology **26:**625-631.
11. **Denis, M.** 1991. Killing of Mycobacterium tuberculosis within human monocytes: activation by cytokines and calcitriol. Clinical and experimental immunology **84:**200-206.
12. **Dishaw, L. J., S. L. Smith, and C. H. Bigger.** 2005. Characterization of a C3-like cDNA in a coral: phylogenetic implications. Immunogenetics **57:**535-548.
13. **Flesch, I. E., J. H. Hess, I. P. Oswald, and S. H. Kaufmann.** 1994. Growth inhibition of Mycobacterium bovis by IFN-gamma stimulated macrophages: regulation by endogenous tumor necrosis factor-alpha and by IL-10. International immunology **6:**693-700.
14. **Forlenza, M., S. Magez, J. P. Scharsack, A. Westphal, H. F. Savelkoul, and G. F. Wiegertjes.** 2009. Receptor-mediated and lectin-like activities of carp (Cyprinus carpio) TNF-alpha. J Immunol **183:**5319-5332.

15. **Fujita, T., Y. Endo, and M. Nonaka.** 2004. Primitive complement system--recognition and activation. Molecular immunology **41:**103-111.
16. **Garcia-Castillo, J., E. Chaves-Pozo, P. Olivares, P. Pelegrin, J. Meseguer, and V. Mulero.** 2004. The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. Cell Mol Life Sci **61:**1331-1340.
17. **Gauthier, M. E., L. Du Pasquier, and B. M. Degnan.** 2010. The genome of the sponge Amphimedon queenslandica provides new perspectives into the origin of Toll-like and interleukin 1 receptor pathways. Evolution & development **12:**519-533.
18. **Grayfer, L., J. G. Walsh, and M. Belosevic.** 2008. Characterization and functional analysis of goldfish (Carassius auratus L.) tumor necrosis factor-alpha. Dev Comp Immunol **32:**532-543.
19. **Hughes, T. K., Jr., E. M. Smith, R. Chin, P. Cadet, J. Sinisterra, M. K. Leung, M. A. Shipp, B. Scharrer, and G. B. Stefano.** 1990. Interaction of immunoactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc Mytilus edulis. Proceedings of the National Academy of Sciences of the United States of America **87:**4426-4429.
20. **Inoue, Y., A. Morinaga, F. Takizawa, T. Saito, M. Endo, C. Haruta, T. Nakai, T. Moritomo, and T. Nakanishi.** 2008. Molecular cloning and preliminary expression analysis of banded dogfish (Triakis scyllia) TNF decoy receptor 3 (TNFRSF6B). Fish & shellfish immunology **24:**360-365.
21. **Johansson, M. W., T. Holmblad, P. O. Thornqvist, M. Cammarata, N. Parrinello, and K. Soderhall.** 1999. A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish. Journal of cell science **112 (Pt 6):**917-925.
22. **Johansson, M. W., and K. Soderhall.** 1996. The prophenoloxidase activating system and associated proteins in invertebrates. Progress in molecular and subcellular biology **15:**46-66.
23. **Konikoff, C. E., R. G. Wisotzkey, M. J. Stinchfield, and S. J. Newfeld.** 2010. Distinct molecular evolutionary mechanisms underlie the functional diversification of the Wnt and TGFbeta signaling pathways. Journal of molecular evolution **70:**303-312.
24. **Krasko, A., V. Gamulin, J. Seack, R. Steffen, H. C. Schroder, and W. E. Muller.** 1997. Cathepsin, a major protease of the marine sponge Geodia cydonium: purification of the enzyme and molecular cloning of cDNA. Molecular marine biology and biotechnology **6:**296-307.
25. **Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu.** 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell **53:**45-53.
26. **Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:**973-983.

27. **Lemaitre, B., J. M. Reichhart, and J. A. Hoffmann.** 1997. Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences of the United States of America **94:**14614-14619.
28. **Lesley, R., and L. Ramakrishnan.** 2008. Insights into early mycobacterial pathogenesis from the zebrafish. Current opinion in microbiology **11:**277-283.
29. **Lotem, J., and L. Sachs.** 1982. Regulation of growth and differentiation by phorbol esters and the mechanism of tumor promotion. Carcinogenesis; a comprehensive survey **7:**385-390.
30. **Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature **388:**394-397.
31. **Metchnikoff, E.** 1905. Immunity in infective diseases. F.G. B, translator. New York: Macmillan Press.
32. **Morrison, D. C., and J. A. Rudbach.** 1981. Endotoxin-cell-membrane interactions leading to transmembrane signaling. Contemporary topics in molecular immunology **8:**187-218.
33. **Moss, M. L., S. L. Jin, J. D. Becherer, D. M. Bickett, W. Burkhart, W. J. Chen, D. Hassler, M. T. Leesnitzer, G. McGeehan, M. Milla, M. Moyer, W. Rocque, T. Seaton, F. Schoenen, J. Warner, and D. Willard.** 1997. Structural features and biochemical properties of TNF-alpha converting enzyme (TACE). J Neuroimmunol **72:**127-129.
34. **Nahrendorf, M., F. K. Swirski, E. Aikawa, L. Stangenberg, T. Wurdinger, J. L. Figueiredo, P. Libby, R. Weissleder, and M. J. Pittet.** 2007. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. The Journal of experimental medicine **204:**3037-3047.
35. **Nair, J. S., C. J. DaFonseca, A. Tjernberg, W. Sun, J. E. Darnell, Jr., B. T. Chait, and J. J. Zhang.** 2002. Requirement of Ca2+ and CaMKII for Stat1 Ser-727 phosphorylation in response to IFN-gamma. Proceedings of the National Academy of Sciences of the United States of America **99:**5971-5976.
36. **Nappi, A. J., E. Vass, F. Frey, and Y. Carton.** 2000. Nitric oxide involvement in Drosophila immunity. Nitric Oxide **4:**423-430.
37. **Neumann, N. F., D. R. Barreda, and M. Belosevic.** 2000. Generation and functional analysis of distinct macrophage sub-populations from goldfish (Carassius auratus L.) kidney leukocyte cultures. Fish & shellfish immunology **10:**1-20.
38. **Neumann, N. F., J. L. Stafford, D. Barreda, A. J. Ainsworth, and M. Belosevic.** 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Developmental and comparative immunology **25:**807-825.
39. **Neumann, N. F., J. L. Stafford, and M. Belosevic.** 2000. Biochemical and functional characterisation of macrophage stimulating factors secreted

by mitogen-induced goldfish kidney leucocytes. Fish & shellfish immunology **10:**167-186.

40. **Newton, R. C., K. A. Solomon, M. B. Covington, C. P. Decicco, P. J. Haley, S. M. Friedman, and K. Vaddi.** 2001. Biology of TACE inhibition. Annals of the rheumatic diseases **60 Suppl 3:**iii25-32.
41. **Nonaka, M.** 2001. Evolution of the complement system. Current opinion in immunology **13:**69-73.
42. **Nonaka, M.** 2000. Origin and evolution of the complement system. Current topics in microbiology and immunology **248:**37-50.
43. **Nonaka, M., and F. Yoshizaki.** 2004. Primitive complement system of invertebrates. Immunological reviews **198:**203-215.
44. **Ottaviani, E., and A. Franchini.** 1995. Immune and neuroendocrine responses in molluscs: the role of cytokines. Acta biologica Hungarica **46:**341-349.
45. **Pahler, S., B. Blumbach, I. Muller, and W. E. Muller.** 1998. Putative multiadhesive protein from the marine sponge Geodia cydonium: cloning of the cDNA encoding a fibronectin-, an SRCR-, and a complement control protein module. The Journal of experimental zoology **282:**332-343.
46. **Pancer, Z., J. Munkner, I. Muller, and W. E. Muller.** 1997. A novel member of an ancient superfamily: sponge (Geodia cydonium, Porifera) putative protein that features scavenger receptor cysteine-rich repeats. Gene **193:**211-218.
47. **Perez, C., I. Albert, K. DeFay, N. Zachariades, L. Gooding, and M. Kriegler.** 1990. A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell **63:**251-258.
48. **Pfeifer, K., H. C. Schroder, B. Rinkevich, G. Uhlenbruck, F. G. Hanisch, B. Kurelec, P. Scholz, and W. E. Muller.** 1992. Immunological and biological identification of tumour necrosis-like factor in sponges: endotoxin that mediates necrosis formation in xenografts. Cytokine **4:**161-169.
49. **Ramakrishnan, L., and S. Falkow.** 1994. Mycobacterium marinum persists in cultured mammalian cells in a temperature-restricted fashion. Infection and immunity **62:**3222-3229.
50. **Ramakrishnan, L., N. A. Federspiel, and S. Falkow.** 2000. Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science (New York, N.Y **288:**1436-1439.
51. **Ramakrishnan, L., H. T. Tran, N. A. Federspiel, and S. Falkow.** 1997. A crtB homolog essential for photochromogenicity in Mycobacterium marinum: isolation, characterization, and gene disruption via homologous recombination. Journal of bacteriology **179:**5862-5868.
52. **Rast, J. P., L. C. Smith, M. Loza-Coll, T. Hibino, and G. W. Litman.** 2006. Genomic insights into the immune system of the sea urchin. Science (New York, N.Y **314:**952-956.

53. **Resnick, D., A. Pearson, and M. Krieger.** 1994. The SRCR superfamily: a family reminiscent of the Ig superfamily. Trends in biochemical sciences **19:**5-8.
54. **Robertson, A. J., J. Croce, S. Carbonneau, E. Voronina, E. Miranda, D. R. McClay, and J. A. Coffman.** 2006. The genomic underpinnings of apoptosis in Strongylocentrotus purpuratus. Developmental biology **300:**321-334.
55. **Rook, G. A., J. Steele, M. Ainsworth, and B. R. Champion.** 1986. Activation of macrophages to inhibit proliferation of Mycobacterium tuberculosis: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. Immunology **59:**333-338.
56. **Rovera, G., D. Santoli, and C. Damsky.** 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proceedings of the National Academy of Sciences of the United States of America **76:**2779-2783.
57. **Rupper, A., and J. Cardelli.** 2001. Regulation of phagocytosis and endo-phagosomal trafficking pathways in Dictyostelium discoideum. Biochimica et biophysica acta **1525:**205-216.
58. **Rupper, A., B. Grove, and J. Cardelli.** 2001. Rab7 regulates phagosome maturation in Dictyostelium. Journal of cell science **114:**2449-2460.
59. **Rupper, A., K. Lee, D. Knecht, and J. Cardelli.** 2001. Sequential activities of phosphoinositide 3-kinase, PKB/Aakt, and Rab7 during macropinosome formation in Dictyostelium. Molecular biology of the cell **12:**2813-2824.
60. **Rupper, A. C., J. M. Rodriguez-Paris, B. D. Grove, and J. A. Cardelli.** 2001. p110-related PI 3-kinases regulate phagosome-phagosome fusion and phagosomal pH through a PKB/Akt dependent pathway in Dictyostelium. Journal of cell science **114:**1283-1295.
61. **Saeij, J. P., R. J. Stet, B. J. de Vries, W. B. van Muiswinkel, and G. F. Wiegertjes.** 2003. Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Dev Comp Immunol **27:**29-41.
62. **Savan, R., S. Ravichandran, J. R. Collins, M. Sakai, and H. A. Young.** 2009. Structural conservation of interferon gamma among vertebrates. Cytokine & growth factor reviews **20:**115-124.
63. **Smith, V. J., and K. Soderhall.** 1983. Induction of degranulation and lysis of haemocytes in the freshwater crayfish, Astacus astacus by components of the prophenoloxidase activating system in vitro. Cell and tissue research **233:**295-303.
64. **Stuart, L. M., and R. A. Ezekowitz.** 2005. Phagocytosis: elegant complexity. Immunity **22:**539-550.
65. **Tafalla, C., R. Aranguren, C. J. Secombes, J. L. Castrillo, B. Novoa, and A. Figueras.** 2003. Molecular characterisation of sea bream (Sparus aurata) transforming growth factor beta1. Fish & shellfish immunology **14:**405-421.

66. **Tafalla, C., J. Gomez-Leon, B. Novoa, and A. Figueras.** 2003. Nitric oxide production by carpet shell clam (Ruditapes decussatus) hemocytes. Developmental and comparative immunology **27:**197-205.
67. **van den Berg, J. M., S. Weyer, J. J. Weening, D. Roos, and T. W. Kuijpers.** 2001. Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. Journal of leukocyte biology **69:**467-473.
68. **Wang, P. H., D. H. Wan, Z. H. Gu, X. X. Deng, S. P. Weng, X. Q. Yu, and J. G. He.** 2010. Litopenaeus vannamei tumor necrosis factor receptor-associated factor 6 (TRAF6) responds to Vibrio alginolyticus and white spot syndrome virus (WSSV) infection and activates antimicrobial peptide genes. Developmental and comparative immunology **35:**105-114.
69. **Zhao, C., H. Zhang, W. C. Wong, X. Sem, H. Han, S. M. Ong, Y. C. Tan, W. H. Yeap, C. S. Gan, K. Q. Ng, M. B. Koh, P. Kourilsky, S. K. Sze, and S. C. Wong.** 2009. Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. J Proteome Res **8:**4028-4038.
70. **Zhu, Y., S. Thangamani, B. Ho, and J. L. Ding.** 2005. The ancient origin of the complement system. The EMBO journal **24:**382-394.
71. **Ziegler-Heitbrock, L.** 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. Journal of leukocyte biology **81:**584-592.