The role of teleost neutrophils in the regulation of inflammation

by

Jeffrey John Havixbeck

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences

University of Alberta

Abstract

The inflammatory response is a complex biological process initiated following the recognition of noxious stimuli. It is composed of an elaborate cascade of both pro- and anti-inflammatory mediators, essential for effective defenses against infection, the removal of damaged cells, and the initiation of tissue repair processes. At the core of these responses is phagocytosis, an evolutionarily conserved mechanism critical to immune defense and the maintenance of homeostasis. Phagocytosis is initiated through the recognition of large particles (>1μM) by receptors on the cell surface, leading to actin polymerization, particle internalization, and a diverse array of downstream responses.

Neutrophils are essential effector cells that form the first line of defense against invading pathogens. The main objective of my thesis research was to characterize the functional roles of teleost neutrophils, where various mechanisms are involved in the induction of pro-inflammatory responses, the regulation of inflammation, and neutrophil-driven mechanisms of resolution.

Similar to mammalian phagocytes, I found that goldfish and lamprey phagocytes contribute to both pro-inflammatory and pro-resolving responses *ex vivo*. However, I found significant differences with regards to the level of responsiveness to zymosan and apoptotic cells. Interestingly, goldfish phagocytes displayed a reduced sensitivity to apoptotic cells, instead displaying a greater induction of antimicrobial respiratory burst responses during co-stimulation. Moreover, teleost phagocytes remained as central contributors to the resolution phase of inflammation, even though they showcased an improved ability to induce strong antimicrobial inflammatory responses.

When I began focusing on neutrophils, I found that the number of circulating neutrophils in teleost fish was far below their mammalian counterparts. Despite this, many other aspects of neutrophil biology and regulation remained the same. Within the hematopoietic tissue, I found that teleost neutrophils exist in a large storage pool, from which they rapidly exit, enter into circulation, and infiltrate the inflammatory site when called upon. It has previously been shown that both mammalian and teleost macrophages exhibit extensive roles in inflammatory control. Interestingly, I demonstrated that neutrophils alter their phenotype throughout the acute inflammatory response, and contribute to both the induction and the resolution of inflammation. Neutrophils isolated during the pro-inflammatory phase displayed elevated ROS responses and released inflammation-associated leukotriene B_4. In contrast, neutrophils isolated during pro-resolution displayed low levels of ROS and released lipoxin A_4. Notably, eicosanoid release by neutrophils played a role, at least in part, in regulating divergent macrophage responses, including inducing their uptake of apoptotic cells.

These observations were then applied to study the teleost inflammatory response following infection with the natural fish pathogens, *Aeromonas veronii* or *Mycobacterium fortuitum*. Both bacteria have been associated with significant outbreaks in aquaculture, often leading to mass die offs. I found that similar to the zymosan model, neutrophils rapidly migrated to the site of infection. In addition, both pathogens stimulated the production of ROS in teleost neutrophils. However, neutrophils were only capable of significantly killing *A. veronii*, unlike *M. fortuitum*, which required the total population of leukocytes for significant elimination. However, most interestingly, was the capacity of neutrophils to internalize dying macrophages previously incubated with *A.*

veronii, despite remaining unable to internalize other forms of apoptotic cells. Notably, following the uptake of dying macrophages, neutrophils remained viable and increased their production of ROS, remaining pro-inflammatory in nature.

Overall, the examination of the teleost inflammatory response mounted against pathogen mimics and natural bacterial infections provides additional insights into the complex mechanisms by which neutrophils operate within an inflammatory site and contribute to the induction and regulation of acute inflammatory responses.

Preface

This thesis is an original work by Jeffrey J. Havixbeck. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board as part of an NSERC Discovery Grant entitled "Comparative biology of phagocytic antimicrobial responses" awarded to Dr. Daniel R. Barreda, held from 2008-2013 and 2013-2018:

1. Comparative biology of fish phagocytic antimicrobial responses; Protocol #706.

Some of the research conducted in this thesis was part of collaborations with local and international groups, leading to publication. Details for each chapter are outlined below:

Chapter 1: A portion of this chapter has been previously published in: Havixbeck JJ and Barreda DR (2015) Neutrophil development, migration, and function in teleost fish. *Biology* 4(4): 715-734. I contributed the design of the review, figure design, and manuscript composition. D.R. Barreda was the supervisory author, and assisted in manuscript composition and editing.

Chapter 3: A version of this chapter has been previously published in: Havixbeck JJ, Rieger AM, Wong ME, Wilkie M, Barreda DR (2014) Evolutionary conservation of divergent pro-inflammatory and homeostatic responses in sea lamprey phagocytes. *PLoS ONE* 9(1):e86255. doi:10.1371/journal.pone.0086255. I was responsible for the

experimental design, figure design, data collection and analysis, and manuscript composition. AM Rieger and ME Wong assisted with data collection and manuscript editing. M Wilkie provided the sea lampreys and contributed to manuscript editing. DR Barreda was the supervisory author, assisted in experimental design, manuscript composition, and editing.

Chapter 4: A version of this chapter has been previously published in: Havixbeck JJ, Rieger AM, Wong ME, Hodgkinson JW, Barreda DR. (2016) Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish. *Journal of Leukocyte Biology* 99(2):241-52 (*Leading Edge Research Article)*. I was responsible for the experimental design, figure design, data collection and analysis, and manuscript composition. AM Rieger assisted with data collection, figure design, and manuscript editing. ME Wong and JW Hodgkinson assisted with data collection and manuscript editing. DR Barreda was the supervisory author and assisted with manuscript composition and editing.

Chapter 5: A version of this chapter has been previously published in: Havixbeck JJ, Rieger AM, Churchill LJ, Barreda DR. (2017) Neutrophils exert protection in early *Aeromonas veronii* infections through the clearance of both bacteria and dying macrophages. *Fish and Shellfish Immunology doi: 10.1016/j.fsi.2017.02.001* (Epub ahead of print). I was responsible for the experimental design, figure design, data collection and analysis, and manuscript composition. AM Rieger was involved in figure design, data collection and analysis, and manuscript composition and editing. LJ Churchill assisted

with data collection. DR Barreda was the supervisory author and assisted with manuscript composition and editing.

Chapter 7: A portion of this chapter has been previously published in: Havixbeck JJ and Barreda DR (2015) Neutrophil development, migration, and function in teleost fish. *Biology* 4(4): 715-734. I contributed the design of the review, figure design, and manuscript composition. D.R. Barreda was the supervisory author, and assisted in manuscript composition and editing.

Appendix I: A version of this chapter has been previously published in: Havixbeck JJ, Wong ME, More Bayona JA, Barreda DR. (2015) Multi-parametric analysis of phagocyte antimicrobial responses using imaging flow cytometry. *Journal of Immunological Methods* 423: 85-92. I was responsible for the experimental design, figure design, data collection and analysis, and manuscript composition. ME Wong and JA More Bayona assisted with data collection. DR Barreda was the supervisory author and assisted with manuscript composition and editing.

Acknowledgements

This journey has not been without its share of highs and lows. Overcoming the difficult times allowed me to truly appreciate the significance of my time here, all while embracing the many high points along the road. This has been a rewarding experience that would not have been possible without the support of many individuals. I cannot thank you enough for everything you have done for me over the past 5 years, and without you, I would not be where and who I am today.

First of, I would like to thank my supervisor Dr. Dan Barreda. Although we may have our differences, you provided me with opportunities and advice throughout my time in your lab, allowing me to grow as both a person and a scientist. We never had a shortage of new technology to play with, helping me pursue some of my craziest experimental ideas. On that note, thank you for funding those same ideas even when you may not have agreed with the approach. It has been a memorable 5 years working with you and in your lab.

To my Ph.D. committee members, Dr. Christine Szymanski and Dr. Mike (Miodrag) Belosevic, your guidance, comments, and suggestions were instrumental in shaping my research throughout the years. To Christine, I will never forget your excitement over my research project in my first committee meeting; it instantly put any nerves about my first committee meeting at ease. To Mike, you were always a short walk across the hall and willing to chat about any aspect of science. It may not have seemed like it at the time, but I do understand and greatly appreciate why you always challenged and pushed me to become a better scientist. I would also like to thank my candidacy: Dr. Edan Foley, Dr. Kathy Magor, and Dr. Keith Tierney, as well as my defense examiners:

Dr. Greg Goss, and Dr. Derek McKay for your challenging questions and stimulating discussions.

I'd like to thank my lab mates and friends; you are what made the entire experience one of a kind. It would not have been the same without you. A special thanks to Dr. Aja Rieger, Mike Wong, Juan More Bayona, Caitlin Thomson, Moira Kiemele, Jeffrey Konowalchuk, Dustin Lillico, and Jordan Hodgkinson- it has been a blast.

I have also had the opportunity to collaborate with a several scientists throughout the course of my degree- Dr. Beatriz Abós, Dr. Carolina Tafalla (INIA, Madrid, Spain) and Dr. Mike Belosevic (U of A) and his lab members (Jordan Hodgkinson, Dr. Barbara Katzenback, Dr. Leon Grayfer). I am truly thankful for having had the opportunity to work with you; you have broadened my research and understanding in other areas of research.

To my family, most importantly my parents- John and Sharon Havixbeck. You will always be the two I look up to most in life, your unending support and encouragement, especially during the difficult times helped me persevere and remain level-headed throughout my degree.

I would also like to thank the financial support I received from the Natural Sciences and Engineering Research Council of Canada (NSERC; CGS-M and Vanier Scholarship), the Department of Biological Sciences (Teaching assistantship and Queen Elizabeth II Scholarship), and the Faculty of Graduate Studies (Walter H Johns Graduate Scholarship and Presidents Doctoral Prize of Distinction).

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List of abbreviations

AA	arachidonic acid
AC	apoptotic cell
AMP	antimicrobial peptides
ANOVA	analysis of variance
AnxV	Annexin V
APC	allophycocyanin
A.v.	*Aeromonas veronii*
β2M	beta-2-microglobulin
Bfp	bundle forming protein
BPI	bactericidal permeability increasing protein
BrdU	bromolated deoxyuridine triphosphate nucleotides
CCM	cell-conditioned media
CD	cluster of differentiation
cDNA	complementary DNA
CE	carp erythrodermatitis
CFU	colony forming units
Chk	chemokinesis
CRH	cytokine receptor homologous
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor
DAMPs	damage-associated molecular patterns
DHR	dihydrorhodamine
DMEM	Dulbecco's modified Eagle media
DNA	deoxyribonucleic acid
dpf	days post fertilization
dpi	days post infection
dsRNA	double stranded ribonucleic acid
EPA	eicosapentanoic acid
eNOS	endothelial NOS
FACS	fluorescence activated cell sorter
FADD	Fas associated death domain
FBS	fetal calf serum
FITC	fluorescein isothiocyanate
FMLP	N-formylmethionine-leucyl-phenyalanine
G-CSF	granulocyte colony stimulating factor
G-CSFR	granulocyte-colony stimulating factor receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
GUD	goldfish ulcer disease
HBSS	Hank's balanced salt solution
HETE	hydroperoxyeicosatetraenoic
Hcp	haemolysin co-regulated protein
HPCs	hematopoietic precursor cells
hpf	hours post fertilization

hpi	hours post injection
Hk	heat-killed
HSCs	hematopoietic stem cells
HSP	heat shock protein
ICAM	intracellular adhesion molecule
ICM	intermediate cell mass
IFN	interferon
IFNG2	interferon-gamma receptor 2
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
Int	internalized
IRF	interferon regulatory factors
kDa	kilo-Dalton
LB	Luria-Bertani
LPS	lipopolysaccharide
LT	leukotriene
LTA4H	leukotriene A4 hydrolase
LTBP	latent TGF binding protein
LX	lipoxin
M-CSF	macrophage-colony stimulating factor
MAMTs	mycolic acid methyl transferases
MAPK	mitogen activated protein kinase
MDP	muramyl dipeptide
MGFL-15	modified goldfish Leibovitz-15
MHC	major histocompatibility complex
MPO	myloperoxidase
mRNA	messenger ribonucleic acid
MSHA	mannose-sensitive haemagglutinin
MTBC	*Mycobacterium* tuberculosis complex
NADP	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
NTM	non-tuberculosis *Mycobacterium*
n-Ph	non-phagocytic
OMP	outer membrane protein
PAF	platelet activating factor
PAMPs	pathogen-associated molecular patterns
PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDIM	phthiocerol dimycocerosates
PE	phycoerythrin

PECAM	platelet endothelial cell adhesion molecule
PG	prostaglandin
PGSL	P-selectin glycoprotein ligand
PI	propidium iodide
PKC	protein kinase C
PKM	primary kidney macrophages
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
Q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SB	surface bound
SOCS	suppressor of cytokine signaling
ssRNA	single stranded ribonucleic acid
TB	tuberculosis
T2SS	type II secretion system
T3SS	type III secretion system
T6SS	type VI secretion system
TACE	TNF-alpha converting enzyme
TF	transcription factor
TGF	transforming growth factor
TLRs	Toll-like receptors
TMS	tricaine methane sulfonate
TNF	tumor necrosis factor
TRADD	TNF receptor associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultraviolet
VCAM	vascular cell adhesion molecule
VF	virulence factor
Zym	zymosan

Chapter 1. Introduction and literature review[1]

1.1 Introduction

Driven by the continuous interactions of hosts and microbes, the immune system has evolved over many millennia to confer effective protection against infection. At the heart of the immune system lies the phagocyte, and since Elie Metchnikoff's seminal discovery of phagocytosis over 134 years ago, phagocytes have become widely embraced as pivotal players in host defense. Phagocytes are essential to both development and defense- participating in embryogenesis, organogenesis, tissue maintenance and homeostasis, nutrition, the recognition and clearance of pathogens, the initiation, regulation, and resolution of inflammation, and the activation of cells through the production and secretion of humoural factors and the presentation of antigens.

The multifaceted immune system requires complex cross-regulatory pathways consisting of divergent pro- and anti- inflammatory responses. At the core of these responses, phagocytosis continues to exhibit increasing roles as both an inducer and regulator of host immunity. These responses are integral to the clearance of pathogens, the resolution of inflammation, and the healing of wounds. In mammalian immunology, it is extensively known that phagocytes are capable of internalizing both pathogenic and homeostatic stimuli, leading to divergent responses. Following the internalization of pathogens, phagocytes are capable of producing a vast array of pro-inflammatory

[1] A portion of this chapter has been previously published in:

Havixbeck JJ and Barreda DR (2015) Neutrophil development, migration, and function in teleost fish. *Biology* 4(4): 715-734.

mediators. In addition, the internalization of pathogens leads to the production of pro-inflammatory antimicrobial products such as reactive oxygen and nitrogen species. Alternatively, the uptake of homeostatic stimuli, such as apoptotic cells, results in the generation of anti-inflammatory biomolecules involved in pro-resolution processes. Further, the uptake of homeostatic stimuli generally leads to decreased pro-inflammatory antimicrobial activities. While this has been studied extensively in macrophages at the mammalian and lower vertebrate levels, little is known about the roles and responses of teleost neutrophils.

Non-classical models have been particularly important in the discovery of novel immune processes. This is underlined by the discovery of phagocytosis in starfish larva and the phagocytic antimicrobial responses of the freshwater crustacean *Daphnia* (Elie Metchnikoff; 1908 Nobel Prize in Physiology or Medicine), and Toll receptors in Drosophila (Jules Hoffman, Ralph Steinman, and Bruce Beutler; 2011 Nobel Prize in Physiology or Medicine).

In this thesis, I begin by examining the divergent responses of phagocytes in early vertebrate fish, the sea lamprey (*Petromyzon marinus)*, and goldfish (*Carrasius auratus)*. Sea lampreys represent one of two living species within the jawless vertebrates, which allowed me to examine the responses of phagocytes at the lowest living level within the vertebrate lineage. I then employed a goldfish model system to characterize the roles of neutrophils in the induction, regulation, and resolution of inflammation following infection from aquatic pathogens.

1.2 Thesis objectives

The main objective of my thesis research was to characterize the functional roles of teleost neutrophils, where various responses are involved in the induction of pro-inflammatory responses, the regulation of inflammation, and the neutrophil-driven resolution of inflammation. I hypothesized that neutrophils play a significant role beyond the pro-inflammatory response, where they are involved in both pro-resolving and tissue regeneration responses. The specific aims of my research were (1) Examine the responses of sea lamprey and goldfish phagocytes to pathogenic versus homeostatic stimuli; (2) characterize the mobilization and effector responses of neutrophils in a self-resolving zymosan peritonitis model; and (3) investigate the role of neutrophils in host defense against two important aquatic pathogens, *Aeromonas veronii* and *Mycobacterium fortiutum*. These aims effectively allowed me to undertake a broad analysis of total phagocyte responses, followed by an in depth analysis of neutrophil responses in both a controlled (zymosan) and uncontrolled (bacterial infection) inflammatory environment.

1.2.1 Thesis outline

This thesis is composed of 7 chapters plus an appendix. The first chapter consists of an introduction and literature review focusing on neutrophils in inflammation. Specifically, chapter 1 will outline the significant steps of acute inflammation, from the mobilization of leukocytes to the resolution of inflammation. Chapter 2 provides detailed methodologies and materials used throughout this thesis. Chapter 3 examined the phagocytic responses of sea lamprey and goldfish phagocytes to pathogenic and homeostatic stimuli *ex vivo*. In chapter 4, I focus specifically on the recruitment,

mobilization, and functional roles of neutrophils in the induction and resolution of acute inflammation. Chapter 5 investigated the importance of teleost neutrophils to the control and clearance of an *Aeromonas veronii* infection, as well as the resolution of the resulting inflammation. In chapter 6, I investigated the responsibilities of teleost neutrophils during both the local and systemic portions of infection from *Mycobacterium fortuitum*. Chapter 7 is a discussion, providing context to my findings within the field of innate immunity and inflammation. This chapter also suggests future directions for this work and the relevance of my findings. Lastly, Appendix I outlines novel techniques using imaging flow cytometry.

1.3 Literature review: The role of teleost neutrophils in the induction, regulation, and resolution of acute inflammation

1.3.1 Introduction

Inflammation is classically described as a response to infection or injury. The acute inflammatory response is composed of an elaborate cascade of both pro- and anti-inflammatory mediators, with the balance between these mediators determining the outcome after injury. For a successful outcome, the inflammatory response must activate tissue resident cells, initiate the recruitment of leukocytes, and produce a series of soluble mediators. A successful response will ultimately include the resolution of inflammation, triggering events that lead to the clearance and uptake of inflammatory cells and wound healing.

Neutrophils are typically the first cells to arrive at an inflammatory site, and are now widely recognized as sophisticated cells that are critical to host defense and the

maintenance of homeostasis (Borregaard, 2010; Havixbeck et al., 2015; Kolaczkowska and Kubes, 2013; Sadik et al., 2011; Summers et al., 2010). In addition, concepts such as neutrophil plasticity are helping to define the range of phenotypic profiles available to cells in this group and the physiological conditions that contribute to their differentiation (Havixbeck et al., 2015; Kohli and Levy, 2009; Serhan, 2014; Serhan et al., 2008). The potent anti-microbial mechanisms elicited by these cells are a testament to their long-standing evolutionary contributions in host defense (Amulic et al., 2012; Brinkmann et al., 2004; Havixbeck et al., 2015; Rieger et al., 2012; Rieger and Barreda, 2011; Wright et al., 2010). In general, intracellular and extracellular antimicrobial defenses are induced by the presence of pro-inflammatory immune products or the internalization of pathogens or pathogen products, providing protection against diverse pathogens. Further, through their divergent effects on immune cells, the products of antimicrobial responses are essential in shaping protective immune responses and in resolving inflammation. In addition, several insights into their active roles in the control and resolution of inflammation prior to induction of apoptosis highlight their importance to the maintenance of host integrity (Esmann et al., 2010; Havixbeck et al., 2015; Yang et al., 2012).

1.3.2 The inflammatory site

1.3.2.1 Resident tissue macrophages

Tissues within the body contain a tissue resident macrophage population. These macrophages are heterogeneous from tissue to tissue, a necessity for developing niche-specific functions that are integral to tissue function and homeostasis. However, these

cells are best known for their role as immune patrols at the forefront of defense where they are uniquely situated and transcriptionally encoded to respond to any intruding pathogen or environmental challenge (Davies et al., 2013). Tissue resident macrophages recognize specific signals within the environment including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Matzinger, 2007, 2002, 1998; Mogensen, 2009; Tang et al., 2012). Major PAMPs include microbial DNA (e.g. unmethylated CpG motifs), double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and 5′-triphosphate RNA, as well as lipoproteins, surface glycoproteins, and membrane components [peptidoglycans, lipoteichoic acid, and lipopolysaccharide (LPS)], which are all derived from infectious pathogens. On the hand, DAMPs are cell-derived molecules that can initiate and perpetuate an immune response. Major DAMPs include HMGB1, heat-shock proteins (HSP) from exosomes, hyaluronic acid from the extracellular matrix, complement components (C3a, C4a, and C5a), ATP, RNA, and DNA. Both PAMPs and DAMPs are recognized by pattern recognition receptors such as Toll-like receptors (TLRs; summarized in Table 1.1) (Blasius and Beutler, 2010; Leulier and Lemaitre, 2008; Medzhitov, 2007; Mogensen, 2009; Takeuchi and Akira, 2010).

Macrophages are traditionally grouped into two functional classes, the M1/M2 paradigm (Martinez and Gordon, 2014). However, this should not be taken as a rigid functional dichotomy as macrophages can fall somewhere in between, reflecting unique phenotypes depending on the specific microenvironment and activating ligands (Martinez and Gordon, 2014). After the initial recognition of pathogen challenge, resident macrophages (along with out tissue resident cells, such as mast cells, dendritic cells, and

stromal cells) drive the influx of inflammatory leukocytes. Classically, the initial infiltrate often consists of primarily neutrophils, however, inflammatory monocytes also migrate as a source of inflammatory macrophages. The initiation of the inflammatory response is dependent on the specific pathogenic insult and its magnitude, allowing for an appropriately tailored immune response.

Table 1.1 Mammalian Toll-like receptors and their main ligands

TLR	PAMP	DAMP
TLR 1/2	Bacterial lipoproteins	
TLR 2	Lipoteichoic acid, peptidoglycan, bacterial lipoproteins, zymosan	
TLR 2/4		HSP-60, HSP-70
TLR 3	dsRNA	Self dsRNA
TLR 4	LPS	Hyaluronic acid, HMGB1
TLR 5	Flagellin	
TLR 2/6	Peptidoglycan	
TLR 7	ssRNA	Self ssRNA
TLR8	ssRNA	Self ssRNA
TLR9	Unmethylated CpG DNA	Self DNA, chromatin IgG complex
TLR 10/11/12	Profillin	

1.3.2.2 Soluble mediators of acute inflammation and resolution

1.3.2.2.1 Chemokines and other recruitment mediators

There are several important neutrophil-recruiting chemokines, lipid mediators, and proteins, including CXCL1, CXCL5, CXCL8, leukotriene B_4 (LTB_4), and the complement protein C5a (de Oliveira et al., 2015, 2013; Lin et al., 2006; Scapini et al., 2004; Schumacher et al., 1992). Chemokines in particular are separated into 4 main classes depending on the location of key cysteine residues involved in disulfide binding: C, CC, CXC, CX_3C (White et al., 2013). Lipid mediators represent a class of bioactive lipids produced by a vast array of cells, and often play roles outside of immunity and inducing chemotaxis. They are broadly broken down into prostaglandins, leukotrienes, lipoxins, resolvins, and protectins (Kohli and Levy, 2009; Levy et al., 2001; Serhan, 2004; Serhan et al., 2008; Serhan, 2014).

1.3.2.2.1.1 CXCL8 (IL-8)

CXCL8 was the first chemokine described. CXCL8 (alternatively known as IL-8) is a small chemokine that plays a critical role in the recruitment of human neutrophils to the site of inflammation (Kaur and Singh, 2013; Yoshimura et al., 1987; Yoshimura et al., 1987). It is generated via the cleavage of a 20 amino acid leader sequences from the 99 amino acid chemokine precursor (Clark-Lewis et al., 1991; Walz et al., 1987). The cleaved protein is processed extracellularly, which results in increasing activity (Schröder et al., 1987; Van Damme and Billiau, 1988; Walz et al., 1987). Among several other functions, including the induction of shape change, upregulation of adhesion molecules, transient increases in intracellular Ca^{2+} concentration, the formation of bioactive lipids,

and respiratory burst production (Baggiolini et al., 1989), CXCL8 is also responsible for guiding neutrophils through the tissue matrix until they reach the site of injury.

Several key papers examining the binding of CXCL8, have revealed that there are two receptors: CXCR1 (or IL-8RA) and CXCR2 (or IL-8RB) (Holmes et al., 1991; Moser et al., 1991; Murphy and Tiffany, 1991). Interestingly, CXCR1, the IL-8 selective receptor, is detectable in a variety of cells types, including lymphocytes and fibroblasts (Moser et al., 1993), while CXCR2 is restricted to myeloid cells (Moser et al., 1993), which explains it's chemotactic effect on other cell types. *In vivo*, the effects of CXCL8 are long lasting, and are likely caused by the resistance of inactivation and binding to matrix glycosaminoglycans (Baggiolini et al., 1995). In mammals, active CXCL8 is produced by endothelial and epithelial cells, keratinocytes, synovial cells, fibroblasts, chondrocytes, and even neutrophils (Baggiolini et al., 1995). Within these cell types, the induction of CXCL8 expression has been linked to IL-1 and tumor necrosis factor (TNF) (Baggiolini et al., 1995).

A diverse array of sequences similar to mammalian CXCL8 have been reported in teleost fish, including zebrafish (de Oliveira et al., 2013; Oehlers et al., 2010; Yang et al., 2012), carp (Li et al., 2013; van der Aa et al., 2012), medaka (van der Aa et al., 2010), Atlantic cod (Seppola et al., 2008), trout (Laing et al., 2002), and goldfish (Grayfer et al., 2010; Havixbeck et al., 2015; Rieger et al., 2013). We now know that the majority of these teleost sequences exhibit a functional homology equivalent to mammalian CXCL8. It has been shown that all teleost fish possess at least one form of CXCL8 (CXCL8-l1), while two distinct forms can be found in carp and zebrafish (CXCL8-l1 and CXCL8-12) (van der Aa et al., 2010). Of note, both forms appear to be functionally equivalent to

mammalian CXCL8 (van der Aa et al., 2010). In addition, a recent study in trout found a third isoform (termed CXCL8-L3), however further characterization is still needed in order to fully determine its function (Chen et al., 2013). Several studies using different teleost models have gone on to find increased levels of *cxcl8* mRNA expression at the site of inflammation (Havixbeck et al., 2015; de Oliveira et al., 2013; Oehlers et al., 2010; de Oliveira et al., 2015). In addition, recombinant CXCL8 molecules from both forms in carp have also been shown to induce chemotaxis in neutrophils at 200 ng/ml *in vitro* (van der Aa et al., 2010).

1.3.2.2.1.2 Leukotriene B_4 (LTB_4)

The leukotrienes are biologically active fatty acids which contain a conjugated triene moiety and were originally isolated from leukocytes. They are produced by the oxidation of the essential fatty acids arachadonic acid (AA) and eicosapentanoic acid (EPA), using the enzyme arachidonate-5-lipoxygenase (O'Byrne et al., 1997; Salmon and Higgs, 1987). More specifically, LTB_4 synthesis is catalysed by 5-lipoxygenase and leukotriene A_4 hydrolase and is increased by inflammatory mediators including complement fragments, endotoxin, TNF, and several interleukins. Further, the production of LTB_4 is often accompanied by the production of histamine and prostaglandins, which are also involved in inflammation.

The primary function of LTB_4 is to recruit neutrophils to areas of tissue damage and inflammation, however, it is also involved in promoting the production of inflammatory cytokines by various immune cells, bronchoconstriction, and increased vascular permeability (Dahlén et al., 1981). In addition, LTB_4 has been shown to induce the

formation of ROS (Havixbeck et al., 2015; Levy et al., 2001), and the release of lysosomal enzymes (Kumar et al., 2015) in neutrophils. Furthermore, it induces the production of IL-8 and LTB_4 in neutrophils, enhances C3b receptor expression on neutrophil surfaces, and primes neutrophil responses to the chemokine FMLP (Fletcher, 1986), which suggests the autocrine amplification of inflammation.

1.3.2.2.1.3 CCL-1

CCL-1 is a small glycoprotein secreted by activated T cells, involved in the recruitment of monocytes (Jun et al., 2011; Miller and Krangel, 1992). In addition, this chemokine has been shown to attract NK cells, immature B cells, and dendritic cells through the interaction with its cognate receptor CCR8 (Gombert et al., 2005; Miller and Krangel, 1992; Roos et al., 1997). In addition, the murine homolog, beta-chemokine TCA3, has been shown to play a role in the recruitment of neutrophils (Luo et al., 1994). CCL-1 has been linked to several diseases including type 1 diabetes (Cantor and Haskins, 2007), atopic dermatitis and psoriasis (Kim et al., 2012), and allergy based asthmas (Bishop and Lloyd, 2003; Montes-Vizuet et al., 2006).

Little work has been done to examine and characterize CLL-1 in fish. However, in goldfish, recombinant CSF-1 (Grayfer et al., 2009) and recombinant IFN-γ (Grayfer and Belosevic, 2009), as well as the presence of recombinant heat shock protein 70 from *Trypanosome carrasii* (Oladiran and Belosevic, 2009), induced the expression and production of CCL-1.

1.3.2.2.2 Pro-inflammatory cytokines and mediators

1.3.2.2.2.1 TNF-α

Tumor necrosis factor-alpha is a potent cytokine with a broad range of biological activities, most importantly in the initiation of the acute inflammatory response (Idriss and Naismith, 2000; Pfeffer, 2003). Several studies employing anti-TNF antibodies demonstrate that the host defense against pathogens is severely impaired in the absence of TNF (Havell, 1989; Nakane et al., 1988). Further, using gene targeting strategies in mice, TNFR1 was shown to be critical for survival following intracellular pathogen infections, including *Mycobacterium tuberculosis* (Flynn et al., 1995), *Mycobacterium avium* (Ehlers et al., 1999), and *Listeria monocytogenes* (Hauser et al., 1990). However, TNFR1 knockouts in mice appeared to have no impact on the antimicrobial defense systems involved in the generation of ROS ($p47^{phox}$/$gp91^{phox}$) and reactive nitrogen species (RNS) (Pfeffer, 2003).

TNF- α was originally discovered in the 1970's as a cytotoxic factor produced by macrophages, with the ability to induce tumor cell apoptosis (Carswell et al., 1975; Helson et al., 1975). However, endothelial cells, fibroblasts, lymphoid cells, and neurons have since demonstrated the capacity to produce TNF (Pfeffer, 2003). The release of large amounts of TNF has been observed following the stimulation with lipooligosaccharide, bacterial products, and IL-1 (Carswell et al., 1975; Helson et al., 1975). TNF is primarily produced as a 233 amino acid type II transmembrane protein arranged in stable homotrimers (Kriegler et al., 1988; Tang et al., 1996). The membrane-integrated form undergoes proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE, also called ADAM17) forming the soluble homotrimeric

cytokine (Black et al., 1997). Both versions are biologically active (Palladino et al., 2003; Pfeffer, 2003), with the soluble form of TNF binding one of two receptors, TNFR1 or TNFR2 (Pfeffer, 2003). TNFR1 is found in nearly all tissues, whereas TNFR2 is found in only cells of the immune system. Upon contact with their ligand, TNF receptors also form trimers, leading to a conformational change and the recruitment of TNF receptor associated death domain (TRADD). Following the binding of TRADD, three potential signaling cascades can be initiated: (1) the activation of NF-κB, (2) the activation of mitogen activated protein kinases (MAPK) pathways, or (3) the induction of death signaling (Chen and Goeddel, 2002; Wajant et al., 2003). The NF-κB pathway ultimately leads to the transcription of a vast array of mediators involved in cell survival, the inflammatory response, and anti-apoptotic factors (Baltimore, 2011; Smale, 2011). The MAPK pathway leads to the activation of JNK, which aids in cell differentiation and proliferation (Kaminska, 2009). Lastly, TRADD can interact with Fas associated protein with death domain (FADD), leading to a high concentration of effector caspases and eventually apoptosis (Gaur and Aggarwal, 2003).

This traditionally pro-inflammatory cytokine has extensive physiological roles. During an immune response, it is known to induce chemotaxis of neutrophils, monocytes, and macrophages (Ming et al., 1987), augment their phagocytic response (Klebanoff et al., 1986; van Strijp et al., 1991), and prime cells for the production of antimicrobial ROS and NOS (Ding et al., 1988; Meyer et al., 1988). Further, rTNF enhanced LTB_4 generation in human neutrophils (Meyer et al., 1988). However, some evidence indicates that at high concentrations, TNF-α lost its protective effects and also reverses the inductive effects of IFN-γ and GM-CSF (van den Berg et al., 2001).

As of now, TNF-α has been examined in several fish species, including rainbow trout (Laing et al., 2001), carp (Saeij et al., 2003), and goldfish (Grayfer et al., 2008a), catfish (Zou et al., 2003), and Japanese flounder (Hirono et al., 2000). Several functions of fish TNF-α have been reported, including the upregulation of IL-1β and CXCL8 expression, the rapid mobilization of phagocytes, increased phagocytosis, and the priming of antimicrobial ROS and NO responses (García-Castillo et al., 2004; Grayfer et al., 2008b; Zou et al., 2003).

1.3.2.2.2.2 IFN-γ

Interferon gamma is an important pleiotropic cytokine with pro-inflammatory and antiviral function (Schroder et al., 2004). It is a dimerized soluble cytokine and the only member of the type II class of interferons (Gray and Goeddel, 1982). The biologically active dimer is formed by the anti-parallel interlocking of two individual monomers (Gray and Goeddel, 1982). It was initially described as a soluble product secreted by human leukocytes stimulated with phytohemagglutinin (Wheelock, 1965), and by several others as a product of antigen-stimulated blood lymphocytes (Green et al., 1969). In both cases, the resulting supernatants were shown to inhibit the growth of vesicular stomatitis virus. IFN-γ is produced primarily by activated Th1 CD4+ cells (Mosmann and Coffman, 1989), CD8+ cells (Sad et al., 1995), and natural killer cells (Wherry et al., 1991). Interesting, apart from displaying modest antiviral properties, IFNγ is essential to host defense against several important obligate and facultative intracellular pathogens (Levy et al., 2001; Malmgaard, 2004; Samuel, 2001). Among others, IFN-γ gene knockout mice are incapable of controlling *Mycobacterium* (Cooper and Orme, 1993; Flynn et al., 1995;

Pearl et al., 2001) and *Leishmania* (Swihart et al., 1995; Wang et al., 1994) infections, which highlights the importance of this cytokine to host antimicrobial responses and immune defense beyond that of viral infections (Berton et al., 1986; Cassatella et al., 1990; Fertsch and Vogel, 1984; Martin et al., 1994).

IFN-γ initiates cellular responses through direct interactions with a heterodimeric receptor consisting of interferon gamma receptor 1 (IFNGR1) and interferon gamma receptor 2 (IFNGR2) (Bach et al., 1997), forming a signalling complex and subsequent signalling cascade. In unstimulated cells, the receptor subunits are not pre-associated with each other, but rather associate through their intracellular domains with inactive forms of specific Janus family kinases (Jak1 and Jak2). Binding of IFN-γ to IFNGR1 induces rapid dimerization of IFNGR1 chains, thus establishing a site recognized by the extracellular domain of IFNGR2 (Ihle and Kerr, 1995). The IFN-γ induced assembly of the receptor complex contains two IFNGR1 and two IFNGR2 subunits, which bring the intracellular domains of these proteins into close association, along with Jak1 and Jak2 (Ihle and Kerr, 1995). Jak1 and Jak2 are subsequently phosphorylated, activating the IFNGR1-associated signal transducer of activation-1 (Stat1) transcription factor (Darnell et al., 1994). This results in a Jak-Stat signalling cascade, ultimately ending in gene regulation through transcription factor (TF) recognition and binding of IFN-γ activated sequences in the promoter regions of specific target genes (Takaoka and Yanai, 2006). This signalling cascade is incredibly fast, and within 30 minutes of IFN-γ receptor binding and heterodimerization observable increases in the transcript levels of several interferon regulatory factors (IRFs) can be seen, which then modulate subsequent waves IFN-γ signaling cascades (Young and Hardy, 1995). In addition, IFN-γ is an important activator

of macrophages and inducer of class II major histocompatibility complex (MHC) molecule expression, eliciting immunostimulatory and immunomodulatory effects within the host immune response (DelaRosa et al., 2009; Krampera et al., 2006; Shankar and Titus, 1997).

IFN-γ was first identified in fish when supernatants from mitogen-simulated leukocytes in trout were demonstrated to possess macrophage activating factors similar to that of mammalian IFN-γ (Graham and Secombes, 1990, 1988). Further evidence was established when the downstream signalling molecules of mammalian IFN-γ (Stats) were also identified in fish (Rycyzyn et al., 1998). Since then, IFN-γ has been sequenced in several fish species (Arts et al., 2010; Grayfer and Belosevic, 2009; Zou et al., 2005). A functional study examined the effect of recombinant IFN-γ on goldfish macrophages and found it to increase ROS and NO responses, enhance phagocytosis, and increase the expression of pro-inflammatory cytokines (Grayfer and Belosevic, 2009). Another isoform of IFN-γ has been identified in goldfish, known as IFN-γrel (IFN-γrelated). Similar to IFN-γ, IFN-γrel induced a rapid ROS production, enhanced phagocytosis, increased nitrc oxide synthase (iNOS) expression, and NO production (Grayfer et al., 2010). In addition, like IFN-γ, IFN-γrel increased the gene expression of a number of pro-inflammaory cytokines including IL-1β and TNF-α (Grayfer et al., 2010).

1.3.2.2.2.3 IL-1β

Interleukin 1 beta, also known as 'leukocytic pyrogen' is a member of the IL-1 family, most well known in mammals for the induction of fever (Kozak et al., 1998). This

family is currently made up of 10 other cytokines, which are central to the induction and regulation of immune defense (Dinarello, 1994).

Myeloid cells (dendritic cells, macrophages, and monocytes) (Dinarello, 1988) are the primary producers of IL-1β. It is currently understood that the activation of myeloid cells by pathogens or pathogen derived factors (eg. PAMPs) induces the expression of pro-IL-1β. IL-1β is then produced when caspase 1 cleaves pro-IL-1β into its biologically active form (Contassot et al., 2012; Sahoo et al., 2011). The release of IL-1β is accomplished through multiple mechanisms, including cell lysis, the export through specific transporters, exocytosis, and vesicular budding (Eder, 2009). IL-1β mediates its biological effects by binding to its cognate receptor, IL-1R (Dinarello, 1996; Kuno and Matsushima, 1994). IL-1R is structurally related to TLRs and uses similar downstream signaling pathways to TNF including the activation of MAP kinases (Dunne and O'Neill, 2003; Kuno and Matsushima, 1994). Opposing the effects of IL-1β is IL-1 receptor agonist (IL-1Ra), which also binds IL-1R completely inhibiting IL-1β from binding and exerting its biological effects (Colotta et al., 1993).

IL-1β is a central mediator of inflammatory defense, involved in a variety of cellular and physiological effects including cell proliferation and differentiation, and apoptosis. It has been shown to exert effects on most cell types within the host, previously reviewed by Dinarello (Dinarello, 1996, 1988). Briefly, the known pro-inflammatory properties of IL-1β include proteoglycan release, the synthesis of lipid mediators such as prostaglandin E_2 (PGE_s), and the enhanced proliferation of fibroblasts. Further, IL-1β also induced the synthesis of thromboxane by neutrophils and monocytes, T and B cell chemotaxis, increased expression of leukocyte adherence receptors on

endothelial surfaces, and the synthesis of type I IFNs (Dinarello, 2011, 1996, 1994, 1988). Given the above wide range of target cells and effects, it is not surprising that IL-1β has been implicated in numerous pathological diseases including cardiac disease (Bujak and Frangogiannis, 2009), rheumatoid arthritis (Khan et al., 2009), neurodegenerative diseases (Griffin et al., 1998), and autoimmune and auto-inflammatory diseases (Masters et al., 2009).

Fish were first suspected of possessing an IL-1 homolog when a mammalian-derived IL-1 enhanced the proliferation of catfish T-lymphocytes in response to concanavalin A (ConA) (Hajjar et al., 1987), and carp macrophages and granulocytes (Verburg-van Kemenade et al., 1995) produced factors with similar properties to that of mammalian IL-1. Among other species of fish (Secombes et al., 1998; Sigel et al., 1986), IL-1β has been found in carp (Engelsma et al., 2003) and catfish (Ellsaesser and Clem, 1994). Similar to mammalian IL-1β, fish IL-1β is also central to inflammatory defense. It has been shown to increase resistance to *Aeromonas salmonicida* and *Aeromonas hydrophila* in carp and trout (Hong et al., 2003; Kono et al., 2002) and enhance the agglutination capacity of carp antibodies in response to infection with *A. hydrophila* (Yin and Kwang, 2000). In addition, a recombinant trout IL-1β enhanced the expression of MHCII β chain, IL-1β, and COX-2 genes in head kidney leukocytes (Hong et al., 2004). Functionally, recombinant IL-1β induced the proliferation of head kidney leukocytes and enhanced their phagocytic uptake of yeast particles (Hong et al., 2004).

1.3.2.2.2.4 G-CSF

Granulocyte colony-stimulating factor is a glycolipid that mediates the proliferation, differentiation, activation, and survival of neutrophils and their progenitors (Lieschke et al., 1994; Panopoulos and Watowich, 2008). The *gcsf* gene has been identified in human (Kanda et al., 1987), mouse (Nicola et al., 1983), cat (Dunham and Onions, 2001), porcine (Kulmburg et al., 1997), bovine (Heidari and Kehrli Jr., 2000), avian (Leutz et al., 1984), and fish species (Liongue et al., 2009). Functionally, G-CSF acts as both a cytokine and a hormone and is produced by a number of different cell types and tissues. Healthy individuals (humans) express low levels (~50-150 pg/mL) of G-CSF in the serum (Cheers et al., 1988; Kawakami et al., 1990; Watari et al., 1989), however, this can increase up to ~3000 pg/mL during infection (Cheers et al., 1988; Kawakami et al., 1990). The increased levels of G-CSF produced during infection are generally coupled with increases in hematopoietic growth factor activity in serum, which stimulates increased proliferation of granulocytic progenitor cells. This correlates with the receptor for G-CSF, which is expressed in both granulocytic precursors and mature neutrophils, however, higher receptor levels are detected at later stages of maturation (Nicola and Metcalf, 1985). Moreover, granulocytic progenitors respond to G-CSF by shortening their passage through the cell cycle, therefore dividing more frequently (Lord et al., 1991). The ability of the bone marrow neutrophil pool to respond to the physiologic demand of infection or pharmacological levels of G-CSF during clinical administration is termed 'emergency' granulopoiesis. In mammals, this response is necessary for a sustained output of circulating neutrophils during infection or G-CSF therapy (Dale, 1998; Lieschke et al., 1994; Zhan et al., 1998).

G-CSF acts through its cognate receptor G-CSFR. Structurally, it is similar to Type 1 cytokine receptors, containing the conserved cytokine receptor homologous (CRH) domain, an Ig-like domain, and three fibronectin type III-like domains in the extracellular region; a transmembrane region; and an intracellular region lacking intrinsic catalytic activity (Bazan, 1990; R Fukunaga et al., 1990; Rikiro Fukunaga et al., 1990; Tamada et al., 2006). Similar to other cytokine signaling pathways, G-CSFR signals through the Jak-Stat pathway (Nicholson et al., 1994; Tian et al., 1994). The ultimate outcome is the up- or down-regulation of genes involved in neutrophil proliferation, differentiation, and activation; however, the exact mechanism(s) of action remain mostly unknown. Furthermore, our current understanding of the specific role(s) of specific signaling pathways under 'emergency' and steady state conditions of granulopoiesis remains rather limited due to the lack of appropriate model systems to explore the plethora of *in vivo* functions.

Perhaps unsurprisingly, G-CSF also plays a role during infection, and displays various functions depending on the infection and its route. For example, following intraperitoneal infection with *L. monocytogenes*, G-CSF was required for maintaining total bone marrow cell numbers (Zhan et al., 1998). In mice deficient in G-CSF, Gregory and colleagues used an intratracheal injection to mimic the pulmonary infection in cystic fibrosis patients; G-CSFR was required for the systemic induction of circulating neutrophil levels, bacterial clearance from the lung, and the prevention of extensive pulmonary damage during infection (Gregory et al., 2007). In addition, neutrophil survival was impaired in the absence of G-CSFR, suggesting that G-CSF signals were important for controlling neutrophil survival at the site of infection (Gregory et al., 2007).

Teleost G-CSF gene (*gcsf*) was first identified in Japanese flounder, green-spotted puffer fish, and fugu (Santos et al., 2006). Two *gcsf* genes were identified in the green-spotted pufferfish and fugu, termed *gcsf-1* and *gcsf-2*, while only an orthologue of *gcsf-2* was identified in flounder (Santos et al., 2006). Further, two g*csf* genes were identified in the black rockfish (Nam et al., 2009) and in zebrafish (Liongue et al., 2009). In flounder, *gcsf-2* mRNA levels were upregulated in the kidney and in peripheral blood leukocytes following treatment with LPS or a mixture of ConA and phorbol-12-myristate-13-acetate (PMA) (Santos et al., 2006). Similarly, *gcsf-2* gene expression in black rockfish was ubiquitously expressed in the peripheral blood leukocytes and kidney (Nam et al., 2009). Currently, functional analyses of teleost G-CSF are quite limited. The major study used an *in vivo* morpholino mediated knockdown of *gcsfr* in zebrafish, which displayed a decrease in both cell numbers and migration of cells expressing both neutrophil and macrophage specific markers during both primitive and definitive hematopoiesis in the embryonic stage (Liongue et al., 2009). Further, injection of wild-type zebrafish with *gcsf* mRNA increased the number of myeloid and GCSF+ cells. Taken together, GCSF-1 may play an important role during myeloid cell development as well as inflammation, similar to that observed in mammalian systems.

1.3.2.2.3 Anti-inflammatory/ resolving cytokines and mediators

1.3.2.2.3.1 TGF-β

TGF-β is a multi-functional cytokine/ growth factor and part of the TGF-β super-family of more than 40 factors (Herpin et al., 2004). In mammals, there are 3 different isoforms (TGF-β1-3). While the three isoforms are structurally similar and do share

overlapping functions, they are expressed within distinct tissues by different cells (Govinden and Bhoola, 2003). However, of these different isoforms, TGF-β1 is the key immunomodulator (Letterio and Roberts, 1998). Secreted TGF-β complexes with latent TGF-β binding protein (LTBP), which is thought to maintain proper folding of TGF-β and target it to extracellular structures (Govinden and Bhoola, 2003). In addition, TGF-β binding to latency-associated protein (LAP) to form the 'small latency complex', which is necessary for TGF-β secretion, preventing early receptor engagement, and maintaining large extracellular reservoirs of the cytokine (Khalil, 1999). The mechanisms of dissociation of the latency complexes and subsequently activation of TGF-β are currently not well understood, however, it is believed to involve a series of proteolytic and non-proteolytic events and cellular interactions (Gleizes et al., 1997; Munger et al., 1997; Nunes et al., 1997). TGF-β binds and forms several serine/threonine kinase complexes that bind to its cognate TGF-β receptors and signal through the SMAD pathway (Bradshaw and Dennis, 2011; Derynck and Zhang, 2003; Massagué, 1998; 2001).

TGF-β displays a multitude of immunoregulatory roles depending on target cell type and activation/ state of differentiation of the cell, cytokine concentration, and environmental milieu. In T lymphocytes, TGF-β1 has been shown to precipitate the expression of Foxp3 and Treg differentiation (Gandhi et al., 2010). TGF-β has mainly inhibitory effects on B lymphocytes, including the inhibition of cell proliferation and the induction of apoptosis in immature or resting B cells (Arsura et al., 1996). It is also important in downregulating NK cell function and IFN-γ production (Bellone et al., 1995; Rook et al., 1986). TGF-β also exerts numerous effects on phagocytes. Interestingly, TGF-β has been shown to stimulate resting monocytes by functioning as a

chemoattractant and upregulating the expression of IL-6, IL-1β, and TNF- α (McCartney-Francis et al., 1990; Wahl et al., 1990; Wiseman et al., 1988). However, the stimulatory effects of TGF-β in monocytes appears to be highly context dependent (Wahl, 2007). In macrophages, TGF-β is thought to drive alternative activation/ anti-inflammatory phenotype (Murray et al., 2014), ablating ROS and NO production (Ding et al., 1988), and reducing its binding capacity for TNF- α and IFN- γ by decreasing receptor expression (Hausmann et al., 1994; Pinson et al., 1992). The result is a decreased activation state and reduction in antimicrobial responses in macrophages. In dendritic cells, TGF-β1 affects their differentiation and antigen-presenting ability, inhibits MHC II expression, and ablates IL-1β and TNF-α induced IL-12 production (Geissmann et al., 1999). Finally, TGF-β1 knockout mice display an excessive/ prolonged inflammatory response and extensive multiple organ infiltration by macrophages and lymphocytes, wherein they typically succumb to these conditions (Kulkarni et al., 1993; Kulkarni and Karlsson, 1993; Shull et al., 1992). Further, these mice also have a tendency to develop severe autoimmunity (Christ et al., 1994; Yaswen et al., 1996).

TGF-β1 was first identified in trout (Daniels and Secombes, 1999; Hardie et al., 1998; Sigel et al., 1986). Since then it has been identified is several other fish species including sea bream (Tafalla et al., 2003), carp (Zhan and Jimmy, 2000), tilapia (Harms et al., 2003), and goldfish (Haddad et al., 2008). In goldfish, recombinant TGF-β was shown to induce proliferation of a fibroblast cell line, CCL-71, in a dose dependant manner, and decrease NO responses in macrophages stimulated with rTNF-α (Haddad et al., 2008).

1.3.2.2.3.2 IL-10

Interleukin-10 is a central anti-inflammatory cytokine that regulates inflammation as well as other immune processes. Our understanding of this important immune mediator has grown through extensive studies employing IL-10 knockout mice (Kühn et al., 1993; Rennick et al., 1997; Rennick and Fort, 2000). Despite being born into pathogen free environments, these mice appear to develop severe enterocolitis (due to natural flora) 2-3 months into their lives. They also exhibit a polarized Th1 bias with excessive pro-inflammatory responses. Further, IL-10 is essential to preventing the pathological development of autoimmune diseases due to inflammation (Hawrylowicz and O'Garra, 2005; Moore et al., 2001; O'Garra et al., 2008). IL-10 is crucial to ameliorating the inflammatory-related pathology caused by IFN-γ and TNF-α during pathogenic infections (eg. *Mycobacterium, Trypanosoma, Plasmodium,* and viral infections) (Bekker et al., 1998; Grau et al., 1987; Magez et al., 2002; Robinson et al., 2007; Suvas et al., 2004; Wu et al., 2007). In fact, the absence of IL-10 has been shown to increase the severity of immunopathology in response to a broad spectrum of pathogenic infections (Gazzinelli et al., 1996; Hunter et al., 1997; Wilson et al., 2005). For this reason, and due to its potent effects during inflammatory responses, many pathogens focus on the induction of IL-10 to ablate the pro-inflammatory response and promote pathogen survival as an immune evasion strategy (Anderson et al., 2005; Belkaid et al., 2001; Roque et al., 2007). In general, the induced over expression of IL-10 invariably led to decreased killing and subsequent growth of infiltrating pathogens (Feng et al., 2002; Groux et al., 1999; Reed et al., 1994).

IL-10 was initially identified in the supernatants of T cells following stimulation with ConA (Fiorentino et al., 1989), where the supernatants inhibited the synthesis of pro-inflammatory cytokine production. In addition, B cells, epithelial cells, eosinophils, monocytes/ macrophages, NK cells, neutrophils, and tumor cells also produce IL-10 (Akbari et al., 2001; Ding et al., 2003; Rieger and Bar-Or, 2008; Siewe et al., 2006). To induce signaling, Il-10 forms a dimer and signals through a receptor complex consisting of two ligand binding IL-10R1 receptors and two accessory subunit IL-10R2 receptors ("Interleukin-10: new perspectives on an old cytokine - Mosser - 2008 - Immunological Reviews - Wiley Online Library," n.d.; Kotenko et al., 1997; Liu et al., 1994; Spencer et al., 1998; Tan et al., 1993), inducing a signaling cascade through the Jak-Stat pathway (Weber-Nordt et al., 1996). 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, with the inhibition of lymphocyte function believed to be a bystander effect (Ding and Shevach, 1992; Fiorentino et al., 1991a, 1991b; Oswald et al., 1992). In addition, to the abrogation of pro-inflammatory cytokine expression, IL-10 decreases macrophage ROS and, to a lower extent, NO responses (O'Farrell et al., 1998; Oswald et al., 1992).

Il-10 has been identified in several species of bony fish including carp (Savan et al., 2003), puffer fish (Jun Zou et al., 2003), trout (Inoue et al., 2005), sea bass (Pinto et al., 2007), cod (Seppola et al., 2008), zebrafish (Zhang et al., 2005), and goldfish (Grayfer et al., 2011). In goldfish, rIL-10 has been shown to decrease TNF-α1, TNF-α2, IL-1β1, IL-10, CXCL-8, and NADPH oxidase component p47 in monocytes activated with heat-killed *A. salmonicida* (Grayfer et al., 2011). In addition, recombinant IL-10

increased suppressor of cytokine signaling-3 (SOCS3) gene expression in monocytes (Grayfer et al., 2011), suggesting that similar to mammals, IL-10 may be a mechanism by which goldfish down-regulate pro-inflammatory immune responses.

1.3.2.2.3.3 Lipoxin A_4 (LXA_4)

Lipoxins are a family of bioactive lipids first identified by Serhan and colleagues (Serhan et al., 1984) from purified fractions of leukocyte suspensions co-incubated with the ionophore A-23187 and 15-hydroperoxyeicosatetraenoic acid. In general, lipoxins are endogenously produced, short-lived eicosanoids with a multitude of immunomodulatory and anti-inflammatory actions (Levy et al., 2001; Serhan et al., 2008). In contrast to the leukotrienes, lipoxins are believed to act as a 'braking' signal to the inflammatory response (McMahon et al., 2001; Serhan, 2002). There are three major routes of lipoxin formation, all of which depend on the cells and enzymes present therein, and can be subjected to modulation by cytokines (Gronert et al., 1998; Hachicha et al., 1999; Serhan et al., 1996; Sodin-Semrl et al., 2000). The first pathway is via platelet-polymorphonuclear neutrophil interactions (Edenius et al., 1988; Serhan and Sheppard, 1990). The second route of synthesis via the action of 15-lipoxygenase in epithelial cells and monocytes, whereby molecular oxygen is inserted into C20:4, resulting in hydroperoxyeicosatetraenoic (HETE) acid (Serhan et al., 1984). This can then serve as a substrate for neutrophil 15-lipoxygenase, yielding LXA_4. The final route of LXA_4 biosynthesis is via the aspirin- triggered 15-epi-lipoxin pathway (Clària and Serhan, 1995). In a cytokine-primed milieu, aspirin-induced acetylation of cylcooxygenase-2

promotes conversion of arachidonate to 15-HETE, which can then be used as a precursor for neutrophilic production of LXA_4.

LXA_4, as well as certain peptides, serve as high affinity ligands for the lipoxin A_4 receptor found on leukocytes, which in turn can regulate their function (Levy et al., 2001). The discovery of LXA_4 receptors can largely be attributed to the development of synthetic ligands of LXA4 (Clish et al., 1999; Schottelius et al., 2002; Serhan et al., 1995; Takano et al., 1997). In particular, LXA_4 plays a key role in the resolution of neutrophilic inflammation. In humans, subnanomolar concentrations of LXA_4 and LXB_4 have been shown to inhibit leukotriene-mediated interactions of neutrophils and endothelial cells (Fierro et al., 2003; Papayianni et al., 1996). In addition, LXA_4 can act as a signal for macrophages to begin the process of efferocytosis (McMahon and Godson, 2004; Mitchell et al., 2002a). During infection, LXA_4 was shown to increase survival of rats during sepsis by simultaneously reducing bacterial spread and systemic inflammatory responses (Walker et al., 2011).

Lipoxin formation has been observed in several fish leukocytes including Atlantic salmon (Rowley, 1991), rainbow trout (Sharp et al., 1992), zebrafish (Tobin and Ramakrishnan, 2013), and goldfish (Havixbeck et al., 2015). In goldfish, LXA_4 was shown to downregulate the production of ROS in zymosan-activated neutrophils isolated from the peritoneal cavity (Havixbeck et al., 2015). Further, the addition of LXA_4 to primary kidney macrophage (PKM) cultures simultaneously decreased ROS production, as well as induced the internalization of apoptotic neutrophils, in a dose dependent manner (Havixbeck et al., 2015).

1.3.3 The life of a neutrophil

Teleost neutrophils are terminally differentiated leukocytes that have evolved to protect the animal host and mount early and potent antimicrobial responses against invading pathogens. They are typically the first leukocytes recruited to an inflammatory site (Havixbeck et al., 2015) and are capable of eliminating pathogens through multiple complementary mechanisms. Upon activation, neutrophils become powerful killers, utilizing toxic intracellular granules (Flerova and Balabanova, 2013; Meseguer et al., 1994), the production of reactive oxygen species (ROS) (Filho, 2007; Katzenback and Belosevic, 2009; Rieger et al., 2012), and deploying neutrophil extracellular traps (NETs) (Palić et al., 2007a; Pijanowski et al., 2013). These characteristics are shared between teleost neutrophils and their mammalian counterparts (Borregaard, 2010; Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012; Nathan, 2006; Prokopowicz et al., 2012; Winterbourn and Kettle, 2012). However, recent data indicate that neutrophil functions extend beyond their historical role of simple pro-inflammatory foot soldiers, expanding our view for the life and death of this long-standing myeloid contributor. Unique features are also arising in different animal models, pointing to a shift in the contributions of this important cell across evolution. In all, neutrophils are critical to the effectiveness of early host antimicrobial responses, the co-ordination of subsequent adaptive mechanisms, the conservation of host integrity, and the maintenance of homeostasis.

1.3.3.1 Neutrophil development

Despite extensive (~400 million years) evolutionary divergence between teleost fish and mammals, the molecular pathways governing hematopoiesis have been highly

conserved. The presence of teleost kidney hematopoietic stem cells (HSCs) and hematopoietic precursor cells (HPCs) responsible for generating all blood lineages was originally demonstrated using transplantation studies in zebrafish and ginbuna crucian carp. Whole kidney marrow from *gata* ^{eGFP} zebrafish or *β-actin*^{eGFP} was transplanted into pre-thymic *gata*$^{-/-}$ zebrafish (Traver et al., 2003) or lethally irradiated zebrafish, respectively, rescuing the phenotype and producing both lymphoid and myeloid cell types. In addition, HSCs from ginbuna crucian carp renal tubules were capable of engraftment into lethally irradiated recipients, resulting in long-term production of all hematopoietic cells (Kobayashi et al., 2008). These studies add further support for the important contributions of the teleost kidney to myelopoiesis.

Successive waves of primitive and definitive hematopoiesis, occurring in anatomically distinct sites, are characteristic of vertebrate embryos (Galloway and Zon, 2003). In fish, myelopoiesis and the development of neutrophils have been studied primarily using the zebrafish model. Primitive hematopoiesis takes place intraembryonically in the intermediate cell mass (ICM) (Davidson and Zon, 2004). It is here that expression of the granulocyte-specific marker myeloperoxidase (*mpo*) in zebrafish is first detected at 18 hours post fertilization (hpf) (Davidson and Zon, 2004). The detection of *mpo*$^{+}$ in the ICM suggests this tissue is capable of producing a small population of myeloid progenitors. From here HSCs from the caudal hematopoietic tissues seed the major hematopoietic organ of teleost fish, the pronephros, followed by the mesonephros, which is functionally analogous to the mammalian bone marrow. It is in this tissue that the generation of neutrophils occurs from 2 dpf (Guyader et al., 2008; Murayama et al., 2006). However, it is currently unknown how long embryonic

granulocytes from the ICM persist or if they contribute to the granulocyte population that is found in the pronephros at 7 dpf (Willett et al., 1999). Over the next several weeks of larval development the hematopoeitic tissue expands, however, development remains restricted to the myeloerythroid cell lineages (Willett et al., 1999). In adult zebrafish, hematopoietic tissue is present throughout the kidney and all leukocyte lineages and their precursors are found as a heterogeneous population.

Studies in mice have confirmed that commitment of pluripotent HSCs to myeloid precursors to mature neutrophils is controlled by both the extracellular cues (growth factors, cytokines, etc) ("Hematopoietic cytokines, transcription factors and lineage commitment," 2002; Metcalf, 2008; Rieger et al., 2009) as well as intracellular transcription factors ("Hematopoietic cytokines, transcription factors and lineage commitment," 2002; Lenny et al., 1997; Rosmarin et al., 2005). In particular, one of the most important cytokines involved in granulopoiesis, the granulocyte-colony stimulating factor (G-CSF), is known to stimulate the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils. Interestingly, mice lacking G-CSFR or patients constitutively expressing hypomorphic G-CSFR mutants are neutropenic, but still possess mature granulocytes, indicating that both G-CSFR-dependent and G-CSFR-independent granulopoiesis occur in mammals (Hermans et al., 2003; Liu et al., 1996). More broadly, the G-CSF/G-CSFR pathway functions to maintain neutrophil viability and function within an inflammatory site (Villunger et al., 2003). A recent study confirmed both G-CSFR-dependent and G-CSFR-independent pathways are also present in zebrafish (Liongue et al., 2009). Morpholino-mediated knockdown of *gcsfr* and overexpression of *gcsf* in zebrafish discovered the presence of an anterior

population of myeloid cells during hematopoiesis dependent on the G-CSF/G-CSFR pathway for development and migration. However, a population of myeloid cells found within the posterior domain developed largely independent of this pathway (Liongue et al., 2009). Unlike G-CSF, however, GM-CSF and IL-3-like molecules have yet to be identified in fish, making further characterization of granulopoiesis difficult. Alternatively, it may point to differences in the soluble drivers for granulopoiesis in these lower vertebrates.

Hematopoietic stem cell commitment to the myeloid lineage is also heavily dependent of the intricate regulation of transcription factors (TFs). TFs are capable of acting independently, co-operatively, or antagonistically when determining lineage fate decisions. The stem cell leukemia (*scl*) gene has been extensively characterized in both mammals and zebrafish, where it plays a critical role in HSC formation (Robb et al., 1995; Shivdasani et al., 1995). In addition, naïve kidney neutrophils from goldfish expressed increased levels of *gcsfr, pu.1,* and *cepba* which are all known to be important in mammalian neutrophil development (Katzenback et al., 2016). However, much like the examination of teleost cytokines and growth factors involved in hematopoiesis, few studies have thoroughly examined the role of neutrophil TFs involved in granulopoiesis.

1.3.3.2 Neutrophil reservoirs

A major difference between many teleost species and mammals relates to the relative numbers of neutrophils in circulation. Whereas in mammals (e.g humans and swine) they represent the most prominent leukocyte in circulation (40-70%, 30-40% respectively), neutrophils constitute less than 5% of circulating leukocytes in several

bony fish (Havixbeck et al., 2015; Rey Vázquez and Guerrero, 2007). However, recent indications from our lab show a large reservoir of neutrophils in the teleost hematopoietic kidney, which can be readily deployed to sites of inflammation (Havixbeck et al., 2015; Katzenback and Belosevic, 2009; Scharsack et al., 2003; Woo et al., 2011). These neutrophils rapidly exit the hematopoietic compartment during the first 12 hours post zymosan administration in the peritoneal cavity (Havixbeck et al., 2015). This points to the relative importance of the hematopoietic storage pool of neutrophils for bony fish, likely representing the majority of the mature neutrophils that can traffic to a site of microbial challenge.

1.3.3.3 Neutrophil migration

Mature neutrophils must migrate across the sinusoidal endothelium that separates the hematopoietic tissue from the circulation. From there, they are rapidly recruited from the blood to sites of inflammation by chemotactic signals derived from the infectious agents (pathogen-associated molecular patterns) and the host (damage/danger-associated molecular patterns). Delineating exactly how individual neutrophils traffic from the bloodstream to tissues *in vivo* has been difficult as a result of the limitations of available mammalian models. Although there have been significant advances using transgenic mice harbouring fluorescent neutrophils, these models are technically complex and require intricate surgical procedures, as well as intravital microscopy (McDonald et al., 2010). Recently, the zebrafish has emerged as a powerful vertebrate model to study *in vivo* chemoattractant gradients (Niethammer et al., 2009) and neutrophil recruitment (Mathias et al., 2006; Renshaw et al., 2006) during inflammation. This organism is

amenable to *in vivo* manipulation with excellent optical clarity and good genetic approaches. In addition, the zebrafish shows remarkable conservation of its immune system at cell and molecular levels (Lieschke and Trede, 2009).

Teleost models have also provided us with the ability to examine the movement of neutrophils using whole animal studies. For example, using an *in vivo* zymosan peritonitis model in goldfish, our lab recently quantified the migration of neutrophils from the hematopoietic compartment to the site of inflammation (Havixbeck et al., 2015). The use of *in vivo* imaging in zebrafish has also been an invaluable tool in examining the migration of neutrophils during acute inflammation. *In vivo* studies using *cxcl8* zebrafish morphants displayed reduced neutrophil recruitment during acute inflammation (de Oliveira et al., 2013). In addition, Renshaw *et al.,* were able to examine fluorescent neutrophils migrating towards the site of injury following tail wounding in transgenic zebrafish embryos (Renshaw et al., 2006). Other studies have also taken advantage of this system to examine the movement of neutrophils during inflammation. For example, the movement of PI3K has been examined within individual neutrophils *in vivo* and showed that PI3K is critical for cell motility and must be present at the leading edge during migration (Yoo et al., 2010).

Mammalian models have been able to describe neutrophil adhesion and transmigration into the inflammatory site in more depth thus far. Adhesion and entry of neutrophils involves five sequential steps, tethering, rolling, adhesion, crawling, and transmigration. Neutrophils use P-selectin glycoprotein ligand-1 (PGSL-1) to rapidly form and break bonds with P-selectin and E-selectin on the blood vessel wall, which allows the neutrophil to 'tether and roll' at the appropriate site within the host (von

Vietinghoff and Ley, 2008). The endothelial cell walls within the blood vessel are also coated with chemokines, which are positively charged and bind to negatively charged heparin sulphates on the surface of the neutrophil (Wang et al., 2005). CD11a firmly binds intercellular adhesion molecule-1 (ICAM-1) allowing for tight neutrophil adhesion to the endothelial wall (Hentzen et al., 2000). This is one of the first steps involved in inducing neutrophil activation at the site of inflammation. Neutrophils can then crawl perpendicularly to the flow of blood through the continuous interactions of ICAM-1 and Mac-1 (Phillipson et al., 2006). At this point, it will take a neutrophil approximated 2-5 minutes to transmigrate into the affected tissue. Endothelial cells will undergo cytoskeletal changes, altering their attachment to the extracellular matrix, which will allow the neutrophil to pass through. We currently know this involves ICAMs, and platelet endothelial cell adhesion molecule-1 (PECAM-1), however, the exact mechanism of diapedesis remains unknown (Butcher, 1991; Kolaczkowska and Kubes, 2013; Phillipson et al., 2006; Williams et al., 2011). Further, the exact mechanisms of this process remain largely unclear in teleost fish.

Conventional thinking has assumed neutrophils are one directional, ending with death at the site of inflammation. This dogma has dominated the field of inflammation for many decades. However, a number of more recent studies have challenged this paradigm, suggesting that neutrophils, in some circumstances, can emigrate from damaged tissues by a process of reverse transmigration (Mathias et al., 2006). *In vivo* time-lapse imaging has shown that neutrophils are capable of retrograde chemotaxis back toward the vasculature, implicating this mechanism as a novel method of inflammatory resolution in zebrafish (Mathias et al., 2006). This mechanism appears to be conserved in the higher

vertebrates, where a specific subset of human neutrophils ($CD54^{high}/CXCR1^{low}$) represent long-lived neutrophils that have migrated though an endothelial monolayer and then re-emerged via reverse transmigration.

1.3.4 Antimicrobial defense mechanisms of teleost neutrophils

Neutrophils are a critical component in the first line of defense against invading pathogens. Multi-receptor recognition of PAMPs and DAMPs define intruding pathogens, resulting in the activation of cellular antimicrobial responses designed to kill infiltrating pathogens. Antimicrobial responses are tailored to the type and location of the pathogen, and can be divided into two main categories: intracellular and extracellular. Intracellular defense mechanisms are designed to provide protection against pathogens found within membrane-enclosed structures. These defenses are not limited to killing pathogens that have been internalized through phagocytosis, but also to provide protection against pathogens actively hiding from humoral immune defenses.

Neutrophils are armed with an extensive antimicrobial arsenal designed to limit the dissemination of a broad range of pathogens. Interestingly, many of the antimicrobial mechanisms present in teleost neutrophils are utilized both as intracellular and extracellular defenses. A key example is the robust production of reactive oxygen and nitrogen species found in fish neutrophils (Forlenza et al., 2008; Mayumi et al., 2008; Moritomo et al., 2003; Scharsack et al., 2003). Unlike the predominantly intracellular release of ROS and NOS in monocytes and macrophages, teleost neutrophils elicit robust responses both intracellularly and extracellularly (Forlenza et al., 2008; Leiro et al., 2001). Further, antimicrobial and cytotoxic substances stored in neutrophilic granules can

be released into the extracellular space (described below), or within the phagosome, where they exert potent antimicrobial actions (Cuesta et al., 2008; Faurschou and Borregaard, 2003; Mulero et al., 2008).

Defenses towards pathogens within the extracellular space provide a method by which the innate immune system can effectively clear pathogens that have escaped internalization or are too large to be internalized. These responses are generally activated by the presence of microbial products or inflammatory mediators and result in the release of antimicrobial factors into the extracellular space. These soluble products are efficient antimicrobial agents, but their mode of action is generally non-specific (Morel et al., 1991; Stuart and Ezekowitz, 2005). As a result, extracellular release of ROS and NOS also leads to collateral damage of host tissue (Forlenza et al., 2008; Leiro et al., 2001). Unlike the release of reactive oxygen and nitrogen species, products in neutrophilic granules are specifically targeted towards microorganisms and cause little damage to host tissues (Faurschou and Borregaard, 2003). Intracellular defenses effectively kill internalized pathogens following phagolysosome fusion, where a toxic degradative environment is established within neutrophils. Targeted production and release of these antimicrobial molecules into membrane-enclosed structures ensures reduced damage to host phagocytes and tissues, while maximizing the degradative potential to internalized pathogens (Thomas et al., 1988).

1.3.4.1 Intracellular mechanisms

1.3.4.1.1 Respiratory burst

The first indication of a respiratory burst was described in mammalian leukocytes in 1933 by Baldridge and Gerard, when it was noted that phagocytosis was associated with increased oxygen consumption (Baldridge and Gerard, 1933). Subsequently, it was found that this increase in oxygen consumption was the result of the formation of superoxide anions (Babior et al., 1973), in a process catalyzed by NADPH-oxidase (Briggs et al., 1975). Phylogenetic analysis of the NADPH-oxidase has been described in several teleost fish species, including rainbow trout (Boltaña et al., 2009), Japanese pufferfish (Inoue et al., 2004), carp (Mayumi et al., 2008), Atlantic salmon (Olavarría et al., 2010), zebrafish, and pufferfish (Kawahara et al., 2007; Kawahara and Lambeth, 2007), indicating homology and a common mammalian/teleost ancestor (Mayumi et al., 2008). Further, each lineage has evolved separately, leading to a cluster containing all fish NADPH components (Olavarría et al., 2010). Although fish and mammals share relatively low sequence homology, the functional domains remain highly homologous (Boltaña et al., 2009; Inoue et al., 2004; Mayumi et al., 2008; Olavarría et al., 2010). Importantly, fish NADPH-oxidase components have been shown to have similar modes of activation and functional activities (Inoue et al., 2004; Mayumi et al., 2008; Olavarría et al., 2010).

Several studies examining the induction of ROS production in mammalian neutrophils indicate an important role for inflammatory cytokines, including TNF-α (Nathan, 1989), IFN-α (Little et al., 1994), G-CSF, and GM-CSF (Nathan, 1989). In addition, neutrophil derived products [PAF and Leukotriene B_4 (LTB_4)] have been shown to induce NADPH activity (Dewald and Baggiolini, 1985). Although the impact of the

majority of these inflammatory molecules have not been examined directly on teleost neutrophils, they have been shown to induce respiratory burst responses in fish phagocytes (Arts et al., 2010; Grayfer et al., 2008a; Grayfer and Belosevic, 2009; Zou et al., 2005). Respiratory burst responses in fish neutrophils can also be strongly activated by PAMPs, including LPS, CpG DNA, flagellin, and muramyl dipeptide (MDP), which have been shown to induce respiratory burst activity in gilthead seabream (Sepulcre et al., 2011, 2007). Finally, several important fish pathogens, *Aeromonas salmonicida* and *Vibrio anguillarum,* have also been shown to induce potent respiratory burst responses in neutrophils (Chaves-Pozo et al., 2005; Katzenback and Belosevic, 2009; Kemenade et al., 1994).

Following stimulation with any of these factors, NADPH-oxidase activates through three sequential steps: (i) activation of PKC, (ii) phosphorylation of $p47^{phox}$, and (iii) the production of reactive oxygen intermediates by NADPH-oxidase (Olavarría et al., 2010). While activation pathways are conserved across teleost fish species, the kinetics and strength of the response showed differences across those species studied. For example, a study comparing the production of ROS from carp and ayu neutrophils found that ayu neutrophils spontaneously activated respiratory burst, however the response was not further enhanced by priming (Moritomo et al., 2003; Serada et al., 2005). In contrast, carp neutrophils displayed low levels of respiratory burst in resting cells that was enhanced in the presence of inflammatory stimuli (Moritomo et al., 2003; Serada et al., 2005). These observations may highlight a differential level of responsiveness across fish that is partially driven through the specific requirements and challenges that face each species within its chosen ecological niche. In light of the available results, it will be particularly

interesting to go beyond the intrinsic mechanisms that regulate these responses at the cellular level, and assess the differential crosstalk that exists between respiratory burst mechanisms and other antimicrobial host responses across distinct cellular niches. Further, we look forward to an increased understanding of the environmental factors that may contribute to the heterogeneity observed in these responses across teleost fish species.

1.3.4.1.2 Nitric oxide

Nitric oxide (NO) is essential to neuronal communications, inhibition of cell proliferation, vasodilation, and intracellular signaling (Clementi, 1998; Hopper and Garthwaite, 2006; Napoli et al., 2013; Paniagua et al., 2001). In addition, it has potent toxic effects and serves as a vital component of antimicrobial defenses. Nitric oxide is formed by the oxidation of l-arginine to l-citrulline by NO synthase (NOS) (MacMicking et al., 1997). Three forms of NOS have been identified in mammals- endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) (Knowles and Moncada, 1994). Of these, only the latter has been shown to be involved in immune defense. iNOS was first identified in the goldfish (Laing et al., 1996), with further characterization in rainbow trout (Wang et al., 2001) and carp (Saeij et al., 2000). The identified carp protein shares 57% similarity with human iNOS and contains putative binding sites for heme, tetrahydrobiopterin calmodulin, flavine mononucleotide, flavine adenine dinucleotide, and NADPH, all of which represent important sites in mammalian iNOS (Saeij et al., 2000). Further, iNOS activity has been demonstrated in teleost granulocytes, including neutrophils (Barroso et al., 2000; Saeij et al., 2000). Similar to mammalian iNOS, transcription appears to require

NF-κB (Saeij et al., 2000) following stimulation with cytokines or PAMPs. In addition, the parasitic haemoflagellate *Trypanoplasma borreli,* has also been shown to induce NO production in head kidney neutrophils of carp (Forlenza et al., 2008; Scharsack et al., 2003). Nitric oxide has been shown to have potent antimicrobial effects against a number of relevant fish pathogens (CamposPrez et al., 2000; F. Wiegertjes and Forlenza, 2010; Tafalla et al., 1999). However, little work has been done to characterize nitric oxide responses in teleost neutrophils, and their ability to use NO as a potent antimicrobial mediator.

Nitric oxide release, like the superoxide anions in respiratory burst responses, are not specifically targeted to microorganisms, thus leading to a potentially toxic environment for host tissues. Due to this, NO production must remain tightly regulated. The antioxidant glutathione has also been shown to play a protective role against nitrosative stress (Saeij et al., 2003). This was most prominent in carp phagocytes that naturally contain higher levels of glutathione compared to other peripheral blood leukocytes (Saeij et al., 2003). In addition, carp neutrophils have been shown to up-regulate genes involved in the glutathione redox cycle following LPS stimulation, resulting in further protection against the deleterious effects of NO products (Saeij et al., 2003).

1.3.4.1.3 Phagolysosome fusion

Phagocytosis triggers the activation of multiple transmembrane signaling pathways, leading to the reorganization of the actin cytoskeleton and the formation of a sealed intracellular vesicle — the phagosome (Kinchen and Ravichandran, 2008). It is now commonly accepted that the phagosome matures by numerous transient interactions

with internal vesicles, including lysosomes, to form a hybrid-like organelle termed the phagolysosome (Desjardins, 1995), with a primary function to degrade the internalized particle. The process of fusing is known to proceed through several tethering and docking steps (Stockinger et al., 2006). *In vitro* experiments have shown requirements for RAB5 (Jahraus et al., 1998), RAB7 and its effector RILP (Harrison et al., 2003), as well as filamentous actin (Kjeken et al., 2004).

Lysosomes contain roughly 50 different enzymes that break down various biological molecules including proteins, nucleic acids, lipids, and carbohydrates (Kornfeld and Mellman, 1989). In neutrophils, phagolysosome biogenesis is essential to kill ingested organisms, and like other cells it occurs through the fusion of newly formed phagosomes with other granules and or endosomes, resulting in the subsequent degradation of the internalized particle (Perskvist et al., 2002).

1.3.4.2 Extracellular mechanisms

1.3.4.2.1 Degranulation

As one of the first infiltrating cells into an inflammatory site, neutrophils are armed with a wide arsenal of intracellular and extracellular antimicrobial tools critical to the defense against pathogens. One of the primary extracellular defense mechanisms of neutrophils is the targeted degranulation of cytoplasmic granules containing preformed antimicrobial mediators (Faurschou and Borregaard, 2003). Granules isolated from mammalian neutrophils have been shown to contain a vast array of antimicrobial enzymes that include, myeloperoxidase, proteinase-3, cathepsin G and elastase, metalloproteinase, and acidic hydrolases, as well as antimicrobial peptides such as

lactoferrin and cathlicidin (Faurschou and Borregaard, 2003). Specifically, neutrophils have been shown to contain four types of granules: (1) azurophilic granules (sometimes call primary lysosomes), (2) specific granules, (3) gelatinase granules, and (4) secretory vesicles.

Azurophilic granules are packed with acidic hydrolases and antimicrobial proteins, and display great heterogeneity in size and shape. These granules undergo limited exocytosis following stimulation, however they are believed to be the primary granules involved in the degradation and killing of engulfed microorganisms in the phagolysosome (Joiner et al., 1989). Alpha-defensins are major constituents of azurophilic granules and have been estimated to make up ~5% of the total protein content of neutrophils (Faurschou and Borregaard, 2003). Another potent antimicrobial peptide found within these granules is bactericidal permeability increasing protein (BPI), which kills gram-negative bacteria at nanomolar concentrations (Elsbach, 1998; Weiss and Olsson, 1987), by binding to the negatively charged residues found within the lipopolysaccharide (LPS) of the outer membrane (Ooi et al., 1987).

Specific granules and gelatinase granules can be grouped together as peroxidase-negative granules (Kjeldsen et al., 1992), however, from a physiological view they are divided due to the significantly different protein content and secretory properties. Specific granules are large and rich in antibiotic substances, participating primarily in antimicrobial activities as the neutrophil mobilizes these granules to either the phagosome or the surrounding environment exterior to the cell (Jesaitis et al., 1990; Joiner et al., 1989; Mollinedo et al., 1997). The smaller gelatinase granules are more

easily exocytosed, and serve primarily as a reservoir of matrix-degrading enzymes and membrane receptors needed during diapedesis (Joiner et al., 1989).

The final group of granules is a group of endocytic vesicles known as secretory granules (Borregaard et al., 1987). These vesicles are mobilized in responses to a wide variety of stimuli, including N-formylmethionine-leucyl-phenyalanine (FMLP), granulocyte-macrophage stimulating factor (GM-CSF), IL-8, LTB_4, and TNF-alpha (Sengeløv et al., 1993), and contain a reservoir of membrane-associated receptors needed during the early phases of neutrophil-mediated immunity.

While granular contents of teleost neutrophils have not been as thoroughly described, an assay has been developed to quantitate myeloperoxidase degranulation in fish neutrophils (Palić et al., 2005). Using this assay, teleost neutrophils have been shown to degranulate following stimulation with various mitogens, zymosan, and *Aeromonas salmonicida* (Forlenza et al., 2008; Katzenback and Belosevic, 2009; Palić et al., 2005). Further, degranulation was not affected by the addition of cytochalasin B, indicating this potent mechanism does not always require prior phagocytic events. Functional studies and protein analysis have shown that teleost fish express homologues of proteinase-3, cathepsin G, elastase, and azurocidin, helping to further dissect the classical granule contents of teleost neutrophils (Wernersson et al., 2006). However, further examination must be done in order to confirm the expression of these enzymes at a protein level and their functionality, as well as to determine their relative contributions to the effector mechanisms mounted against invading pathogens.

1.3.4.2.2 Neutrophil extracellular traps (NETs)

Along with degranulation, mammalian neutrophils can also kill extracellular pathogens by NETs (Brinkmann et al., 2004). The ultrastructure of NETs consists of smooth filaments with a diameter of ~17 nm (Brinkmann et al., 2004), containing stacked and likely modified nucleosomes (Urban et al., 2009). This backbone is dotted with granular proteins forming globular domains with a diameter of ~50 nm (Brinkmann et al., 2004). NETs are capable of multiple mechanisms of action- binding microorganisms, degrading virulence factors, preventing dissemination, and killing bacteria by maintaining a high local concentration of antimicrobial granule components (Brinkmann et al., 2004). The production of NETs by teleost neutrophils has been recently described in carp, zebrafish, and fathead minnows (Palić et al., 2007a, 2007b, 2005; Pijanowski et al., 2013). Similarly to mammalian NETs, fish NETs are composed of neutrophil granule proteins associated with extracellular DNA fibers, but not cytoskeleton (Brinkmann et al., 2004; Palić et al., 2007b, 2007a). However, much remains to be learned about the contribution of different neutrophilic granule classes to the composition of teleost NETs, as well as the ability of these NETs to prevent pathogen spread and kill invading microorganisms.

1.3.4.2.3 Antimicrobial peptides

Antimicrobial peptides (AMPs), also referred to as cationic host defense peptides (Brown and Hancock, 2006) are an integral part of the innate immune. AMPs are potent broad spectrum antimicrobial proteins with targets on organisms ranging from viruses to parasites (Bahar and Ren, 2013). Thus far, more than 5,000 AMPs have been described in

both prokaryotes (bacteria) and eukaryotes (protozoan, fungi, plants, insects, and animals) (Conlon and Sonnevend, 2010; Leippe, 1999; Peters et al., 2010). In 1956 the first animal-originated AMP, defensin, was isolated from rabbit leukocytes (Hirsch, 1956). Shortly there after, human leukocytes were shown to contain AMPs in their lysosomes (Zeya and Spitznagel, 1963).

In teleost fish, a number of AMPs have been described in immune important tissues including the kidney, spleen, intestine, blood, and gills. However, the identification of AMP expression in azurophilic neutrophils (functionally equivalent to neutrophils) of gilthead seabream is of significant importance (Cuesta et al., 2008; Mulero et al., 2008). One such AMP, hepcidin, acts as an intestinal iron regulator, reducing iron availability for invading bacteria (Verga Falzacappa and Muckenthaler, 2005; Vyoral and Petrák, 2005). Another, piscidin, has been shown to have both potent antimicrobial activities against both gram-positive and gram-negative bacteria in striped bass (Lin et al., 2012). In addition, the expression of AMPs was upregulated following stimulation with various mitogens, PAMPs, or antigens both *in vivo* and *in vitro* (Mulero et al., 2008), suggesting that granulocyte activation increased the production of AMPs and likely antimicrobial killing potential. Interestingly, neither AMP was shown to be expressed in lymphocytes or monocytes/macrophages (Cuesta et al., 2008; Mulero et al., 2008). Though further research is needed, it appears that AMPs are critical to teleost immune defenses.

1.3.5 Neutrophils and the resolution of inflammation

Resolution of inflammation and the return of tissues to homeostasis are essential to host health and survival. Excessive inflammation is widely recognized as a major component in many chronic diseases, including vascular diseases, metabolic syndrome, and neurological diseases and is thus is a public health concern. Inflammation functions to neutralize and eliminate pathogenic invaders as well as to clear damaged tissue. Understanding endogenous control points within the inflammatory response could provide us with new perspectives on disease pathogenesis and treatment approaches.

Neutrophils have typically been viewed as necessary, but detrimental, short-lived cells that are recruited early in the inflammatory response. Once the pathogenic threat was eliminated, neutrophils were simply thought to undergo apoptosis, thus signaling resolution mechanisms to begin. However, recent work has altered our preconceived notions of neutrophilic contributions to inflammatory processes. In particular, significant evidence implicates a central role for neutrophils in triggering inflammatory resolution. Such mechanisms involve both metabolic and biochemical crosstalk pathways during the intimate interactions of neutrophils with other cell types at the site of inflammation.

1.3.5.1 Production of lipid mediators

The resolution of inflammation requires a reduction or removal of leukocytes and debris from inflamed sites and the initiation of wound healing mechanisms, enabling a return to homeostasis. It is now understood that uncontrolled inflammation is a unifying component in many diseases and recent evidence indicates that inflammatory resolution is a biosynthetically active process (Serhan et al., 2008) and not simply passive. These

new findings direct decision processes wherein acute inflammation, chronic inflammation, or inflammatory resolution outcomes are governed based on the endogenous mechanisms employed to control the magnitude and duration of the response (Serhan, 2014; Serhan and Savill, 2005). Following an immune challenge, the resolution process is rapidly initiated by cellular pathways that actively biosynthesize local specialized dual-acting anti-inflammatory and pro-resolution lipid mediators, such as lipoxins, resolvins and protectins (Serhan et al., 2008).

In mammals, an active switch of the lipid mediators released into the exudate accompanies the resolution of inflammation. Initially pro-induction mediators are generated, such as leukotrienes (LT) and prostaglandins (PG), which activate and amplify the cardinal signs of inflammation. Next, two major prostaglandins, PGE_2 and PGD_2 gradually induce key enzymes involved in the production of mediators with both anti-inflammatory and pro-resolving function, such as the lipoxins (Levy et al., 2001), resolvins and protectins (Hong et al., 2003; Serhan et al., 2000). These families of endogenous pro-resolution molecules activate specific mechanisms to promote homeostasis by stimulating and accelerating resolution at the tissue level. Specific lipoxins and members of the resolvin and protectin families are potent stimuli that selectively stop neutrophil infiltration; stimulate nonphlogistic recruitment of monocytes, induce macrophage efferocytosis, increase lymphatic removal of phagocytes, and stimulate expression of antimicrobial defense mechanisms (Schwab et al., 2007; Canny et al., 2002; Serhan et al., 2002).

Mammalian research has now shown that neutrophils alter their phenotype to produce different lipid mediator profiles depending on the environmental milieu (Levy et al.,

2001; Serhan et al., 2000). For example, neutrophils found in exudates during inflammatory resolution switch from the production of leukotrienes to lipoxins and resolvins, whereas those in peripheral blood generate and release leukotriene B_4 upon activation (Levy et al., 2001). Interestingly, we have also shown this to be the case in teleost neutrophils. Neutrophils, isolated during the induction of inflammation were found to produce elevated levels of the pro-inflammatory and chemotactic lipid, LTB_4, whereas neutrophils isolated during the initiation of resolution displayed increased levels of the pro-resolving lipid LXA_4 (Havixbeck et al., 2015). We showed that LTB_4 increased ROS production in both naïve and pro-inflammatory neutrophils, while LXA_4 increased the uptake of apoptotic neutrophils by macrophages (Havixbeck et al., 2015). In another study, PGD_2 and its metabolites contribute to the resolution of inflammation in the gilthead seabream (Gómez-Abellán et al., 2015).

1.3.5.2 Death of a neutrophil

Neutrophils are central to the clearance of pathogens. However, despite indications for neutrophil reverse transmigration, the timely entry of neutrophils into apoptosis remains critical for the resolution of inflammation (Erwig and Henson, 2007; Fadok et al., 1998a; Fox et al., 2010a; Haslett et al., 1994). Apoptosis renders neutrophils unresponsive to extracellular stimuli and leads to the expression of molecules signaling their removal by scavenger macrophages (Savill et al., 1990; Savill and Haslett, 1995). The life and death of neutrophils can be profoundly influenced by signals from the inflammatory milieu (Luo and Loison, 2008; Simon, 2003). Pro-inflammatory mediators,

including granulocyte macrophage colony stimulating factor (GM-CSF), IL-8, or bacterial components (LPS, bacterial DNA-CpG motifs, etc) could markedly prolong the longevity of neutrophils, whereas pro-apoptotic stimuli, such as tumor necrosis factor alpha (TNF-α), TNF-related apoptosis-inducing ligand (TRAIL), or Fas ligand can shorten their life span (Colotta et al., 1992; Lee et al., 1993; Renshaw et al., 2003). Precise control of the neutrophil death program provides an essential balance between their defense functions, appropriate apoptotic clearance, and tissue homeostasis.

A complex network of intracellular death/survival signaling pathways regulates entry into apoptosis ultimately determining the fate of neutrophils. Extensive reviews have already been written regarding the mechanisms of neutrophil apoptosis (El Kebir and Filep, 2010; Fox et al., 2010b; Luo and Loison, 2008). Briefly, intrinsic mechanisms are mediated through mitochondrial pathways and likely involve ROS production, although the mechanism of ROS generation is not well understood in aging neutrophils. The extrinsic pathway triggers apoptosis following ligation of cell surface death receptors (TNF-α, TRAIL receptors or Fas) to form the death-inducing signaling complex. Further, Fas signaling overrides the anti-apoptotic effects of GM-CSF (Daigle et al., 2002).

Neutrophil apoptosis is another critical point in the resolution of inflammation, where many cell types including 'professional' phagocytes such as macrophages can accomplish the clearance of apoptotic corpses. The process of clearing apoptotic cells can be broken down into a succession of steps, from the recognition of "eat-me signals" on dying cells, internalization and processing of the apoptotic cell, to the downstream consequences following engulfment. Apoptotic cell uptake stimulates the production of

lipoxin A_4, further enhancing the uptake of apoptotic cells (Mitchell et al., 2002b) and, in conjunction with resolvins and protectins, dominates the resolution phase of the inflammatory response (Serhan and Savill, 2005). In addition to autocrine and paracrine effects mediated through cytokines and lipid mediators, *in vitro* and *in vivo* studies have shown that the clearance of apoptotic cells within an inflammatory site decreases the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-17, IL-23, as well as the lipid mediators PGE_2 and LTC_4, promoting the production of anti-inflammatory immune mediators, notably IL-10 and transforming growth factor beta (TGF-β) (Fadok et al., 1998b; Maderna and Godson, 2003; Stark et al., 2005; Voll et al., 1997). Further, the shift in the balance from TNF-α to TGF-β contributes to the quenching of reactive oxygen and nitrogen species (Serinkan et al., 2005). Although, the downstream events following the engulfment of apoptotic neutrophils by macrophages in teleost fish have not been examined to the same extent, several important studies have assessed the functional outcomes of this process. For example, we have found that neutrophils incubated with apoptotic cells are able to down-regulate the generation of ROS in macrophages (Havixbeck et al., 2015). In addition, macrophages that have internalized apoptotic neutrophils are capable of down-regulating ROS production in all phagocytes (Rieger et al., 2012). Interestingly, similarly to mammalian studies, we found lipoxin A_4 induced an increase in apoptotic cell uptake by macrophages (Havixbeck et al., 2015).

1.3.6 *Aeromonas* spp.

1.3.6.1 *Aeromonas*- an overview of the genus

Aeromonads are inhabitants of aquatic environments and able to cause disease in a range of species, but are primarily associated with infections in aquatic poikilothermic animals. They are a genus of Gram-negative, facultative anaeroebic, rod-shaped bacteria (Graf, 2015), currently with 25 described species. The genus is taxonomically complex, however, it is divided into two main groups. The first group consists of non-motile psychrophilic aeromonads, with optimal growth temperatures of 22-28 °C (Beaz-Hidalgo and Figueras, 2013). This group is highlighted by *A. salmonicida*. The second, and much larger group, houses the motile, mesophilic aeromonads, with optimal growth temperatures of 30-37 °C (Beaz-Hidalgo and Figueras, 2013). This group is highlighted by *A. hydrophila.*

1.3.6.2 Virulence factors in *Aeromonas*

1.3.6.2.1 Lipopolysaccharide (LPS) and capsule

Similar to other Gram-negative bacteria, the exterior leaflet of the outer membrane is composed of LPS. LPS is divided into three domains, the extremely variable O-antigen, the core oligosaccharide, and the toxic lipid A component (Tomas et al., 2012). Originally, 44 O-antigen serotypes were found within 307 strains of *Aeromonas* (Sakazaki and Shimada, 1984), however, since then an additional 52 serotypes have been reported (Thomas et al., 1990). Initial electrophoretic studies on *Aeromonas* LPS demonstrated three main patterns. The first is a major ladder-like pattern often observed in enterobacteria and is associated with smooth LPS. The second is

characterised by the presence of some O-antigen chains demonstrating a homogeneous pattern, and the third a rough pattern in which no O-antigen is present (Khashe et al., 1996; Kokka et al., 1990). Interestingly, smooth LPS is primarily prevalent at 20 °C and higher osmolarity, whereas the rough LPS is more common at 37 °C and lower osmolarity (Tso and Dooley, 1995). The thermoregulation of LPS has been linked to colonisation, where smooth LPS has been associated with adherence to HEp-2 cells by *A. hydrophila* (Merino et al., 1992). When strains of *A. hydrophila* were grown at 20 °C, the presence of their smooth LPS was correlated with higher virulence in fish and mice (Merino et al., 1992). Further, smooth LPS has been implicated to promote adhesion of *A. veronii* bv. *sobria* to HEp-2 cells (Nandapalan and Chang, 1989).

In bacteria, the capsule is usually a hydrated polysaccharide structure covering the outer layer of the cell wall. It is generally composed of monosaccharides linked together via glycosidic bonds (Merino and Tomás, 2015). Due to its environmental exposure, capsules are thought to have roles in virulence and colonisation, prevention of bacterial desiccation, foiling phagocytosis by host cells, or reducing complement-mediated lysis (Merino and Tomás, 2015; Schembri et al., 2004). Although aeromonads have traditionally been described as non-capsulate, capsules have been identified in both non-motile and motile species, with the first capsule described in *A. salmonicida* when grown *in vitro* in glucose-rich media (Garrote et al., 1992), and *in vivo* in Atlantic salmon (Garduno and Kay, 1995). Since then, capsule formation has also been described in the mesophilic *A. hydrophila* (Martínez et al., 1995) when grown in glucose rich media *in vitro*. Capsules are produced in response to specific environmental conditions, for example, environments with high glucose concentration (Reckseidler-Zenteno, 2012).

Thus capsular formation is likely to have a role in the virulence of *Aeromonas* species capable of causing septicaemia, with capsule expression being induced upon entry into the host blood stream where the environment is glucose rich.

1.3.6.2.2 Pili, outer membrane proteins, and S-Layer

Pili are filamentous, extracellular structures that allow for bacteria adherence. Adhesion and colonization of host tissues are critical to *Aeromonad* virulence (Hadi et al., 2012). A variety of pili have been described for *Aeromonas* spp., with initial studies defining two specific groups based on structural differences- short-rigid pili and long-wavy pili (Carrello et al., 1988; Ho et al., 1990). Interestingly, environmentally isolated *Aeromonas* strains displayed a preference for short-rigid pili, while those isolated from clinical samples generally produced long-wavy pili (Carrello et al., 1988), suggesting long-wavy pili are important in *Aeromonad* virulence. Further evidence was provided when the mechanical removal of long-wavy pili from the bacterial surface, or pre-exposure to an anti-pilin antibody, was shown to block *Aeromonad* adherence to a variety of both human and animal cell lines (Barnett and Kirov, 1999; Hokama et al., 1990; Honma and Nakasone, 1990; Kirov et al., 1995; Nakasone et al., 1996). Notably, some long-wavy pili are capable of forming filamentous networks or bundles, and it is these bundle forming pili (Bfp) that are truly major colonization factors in mesophilic *Aeromonads* (Kirov et al., 1995). The first Bfp was isolated in *A. veronii* biovar sobria (Kirov and Sanderson, 1996). When examined, the N-terminal sequence of the major pilin found in the Bfp demonstrated high sequence homology with mannose-sensitive haemagglutinin (MSHA) pilin from *Vibrio cholerae* (Kirov and Sanderson, 1996).

Several years later, with the advent of new genetic techniques, this Bfp was subsequently shown to be a member of the MSHA pilus family (Hadi et al., 2012). Interestingly, *A. veronii* biovar sobria displayed optimal Bfp expression at temperatures below 22 °C in liquid media (Kirov et al., 1995), which may explain the efficiency of this species when infecting fish. Two other pili have been identified in *Aeromonas*, the Tap pilus and the Flp pilus, however deletion studies demonstrated that the lack of these pilin genes had no effect on host colonization and virulence in Atlantic salmon (Boyd et al., 2008; Kirov et al., 2000).

A number of outer-membrane proteins (OMPs) have been identified in Aeromonads as monomeric adhesins. Early studies in *A. hydrophila* demonstrated that upon pili removal, some strains retained their ability to adhere (Atkinson and Trust, 1980). Eventually carbohydrate-reactive proteins were found on the bacterial surface, and when down-regulated, a decrease in adherence was observed (Quinn et al., 1994). Many more OMPs have since been discovered, with variation in surface expression seen during changes in environment, temperature, osmolarity, and iron availability. The most important OMP, Omp48, was originally identified in *A. veronii* biovar sobria (Torres et al., 2005). Omp48 binds proteins of the extracellular matrix (collagen and fibronectin), as well as several mucus glycoproteins (lactoferrin and mucin). Further, *A. veronii* incubated with anti-Omp48 showed a decreased ability to bind HeLa cells (Torres et al., 2005), confirming its importance to the host colonization process. Interestingly, Omp48 may also represent a potential candidate for vaccine development, as recombinant *A. hydrophila* Omp48 was recently shown to induce a protective response in rohu (Khushiramani et al., 2012).

Surface layers or S-layers consist of a two dimensional crystalline protein array covering the entire organism (Beveridge and Graham, 1991). S-layers are composed of proteins and glycoproteins and are found in Gram-positive and Gram-negative bacteria, as well as *Archaea*. In *Aeromonas,* a unique protein, VapA, forms the S-layer. It was first described in *A. salmonicida* and was recognized as a major virulence factor causing septicaemia in fish (Kay et al., 1981). Although less common in mesophilic species, an S-layer has been found in both *A. veronii* and *A. hydrophila* (Esteve et al., 2004; Kokka et al., 1990). However, the role in pathogenicity for these species still remains clouded.

1.3.6.2.3 Secreted factors

Aeromonads secrete/ inject a plethora of virulence factors involved in both host colonization, as well as further invasion to stabilize infection. Thus far, five secretion systems have been identified in *Aeromonas* spp., Type I, II, III, IV, and VI) (Burr et al., 2002; Li et al., 2011; Rangrez et al., 2006; Seshadri et al., 2006; Suarez et al., 2008). Of these, the type 3 and type 6 secretion systems (T3SS/T6SS), are most associated with virulence and disease (Chacón et al., 2004; Suarez et al., 2008). T3SS are often referred to as needle-like structures and described as an 'injectisome', that directly inject effector molecules into the cytoplasm of target cells (Chacón et al., 2004; Vilches et al., 2004). Of note, loss of a functional T3SS in *A. hydrophila* leads to attenuated virulence during infection in both fish and mice (Vilches et al., 2012). To date, five main virulence factors (VF) have been described for the type III secretion system in *Aeromonas*: AexT, AexU, AopP, AopH, and AopO. AexT and AexU are multi-functional toxins that contain a GTPase-activating domain capable of disrupting host cytoskeletal assembly and

movement, leading to the induction of apoptosis (Silver and Graf, 2009; Vilches et al., 2009). To support the role of these VFs in pathogenicity, Braun and colleagues found that an *aexT A. salmonicida* mutant had no toxic effect on fish RTG-2 cells (Braun et al., 2002). Further, mice infected with an *aexU A. hydrophila* mutant displayed increased survival rates compared to those infected with wild-type bacteria (Sha et al., 2007a; Sierra et al., 2007). Several strains of *A. salmonicida* have been shown to secrete AopP (NF-κB inhibitor), AopH (tyrosine phosphatase), and AopO (serine/threonine kinase), which are all involved in disrupting host cell signaling pathways (Dacanay et al., 2006; Reith et al., 2008). The T6SS has also been shown to play a role in pathogenicity by injecting VFs directly into the target cell cytoplasm (Suarez et al., 2010), secreting haemolysin co-regulated protein (Hcp) (Suarez et al., 2008), Vrg1, Vrg2, and Vrg3 (Suarez et al., 2010, 2008). Hcp has been implicated in modifying phagocytic uptake of this bacterium, however the mechanism of action remains unknown (Suarez et al., 2008). Although Vrg2 and Vrg3 currently have unknown functions, Vrg1 has been identified as an ADP-ribosylating toxin, which disrupts the host cell cytoskeleton and induces apoptosis in HeLa cells (Suarez et al., 2010, 2008).

1.3.6.3 Identification of *Aeromonas*

There are many agents capable of ichthyopathology and the accurate and rapid identification is essential in establishing the correct course of treatment. Outbreaks of *Aeromonas* can spread rapidly, requiring swift and consistent identification in order to minimize the impact of infection. Typically, a bacterium is evaluated on 63 phenotypic and biochemical properties to identify the particular species. Interestingly, of the 63 tests,

only 9 yielded uniform results when typing *Aeromonas* (Abbott et al., 2003). These included, the presence of cytochrome oxidase and nitrate reductase, fermentation of D-glucose and trehalose, failure to utilize mucate, and the inability to generate acid from D-arabitol, dulcitol, erythritol, and xylose (Abbott et al., 2003). For a complete view of all biochemical and phenotypic tests, please reference Abbott and colleagues (Abbott et al., 2003). These phenotypic and biochemical characteristics allowed for the discrimination between three main groups: 1) the 'Aeromonas hydrophila complex' (*A. hydrophila, A. bestarium, A. salmonicida, A. popoffii*), 2) the 'Aeromonas caviae complex' (*A. caviae, A. media, A. eucrenophila*), and 3) the 'Aeromonas sobria complex' (*A. sobria, A. veronii, A. jandaei, A. trota*) (Abbott et al., 1998, 1992; Borrell et al., 1998; Kozińska et al., 2002). Today, PCR and sequence analysis, using 16S rRNA, *gyrB,* and *rpoD* are the typical methods by which we identify Aeromonads. *GyrB* and *rpoD* are used in conjunction due to the lack of heterogeneity in the 16s rRNA sequence, and thus the inability to identify species sole based upon this gene. Most importantly these methods have been used to identify *Aeromonas* from various fish tissues including spleen, kidney, blood, skin, liver, intestines, and furuncles (Beaz-Hidalgo et al., 2010; Godoy et al., 2010; Gustafson et al., 1992; Kozińska, 2007; O'Brien et al., 1994).

1.3.6.4 *Aeromonas* infections in fish

Many species of Aeromonads have been shown to cause infections in both wild and farmed fish including, but not limited to trout (Paniagua et al., 1990), African catfish (Shodeinde Shoyika, 2015), artic char (GoldschmidtClermont et al., 2009), flounder (Wiklund, 1995), and goldfish (Wiklund and Dalsgaard, 1998). The most commonly

reported species are *A. veronii* biovar sobria, *A. salmonicida, A. hydrophila, A. sobria, A. encheleia, A. allosaccharophila,* and *A. jandaei* (Kozińska, 2007). Interestingly, the prevailing isolate found in goldfish and carp, *A. veronii* biovar sobria, could be isolated in both healthy and diseased fish (Kozińska, 2007). It is widely recognized that *Aeromonas* infections in fish often result in furunculosis, septicaemia, and ulcerative and haemorrhagic diseases, leading to significant mortality in both wild and farmed fish, and noteworthy loses in the aquaculture sector (Austin and Austin, 2007; Bernoth et al., 1997).

Early symptoms of *Aeromonas* often include lethargy, decreased appetite, scale protrusions and shedding. As infection progresses, fish may become septicaemic and/ or develop organ failures and furuncles (Austin and Austin, 2007; Bernoth et al., 1997; Wiklund and Dalsgaard, 1998), with the most common and recognizable symptom being furunculosis. Furunculosis was first described by Emmerich and Weibel in 1890 as a serious, septicemic, disease found to affect salmonids (Emmerich and Weibel, 1890). Since then, furunculosis has been described in a number of other fish species including common carp and goldfish (Austin and Austin, 2007; Bernoth et al., 1997; Kozińska, 2007). Interestingly, both healthy and infected fish are capable of shedding large amounts of bacteria into the water (10^5-10^6 CFU/hour) (Bernoth et al., 1997; Noga, 2010). Outbreaks of infection tend to arise during periods of undue stress (changes in water quality and temperature, transport, crowding, and improper handling), as well as during spawning season (Bernoth et al., 1997; Gustafson et al., 1992; Noga, 2010).

Furunculosis presents slightly different clinical symptoms depending upon the infected species. However, similarities in external sore development and progression are

observed. In carp, *Aeromonas* infections are known as carp erythrodermatitis (CE), with the first signs of infection appearing as small, inflamed haemorrhagic areas, or small white erosions surrounded by red tissue. Common carp with extensive lesion development also exhibit exophthalmia, distended abdomens, and hemorrhages in the gills (Bootsma et al., 1977). During infection, *Aeromonas* is present exclusively in lesions between the dermis and epidermis (Bootsma et al., 1977). In goldfish, pathology was originally described as goldfish ulcer disease (GUD) (Mawdesley-Thomas, 1969). In contrast to CE, bacterial involvement is predominantly external with GUD and initiated by atypical variants of *A. salmonicida* (Elliott and Shotts, 1980a, 1980b). Evidence of early infections present with the appearance of white proliferations of the epithelium of fish, slowly developing into peripheral hemorrhages, necrotic dermal tissues, and muscle degeneration (Elliott and Shotts, 1980a, 1980b). In addition, marked infiltration of leukocytes (acidophilic, basophilic, and heterophylic granulocytes, lymphocytes, monocytes and macrophages) was observed at the infection site (Elliott and Shotts, 1980a, 1980b). Similar to CE, the causative bacteria was isolated from the peripheral infected tissue (Elliott and Shotts, 1980a, 1980b).

1.3.6.4.1 Transmission of disease

Disease transmission occurs laterally through contact with contaminated water sources or infected fish (Horne, 1928; Scott, 1968). Although reports vary, Klontz and colleagues observed the transmission of furunculosis in sablefish through the consumption of infected coho salmon (Klontz and Wood, 1973). Vertical transmission from parent to offspring is not a significant route of infection, as *A. salmonicida* is

unlikely to survive the egg stage of development (McCarthy, 1975). Elliot and Shotts found that goldfish died when *A. salmonicida* was directly injected (Elliott and Shotts, 1980b). However, goldfish would develop ulcers when exposed to *Aeromonas* contaminated water or scarred (patch of scales removed) (Elliott and Shotts, 1980b). In addition, the removal of the mucus layer, followed by skin inoculation produced ulcers in brook trout and Atlantic salmon, but not rainbow trout (unpublished U.S. Fisheries research).

1.3.6.4.2 Current methods of disease control

The health significance of detecting mesophilic aeromonads in public water supplies is not well understood, and no clearly defined point-source outbreak has been documented, thus establishing epidemiological links is very difficult. In aquaculture, controlling the spread of infection is critical to maintaining healthy stocks. Preventing the migration of infected fish between farms, the sharing of potentially contaminated equipment, and housing infected fish near uncontaminated fish are all cited as important factors when trying to limit the spread of bacteria. The main mechanisms to prevent spread include personnel education (Tucker and Hargreaves, 2009), the UV irradiation of water (Bullock and Stuckey, 1977), and the fallowing of net pens (Tucker and Hargreaves, 2009).

The biological control of *Aeromonas* has also been shown to play a potential role is preventing dissemination. Co-culturing *A. salmonicida* and *A. salmonicida* phage PAS-1 in rainbow trout displayed a temporal neutralizing and protective ability, increasing survival rates and mean times to death of fish (Kim et al., 2015). In addition,

intraperitoneal immunizations of *A. salmonicida* have shown some induced protection and the induction of immunity in fish (Ellis, n.d.; Lillehaug et al., 1992). Finally, outer membrane protein 48 (Omp48) may also represent a potential candidate for vaccine development, as recombinant Omp48 from *A. hydrophila* has been shown to induce a protective response in rohu (Khushiramani et al., 2012).

1.3.6.5 *Aeromonas* infections in other organisms

Aeromonads are ubiquitous in nature and possess the ability to colonize a plethora of hosts. These associations can be detrimental to the host (disease in fish and humans) or in other cases beneficial, as aeromonads can subsist in a symbiotic nature with their host (medicinal leech and possibly the mosquito).

With respect to human diseases, *Aeromonas* species span a wide range of localized and systemic illnesses. However, *Aeromonas* generally infects immunocompromised individuals and are often referred to as opportunistic pathogens, with diseases including, acute gastroenteritis, necrotizing fasciitis, and septicemia (Merino et al., 1995). For this reason, infections are often slotted into four major categories: 1) gastrointestinal disorders, 2) wound and muscle tissue infections, 3) blood-based diseases, and 4) diseases less frequently encountered (Janda and Abbott, 2010). Of these, acute gastroenteritis is by far the most common site of infection and Aeromonad isolation (Deodhar et al., 1991). *Aeromonas* spp. have also been recovered from respiratory tract infections, postoperative infections, or exposure to environment sources harboring the bacteria (Washington, 1972). Other specimens less commonly observed include blood cultures, and other bodily fluids (peritoneal fluid and bile).

Conversely, *Aeromonas* colonization can be beneficial, where bacterium and host can live in symbiosis. For example, Aeromonas is present as a symbiont in the gut of the medical leech (*Hirudo verbana*) with *A. veronii* being the most abundant species found. The purpose of this symbioses has been examined through transcriptome analysis and suggests that *Aeromonas* may feed from nutrients released by another symbiont, *Riknella*-like bacterium (Bomar et al., 2011). In addition, it is also possible nutrients released by symbiotic bacteria may be utilised by the leech, providing optimum nutrition (Bomar et al., 2011). Finally, similar to flora in the human gut, symbionts may be present to protect the host organism from pathogenic bacteria. Another study concluded that the T2SS and its capacity to secrete haemolysin is critical for symbiosis and gut colonization, allowing for erythrocyte lysis in the leech (Maltz and Graf, 2011). Recently, the analysis of gut microbiota from wild Kenyan mosquitos have revealed *Aeromonas* spp. as the most prevalent bacteria present (Osei-Poku et al., 2012). It has been suggested the presence of Aeromonads aid in disease transmission. Further, studies have already demonstrated the use of genetically engineered symbionts as a tool to prevent malaria dissemination (Sibao Wang, 2012).

1.3.6.6 Immune evasion strategies used by *Aeromonas*

Like other pathogenic bacteria, *Aeromonas* spp. have been continuously co-evolving with their wide range of hosts. In response to immune cell challenges, bacterial pathogens have developed diverse mechanisms enabling their survival, and at times, exhibiting dominance over various host immune defense systems. As previously mentioned, *Aeromonads* harbor a wide range of effector molecules injected via their

T3SS/ T6SS, modulating host defenses and even inducing cell death (Beaz-Hidalgo and Figueras, 2013; Burr et al., 2002; Chacón et al., 2004; Dacanay et al., 2006; Paniagua et al., 1990; Rosenzweig and Chopra, 2013; Sha et al., 2007b; Sierra et al., 2007; Suarez et al., 2010; Tomas et al., 2012). In addition to immune modulation, *Aeromonas* possess several mechanisms of immune evasion.

Li and colleagues found that the expression of 2,168 unique genes was significantly altered during the first 12 hours of infection with *A. hydrophila* in channel catfish skin (Li et al., 2013). This included genes involved in antioxidant formation, cytoskeletal elements, immune function, and nervous system pathways were all disturbed (Li et al., 2013). Specifically, *A. hydrophila* infection rapidly altered a number of critical lectins, chemokines, interleukins, and other mucosal factors in a manner to enhance its ability to adhere and invade the catfish (Li et al., 2013).

Bacteria can also target the innate complement pathway, allowing for dissemination throughout the host. *Aeromonas salmonicida* has been shown to target the complement protein C1q, using a 40 kDa C1q-binding outer membrane protein, *porin,* which binds C1q in an antibody independent process (Merino et al., 2005). The 40 kDa porin protein was present in all *A. salmonicida* and *A. veronii* strains examined, and *in vivo* data indicated a role for porin in serum resistance (Merino et al., 2005). In addition, resistance to complement lysis has been linked to the degradation of C3b, averting the formation of the membrane attack complex (Janda et al., 1994; Merino et al., 1996).

Several studies in mice have also examined the interaction between macrophages and *Aeromonas*. Interestingly, *Aeromonas veronii* and *Aeromonas hydrophila* were

poorly internalized by J774 macrophages (Krzymińska, 2008). Further, those bacteria that were internalized were capable of replicating up to three hours post infection, indicating they likely possess several immune evasion techniques allowing them to act as a intracellular pathogen (Krzymińska, 2008). One such mechanism is the expression of catalase genes, which protect against reactive oxygen species (Rio et al., 2007). In addition, *Aeromonas* has been shown to induce apoptosis in both macrophages and epithelial cells (Krzymińska et al., 2011, 2009), suggesting *Aeromonas* uses the induction of immune cell death as a method of immune evasion.

1.3.7 *Mycobacterium* spp.

1.3.7.1 *Mycobacterium* – an overview of the genus

The *Mycobacterium* genus contains more than 120 members (Tortoli, 2003), which were identified and classified based on 16S rRNA (Rogall et al., 1990). In addition, members are structurally distinguishable by a uniquely complex cell wall envelope (Niederweis et al., 2010), which can be identified by the Ziehl-Neelsen acid-fast stain (Ellis and Zabrowarny, 1993). Currently, the *Mycobacterium* genus is separated into three distinct groups for the purpose of diagnosis and treatment: 1) the *Mycobacterium* tuberculosis complex (MTBC) consists of closely related members that cause tuberculosis in their respective hosts (*M. tuberculosis, M. bovis,* and *M. pinnipedii*) (Smith et al., 2006); 2) the non-cultivable *M. leprae* which causes leprosy in humans; and 3) the non-tuberculous *Mycobacterium* (NTM) group (*M. kansasii, M. malmoense, M. avium,* which are capable of causing tuberculosis-like symptoms in immunocompromised individuals) and (*M. marinum, M. chelonae,* and *M. fortuitum* which can cause skin and

subcutaneous granulomatous infections) (Horsburgh, 1992; Ishikane and Tanuma, 2014; Petrini, 2006; Portaels, 1995).

1.3.7.2 Virulence factors of *Mycobacterium*

It is interesting that mycobacteria lack the classical virulence factors such as toxins and secreted pathogenic products, and that many of the virulence genes of pathogenic species are also conserved in non-pathogenic mycobacteria. This suggests pathogenic *Mycobacterium* have adapted to the intracellular environment with minimal acquisition of exclusive virulence genes, having instead altered their genomes to express multifunctional virulence factors.

1.3.7.2.1 Cell envelope factors

The cell wall is considered to be a major factor in pathogen virulence, especially that of mycobacteria. *Mycobacterium* synthesizes a family of long-chain fatty acids, termed mycolic acids, which are located in the cell envelope. Over the past decade, a vast amount of research has been conducted on mycolic acid methyl transferases (MAMTs), S-adenosyl methionine dependent enzymes which function to introduce methyl groups, which are then further altered to cyclopropane rings, methoxy or keto groups (Schroeder and Barry 3rd, 2001). As a whole, these enzymes function to create subtle structural differences with very specific roles. For example, a *M. tuberculosis* mutant with an inactivated *mmaA4* gene displayed a drastic alteration in envelope permeability, as well as a loss of oxygenated mycolic acids (Dubnau et al., 2000). Further, this mutant displayed an attenuated phenotype when tested in C57BL/6 mice (Dubnau et al., 2000). Continuing this line of research, Glickman and colleagues demonstrated that the deletion

of *pcaA*, an enzyme involved in catalyzing the proximal cyclopropanation of α-mycolates, led to a loss of cording (end-to-end growth), which contributes to mycobacterial virulence (Glickman et al., 2000). Once again, when tested in a murine model, this mutant failed to persist and kill infected C57BL/6 mice. These studies on mycolic acid synthesis correlated with others performed on their mechanisms of transport and assembly in the mycobacterial cell envelope (Armitige et al., 2000; Belisle et al., 1997). Moreover, these studies demonstrated that subtle mycolic acid structural modifications and their locations (esterified in the cell wall or loosely attached to the outer membrane) are fundamental to preserve cell wall structure and functionality, as well as to modulate the interaction with the host immune system.

Another cell wall virulence factor is the product of *Rv2869c*, a membrane-bound zinc metalloprotease. Its disruption translated into numerous alternations in mycolic acid biosynthesis and phosphatidylinositol mannoside composition, which is consistent with the model that Rv2869c protease participates in multiple biosynthetic pathways through the cleavage of membrane bound transcriptional regulators (Makinoshima and Glickman, 2005). When the mycobacterial Rv2869c mutant was tested, the strain was defective for initial replication in the lungs and persistence, indicating its importance to mycobacterial virulence (Makinoshima and Glickman, 2005).

In addition to mycolic acids, many members of the MTBC are also characterized by a plethora of complex lipids and glycolipids present in the cell envelope (Forrellad et al., 2013). These lipids and glycolipids are loosely attached to the cell envelope, and thus may play a role as diffusible factors involved in modulating host immune responses. Specifically, phthiocerol dimycocerosates (PDIM) constitute major virulence factors for

M. tuberculosis, as they are crucial components during the early stage of infection when mycobacteria encounter the host macrophages (Astarie-Dequeker et al., 2009; Forrellad et al., 2013). Interestingly, this was further evidenced in a zebrafish model, where Torraca and colleagues found that the initial recruitment of macrophages was largely dependent upon *M. marinum* (a non-tuberculous group member) PDIM, which masks the underlying mycobacterial PAMPs (Torraca et al., 2014). Further, non-pathogenic mycobacteria lack PDIM, and although capable of inducing robust immune responses, the mycobacteria are efficiently contained, which indicates PDIM may also play an important role in sustained infections.

Another component of the cell envelope is the capsule, which consists of a mixture of polysaccharide, protein, and lipid. The capsule plays an important role in determining which mycobacterial components are seen by the host cells, as well as what host components can reach mycobacteria. In addition, the capsule also determines how readily antimycobacterial drugs can reach the cytoplasm. During the early stages of pathogenesis, mycobacterial capsules are essential in aiding adhesion and penetration/ internalization in host macrophages. For an extensive review of the proteins and lipids found within the capsule, please refer to Daffé et al. (Daffé and Etienne, 1999;). In short, capsules contain inducible proteases, lipases, and secreted enzymes that are potentially involved in detoxifying reactive oxygen intermediates (catalase, peroxidase, and superoxide dismutase). The capsule also contains specialized contact-dependent lytic substances (e.g. phospholipases) that are involved in haemolysis and phospholipid degradation (Johansen et al., 1996; Leão et al., 1995).

1.3.7.2.2 Secreted factors

Protein secretion is critical to proper bacterial function and operation in their natural environment for survival. Moreover, these systems are also essential during infection for the interaction/ manipulation of host cells by exporting toxins/signal proteins. Recent evidence has shown that *Mycobacteria* have developed a specialized secretion system to transport extracellular proteins across their hydrophobic, highly impermeable cell envelope (Abdallah et al., 2007).

Notably, *Mycobacterium* encodes up to 5 of these transport systems, with two, ESX-1 and ESX-5 involved in virulence. ESX-1 has been analyzed in great detail in *M. marinum*, where it is involved in virulence and haemolysis (Gao et al., 2004). It is hypothesized that, similar to other secretion systems (T1SS - T6SS), ESX-1 forms a multi-subunit cell-envelope spanning complex, however, structural data to support these claims are lacking. Although, protein-protein interaction studies have demonstrated that ESAT-6 and CFP-10 (secreted effector molecules) are dependent on each other, forming a tight dimer (Brodin et al., 2005; Renshaw et al., 2005; Stanley et al., 2003). Recent evidence has found that ESAT-6 is secreted in abundant levels from *Mycobacterium*, interacting with beta-2-microglobulin (β2M) and affecting antigen presentation in macrophages (Sreejit et al., 2014). Further, the inactivation of ESAT-6 leads to attenuated virulence in mouse models (Sreejit et al., 2014). Like, ESX-1, ESX-5 has also been studied in *M. marinum*, and found to be a PPE secretion system, aiding in cell-to-cell spreading of pathogenic bacteria (Abdallah et al., 2006). PPE41 is a small hydrophilic protein secreted by *M. marinum* inside macrophages. Interestingly, when the entire ESX-5 gene cluster was introduced into *M. smegmatis*, it resulted in the secretion of

heterologously expressed PPE41 (Abdallah et al., 2006). Moreover, in specific transposon insertion mutant of *M. marinum*, PPE41 was no longer secreted and decreased cell-to-cell spreading was oberserved, indicating a potential role in virulence. However, mutants were still capable of infecting and persisting, thus more work is needed to truly understand the virulent nature of the ESX-5 secretion system.

1.3.7.3 *Mycobacterium* infections in fish

Fish have been gaining in notoriety as a model to study *mycobacterial* pathogenesis. *Mycobacterium marinum* was first isolated from saltwater fish in 1926 (Aronson, 1926) as an agent of tuberculosis. Since then, several species of mycobacteria (*M. marinum, M. fortuitum, M. chelonae,* and *M. smegmatis*) have been shown to cause infections in both wild and farmed fish including zebrafish (Meijer, 2015; Parikka et al., 2012; Swaim et al., 2006; Watral and Kent, 2007; Yang et al., 2012), trout (Chen et al., 1998; El-Etr et al., 2001), salmon (Pourahmad, 2013), yellow perch (Daoust et al., 1989), striped bass (Hedrick, 1987)., and goldfish (Hodgkinson et al., 2012; Pourahmad et al., 2014; Talaat et al., 1999, 1998).

Disease is usually accompanied by emaciation, lethargy, and often death in infected fish. The typical lesion, observed using histopathological methods, is the granuloma, and is present throughout infected fish (Parisot, 1958). Moreover, the histopathology of granulomas observed in fish (Daoust et al., 1989; Hedrick, 1987) is similar to that seen in infected humans (Fenton and Vermeulen, 1996; Lucas, 1988). Recently, fish have become model organisms to study other non-tuberculosis mycobacterial diseases including *M. chelonae* and *M. fortuitum.*

In goldfish, chronic mycobacterial infections are induced through intraperitoneal injections of 10^2 to 10^7 CFU/fish (Talaat et al., 1998). Infections were characterized by progressive, systemic granuloma formation with different histopathological features including necrotizing, non-necrotizing, and caseous, which is consistent with the typical granulomas formed in other animals (Hedrick, 1987). Notably, the histopathology of caseous granulomas appears similar to that seen in humans infected with *M. tuberculosis* (Lucas, 1988; Nau et al., 1997), which cannot be said for the murine model (Dannenberg, 1994). In addition, isolation of *M. marinum* from infected fish could be conducted up to 16 weeks post infection (Talaat et al., 1998).

Several studies have also begun to examine the immune response of goldfish when challenged with *Mycobacterium.* Upon infection, Hodgkinson and colleagues found notable increases in IL-β1 and IFN-γ 7 days post infection in goldfish (Hodgkinson et al., 2012). Further, goldfish primary kidney neutrophils were capable of migrating to and internalizing *M. marinum in vitro* (Hodgkinson et al., 2015). However, ROS production wasn't induced until *M. marinum* outnumbered the neutrophils 50:1 (Hodgkinson et al., 2015). Another study, conducted by Yang et al. found zebrafish neutrophils to be mildly chemotactic toward *M. marinum in vivo* (Yang et al., 2012). In addition, *in vivo* imaging demonstrated that neutrophils were recruited to nascent granulomas in response to signals from dying macrophages within the granuloma, which they phagocytose (Yang et al., 2012). Notably, neutrophils exert a protective effect through oxidative killing of the mycobacteria from infected dying macrophages. This is of particular interest considering this activity was lost when genetically engineered neutropenic zebrafish and zebrafish deficient in phagocytic oxidase activity were used.

1.3.7.3.1 Transmission of disease

Pathogenic mycobacteria can be found outside of the infected host (fish) in the water, on other organisms, on plant matter, and on abiotic substances within tanks and aquariums (Beran et al., 2006). The water temperature plays the largest role of species survival, as pathogenic mycobacteria grow ideally above 18-20 °C, but can also survive below these temperatures for months (Beran et al., 2006). Environmental infestations, contaminated home aquariums, and aquaculture farms appear to cause the majority of infections transferring to humans; and much like *Mycobacterium* can transfer from fish to fish via close contact and consumption, transmission to humans occurs by the touching or ingesting of any infected object or organism. Further, *mycobacteria* have even been found on clinically healthy fish at low concentrations.

1.3.7.3.2 Mechanisms of disease control

There are several methods to prevent *Mycobacterium* infections from the onset, including frequent water changes, quarantining newly acquired fish, and maintaining healthy well-fed fish, making it possible to handle limited exposure to mycobacteria.

Treatment of mycobacterial infections (from fish pathogens) in humans has proven difficult. Generally, topical antibiotics (solo or in combination) are applied for several weeks to months (Wu et al., 2012). Even then, total elimination of the infection is not always guaranteed, in which surgical debridement becomes necessary (Wu et al., 2012).

1.3.7.4 *Mycobacterium* infections in other species

Among others, *M. fortuitum, M. chelonae* and *M. marinum*, are known to be zoonotic pathogens (Barker et al., 1997; Talaat et al., 1999). In humans, the infection tends to remain near the extremities due to the cooler temperatures they provide as opposed to the internal body temperatures (Barker et al., 1997). Fortunately, even though these bacterium can prove fatal to fish, it usually only results in a cutaneous infection for humans. However, these infections can become semi-chronic, develop into ulcerations, and often leading to granulomas lasting two to four weeks (Barker et al., 1997). In some cases, long-term effects may develop including osteomyelitis, arthritis, and a compromised immune system (Laing et al., 1997).

The fish pathogen *M. fortuitum* has also been associated with masses found in cows and sheep, abscesses in dogs, granulomas in cats, and the neurological disorder, spinning disease, in mice (Jubb, 1985). Moreover, *M. fortuitum* becomes particularly potent if the host already has an open wound (Nordén and Linell, 1951).

The most common *Mycobacterium* infection in world comes from *M. tuberculosis*, which leads to tuberculosis (TB) in humans (Baron, 1996). In general, only about 10% of latent infections progress to active TB, which if left untreated, results in death in roughly half of those infected. Despite newer diagnostics and treatment, people are still suffering worldwide and it sits among the top ten killer infectious diseases (Sandhu, 2011). In addition, it is the leading killer of those infected with HIV.

TB infection begins with *Mycobacterium* reaching the pulmonary alveoli. Here they invade/ are internalized by alveolar macrophages, where they begin to replicate within the cells phagosomes (Houben et al., 2006; Lucas, 1988). At this point,

Mycobacterium invokes one/ or several of its immune evasion mechanisms (discussed in the next section) to avoid being killed by host reactive oxygen species. TB is classified as a chronic granulomatous inflammatory disease, where macrophages, B and T lymphocytes, and fibroblasts migrate to form granulomas/ tubercles. At the centre of these tubercles, the host tissue becomes necrotic, termed caseous necrosis (Grosset, 2003).

Symptoms take weeks to arise (if active) and are described as a chronic cough with blood-containing sputum, fever, night sweats, and weight loss (Sandhu, 2011). *M. tuberculosis* can only be spread through air droplets originating from a person either coughing, sneezing, speaking that has the disease. In order to diagnose latent infections, a tuberculin skin test or blood test is performed, while chest X-rays and body fluid analysis must be performed to diagnose an active infection (Sia and Wieland, 2011). The main method of TB prevention is through vaccination with the bacillus Calmette-Guérin vaccine (Hawn et al., 2014), however, early screening of those at high-risk can also help in early detection. Treatment of *M. tuberculosis* infections is becoming a growing problem, as the rates of multiple drug-resistant tuberculosis is on the rise (Gandhi et al., 2006; Gandhi et al., 2010; Jacobs, 1994). Once infected, treatment requires the use of multiple antibiotics over a significant time period (9-12 months) (Lange et al., 2014; Zumla et al., 2013).

1.3.7.5 Immune evasion strategies of *Mycobacterium*

Tuberculosis forming *Mycobacteria* usually enters the alveolar space of their hosts in an aerosol droplet, where its first contact is thought to be with resident

macrophages (Shinnick, 2013; Stokes et al., 1993). *Mycobacteria* can also be internalized by alveolar type II pneumocytes (Bermudez and Goodman, 1996; Mehta et al., 1996), which are found in greater numbers than macrophages in alveoli. In addition, *M. tuberculosis* can infect and grow in pneumocytes *ex vivo* (Bermudez and Goodman, 1996; Mehta et al., 1996). The bacteria are internalized in a manner initiated by bacterial contact with macrophage mannose or complement receptors (Schlesinger, 1993). A glycoprotein found on alveolar surfaces, surfactant protein A, can further enhance the binding and uptake of *M. tuberculosis* by upregulating mannose receptor activity on the cell surface (Gaynor et al., 1995). Once internalized, there are two potential outcomes: 1) the macrophage kills the internalized bacteria through antimicrobial reactive oxygen and nitrogen species, or 2) the bacterium employs it's immune evasion strategies to replicate within the cell. These mechanism(s) include, the inhibition of phagolysosome fusion (Goren et al., 1976), the inhibition of phagosome acidification (Hackam et al., 1997), and the recruitment and retention of tryptophan- aspartate containing coat protein on phagosomes to prevent phagosome maturation (Tanigawa et al., 2009). In order to prevent phagolysosome formation, individual *M. tuberculosis* bud out from the phagolysosomes into vacuoles that fail to fuse to the secondary lysosomes, thus allowing for survival. The temporary residence within a phagolysosome stimulates long-term survival and reproduction. This is aided by anionic trehalose glycolipids, termed sulfatides, which have demonstrated an inhibitory effect on phagolysosomal fusion (Goren, 1977). In addition, studies have reported that vacuoles containing *A. avium* are less acidic than neighboring lysosomes (Crowle et al., 1991; Gordon et al., 1980). These lysosomes appear to lack a vesicular proton-ATPase pump, resulting in a lack of

acidification (Sturgill-Koszycki et al., 1994). Finally, Rab5, an essential protein involved in the biogenesis of endocytic particles, has been shown to be aberrantly expressed on the phagosomes containing *M. tuberculosis*, leading to arrested maturation at the early endosomal stage (Clemens et al., 2000).

Mycobacterium also possesses the capacity to evade immune response during the later stages of infection. One such mechanism can be observed in *M. tuberculosis,* who has the ability to remain dormant within host cells for years, while simultaneously retaining its potential to be activated. This was demonstrated by McKinney and colleagues, who determined that the persistence of *M. tuberculosis* in murine macrophages requires the glyoxylate shunt enzyme, isocitrate lyase, an essential enzyme involved in the metabolism of fatty acids (McKinney et al., 2000). Disruption of this gene attenuated bacterial persistence and virulence in mice during the acute phase of infection. During infection with *M. marinum,* several genes (PE and PE-PGRS) have been identified as being preferentially expressed when bacteria resides in host macrophages and granulomas (Ramakrishnan et al., 2000). Mutation of these genes resulted in *M. marinum* mutants incapable of replicating in macrophages. In addition, these mutants exhibited decreased persistence within granulomas.

The final major immune evasion mechanism of *Mycobacterium* is the ability to protect itself against reactive oxidative radicals. Many *Mycobacterium* species express superoxide dismutase, an important enzyme in the protection against oxidative stress (Dussurget et al., 2001). In addition, *Mycobacterium* expresses the *oxyR* gene, an oxidative stress sensor and transcriptional activator that induce the expression of catalase and hydroperoxidase (Sherman et al., 1995). Finally, some *M. tuberculosis* strains

overexpress a protein that cyclopropanates mycolic acid double bonds, resulting in a significant decrease in susceptibility to peroxide (Yuan et al., 1995).

1.4 Summary

The inflammatory process is a complex cascade critical to host defense. A successful response must permit the removal of harmful foreign invaders and prevent over-stimulation resulting in immune mediated injury, all while simultaneously allowing for wound and tissue healing. Phagocytes are essential to this process, where they play a major role in the initiation, regulation, and resolution of the inflammatory processes. They are capable of deciphering each encountered stimuli and responding with the required precision to enact the appropriate response.

The regulation and control of the vertebrate inflammatory response involves a plethora of complex mechanisms, some of which still remain poorly understood. This is particularly true for the teleost and agnathan model systems, where a lack of specific reagents in many species hampers our capacity to examine the regulation of inflammation at a cellular and/ or mechanistic level. Despite this, there is increasing evidence that many of the key components required to mount an effective inflammatory response are present in early vertebrates. By examining the unique methods of inflammatory control employed by teleost neutrophils, we will gain significant insight into the evolution of the inflammatory response, and most importantly host defenses. In addition, utilizing non-classical models may allow us to uncover novel mechanisms of control unique to lower vertebrates or not yet identified in mammals, providing a broader and more profound understanding of the inflammatory response.

Chapter 2. Materials and Methods

2.1 Animals

Fish were maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. All fish were acclimated for at least two weeks prior to use in experiments. Fish were monitored daily for any signs of disease and only fish that appeared to be healthy were used, unless otherwise noted. Lamprey and goldfish were held at 18 °C in a continuous flow-through water system on a simulated natural photoperiod. All fish were terminated via cervical dislocations using approved procedures; with all efforts made to minimize animal stress.

2.1.1 Lamprey

Ammocoete larvae (8–11 cm in length) of the sea lamprey (*Petromyzon marinus*) were received from Wilfred Laurier University. They were originally captured by electrofishing from freshwater streams in New Brunswick, Canada. Lamprey were maintained in sand-lined aquaria with constant aeration and fed brewer's yeast weekly. Prior to handling, fish were sedated using tricaine methanosulfonate (TMS) solution of 10-20 mg/L of water. When necessary, lampreys were separated using flow through Plexiglas dividers.

2.1.2 Goldfish

Goldfish (*Carassius auratus* L.) 10–15 cm in length were purchased from Mount Parnell (Mercersburg, PA) and obtained through Aquatic Imports (Calgary, AB). Prior to

handling, fish were sedated using TMS solution of 40-50 mg/L of water. When necessary, individual fish were marked by fin clipping.

2.2 Serum

Fish serum was obtained by bleeding common carp (*Cyprinus carpio*) every 6-8 weeks using a 21-guage needed attached to a 3 mL syringe. Carp were anaesthetized with TMS (approximately 40 mg/L) and bled from the caudal vein. Collected carp blood was pooled and allowed to clot overnight at 4 °C. The following day, blood was centrifuged at 1000 x *g* for 25 min and serum removed. The resulting serum was heat-inactivated for 30 min at 56 °C, filter sterilized using a 0.22 µm syringe tip filter, and then stored at – 20 °C until used in the experiments.

2.3 Reagent generation

2.3.1 Zymosan labeling

2.3.1.1 FITC

Unlabeled zymosan particles (Molecular Probes) were labeled overnight with 250 ng/mL fluorescein isothiocyanate (FITC; Sigma) with continuous shaking at 4 °C in carbonate buffer (Table 2.1). After staining, zymosan-FITC was washed twice with 1xPBS$^{-/-}$ at 863 x *g* for 10 minutes at room temperature. Particles were stored at 4 °C in the dark until used.

2.3.1.2 APC

Unlabeled zymosan particles (Molecular Probes) were labeled overnight with 75 mg/mL allophycocyanin (APC; Sigma) with continuous shaking at 4 °C in 1xPBS$^{-/-}$. Zymosan-APC was prepared fresh each use in order to ensure proper stain retention. Prior to use, zymosan particles were washed twice with 1xPBS$^{-/-}$ at 863 x *g* for 10 minutes at room temperature.

2.3.2 Apoptotic cells

2.3.2.1 Catfish 3B11 cells

Apoptotic 3B11 cells were generated by incubating cells for 24 hours in the presence of 10 mg/mL cycloheximide (Sigma). Treated cells were harvested and washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes. In some instances, apoptotic cells were stained with 1.5 mg/mL wheat germ agglutinin Alexa Fluor 555 (Molecular Probes) for 90 minutes with continuous shaking at room temperature. Apoptotic cells were then washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes. We have previously shown that apoptotic cells derived from primary or cell line leukocytes induce equivalent phagocyte responses (Rieger et al., 2012).

2.3.2.2 Lamprey primary typhlosole leukocytes

Apoptotic lamprey cells were generated by incubating primary typhlosole cells for 24 hours in the presence of 10 mg/mL cycloheximide (Sigma). Treated cells were harvested and washed twice in 1xPBS$^{-/-}$ at 400 x g for 8 minutes. In some instances, apoptotic cells were stained with 1.5 mg/mL wheat germ agglutinin Alexa Fluor 555

(Molecular Probes) for 90 minutes with continuous shaking at room temperature. Apoptotic cells were then washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes.

2.3.2.3 Apoptotic goldfish primary kidney macrophages (PKMs)

2.3.2.3.1 Cyclohexamide induced

Apoptotic PKMs were generated by incubating cells for 24 hours in the presence of 10 mg/mL cycloheximide (Sigma). Treated cells were harvested and washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes. In some instances, apoptotic cells were stained with 1.5 mg/mL wheat germ agglutinin Alexa Fluor 555 (Molecular Probes) for 90 minutes with continuous shaking at room temperature. Apoptotic cells were then washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes.

2.3.2.3.2 *Aeromonas veronii* induced

Apoptotic cells were also generated in a more physiologically relevant manner using *Aeromonas veronii*. PKMs were incubated with *A. veronii* at a 3:1 (bacteria: cell) ratio for 24 hours. Following incubation, PKMs were washed 3 times in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes. Using growth plates (CFU counts), this method of washing removed 99% of all bacterial cells. In some instances, apoptotic cells were stained with 1.5 mg/mL wheat germ agglutinin Alexa Fluor 555 (Molecular Probes) for 90 minutes with continuous shaking at room temperature. Apoptotic cells were then washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes.

2.3.2.4 Apoptotic goldfish neutrophils

Apoptotic neutrophils were generated by incubating primary kidney neutrophils cells for 24 hours in the presence of 10 mg/mL cycloheximide (Sigma). Treated cells were harvested and washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes. In some instances, apoptotic cells were stained with 1.5 mg/mL wheat germ agglutinin Alexa Fluor 555 (Molecular Probes) for 90 minutes with continuous shaking at room temperature. Apoptotic cells were then washed twice in 1xPBS$^{-/-}$ at 400 x *g* for 8 minutes.

2.4 Cell media

2.4.1 Modified goldfish Lebovitz's-15 medium

The culture medium used for generation of goldfish primary kidney macrophages, modified goldfish Lebovitz-15 (MGFL-15), has been previously described (Neumann et al., 2000). The composition of precursor GFL-15 is shown in Table 2.2. The composition breakdown of MGFL-15 (Table 2.3) includes GFL-15, nucleic acid precursor solution (Table 2.4), and 10x Hanks Balanced Salt Solution (Table 2.5). MEM sodium pyruvate, MEM non-essential amino acid solution, MEM amino acid solution, and MEM vitamin solution were purchased from Gibco. Bovine insulin was also purchased from Sigma. Complete MGFL-15 medium contained all of the above with the addition of 10% heat-inactivated fetal calf serum, 5% heat-inactivated carp serum, 100 U/mL penicillin/100 µg/mL streptomycin, and 100 µg/mL gentamicin. In experiments examining the interaction of PKMs and *A. veronii* antibiotics were removed.

2.4.2 Neutrophil Hanks balanced salt solution

A plethora of media (Figure 2.1) was examined to determine the most ideal media for experiments containing isolated goldfish neutrophils. 1x Hank's balanced salt solution ($HBSS^{-/-}$; Table 2.6) was ultimately selected to maintain neutrophil viability during washing and $HBSS^{+/+}$ (addition of magnesium and calcium) was used to maintain function during bioassays. Complete $HBSS^{+/+}$ contained 10% heat-inactivated carp serum, 100 U/mL penicillin/100 µg/mL streptomycin, and 100 µg/mL gentamicin. In experiments examining the interaction of neutrophils and *A. veronii* or *M. fortuitum* antibiotics were removed.

2.5 Pathogens and pathogen mimics

2.5.1 *Aeromonas veronii* biovar sobria

Aeromonas veronii was isolated in the Department of Biological Sciences Aquatic Facilities from a naturally infected goldfish presenting with a furuncle. The infected fish furuncle was swabbed with a cotton swab and used to inoculate a tryptic soy agar (TSA) plate. Single colonies were isolated, grown up, and typed using 16s rRNA and gyrB (primers can be found in Table 2.7). Each examined colony was identified to be *Aeromonas veronii* biovar sobria based on sequence analysis. A growth curve for *A. veronii* can be found in Figure 2.2.

Heat-killed *A. veronii* was generated by growing the bacteria to log phase growth and then incubating at 80 °C for 1 hour. The resulting heat-killed bacteria was washed twice by centrifuging at 10,000 x *g* for 2 minutes, and resuspended in 1× $PBS^{-/-}$ Following heat inactivation, an aliquot of heat-killed bacteria was plated onto TSA plates

to ensure that cultures had been adequately killed. No cultures developed. Heat-killed *A. veronii* could be stored at 4 °C for short periods, however, in general the bacterium was prepared fresh for each experiment.

2.5.2 *Mycobacterium fortuitum*

M. foruitum was obtained from the lab of Dr. Mike Belosevic. The bacteria was grown at 30 °C in Middlebrook 7H9 broth (Difco) supplemented with 10% OADC (BD) and 0.05% Tween 80. Single cell suspensions were generated during log phase of growth by passaging through a 25-gauge needle and subsequently through 5 mm filters (Millipore). Bacteria were enumerated by measuring the optical density at 600 nm, and colony forming units were confirmed by plating serial dilutions of cultures on Middlebrook 7H10 agar (Difco) supplemented with 10% OADC (BD). Heat-killed *Mycobacteria* were generated by incubating at 80 °C for 1 hour. The resulting heat-killed *Mycobacteria* was washed twice by centrifuging at 10,000 x *g* for 2 minutes, and resuspended in 1× PBS $^{-/-}$ Following heat inactivation, an aliquot of heat-killed bacteria was plated onto Middlebrook 7H10 agar to ensure that cultures had been adequately killed. No cultures developed. Heat-killed *M. fortuitum* could be stored at 4 °C for short periods, however, in general the bacterium was prepared fresh for each experiment.

2.5.3 Zymosan

Zymosan was used as an inflammatory model to induce acute, self-resolving peritonitis. Soluble zymosan was purchased from Sigma and resuspended at a concentration of 50 mg/mL in 1xPBS$^{-/-}$. The zymosan powder was vortexed for several

minutes at high speed to ensure adequate mixing. Resuspended zymosan was stored at 4°C for up to 1 month.

2.6 Intraperitoneal injections

Goldfish were anesthetized with TMS (as indicated above). Intraperitoneal injections were done in the soft tissue under the left pectoral fin. Goldfish were then returned to water with oxygenation, allowed to recover, and monitored closely to ensure there was no undue stress or delayed deaths.

2.6.1 Zymosan

Zymosan stock (50 mg/mL) was diluted 1:1 with $1xPBS^{-/-}$ to create a 25 mg/mL diluted stock. 100 µL (2.5 mg) of diluted stock was aspirated in a 25-gauge needle attached to a 1 mL syringe and all bubbles were removed prior to injection.

2.6.2 *Aeromonas veronii*

Heat-killed *A. veronii* was prepared as previously described. Following preparation, $5x10^6$ bacterial cells were resuspended per 100 µL. Using a 25-gauge needle attached to a 1 mL syringe, a 100 µL injection was prepared.

2.6.3 *Mycobacterium fortuitum*

M. fortuitum was prepared as previously described. Following preparation, $10x10^6$ bacterial cells were resuspended per 100 µL. Using a 25-guage needle attached to a 1mL syringe, a 100 µL injection was prepared.

2.7 Isolation of primary cells

2.7.1 Lamprey typhlosole leukocytes

Ammocoete larvae (8–13 cm long) of the sea lamprey, *Petromyzon marinus,* were dissected along the ventral side to extract the intestine and the associated typhlosole (spiral valve; Figure 2.3). Typhlosole leukocytes were harvested by maceration of these tissues between two glass slides while suspended in one part water and two incomplete MGFL-15. The resulting cell suspension was washed twice in one part water/ two parts incomplete MGFL-15 by centrifuging at 311 x *g* for 10 minutes at 4 °C. Cells were subsequently used for experiments. Lamprey neutrophils were isolated as previously described (Fujii, 1981). Briefly, total primary typhlosole leukocytes were layered on Ficoll-Paque and centrifuged at 600 x *g* for 30 minutes at 4 °C. The interface band containing neutrophils was isolated at washed 3 times using $HBSS^{-/-}$. Cells were subsequently used in experimental assays.

2.7.2 Goldfish primary kidney leukocytes

Goldfish kidneys were macerated through mesh screens with incomplete MGFL-15 medium (Neumann et al., 1998). Debris was allowed to settle and supernatant was removed. The leukocytes were washed twice with incomplete MGFL-15 medium via centrifugation for 10 minutes at 311 x *g* at 4 °C. The resulting cells consist of total kidney leukocytes. To isolate kidney neutrophils, total kidney leukocytes were layered onto 51% Percoll (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 25 minutes at 400 x *g* and 4 °C (Katzenback and Belosevic 2009). The pellet containing RBCs and neutrophils was collected, and RBCs were lysed with ACK lysis buffer (Lonza, Basal, Switzerland)

for 3 minutes. The remaining neutrophils were washed twice with incomplete $HBSS^{-/-}$ via centrifugation for 10 minutes at 311 x *g* at 4 °C and resuspended in complete $HBSS^{+/+}$ (HBSS supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% carp serum, calcium, and magnesium) for subsequent assays. To isolate mononuclear cells, the buffy layer above Percoll was removed following centrifugation. Cells were washed twice in incomplete MGFL-15 by centrifuging at 311 x *g* for 10 minutes at 4 °C.

2.7.3 Peritoneal leukocytes

Peritoneal cells were isolated from goldfish injected with 2.5 mg of zymosan or heat-killed *A.* veronii by lavaging the fish with $1xPBS^{-/-}$ (no calcium, no magnesium). The cells were spun down at 311 x *g* for 10 minutes and washed with $1xPBS^{-/-}$ before use, as previously described (Rieger et al., 2012). To isolate neutrophils, exudates were subsequently layered onto 51% Percoll and centrifuged for 25 minutes at 400 x *g* at 4 °C. The pellet containing RBCs and neutrophils was collected, and RBCs were lysed with ACK lysis buffer (Lonza) for 3 minutes. The remaining neutrophils were washed twice with incomplete $HBSS^{-/-}$ via centrifugation for 10 minutes at 311 x *g* at 4 °C and resuspended in complete $HBSS^{+/+}$ for subsequent assays. Neutrophil viability was consistently 95% according to annexin V/PI staining, and the level of purity for isolated neutrophils was 93–97%. The buffy coat was removed and used for the isolation of mononuclear cells. Cells were washed twice in incomplete MGFL-15 by centrifuging at 311 x *g* for 10 minutes at 4 °C. The resulting cells were subsequently resuspended in the

appropriate medium depending on the bioassay.

2.7.4 Primary kidney macrophages (PKMs)

Primary kidney macrophages were generated as previously described (Neumann et al., 2000, 1998). Isolated goldfish kidney leukocytes were seeded in 15 ml of complete MGFL-15 medium [MGFL-15 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL gentamicin, 10% newborn calf serum (Gibco, Burlington, ON, Canada), and 5% carp serum] and 5 mL of cell-conditioned medium from previous cultures. PKM cultures were developed over 7 days at 20 °C and were used at the proliferative phase of culture development. This phase is dominated by the proliferation of macrophage progenitors along with their differentiation into mature macrophages (Barreda et al., 2000; Barreda and Belosevic, 2001).

2.8 Tissue sectioning

2.8.1 Furuncles

Goldfish were sacrificed by cervical dislocation at specific points during infection. The furuncle was removed by excising a 2 x 2 cm square of goldfish tissue. The tissue is then fixed in 10% neutral-buffered formalin (Sigma) for 24 hours at 4 °C. Following fixation the tissue was washed in 1× PBS $^{-/-}$ for 1 hour, followed by a decalcification step in Cal-Ex Decalcifier (Fisher Scientific) for 4 hours at room temperature with continuous rocking. Tissues are processed overnight in a series of ethanol, toluene, and wax washes using a Leica TP1020 tissue processor (Leica).

Following the series of washes, tissues were embedded in paraffin wax and allowed to harden. Sections (7 µm thick) were performed using a Leica RM2125 RTS microtome and allowed to set on the slide at 37 °C overnight.

2.9 Cytochemical staining

2.9.1 Cell cytospins

For all cytochemical stains, 5 x 10^4 cells were spun onto glass slides at 55 x *g* for 6 minutes at room temperature using a cytocentrifuge (Shandon Instruments). Images were generated using a DM1000 microscope (Leica) using a bright field 100 x objective (1000 x magnification), and acquired using QCapture software (QImaging).

2.9.1.1 Hema3

Cells were fixed by incubation in 70% methanol for 5 seconds. Cells were then stained with hematoxylin for 5 seconds and counter-stained with eosin for 1 second (all stain components were purchased from Fisher Scientific). Slides were rinsed with tap water and air-dried prior observation using bright field microscopy.

2.9.1.2 Sudan Black

For Sudan Black staining (Sigma-Aldrich), the cells were fixed for 1 minute in a 25% acetone/ 75% glutaraldehyde solution, followed by several rinses in distilled water. The cells were then stained for 5 minutes with Sudan Black with continuous gentle

agitation, followed by several washes in 70% ethanol to remove excess stain. Slides were subsequently stained for 5 minutes in Hematoxylin counterstain.

2.9.2 Tissue sections

2.9.2.1 Hematoxylin & Eosin (H&E)

Following paraffin embedding, tissue sections were washed twice in toluene, twice in 100% ethanol, then once in 90%, 70%, and 50% ethanol, followed by water, for 2 minutes each. Tissues were then stained with hematoxylin Gill solution III (Surgipath) for 2 minutes. Cells are subsequently washed for 15 minutes in running cold water. Tissues are washed once in 70% ethanol for 2 minutes, followed by Eosin stain (Surgipath) for 30 seconds. Staining is completed with two more washes at 100% ethanol and toluene, each for 2 minutes. Tissues are then coverslipped with DPX and incubated at 37 °C overnight to solidify. Images were taken on a Leica microscope at 40 x magnification.

2.10 Quantitative PCR

2.10.1 RNA Isolation

2.10.1.1 Trizol

RNA was isolated from goldfish tissues using Trizol (Invitrogen) under manufacturers recommendations. Isolated tissues were placed in 12 mL round-bottom tubes, flash frozen using dry ice and ethanol, then stored at -80 °C until use. Trizol was then added depending on tissue size (approximately 1 mL for kidney, 1 mL for blood, and 2 mL for furuncle tissues), followed by homogenization using blade disruption along

with intermittent cooling on ice. Chloroform (0.1 mL/ 1 mL Trizol) was added and the entire solution was subsequently transferred into a 1.5 mL microcentrifuge tube, then vortexed. Tubes were centrifuged at 10,800 x *g* for 30 minutes at 4 °C. The aqueous layer was extracted and transferred to a new microcentrifuge tube. One volume of isopropanol was added and suspensions were incubated at -20 °C overnight. The following day, tubes were centrifuged at 10,800 x *g* for 60 minutes at 4 °C to pellet the RNA. Supernatants were aspirated, and the RNA pellet was washed with 1 mL of 75% reagent grade ethanol followed by centrifugation at 10,800 x *g* for 30 minutes at 4 °C. The ethanol was aspirated and RNA pellets were allowed to air-dry. RNA was then resuspended in nuclease-free water. The RNA concentration was quantified using a Nanodrop at an absorbance of 260 nm. Samples were also read at 230 nm and 280 nm to determine phenolic and protein contamination, respectively.

2.10.1.2 Qiagen RNeasy column

RNA was isolated from peritoneal lavage cells with the use of a RNeasy kit (Qiagen) according to the manufacturers recommendations. In short, cells were lysed in RLT Buffer. One volume of 70% ethanol was added to the lysate and the entire suspension applied to the spin column. Columns were spun at 8,000 x *g* for 15 seconds at room temperature and washed with RW1 Buffer. Columns were spun again at 8,000 x *g* for 15 seconds at room temperature. Columns were then washed using RPE Buffer and centrifuged at 8,000 x *g* for 2 minutes at room temperature. The column was then placed into a new 1.5 mL microcentrifuge tube and 30-50 µL of nuclease-free water (Ambion) was added. The water was allowed to stand for 1 minute prior to centrifuging. To elute

RNA, columns were spun at 8,000 x *g* for 1 minute room temperature. The RNA concentration was quantified using a Nanodrop at an absorbance of 260 nm. Samples were also read at 230 nm and 280 nm to determine phenolic and protein contamination, respectively.

2.10.2 cDNA synthesis

cDNA synthesis was performed using SMARTScribe Reverse Transcriptase from Clontech. First strand synthesis was cycled at 72 °C for 3 minutes followed by 42 °C for 60 minutes. Second strand underwent a cycle as follows: 95 °C for 30 seconds; 55 °C for 30 seconds; 72 °C for 15 minutes. Prior to cDNA synthesis, RNA levels were quatified and normalized (2 mg for tissues and 250 ng for peritoneal lavage cells). Primers used in cDNA synthesis reactions can be found in Table 2.8.

2.10.3 Primers

All primers used were previously validated in goldfish. Q-PCR primers were validated by creating a standard curve to determine the R^2 value, y-intercept, and efficiency of the primer set. Primer sets were chosen with an R^2 value equal or greater than 0.997, a y-intercept value of -3.0 to -3.2, and an efficiency of 85% or higher. Q-PCR primers can be found in Table 2.9. Amplified Q-PCR products were run on a gel, excised, and sequenced to ensure the correct amplicon was being amplified.

2.10.4 Quantitative PCR program

Q-PCR was performed with SYBR green reagents on an Applied Biosystems 7500 Fast Real-Time PCR machine. Elongation factor 1 alpha (EF-1α) was used as the endogenous control for all experiments. Q-PCR conditions are as follows: 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data was analyzed using 7500 fast software (Applied Biosciences) and is represented as the average of triplicate wells, followed by experimental replicates with standard error shown. The RQ values were normalized against control fish.

2.11 Cellular bioassays

2.11.1 Cellular infiltration

Goldfish were injected intraperitoneally with 2.5 mg of zymosan (Sigma-Aldrich), $5x10^6$ heat-killed *A. veronii*, or $1x10^7$ *M. marinum* in 100 µL of $1xPBS^{-/-}$. The goldfish were terminated via approved procedure at the indicated timepoints, and the cells were harvested by peritoneal lavage to determine the number of cells at the site of inflammation. Within these time points, the changes in cellular numbers were largely associated with cellular infiltration. The cells were used in the assays as described in the subsequent sections.

2.11.2 Viability (AnnexinV/Propidium iodide) assay

Cell viability was determined as previously described (Rieger et al., 2010) with minor modifications. PKMs and naïve neutrophils were incubated with either

cyclohexamide or *Aeromonas veronii* [1:1, 3:1, 10:1 ratios (bacteria: cell)] for 24 hours at 18 °C. Following incubation, cells were washed twice in Annexin V binding buffer (BD Pharmingen) and resuspended in 100 µL Annexin V binding buffer. Annexin V FITC (eBioscience) was added according to manufacturers protocols. Propidium iodide (Sigma) was added to a final concentration of 4 µg/mL. Cells were then incubated for a further 30 minutes and data was acquired on a BD FacsCanto II or ImageStream MK II flow cytometer (Amnis; EMD Millipore).

2.11.3 Phagocytosis

2.11.3.1 Light microscopy

Latex beads (3 mm; Polysciences) were added at ratios of 1:1, 5:1 and 10:1 (bead: cell) to $1x10^5$ cells in 24 well plates and incubated at 18 °C for the specified times. Cells were stained by Hema3 stain set (Fisher Scientific) and counted by light microscopy. Phagocytic index was calculated based on the number of beads internalized per phagocyte.

2.11.3.2 ImageStream

Latex beads (3 mm; Polysciences), *E. coli* DH5a-GFP, zymosan-FITC, or apoptotic cells were added to cells at various ratios (depending on experiment) and incubated for the specified times. Following incubation the cells were washed twice in $1xPBS^{-/-}$ and fixed in 1% formaldehyde at 4 °C overnight. Phagocytic index was calculated based on the number of particles internalized per phagocyte. Data was acquired on an ImageStream IS100 or MKII flow cytometer and analyzed using INSPIRE

software.

2.11.4 Respiratory burst

Respiratory burst was used as a cellular marker to examine inflammatory state, by taking advantage of the molecule dihydrorhodamine (DHR), which passively diffuses across the cell membrane. When oxidized inside the cell, it becomes rhodamine and fluoresces. This allowed for the analysis of the reactive oxygen species production within the cell. This assay was performed as previously described with minor modifications (Rieger et al., 2012). The cells were harvested and collected into 5-mL polystyrene, round bottom tubes (BD Falcon). Dihydrorhodamine (DHR; Molecular Probes) was added to the cells at a final concentration of 10 mM and incubated for 5 minutes to allow the cells to take up the DHR. PMA (Sigma-Aldrich) was then added at a final concentration of 100 ng/ml. The cells were further incubated for 30 minutes to allow oxidation of the DHR. All samples were appropriately staggered with respect to timing to accommodate the transient state of oxidized DHR fluorescence. Live cells were gated according to the forward scatter and side scatter parameters. DHR fluorescence was detected in the FITC channel, with positive cells having a shift >1 log compared with the unstimulated controls.

2.11.5 Total killing assay

Neutrophils were isolated as described above. Cells ($5x10^5$) were then seeded into 5 mL polystyrene round bottom tubes at a 1:1 ratio with *Aeromonas veronii*. At each respective time point all cells were lysed in distilled water for 45 seconds and then

returned to isotonic conditions with 10x $PBS^{-/-}$. To determine the appropriate time and conditions for lysis cells were observed under microscopy. Following lysis, the suspension was serially diluted and drip plated on TSA drip plates. The following day, the number of CFUs per row was counted and the amount of viable bacteria was determined.

2.11.6 Transwell assay

To determine the role of soluble factors in mediating responses, transwells (0.4 μm pore size, Corning) were used. Transwells permit the free flow of soluble factors while preventing cell transmigration and cellular interactions across the barrier. Neutrophils isolated at 18 hours and 48 hours post injection were combined with various stimuli (apoptotic cells or zymosan at a 3:1 particle to neutrophil ratio) into the top well of a 6-well plate in 1 mL of complete media, while the bottom of the well was used for responding macrophages. Cells were then incubated for 2 hours at 18 °C. Following incubation, responding cells were harvested and ROS production was assayed with DHR and phagocytosis was analyzed as described above on the ImageStream MK II flow cytometer.

2.11.7 Lipid mediators

2.11.7.1 Lipid mediator production

Commercial ELISA kits were purchased from Oxford Biomedical Research. This kit operates via competition between the commercial enzyme conjugate with HRP and the lipoxin A_4 in the isolated sample for a limited number of antibody binding sites on the

plate. ELISAs were performed as previously described (Levy et al., 2001), with minor modifications. In brief, goldfish were injected intraperitoneally with 2.5 mg of zymosan. At each time point, neutrophils were isolated, incubated for 1 hour in peritoneal exudate, and then resuspended in 1x $PBS^{-/-}$. Samples were then analyzed for the presence of Leukotriene B_4 (LTB_4) or Lipoxin A_4 (LXA_4) (Oxford Biomedical Research), according to the manufacturer's protocols. In short, 50 µL aliquots were loaded in the microplate in duplicate, followed by 50 µL of LTB4-HRP or LXA4-HRP and incubated for 1 hour. After incubation, the microplate was washed 3 times with wash buffer. After washing, 150 µL of 3,3',5,5'-tetramethylbenzidine substrate was added to each well and incubated for 15 minutes. The microplate was then read at 650 nm on a SpectraMax M2 plate reader (Molecular Devices). The data was analyzed by determining the ratio of sample binding to the percentage of maximal binding (blank well with control lipid) according to a standard curve.

2.11.7.2 Lipid stimulation on primary neutrophils and macrophages

Neutrophils were isolated from either the hematopoietic tissue (kidney) or peritoneal cavity (18 hpi), as described previously. Isolated neutrophils were seeded into 24-well plates at $5x10^5$. PKM cultures were grown for 7 days and seeded into 24-well plates at the same density. Each well was treated with either LTB4 (10, 42.5, or 75 pg) or LXA4 (90, 190, or 290 pg) for 2 hours. After stimulation, the cells were removed from each well and placed in 5-ml polystyrene round bottom tubes (BD Falcon). Respiratory burst was measured via the percentage of DHR+ fluorescence on a FACSCanto II flow cytometer (BD Biosciences). To examine the internalization of apoptotic neutrophils after

stimulation stained apoptotic neutrophils were added at a 3:1 ratio and incubated for another 2 hours. Macrophages were then fixed overnight in 1% formaldehyde at 4 °C. The samples were then acquired on an ImageStream MKII flow cytometer.

2.11.8 Chemotaxis assay

The chemotaxis assay was performed using blind well leucite chemotaxis chambers (Nucleoprobe Corp.). Zymosan, *A. veronii*, or *M. fortuitum* at varying numbers was applied to the lower wells of a leucite chemotaxis chamber and overlaid with polycarbonate membrane filters (5 µm). To the upper well of the chemotaxis apparatus, $5x10^5$ neutrophils were added and incubated for 1 hour at 20 °C. The chemokinesis (ChK) control allowed for control of neutrophil chemotaxis to an inflammatory stimulus in the absence of a chemotactic gradient and consisted of the addition of zymosan, *A. veronii*, or *M. fortuitum* in both the upper and lower chambers of the chemotaxis apparatus. Following 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 in methanol for 1 min and stained using Gill's solution 3 (Sigma). Chemotactic activity was determined by counting the total number of cells present in 25 randomly selected fields of view under oil immersion (1000 x magnification).

2.11.9 *In vivo* BrdU proliferation assay

BrdU (eBioscience) was used *in vivo* to determine cellular proliferation within the hematopoietic compartment (kidney) of the goldfish. BrdU (10 mM) was injected into the goldfish peritoneal cavity and allowed to incorporate into tissues for 1 hour. After this

period, the fish were killed and their kidneys removed. Total kidney leukocytes were isolated as described and fixed in 1% formaldehyde for a minimum of 24 h in 5 mL polystyrene round bottom tubes (BD Falcon). The cells were stained according to the manufacturer's specifications. In short, after fixation, the cells were washed once in $1xPBS^{-/-}$ followed by once in flow cytometry buffer (2% FBS in $1xPBS^{-/-}$). The cells were resuspended in 1 mL of BrdU staining buffer and incubated at 4 °C for 1 hour. After incubation, the cells were resuspended in 100 µL flow cytometry staining buffer containing DNase I (30 mg; Sigma-Aldrich) and incubated at 37 °C for 1 hour. The cells were then washed in flow cytometry buffer to remove DNase I. After centrifugation, the cells were resuspended in 100 µL flow cytometry staining buffer containing anti-BrdU FITC antibody and incubated at room temperature for 1 hour. The cells were washed twice in flow cytometry staining buffer. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FACSDiva. A minimum of $5x10^4$ cells was acquired. The cells were gated according to the forward scatter and side scatter parameters. BrdU fluorescence was detected in the FITC channel, with positive cells showing increased fluorescence compared with that of unlabeled controls.

2.12 Statistical analysis

Flow cytometry data was analyzed with FCS Express software v4 (DeNovo Software) or FACSDiva v6.1.3 (BD Biosciences). ImageStream data was analyzed using IDEAS v6 (Amnis Corporation, EMD Millipore). Statistics were performed on Prism 6 software (GraphPad Prism). For statistical analyses involving two comparisons a Students' t-test was preformed, while a one-way ANOVA was used for statistical

analysis involving greater than two comparisons. For post-hoc analyses, a Tukey's test was used when all-pairwise analyses were desired, whereas when comparing to control, a Dunnet's post-hoc test was used. For statistical analyses involving more than two comparisons over time, a two-way ANOVA was used, followed by a Bonferroni post-hoc test.

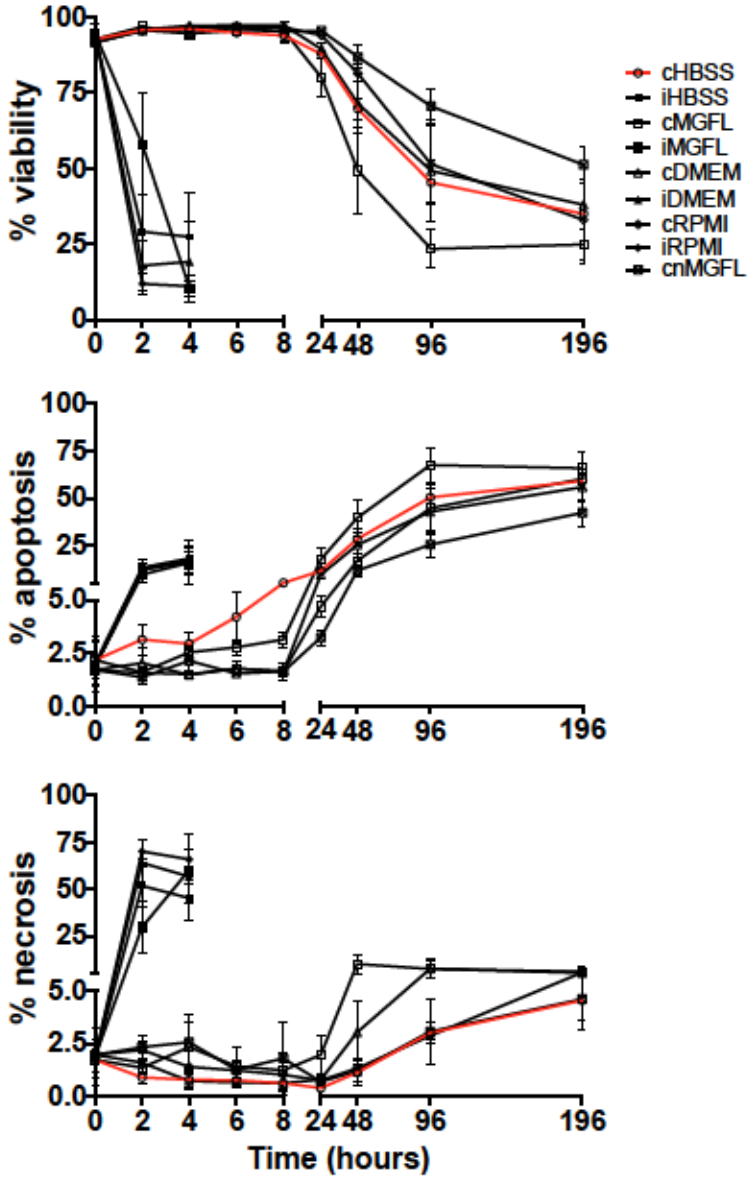


Figure 2.1 Analysis of neutrophil viability

Neutrophils were isolated from goldfish kidneys as described above. Cells were seeded into T25 culture flasks at $4x10^6$ cells per flask. Percent viable, apoptotic, and necrotic was assessed at each time point indicated using AnnexinV/ Propidium iodide staining.

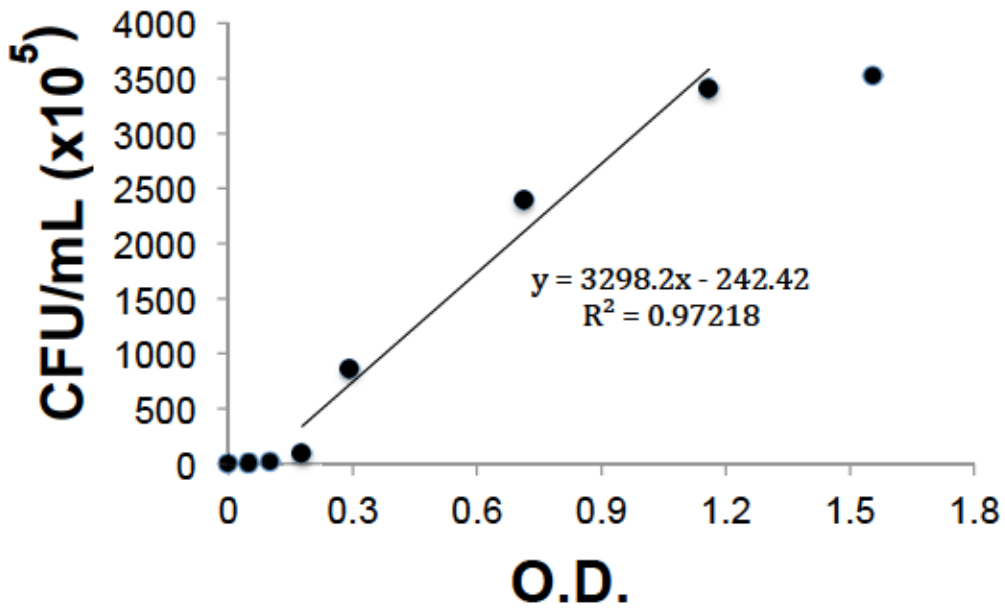


Figure 2.2 Growth curve of *Aeromonas veronii* biovar sobria

Aeromonas veronii was grown for 8 hours at room temperature. Every hour,1 mL aliquot was removed from the culture to determine optical density (OD) and colony forming units (CFU).

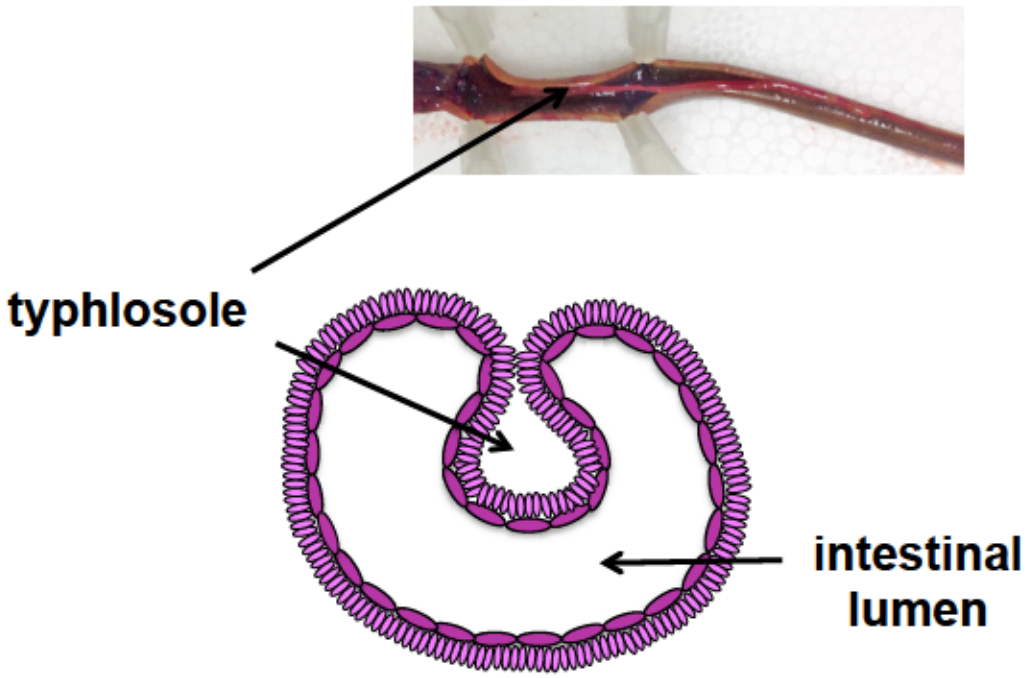


Figure 2.3 Diagram depicting location of lamprey typhlosole

Lampreys were dissected along the central side and the intestine and associated typhlosole tissue were removed. Bottom diagram depicts the location of the typhlosole in relation to the intestinal lumen.

Table 2.1. Carbonate buffer composition

COMPONENT	AMOUNT
0.5 M sodium carbonate buffer*	0.8 mL
0.5 M sodium bicarbonate buffer**	1.7 mL
MilliQ water	top up to 10 mL

* Add 0.529 g of sodium carbonate to 10 mL MilliQ water
** Add 0.420 g of sodium bicarbonate to 10 mL MilliQ water

Set final pH to 9.6

Table 2.2. GFL-15 media composition

COMPONENT	AMOUNT (2L)
Leibovitz's L-15 medium	1 package
Dulbecco's Modified Eagle Medium	1 package

Packages (10 x1L) are purchased from Sigma-Aldrich. Leibovitz's L-15 medium is catalog number 41300039 and Dulbecco's Modified Eagle Medium is catalog number 12100046.

Table 2.3 Components of MGFL-15 medium

COMPONENT	AMOUNT
HEPES	7.00 g
KH_2PO_4	0.688 g
K_2HPO_4	0.57 g
NaOH	0.75 g
$NaHCO_3$	0.34 g
10x Hanks Balanced Salt Solution	80 mL
MEM amino acid solution	25 mL
MEM non-essential amino acid solution	25 mL
Sodium pyruvate	25 mL
MEM vitamin solution	20 mL
Nucleic acid precursor solution	20 mL
L-glutamine	0.5844 g
Insulin	0.01 g
GFL-15*	1000 mL
β-mercaptoethanol	7 µL
MilliQ water	up to 2 L

Table 2.4 Components of nucleic acid solution

COMPONENT	AMOUNT
Adenosine	0.067 g
Cytidine	0.061 g
Hypoxanthine	0.034 g
Thymidine	0.061 g
Uridine	0.061 g
MilliQ water	100 mL

Table 2.5 Components of 10x Hanks balanced salt solution

COMPONENT	AMOUNT
KCl	2.00 g
KH_2PO_4	0.30 g
NaCl	40.00 g
$Na_2HPO_4 \cdot 7H_20$	0.45 g
D-glucose	5.00 g
Phenol red	0.05 g
MilliQ water	top to 500 mL

Table 2.6 Components of 1x neutrophil Hank's balanced salt solution

COMPONENT	AMOUNT (1L)
KCl	0.4 g
KH_2PO_4	0.06 g
NaCl	8.0 g
$Na_2HPO_4 \cdot 7H_20$	0.048 g
D-glucose	1.0 g
MilliQ water	top to 1 L
For $HBSS^{+/+}$ add:	
$CaCl_2$	0.14 g
$MgCL_2 \cdot 6H_20$	0.1 g
$MgSO_4 \cdot 7H_20$	0.1 g

Table 2.7 Primers used to identify *A. veronii* biovar sobria

COMPONENT	SEQUENCE (5'-3')
16S rRNA forward (26)	AGA GTT TGA TCA TGG CTC A
16S rRNA reverse (1391)	GTG TGA CGG GCG GTG TGT A
16S rRNA forward (308)	GCT GGT CTG AGA GGA TGA TC
16S rRNA reverse (556)	CTT TAC GCC CAG TAA TTC CG
gyrB UP3	ACT ACG AGA TCC TGG CCA AG
gyrB UP4	TCC TCC CAG ACC AAG GAC
gyrB UP5r	GCC TTC TTG CTG TAG TCC TCT
gyrB UP6r	GCA GAG TCC CCT TCC ACT ATG

Table 2.8 Primers used in cDNA synthesis

COMPONENT	SEQUENCE (5'-3')
5' oligo	AAG CAG TGG TAT CAA CGC AGA GTA CG
3' CDS poly T	AAG CAG TGG TAT CAA CGC AGA GTA TT
5' PCR	AAG CAG TGG TAT CAA CGC AGA GT

Table 2.9 Primers used in Q-PCR

COMPONENT	SEQUENCE (5'-3')
EF-1α forward	CCG TTG AGA TGC ACC ATG AGT
EF-1α reverse	TTG ACA GAC ACG TTC TTC ACG TT
CXCL-8 forward	CTG AGA GTC GAC GCA TTG GAA
CXCL-8 reverse	TGG TGT CTT TAC AGT GTG AGT TTG G
CCL-1 forward	AAG GTC ACC GAA CCC ATC AG
CCL-1 reverse	TCG TCA CAT GAT GGC CTT CA
TNFα2 forward	TCA TTC CTT ACG ACG GCA TTT
TNFα2 reverse	CAG TCA CGT CAG CCT TGC AG
IL-1β1 forward	GCG CTG CTC AAC TTC ATC TTG
IL-1β-1 reverse	GTG ACA CAT TAA GCG GCT TCA C
IL-10 forward	CAA GGA GCT CCG TTC TGC AT
IL-10 reverse	TCG AGT AAT GGT GCC AAG TCA TCA
TGF-β forward	GTA CAC TAC GGC GGA TTG
TGF-β reverse	CGC TTC GAT TCG CTT TCT CT

Chapter 3. Conservation of divergent pro-inflammatory and homeostatic responses in lamprey and goldfish phagocytes[1]

3.1 Introduction

The immune system has evolved to confer effective protection against infection, driven by continuous interactions between hosts and microbes. The resulting multi-layered system increasingly requires complex cross-regulatory networks. Phagocytosis serves a diverse array of functions from unicellular eukaryotes 'feeding' on bacteria (Pereira-Neves and Benchimol, 2007), to tissue remodeling (Desjardins et al., 2005), immune regulation (Aderem, 2003; Havixbeck et al., 2015; Rieger et al., 2012), and apoptotic cell removal (Maderna and Godson, 2003; Rieger et al., 2012). At the core of these roles, phagocytosis continues to fill increasing roles as an inducer and regulator of host immunity. Internalization of pathogens by phagocytes leads to stimulation of potent killing mechanisms such as the production of reactive oxygen species (ROS) that have evolved to degrade and kill foreign invaders and contribute to downstream adaptive

[1] A portion of this chapter has been previously published in:

Havixbeck JJ, Rieger AM, Wong ME, Wilkie MP, and Barreda DR (2014) Evolutionary Conservation of Divergent Pro-Inflammatory and Homeostatic Responses in Lamprey Phagocytes. PLoS ONE 9(1): e86255.

mechanisms (Dupré-Crochet et al., 2013). Further, the importance for effective internalization and clearance of apoptotic cells is already well established in developmental pathways of early multi-cellular organisms (Franc et al., 1999; Fristrom, 1969; Reddien and Horvitz, 2004). Phagocytic receptors in *Caenorhabditis elegans* (CED-1) and *Drosophila melanogaster* (Draper), for example, drive recognition and internalization of apoptotic corpses and activate downstream processing pathways that are central to morphogenesis and the maintenance of tissue integrity and function (Manaka et al., 2004; Zhou et al., 2001). Further, both CED-1 and Draper appear to play an immunological role as well. Loss of function studies found that CED-1 is required for *C. elegans* immunity to *S. enterica* (Haskins et al., 2008), and a Draper knockdown in *Drosophila* was responsible for impaired migration of hemocytes toward laser induced wounds (Evans et al., 2015).

At the site of infection, mammalian phagocytes effectively shape the environmental milieu for destruction of invading pathogens or resolution of tissue inflammation. Pathogen engagement leads to rapid production of pro-inflammatory mediators including the production of reactive oxygen and nitrogen species (Rieger and Barreda, 2011), release of antimicrobial peptides (Noga et al., 2009), and the secretion of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and IL-1 beta (IL-1β) (Jung et al., 1995). In contrast, internalization of apoptotic cells initiates the shift towards resolution mechanisms that promote tissue repair and a return to homeostasis once the pathogen has been effectively cleared. This is marked by increases in interleukin 10 (IL-10), transforming growth factor beta 1 (TGF-β1), prostaglandin E2 and platelet activating factor (Fadok et al., 1998; Maderna and Godson, 2003; Voll et al., 1997), combined with

decreases in pro-inflammatory mediators, including tumor necrosis factor alpha (TNF-α), IL-6, IL-8, IL-12, IL-17, IL-23, leukotriene C4 and thromboxane B2 (Kim et al., 2004; Maderna and Godson, 2003; Stark et al., 2005).

I previously showed that phagocytes of teleost fish contributed to both pro-inflammatory and anti-inflammatory (resolution) responses at infectious foci (Rieger et al., 2012). Like murine phagocytes, they possessed the capacity to balance between these two seemingly contradictory processes. However, teleost phagocytes displayed significant differences *in vivo* with regards to the level of responsiveness to zymosan and apoptotic bodies, the identity of leukocytes infiltrating the infectious site, their rate of infiltration, and the kinetics and strength of resulting antimicrobial responses (Rieger et al., 2012). The striking evolutionary differences observed in inflammatory control between mice and goldfish provided a platform to investigate these differences down the evolutionary scale in a primordial vertebrate.

In this study, I compared the effects of inflammatory and/ or homeostatic stimuli on the induction and regulation of pro-inflammatory responses in goldfish (*Carassius auratus*) and sea lamprey (*Petromyzon marinus*). As one of the earliest vertebrates (divergence of ~450-500 mya), along with hagfish (Myxinidae), sea lampreys were an appropriate model to investigate the conservation of this dichotomy. Through the use of zymosan, a pro-inflammatory stimulus known to induce production of reactive intermediates (Ariel et al., 2006; Bannenberg et al., 2005; Brown et al., 2002; Cash et al., 2009; Chadzinska et al., 2008; Kolaczkowska et al., 2008; Schwab et al., 2007), the impact of apoptotic cells on the production of reactive oxygen species was examined. Interestingly, I found notable differences between goldfish and lamprey ROS responses

in phagocytes that had internalized both zymosan and apoptotic bodies, though responses in cells that had internalized only zymosan or only apoptotic bodies were largely conserved. This work underscores the importance of phagocytosis as a phylogenetically ancient process essential to the innate immune response.

3.2 Results

3.2.1 Goldfish phagocytes display a greater capacity for the internalization of pro-inflammatory particles compared to lamprey phagocytes

As a first step in the characterization of differences between the contributions of agnathan (jawless fish) and teleost (bony fish) phagocytes to the control of inflammation, I compared the phagocytic capacity of primary leukocytes from sea lamprey typhlosole and goldfish kidney. These corresponded to the primary hematopoietic tissues of the animals examined and provided sufficient numbers of their primary phagocyte populations for *ex vivo* examination (Jordan and Speidel, 1930; Percy and Potter, 1976; Piavis and Hiatt, 1971; Zapata, 1979). Phagocytosis of three commonly used model particles was examined: 1) 3 μm latex beads; 2) *E. coli;* and 3) zymosan (Figure 1A). Phagocytosis was assessed by imaging flow cytometry, which allowed discrimination of bound and internalized particles [Figure 3.1B; (Rieger et al., 2012)]. From the outset, my experiments indicated differences in the efficiency of phagocytosis for goldfish and lamprey leukocytes *ex vivo*. Two hours was sufficient to examine basal levels of phagocytosis in goldfish (Figure 3.1A). In contrast, 6 hours were required to achieve equivalent levels of phagocytosis among sea lamprey leukocytes. The time needed was

further corroborated by a conventional light microscopy phagocytosis assay (Figure 3.2), and the determination of optimal respiratory burst levels (Figure 3.3). However, results were not consistent across the three pro-inflammatory particles examined. Lamprey leukocytes continued to display lower levels of zymosan phagocytosis than goldfish even after six hours of incubation (Figure 3.1A, bottom row).

A greater capacity for phagocytosis of zymosan suggested an increased ability of goldfish phagocytes to effectively mount antimicrobial defenses against fungal pathogens. I wondered whether this difference was associated with differences in the relative abundance of distinct phagocyte groups within the hematopoietic leukocyte pool and/or differences in their phagocytic capacity compared to those in lamprey (Figure 3.4). I focused on the granulocyte, monocyte, and macrophage populations as the classical professional phagocytes of fish, and the lymphocyte population as the newest members of the phagocyte group (Li et al., 2006; Øverland et al., 2010; Zhang et al., 2010). Although leukocyte populations in lamprey displayed similar cellular characteristics to those of goldfish based on size, morphology, and internal complexity (Figure 3.4), the range of reagents available to help define various subsets is still limited. As such, they remain as lymphocyte-like, granulocyte-like, monocyte-like and macrophage-like cells. For goldfish, granulocytes/monocytes represented the majority of the leukocytes isolated from kidney hematopoietic tissues (73%), with smaller contributions from macrophage and lymphocyte populations (19% and 9% respectively; Figure 3.5A). For the lamprey, lymphocyte-like cells comprised the majority of the total typhlosole leukocyte pool (50%; Figure 3.5A, 6 h). Granulocyte/monocyte and macrophage-like cells followed and contributed approximately 32% and 18%, respectively. Evaluation of phagocytosis

indicated that macrophage and macrophage-like cells were the primary mediators of zymosan internalization in goldfish and lamprey, respectively (Figure 3.5B). However, these cells represented different proportions of the hematopoietic phagocyte pool that internalized zymosan in goldfish and lamprey (11% and 35%, respectively; Figure 3.5C). Further, they also displayed a differential ability to internalize zymosan (28% and 17% phagocytosis, respectively; Figure 3.5B). Despite their lower abundance within the hematopoietic tissues examined, goldfish macrophages showed a marked greater capacity to internalize zymosan, which contributed to overall greater levels of zymosan phagocytosis for goldfish kidney leukocytes (16% in goldfish versus 6% in lampreys; Figure 3.1A). The increased relative efficacy for zymosan internalization in goldfish was not limited to the macrophage population. Despite their lower contribution to the internalization of zymosan, monocytes/granulocytes also displayed a four-fold greater capacity for zymosan phagocytosis in goldfish compared to the monocyte/granulocyte-like pool in lamprey (8% versus 2%, respectively; Figure 3.5B). Thus, professional phagocytes (macrophages, monocytes, neutrophils) are the primary contributors to the internalization of zymosan in both goldfish and lamprey hematopoietic leukocytes; however, teleost phagocytes displayed a significantly greater ability for zymosan phagocytosis when compared to their lamprey counterparts. Of note, I observed low levels of phagocytosis among goldfish lymphocytes and lamprey lymphocyte-like cells in these experiments (0.27% and 0.8%, respectively; Figure 3.5B).

3.2.2 Goldfish phagocytes display prominent respiratory burst responses, even when apoptotic cells are internalized

Our lab previously determined that teleost phagocytes, like those of mice, displayed the capacity for divergent pro-inflammatory and homeostatic responses, following internalization of zymosan and apoptotic cells, respectively (Rieger et al., 2012). Further, internalization of zymosan, apoptotic cells, or both by individual phagocytes contributed differentially to the modulation of antimicrobial inflammatory responses (Rieger et al., 2012). Accordingly, I investigated whether or not a similar mechanism of inflammatory control at the level of the individual phagocyte was already displayed in the lamprey. As expected, zymosan internalization resulted in an increase in the level of the respiratory burst response in both goldfish and lamprey (Figure 3.6A). Conversely, internalization of apoptotic cells led to a significant reduction in ROS production in both species. Thus, the results indicated that sea lamprey phagocytes were already capable of mediating divergent pro- and anti-inflammatory responses. Interestingly, when I analyzed the level of ROS production in cells that had internalized both zymosan and apoptotic cells, I saw a striking difference between goldfish and lamprey phagocytes. Goldfish phagocytes that had internalized both zymosan and apoptotic cells showed equivalent levels of ROS production compared to those that internalized zymosan alone ($p = 0.88$; Figure 3.6A). In sharp contrast, lamprey phagocytes that internalized apoptotic cells displayed low levels of ROS production even when zymosan was co-internalized (Figure 3.6A). Given that the proportion of phagocytes internalizing zymosan, apoptotic cells, or both were consistent between

goldfish and lampreys (Figure 3.6B), this may reflect a greater drive for induction of inflammatory antimicrobial responses in goldfish in response to zymosan.

Examination of the Zym + AC group presented an opportunity to examine the mechanism(s) by which individual phagocytes regulate inflammatory processes following internalization of pro-inflammatory or homeostatic particles. One possibility for the greater capacity of goldfish phagocytes to display robust respiratory burst responses was that individual goldfish phagocytes internalized a greater number of zymosan particles. Alternatively, they may have internalized fewer apoptotic cells. However, the results showed that goldfish and lamprey primary hematopoietic tissue phagocytes did not display a differential capacity to internalize zymosan or apoptotic cells (Figure 3.6C). Instead, subsequent analysis revealed that goldfish phagocytes were more responsive to zymosan internalization than those in lamprey, as evidenced by the relative strength of respiratory burst responses (Figure 3.6D). When the number of internalized apoptotic cells was kept constant, relative increases in the internalization of zymosan (1 apoptotic cell with 1-2, 3-4, or >5 zymosan particles) translated to a dramatic increase in ROS production in goldfish phagocytes (Figure 3.6D). In contrast, lamprey phagocytes displayed much less prominent respiratory burst responses in all groups examined when compared to that of zymosan alone. Thus, internalization of even a single apoptotic cell impaired the ability of lamprey, but not goldfish phagocytes, to mount robust pro-inflammatory respiratory burst antimicrobial responses when a phagocyte encountered both pro-inflammatory and homeostatic particles, as would commonly occur within an infection site.

Importantly, the increased strength in goldfish phagocyte respiratory burst responses described above did not preclude their effective inhibition by homeostatic stimuli. Pre-incubation of primary leukocytes with apoptotic cells (-2 h) was sufficient to impair the respiratory burst response of goldfish leukocytes (Zym + AC group; Figure 3.7A). The relative proportion of phagocytes internalizing zymosan, apoptotic cells, or both remained consistent between goldfish and lamprey (Figure 3.7B), as was observed when both particles were added at the same time (Figure 3.7B). Further, the phagocytic index of the Zym + AC population remained constant in both organisms even when apoptotic cells were added two hours prior to zymosan (Figure 3.7C). Thus, despite the greater ability of goldfish phagocytes to mount robust respiratory burst responses to zymosan, a model develops whereby goldfish phagocytes can remain as important contributors to the resolution phase of inflammation following early induction of potent antimicrobial pro-inflammatory mechanisms.

The experiments presented also showcase the responses of individual phagocytes amidst a microenvironment that contains mixed populations of phagocytes that have internalized zymosan, apoptotic cells, both particle types or none. Both goldfish and lamprey displayed marked compartmentalization in the activation of respiratory burst responses among phagocyte subgroups following the internalization of pro-inflammatory and/or homeostatic particles. As such, these results highlight the importance of intrinsic mechanisms of inflammation control at the level of the individual phagocyte in both of these animal groups.

3.2.3 Decreased goldfish phagocyte sensitivity to apoptotic cells contributes to pronounced antimicrobial ROS production but decreased efficacy in leukocyte homeostatic responses

Examination of ROS production amidst mixed cellular populations allowed us to assess the broader impact of phagocyte responses on the total leukocyte pool. Specifically, I sought to determine how the differential sensitivity to pro-inflammatory and homeostatic particles for goldfish and lamprey phagocytes identified above contributed to the control of antimicrobial respiratory burst responses. Varying ratios of zymosan to apoptotic cells (pro-inflammatory and homeostatic particles, respectively) were co-incubated with goldfish and lamprey total hematopoietic leukocyte isolates prior to evaluation of respiratory burst responses (Figure 3.8). The goal was to mimic the natural shift that occurs at an infection site, where phagocytes initially encounter greater proportions of pro-inflammatory particles (pathogens) followed by increasing proportions of homeostatic particles (apoptotic cells), which ultimately contribute to the activation of tissue repair mechanisms and a return to homeostasis (Devitt and Marshall, 2011; Erwig and Henson, 2007; Maderna and Godson, 2003). Consistent with a higher capacity for pro-inflammatory ROS production, goldfish leukocytes displayed greater efficacy in the induction of respiratory burst responses than those of lamprey. However, examination of decreasing Zym to AC ratios showed that this further translated into a lower sensitivity to apoptotic cell homeostatic signals in goldfish compared to lamprey. Goldfish leukocytes required a three-fold greater amount of apoptotic cells than zymosan (1:3 Zym to AC group, Figure 3.8) to reach basal levels of ROS production. In contrast, lamprey leukocytes reached these levels even when zymosan out-numbered apoptotic cells three

to one (i.e. not statistically significant to basal levels of ROS production). For the experiments, basal levels of ROS production (grey dashed line, Figure 3.8) were derived from those phagocytes that exclusively internalized apoptotic cells (Figure 3.8A). As such, the results suggest that the reduced sensitivity of goldfish phagocytes to apoptotic cells translates to overall greater capacity for induction of antimicrobial respiratory burst responses but also a decreased efficacy in leukocyte homeostatic mechanisms that attenuate this pro-inflammatory process.

3.2.4 Lamprey and goldfish neutrophils do not internalize cyclohexamide induced apoptotic cells

It has previously been demonstrated that mammalian, but not teleost, neutrophils are capable of internalizing apoptotic cells (Esmann et al., 2010; Rieger et al., 2012). In light of the results indicating that lamprey phagocytes respond at a higher efficacy to homeostatic stimuli (apoptotic cells), I wanted to determine if lamprey neutrophils were capable of internalizing apoptotic cells. Interestingly, although total lamprey phagocytes respond to homeostatic stimuli with a higher sensitivity, naïve typhlosole neutrophils are unable to internalize apoptotic cells, similarly to naïve goldfish neutrophils isolated from the hematopoietic kidney (Figure 3.9). As such, the results suggest that the increased sensitivity of lamprey phagocytes to apoptotic cells may be due to the monocyte/macrophage populations, or, alternatively due to surface binding events leading to downstream homeostatic responses.

3.3 Discussion

Phagocytosis is a phylogenetically ancient innate defense strategy that has served as an important platform for the evolution of mechanisms of inflammation control (Bianchi et al., 2008; Erwig and Henson, 2007; Fullard et al., 2009; Maderna and Godson, 2003; Rieger et al., 2012). Previous studies from our lab focused on the divergent responses of teleost and murine phagocytes following internalization of pathogen-derived and homeostatic particles (Rieger et al., 2012). In the present study, I show that individual phagocytes of the jawless vertebrate *Petromyzon marinus* (sea lamprey), like those of teleost fish and mice, display the capacity for divergent pro-inflammatory and homeostatic responses. Phagocytes isolated from sea lamprey typhlosole and goldfish kidney hematopoietic tissues were able to internalize a range of particles including latex beads, *E. coli*, and zymosan. However, goldfish leukocytes displayed greater efficiency in the internalization of these pro-inflammatory particles. Examination of phagocytic subsets at the single cell level indicated that, for zymosan, macrophages displayed the greatest capacity of internalization despite representing a significantly lower proportion of the phagocytes within the hematopoietic leukocyte pool. Thus, although professional phagocytes (macrophages, monocytes, neutrophils) were important contributors to the internalization of zymosan in both goldfish and lamprey hematopoietic leukocytes, teleost phagocytes displayed a significantly greater ability for zymosan phagocytosis when compared to their agnathan counterparts. The low levels of phagocytosis observed among goldfish lymphocytes and lamprey lymphocyte-like cells in these experiments (0.27% and 0.8%, respectively) may stem from their limitation for internalization of larger particles (2.5 - 3 μm for zymosan) and not an overall inability for

phagocytosis. Alternatively, this may be associated with a reduced capacity to interact with zymosan. Indeed, evaluation of the capacity for *E. coli* internalization among the hematopoietic leukocyte subsets examined showed increased levels of phagocytosis among lamprey lymphocyte-like cells when compared to zymosan. Whereas 0.8% of lamprey lymphocyte-like cells showed zymosan internalization under the experimental conditions tested, 2.3% showed *E. coli* uptake (data not shown). As lymphocyte-like cells corresponded to approximately 50% of the leukocyte population examined in lamprey, lymphocyte-like cells accounted for ~13% of *E. coli* internalization. This indicates a significant contribution by these cells to bacterial phagocytosis and potential clearance. Interestingly, this is far from the first time lymphocyte-like cells have been implicated in the uptake of particles. Bacteria internalized by trout phagocytic IgM^+ cells induced phagolysosome formation and the induction of downstream killing mechanisms (Li et al., 2006). The role of phagocytic lymphocytes extends even further, with a considerable portion of B cells in *X. laevis* also displaying phagocytic capacities (Li et al., 2006). Collectively, it is conceivable that specific subsets of lymphocyte populations play a large role in the clearance of bacterial pathogens. Further, as new findings are presented, the strict division of leukocytes under the innate or adaptive arms of the immune response becomes blurred.

Although I found some conservation of phagocyte functional responses between lamprey and goldfish, I also saw significant differences in the level and control of these responses to pro-inflammatory and homeostatic stimuli. Goldfish phagocytes that had internalized both zymosan and apoptotic cells showed ROS levels similar to those induced in cells that internalized only zymosan. In sharp contrast, lamprey phagocytes

that internalized both stimuli displayed basal levels of ROS production. Goldfish and lamprey primary hematopoietic tissue phagocytes did not display a differential capacity to internalize zymosan or apoptotic cells - the phagocytic index of each particle was similar in phagocytes derived from each animal. Instead, the results suggest that goldfish phagocytes are more responsive to internalized zymosan than those in lamprey based on the relative strength of respiratory burst responses observed. Internalization of even a single apoptotic cell impaired the ability of lamprey, but not goldfish phagocytes, to mount robust pro-inflammatory respiratory burst antimicrobial responses when a phagocyte encountered both pro-inflammatory and homeostatic particles, as would commonly occur within an infection site. Importantly, priming goldfish and lamprey leukocytes in an anti-inflammatory environment (pre-incubation with apoptotic cells) resulted in similar responses in both animal groups. This was particularly relevant in the Zym + AC group, where the suppression of respiratory burst responses was now observed in both goldfish and lamprey, in contrast to experiments where both particles were added at the same time. As such, the results suggest that goldfish phagocytes remain as central contributors to the resolution phase of inflammation, even though they showcased an improved ability to induce strong antimicrobial inflammatory responses.

Following an infectious challenge, pathogen load and the number of apoptotic cells vary at each point along the inflammatory process. By altering the density of zymosan to apoptotic cells I was able to mimic this natural progression. Analysis of ROS production among mixed cell populations allowed us to assess the broader impact of phagocyte responses on the total leukocyte pool. Goldfish leukocytes required three times the number of apoptotic cells to zymosan to return to basal levels of ROS, whereas

phagocytes from the sea lamprey reached basal levels when zymosan outnumbered apoptotic cells three to one. Consistent with an increased capacity for antimicrobial pro-inflammatory responses in goldfish, the results suggested a reduced sensitivity of their phagocytes to apoptotic cell homeostatic signals, and a greater potency for ROS production compared to lamprey phagocytes following zymosan stimulation. It remains to be determined if these features are shared across the range of potential pathogenic challenges (bacterial, fungal, viral, parasitic) and whether they are consistent across all phagocyte subsets. It is possible that each phagocyte population (e.g. macrophage, monocyte, neutrophil) may display distinct abilities to take up zymosan or apoptotic cells, or that the capacity of apoptotic cells to inhibit the ROS potential for each phagocyte population is different. As such, the overall capacity to elicit or inhibit ROS responses may depend on the type of phagocyte that is primarily present at that site of infection or injury, and their relative contributions to this control. Similarly, it remains to be determined if these features are part of a broadly used strategy for the regulation of phagocyte-driven inflammatory processes beyond ROS antimicrobial responses.

A reduced sensitivity of goldfish phagocytes to apoptotic cells coupled to greater potency for ROS production would presumably translate into increased efficacy for killing of invading pathogens by respiratory burst responses. However, a decreased efficacy in apoptotic cell-driven phagocyte mechanisms that attenuate this pro-inflammatory process could come at a cost unless complementary regulatory strategies are developed to ensure continued maintenance of host integrity. Collectively, the results suggest an evolving contribution of intrinsic phagocyte mechanisms to control of inflammation, and illustrate one effective strategy that allows for increased

responsiveness against invading pathogens while ensuring continued participation in its resolution phase. Importantly, future studies should expand on the contributions of phylogeny and ontogeny to the relative sensitivities of phagocytes to pro-inflammatory and homeostatic signals. Differing life cycles pose unique physiological challenges that drive the development of novel strategies for inflammation control. For example, during the first 4-6 years of their life, larval sea lampreys burrow in tributary sediment where they interact with their environment as blind filter feeders (Morkert et al., 1998). Subsequently, these lampreys undergo an 8-month metamorphosis period, where tissue loss and reorganization is accompanied by extensive cell death (Youson, 2003). Finally, in their 12-month adult stage, the parasitic nature of adult lampreys is likely to set unique requirements for induction and control of inflammatory reactions, given that a compromise must be struck between the continued surveillance of natural infections while ensuring that sustained immune competence does not impinge on the fragile host-parasite relationship. One cannot debate the likely implications of ontogeny in defining the numerous roles of phagocytes without also discussing phylogeny. As eukaryotes continue to evolve into more and more complex species, the complexities of our immune system and host defense must evolve as well. This increase in complexity leads to a two-fold problem for phagocytes, 1) they are now required to maintain a more complex organism at homeostasis; and 2) they are likely to encounter an increasing number of potential pathogens.

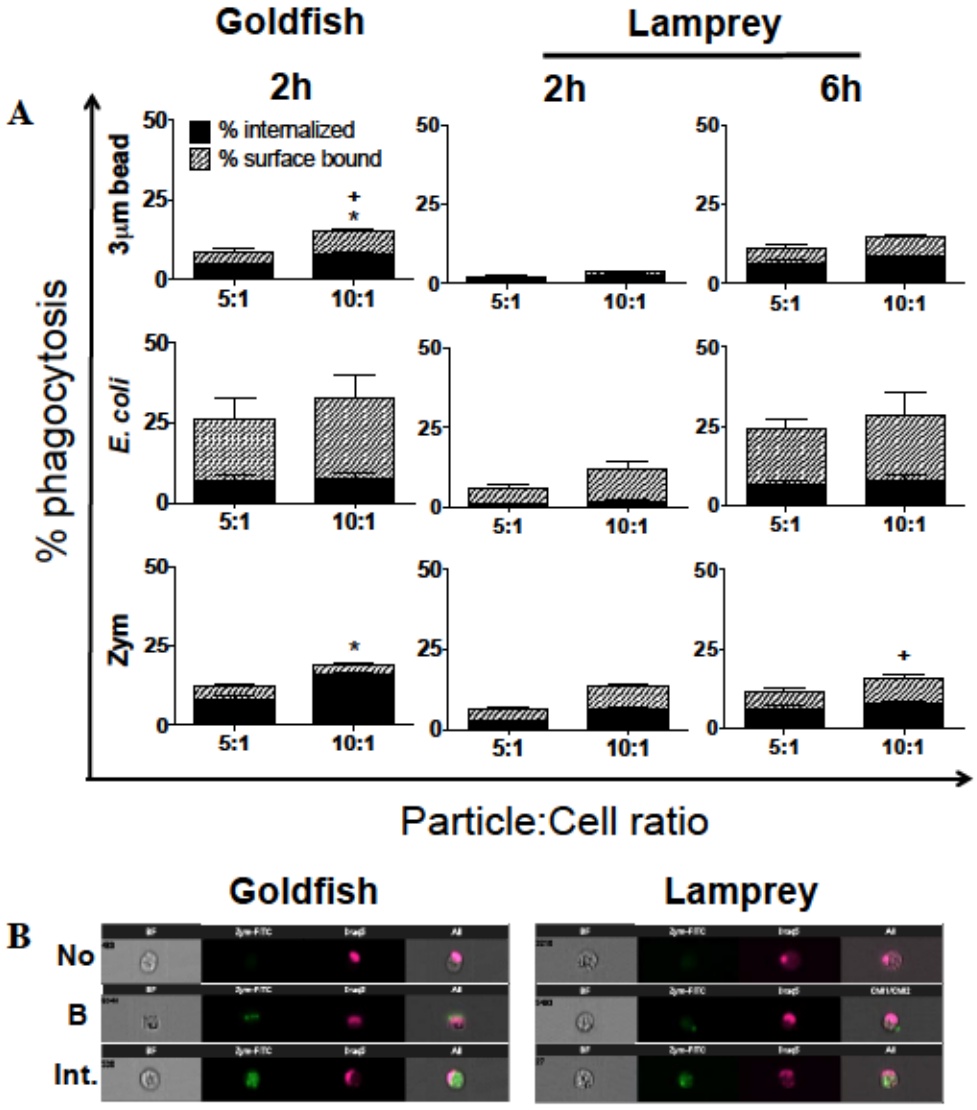


Figure 3.1. Phagocytosis of different target particles by goldfish and lamprey primary leukocytes.

(A) Goldfish primary kidney leukocytes (PKL) or lamprey primary typhlosole leukocytes (PTL) were incubated with 3 μm YG latex beads, *E. coli* DH5α-GFP, or zymosan-FITC at the indicated concentrations for the specified times. Cells were then fixed and phagocytosis was quantified by flow cytometry. Grey bars represent percent internalized. Hatched white bars represent percent surface bound. For all n=4, over 2 examined over a minimum of two independent experiments. * $p<0.05$ for % internalized, + $p<0.05$ for % surface bound- between 10:1 and 5:1 particle to cell ratios in each graph. (B) Representative images of no internalization (No), surface bound (B), and internalized beads (Int.) from ImageStream MkII flow cytometer (Amnis).

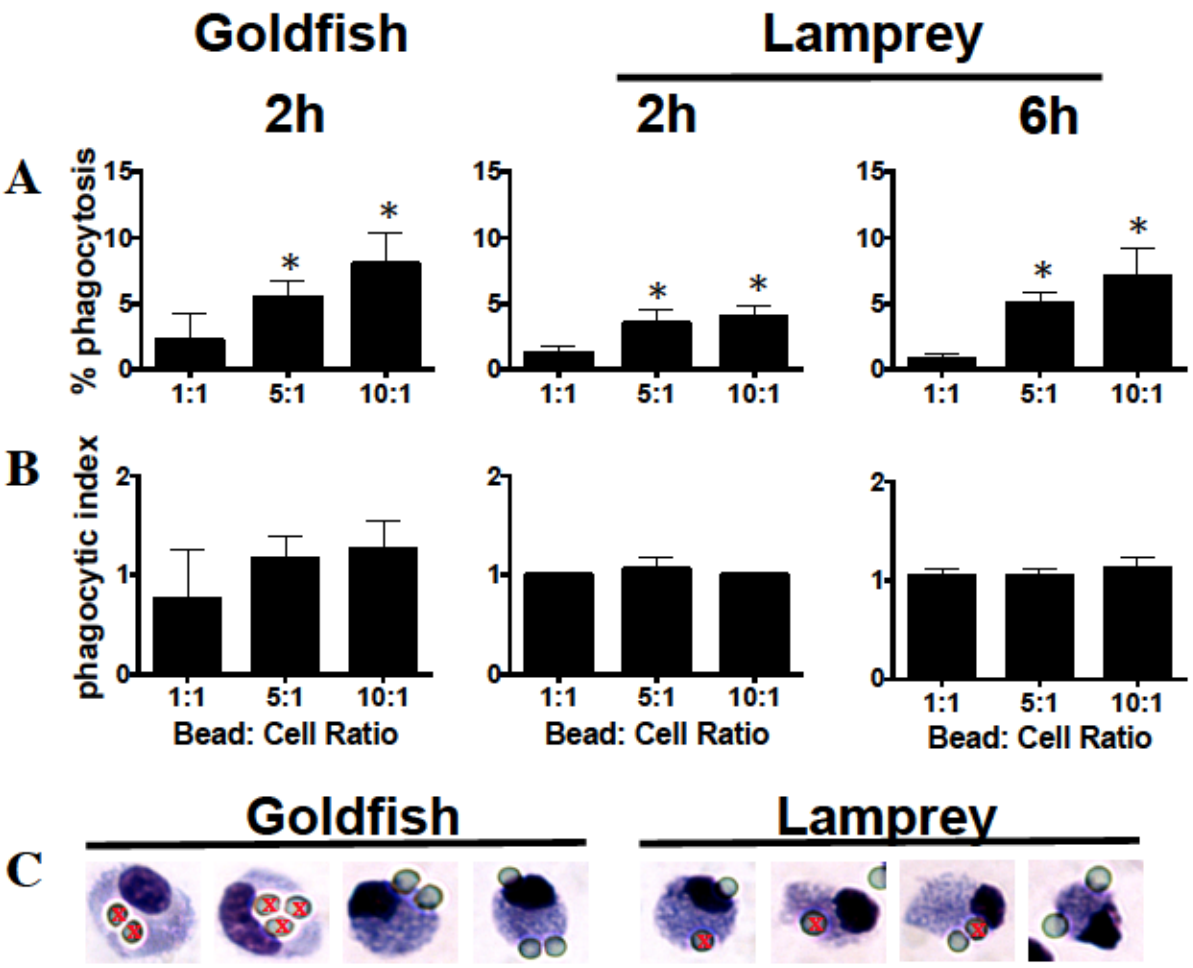


Figure 3.2. Comparative analysis of goldfish and lamprey primary leukocyte phagocytosis by light microscopy.

(A) Goldfish primary kidney leukocytes (PKLs) or lamprey primary typhlosole leukocytes (PTLs) were plated in a 6-well plate and incubated with 3 μm latex beads at the indicated concentrations for the specified times. Phagocytosis was quantified by light microscopy at 100x magnification. (B) Phagocytic index represents the average number of beads internalized per phagocytic cell in the sample. (C) Representative images are of positive phagocytosis (internalized beads marked with x) and surface bound beads at 100x magnification. For all n=4 animals examined over a minimum of two independent experiments, * $p<0.05$.

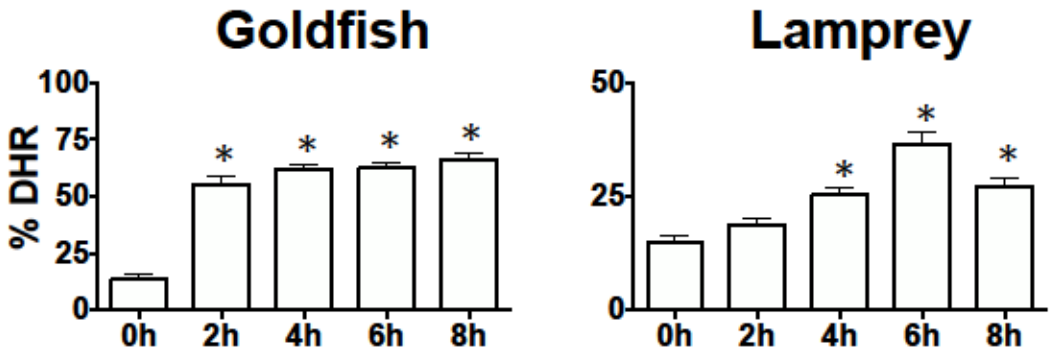


Figure 3.3. Kinetics of goldfish PKL and Lamprey PTL activation as measured by ROS production.

Goldfish PKL and lamprey PTL were incubated with zymosan (5:1 ratio) for the indicated times. Respiratory burst was measured in the total PKL and PTL population. For all n=4, examined over a minimum of two independent experiments. * $p<0.05$ compared to 0 h.

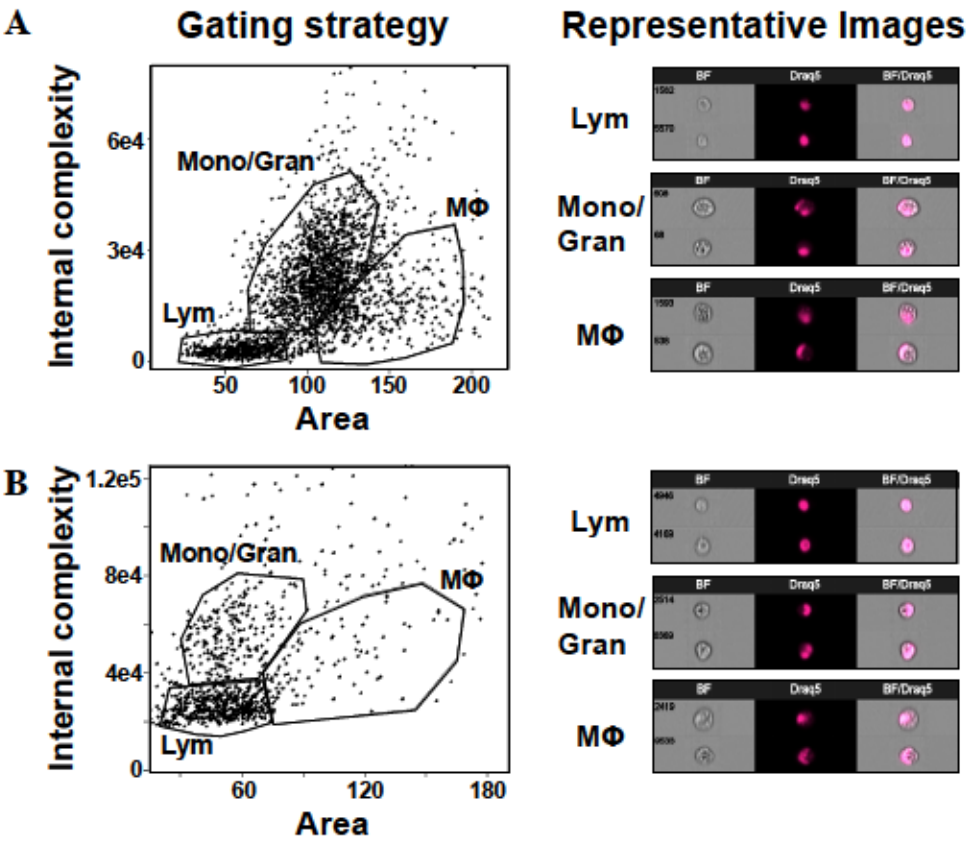


Figure 3.4. Gating strategy for cell subpopulations isolated from hematopoietic tissues.

(A) Primary hematopoietic leukocytes isolated from goldfish kidney and representative images of cells from within each gate. (B) Primary hematopoietic leukocytes isolated from lamprey typhlosole and representative images of cells from within each gate. Cell populations were determined based on internal complexity (dark field) and area. L- lymphocytes; M/G- monocytes and granulocytes; Mac- macrophage.

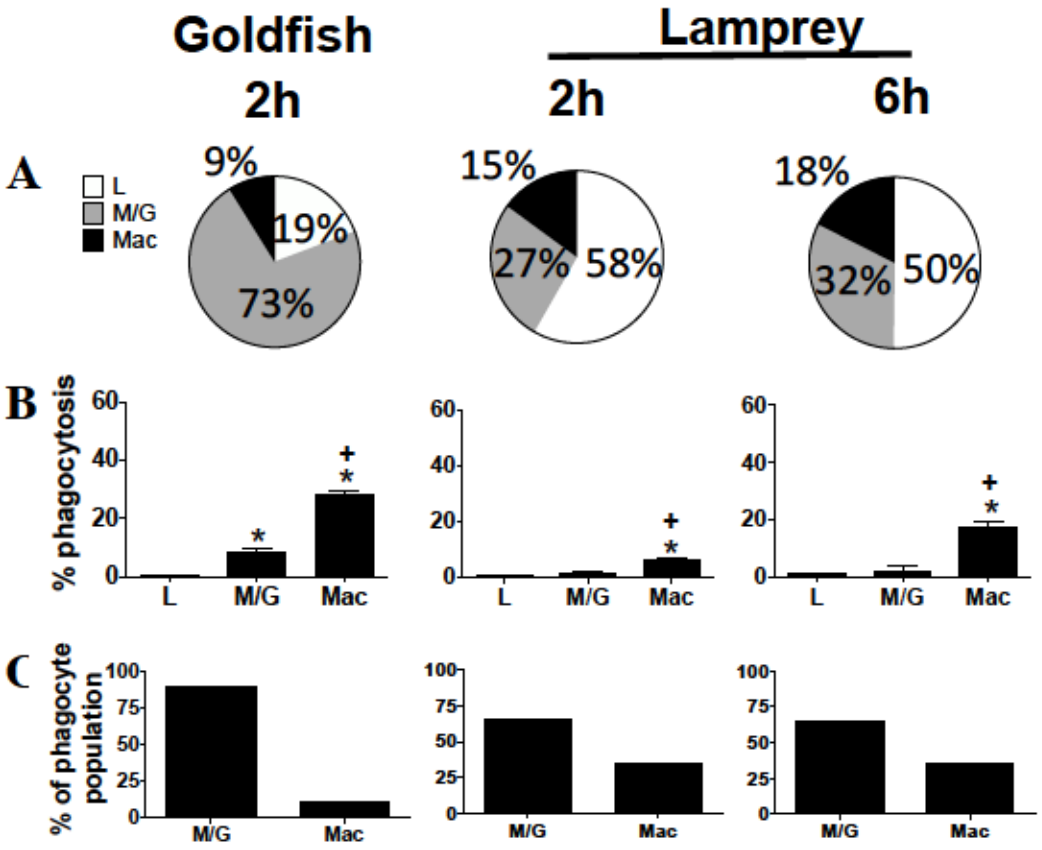
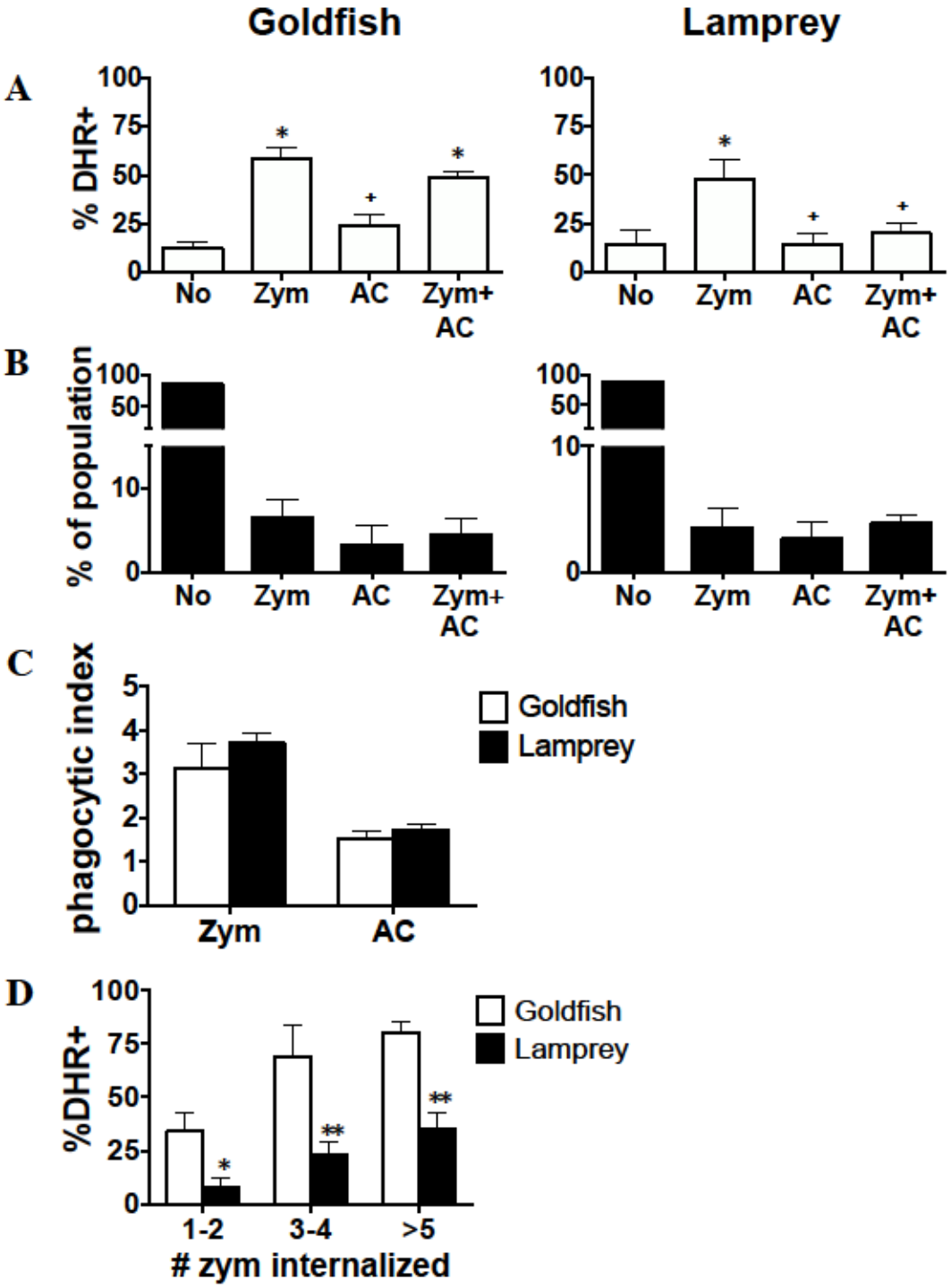


Figure 3.5. Macrophages are the dominant phagocyte of goldfish and lamprey primary hematopoietic tissues.

A) Goldfish PKL and lamprey PTL were incubated with zymosan (5:1 ratio) for the specified times. The total leukocyte pool was broken down into cellular subpopulations based on morphology and flow cytometry forward and side scatter parameters. (B) The percent of phagocytic cells from each subpopulation previously determined in (A). * $p<0.05$ compared to lymphocytes; + $p<0.05$ compared to monocytes/granulocytes. (C) The percent of monocytes/ granulocytes and macrophages that make up the professional population of phagocytes. For all n=4, examined over a minimum of two independent experiments.


Goldfish
Lamprey
A
% DHR+
100
75
50
25
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No
Zym
AC
Zym+ AC
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% of population
100
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5
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Goldfish
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1-2
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Figure 3.6. Divergent pro-inflammatory and homeostatic responses of lamprey and goldfish phagocytes.

Goldfish PKL and lamprey PTL were incubated with both zymosan and apoptotic cells (5:1 ratio for each) for 2 h and 6 h, respectively. (A) Respiratory burst (measured as % DHR positive) was then analyzed based on phagocytic capacity across the four resulting sub-populations: non-phagocytic cells, phagocytes containing only zymosan, phagocytes containing only apoptotic cells, and phagocytes that contain both. (B) The percent of total population found in each of the four sub-populations of (A); no internalization, zymosan only, apoptotic cells only, zymosan and apoptotic cells. * $p<0.05$ compared to No; + $p<0.05$ compared to Zym. (C) The phagocytic index of the Zym+AC group in (A). (D) Respiratory burst analyzed according to the number of zymosan particles internalized in the Zym+AC group. * $p<0.05$ compared to goldfish. No- no internalized particle; AC- apoptotic cells; Zym- zymosan. For all n=4, examined over a minimum of two independent experiments.

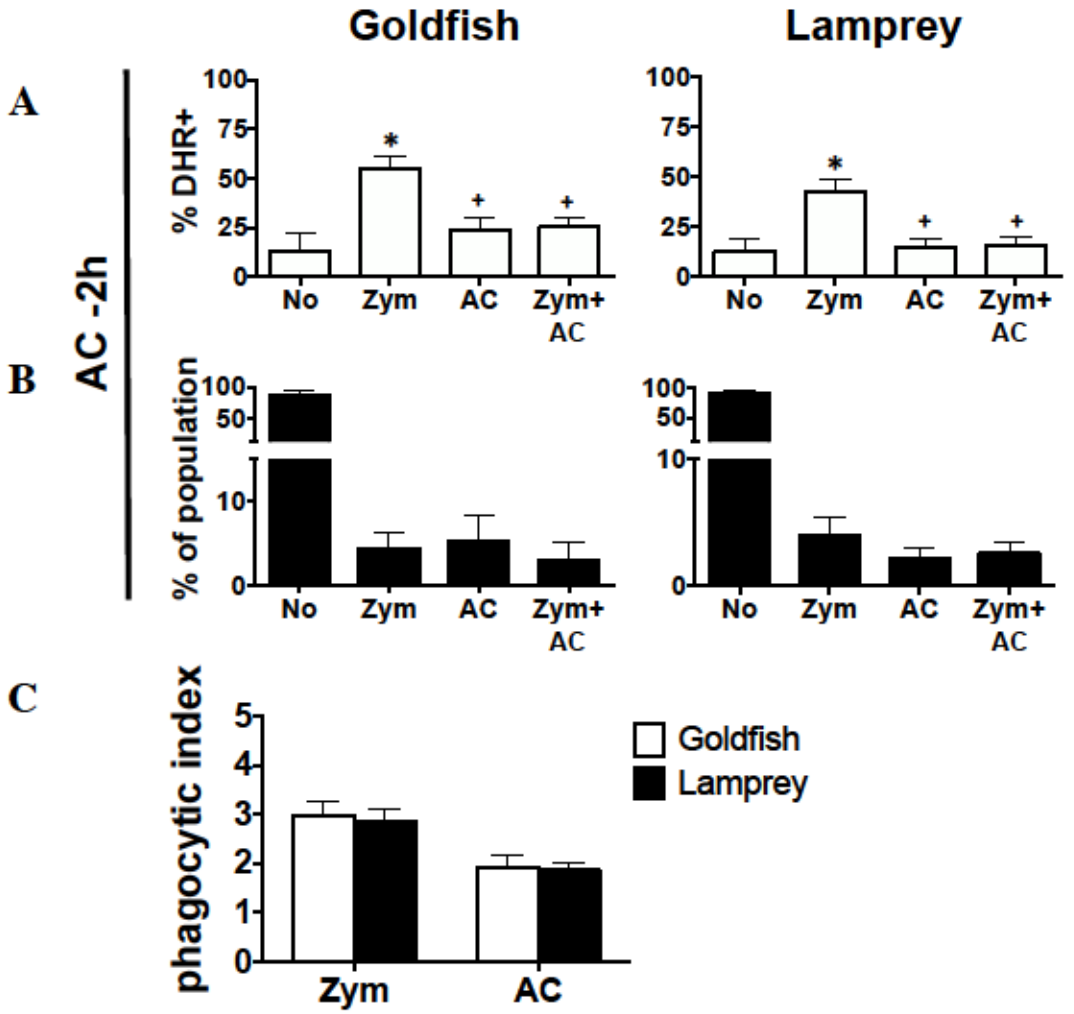

Figure 3.7. Effect of pre-incubation with zymosan and apoptotic cells on respiratory burst responses of individual phagocytes.

Goldfish PKL and lamprey PTL were incubated with both zymosan and apoptotic cells (5:1 ratio for each) for 2 h and 6 h, respectively. (A) To investigate the effects of pre-incubation with apoptotic cells, apoptotic cells were added 2 h prior to zymosan. Respiratory burst (measured as % DHR positive) was then analyzed based on phagocytic capacity across the four resulting sub-populations: non-phagocytic cells, phagocytes containing only zymosan, phagocytes containing only apoptotic cells, and phagocytes that contain both. (B) The percent of total population found in each of the four sub-populations of (A); no internalization, zymosan only, apoptotic cells only, zymosan and apoptotic cells. (C) The phagocytic index of the Zym+AC group in (A). For all n=4, examined over a minimum of two independent experiments. * $p<0.05$ compared to No; + $p<0.05$ compared to Zym.

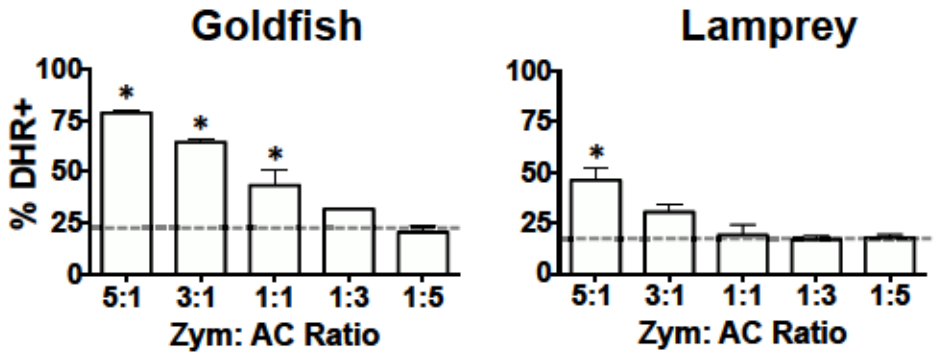

Figure 3.8. Respiratory burst responses of goldfish and lamprey hematopoietic leukocytes following zymosan and apoptotic cell stimulation.

Goldfish PKL and lamprey PTL were incubated with both zymosan and apoptotic cells (5:1 ratio for each) for 2 h and 6 h, respectively. To examine the effects of a dose response, zymosan and apoptotic cells were added at varying concentrations. Respiratory burst (measured as % DHR positive) was then analyzed for the entire leukocyte population. For all n=6, examined over a minimum of two independent experiments. Grey dashed line represents the respiratory burst of phagocytes internalizing only AC cells. * $p<0.05$ compared to grey dashed line (% DHR of phagocytes internalizing only apoptotic cells).

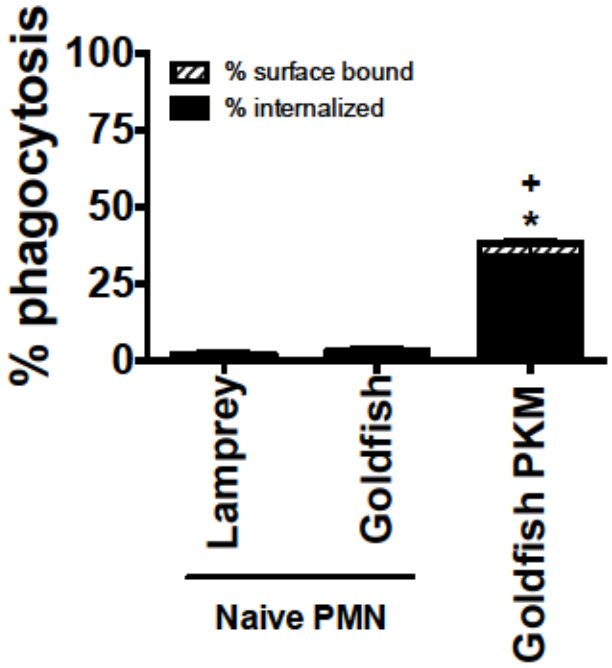

Figure 3.9. Internalization of apoptotic cells by naïve lamprey and goldfish neutrophils / neutrophil-like cells

Neutrophil-like cells from lamprey typhlosole and neutrophils from the goldfish kidney were isolated as described. Neutrophils were incubated with cells isolated from PTLs and PKLs, respectively, rendered apoptotic via cyclohexamide at a 3:1 (AC: neutrophil) ratio. For all n=4, examined over a minimum of two independent experiments. * $p<0.05$ and + $p<0.05$ compared to lamprey and goldfish % internalized, respectively.

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Chapter 4. Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish[1]

4.1 Introduction

Neutrophils are important innate effector cells that dominate the initial influx of leukocytes into a site of inflammation (Henderson et al., 2003). Upon activation within the inflammatory site, neutrophils become powerful killers, release toxic intracellular granules, produce reactive oxygen species (Nathan, 2006; Winterbourn and Kettle, 2012), and deploy neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012). This notion is consistent with early descriptions of the microphage by Elie Metchnikoff, which he identified as highly phagocytic leukocytes important in defense against microorganisms (Metchnikoff, 1905). Historically, it has been these pro-inflammatory roles that have served as our focus for this immune cell; their contributions to the resolution of inflammation limited to apoptotic cell death and subsequent clearance by macrophages. However, more recently I and others have showed that both human and murine neutrophils internalize apoptotic cells and actively contribute to the decreased

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[1] A portion of this chapter has been previously published in:

Havixbeck JJ, Rieger AR, Wong ME, Hodgkinson JW, and Barreda DR (2016) Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish. *JLB* 99(2): 241-252.

production of reactive oxygen species in other leukocytes (Esmann et al., 2010; Rieger et al., 2012). Acquisition of this mechanism would presumably offer mammals a novel alternative for the control of inflammation.

In humans, the generation of neutrophils is a key activity of the hematopoietic compartment, where approximately $5x10^{10}$ - $10x10^{10}$ new neutrophils are generated each day (Summers et al., 2010). Among others, these contribute to the establishment of storage pool of mature neutrophils within the bone marrow (Boxio et al., 2004; Furze and Rankin, 2008; Rankin, 2010). Upon injury or infection, mature neutrophils migrate through the sinusoids and are rapidly released into circulation, trafficking to sites of infection and/or inflammation (Boxio et al., 2004; Furze and Rankin, 2008; Rankin, 2010). The mobilization and recruitment of neutrophils is dependent upon signals that originate from the site of inflammation. A diverse panel of stimuli, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) activate the tissue-resident cells to release pro-inflammatory mediators (e.g. TNF-α, IFN-γ, and IL-1β), as well as neutrophil-specific chemoattractants (e.g. CXCL-8) and lipid mediators (e.g. LTB_4) (Arancibia et al., 2007; Williams et al., 2011; Zeytun et al., 2010). Within minutes of tissue injury, neutrophils begin "rolling" along the walls of venules surrounding the affected site (Butcher, 1991). An interaction between neutrophil L-selectin and vascular E- and P-selectins then allows for extravasation (Butcher, 1991). These mediators also activate neutrophils, increasing their capacity for phagocytosis and antimicrobial effector mechanisms upon recruitment to the inflammatory site (Sørensen et al., 2001).

The characterization of immune cell populations in a range of animal models suggests that neutrophils arose among the osteichthyans about 420 million years ago, prior to the divergence of the last common ancestor for ray- and lobe-finned fish (Flerova and Balabanova, 2013; Henry et al., 2013; Katzenback and Belosevic, 2009). As teleosts represent the most basal animal group to contain bona fide neutrophils, they represent a viable platform to better understand the origins of their contributions to inflammation. However, significant gaps remain with regards to the effector and potential regulatory contributions of teleost neutrophils at the inflammatory site, as well as the mechanisms that promote and control their effective recruitment from hematopoietic tissues. In this chapter, I examined the contributions of neutrophils to the acute inflammatory response in teleost fish using a self-resolving zymosan peritonitis model. This model allowed both biochemical and cellular analyses of local inflammatory responses, which resembled the PMN-rich exudates of human disease. Using this model, I investigated the mobilization of neutrophils and the regulation of inflammatory processes during an acute inflammatory response. I report here that neutrophils play a significant role in the pro-inflammatory and resolution phases of the acute inflammatory response in teleost fish. Consistent with mammalian studies, my data shows that in goldfish a large pool of mature neutrophils rapidly exit the hematopoietic tissue, enter circulation, and infiltrate the inflammatory site. I found that neutrophil infiltration into the peritoneum peaked 18 hpi (hours post injection). Interestingly, once at the inflammatory site, I found that teleost neutrophils possess the capacity to mediate divergent pro- and anti-inflammatory responses. Teleost neutrophils isolated at 18 hpi, a period where the inflammatory site exhibited a classical pro-inflammatory phenotype, displayed increased ROS production

when compared to neutrophils isolated at 48 hpi, where the phenotype had shifted to the pro-resolving phase. Neutrophils from this pro-resolution time point also demonstrated a greater ability to modulate macrophage function. These neutrophils were able to mediate divergent responses, both increasing and decreasing ROS production in macrophages following stimulation with zymosan or apoptotic cells, respectively. Interestingly, I found lipoxin A_4 (LXA_4) to be a significant contributor to the uptake of apoptotic cells by teleost macrophages. In addition, LXA_4 also played a role, at least in part, in the down-regulation of ROS production in macrophages. Our results highlight the evolving roles of neutrophils in both the promotion and regulation of the inflammatory response.

4.2 Results

4.2.1 Kinetic analysis of teleost acute inflammation using an *in vivo* peritonitis model

My goal in this study was to define the acute inflammatory response in teleosts, as a way to better understand the evolutionary origins of neutrophil-based mechanisms of immunity. As a first step to define the pro-inflammatory phase of acute inflammation in teleost fish, I examined the kinetics of cellular infiltration into an inflammatory site, the production of antimicrobial reactive oxygen species (ROS), and the expression of pro-inflammatory cytokines using an *in vivo* zymosan peritonitis model. I found recruitment of leukocytes into the peritoneum as early as 8 hpi, with peak infiltration observed at 18 hpi. Total cell numbers at the site of inflammation remained elevated through 36 h when compared to non-injected fish (0 h; Figure 4.1A). By 72 hpi, the number of cells present at the inflammatory site had returned to basal levels. Consistent with the kinetics of

cellular infiltration, ROS production increased significantly between 8 and 36 h, with a peak at 18 hpi (Figure 4.1B).

Cytokine expression analysis was performed by qPCR using the primers in Table 2.9. RNA was isolated from total leukocytes within the peritoneal cavity and examined for the expression of TNF-α2, IL-1β1, and IFNγ, cytokines known to be important in the inflammation process (Grayfer et al., 2008; Grayfer and Belosevic, 2009). Overall, I identified high local expression of TNF-α2, IL-1β1, and IFN-γ within the first 24 hpi, with a peak between 8 and 12 hpi, and a return to basal levels by 48 hpi (Figure 4.1C). Importantly, even though individual animals were derived from an out-bred population, I found significant homogeneity among the kinetics of pro-inflammatory cytokine gene expression profiles across all fish. Characterization of the leukocyte populations within the exudate identified neutrophils as the dominant subset, representing nearly 50% of all infiltrating leukocytes at the site of inflammation at 18 hpi (Figure 4.1D).

4.2.2 Teleost inflammatory neutrophils exhibit robust antimicrobial responses in comparison to their hematopoietic counterparts

Upon entry into an inflammatory site, mammalian neutrophils are rapidly activated from the naïve phenotype seen in the hematopoietic compartment, and exhibit potent histotoxic capabilities including phagocytosis and downstream killing mechanisms (Mócsai, 2013; Sadik et al., 2011; Wright et al., 2010). To determine if teleost neutrophils share this dichotomy, I compared the antimicrobial responses among teleost neutrophils isolated from the kidney hematopoietic compartment and the peritoneal challenge site of goldfish injected with 1× $PBS^{-/-}$ or zymosan. Neutrophils isolated from the inflammatory

site exhibited significantly higher rates of phagocytosis and marked increases in ROS production in comparison to their hematopoietic counterparts (Figure 4.2A and 4.2B). As such, I chose to focus on neutrophils isolated from the inflammatory site in order to examine their contributions throughout the acute inflammatory response.

4.2.3 Induction of teleost acute inflammation results in marked mobilization of neutrophils from the hematopoietic compartment to the peripheral blood

It is widely recognized that many inflammatory reactions are associated with a prompt and selective mobilization of neutrophils from the bone marrow to the inflammatory site in mammals (Boxio et al., 2004; Furze and Rankin, 2008; Rankin, 2010; Sadik et al., 2011). Further, teleost neutrophils have also been shown to mobilize from the hematopoietic compartment in models such as the zebrafish and gilthead seabream (Chaves-Pozo et al., 2012, 2005; Deng et al., 2013; García-Castillo et al., 2004; Hall et al., 2012). Among others, C/EBPβ and Nos2a are critical to the initiation of granulopoiesis following infection, and CXCL8-CXCR2 signaling is required for the motility and mobilization of neutrophils from hematopoietic tissues (Deng et al., 2013; Yang et al., 2012). As the next step in examining the acute inflammatory response in teleost fish, I investigated the contribution of the hematopoietic compartment to the mobilization of neutrophils in goldfish. I found that a dramatic increase in the peritoneal leukocytes (Figure 4.1A) correlated to a decrease in total leukocytes within the hematopoietic compartment (Figure 4.8A). When I examined the number of leukocytes remaining in the hematopoietic compartment, I found that the number of neutrophils decreased dramatically by 70% at 12 hpi (Figure 4.3A). This further correlated with a

marked early decrease in leukocyte proliferation (8 hpi; Figure 4.3B), which returned to basal levels by 72 hpi. The steady increase in proliferation between 8 and 72 hpi likely contributed to the replenishment of the depleted pool of hematopoietic neutrophils, eventually returning to basal levels between 48 and 72 hpi.

Following an *in vivo* challenge with zymosan, the proportion of circulating neutrophils increased nearly 10-fold, from less than 5% at 0 hpi to nearly 50% of circulating leukocytes between 8 and 18 hpi (Figure 4.3C). In contrast, peripheral blood monocytes peaked at 36 hpi (Figure 4.4C), whereas peripheral blood lymphocytes dropped significantly between 8 and 36 hpi (Figure 4.4D). Interestingly, total blood leukocyte counts remained relatively constant throughout the 72 h time course (Figure 4.4B). However, the significant increase in the proportion of neutrophils within the blood between 8 and 36 hpi (Figure 4.3C), also resulted in a significant increase in total neutrophil numbers, which peaked at 8 hpi (8.4 fold over basal levels; Figure 4.3D), identifying a period of neutrophilia within the circulating blood of goldfish.

4.2.4 Following an increase in cxcl8 expression, neutrophils rapidly infiltrate the site of inflammation and activate potent respiratory burst responses

In mammals, chemotactic mediators such as CXCL8 are produced within inflammatory sites and recruit neutrophils from circulation (Kaur and Singh, 2013; Scapini et al., 2005). Teleost models including zebrafish and carp have also shown CXCL8 to mediate neutrophil recruitment (de Oliveira et al., 2013; Deng et al., 2013; van der Aa et al., 2012). Using this as our foundation, I was interested in determining if CXCL8 played a role in our model. Examination of CXCL8 expression during the acute

inflammatory response to zymosan-induced peritonitis showed a significant increase at the site of inflammation, peaking at 12 hpi and returning to basal levels by 18 hpi (Figure 4.4A). This rise in CXCL8 expression directly preceded the rapid influx of neutrophils into the peritoneum, which peaked at 18 hpi (Figure 4.5B). Together with the data presented above, this allowed us to define 0-18 hpi as the induction phase of the acute inflammatory response in this self-resolving peritonitis model.

As described above, neutrophils isolated from the inflammatory site displayed more prominent antimicrobial responses when compared to their naïve counterparts. To gain further insights into the antimicrobial potential of neutrophils during the teleost acute inflammatory response, I examined kinetics of ROS production among infiltrated neutrophils. As expected, I found that neutrophils displayed prominent respiratory burst responses upon entry into the peritoneal inflammatory site. Similar to the total cell population, respiratory burst responses peaked at 18 hpi (Figure 4.5C). However, unlike ROS production by total leukocytes, which declined steadily until 72 hpi (Figure 1B), the respiratory burst responses of neutrophils returned to basal levels by 36 hpi (Figure 4.5C).

4.2.5 Increase in neutrophil apoptosis marks transition to pro-resolution phase

Neutrophil apoptosis and their subsequent uptake by phagocytes is central to the successful resolution of inflammation in mammals (Leitch et al., 2011). Thus, I addressed if the induction of neutrophil apoptosis may also mark the transition between pro-inflammatory and pro-resolution responses in teleost fish. I found that the presence of apoptotic neutrophils peaked at 24 hpi, subsequently decreasing to basal levels by 72 hpi

(Figure 4.5D). This transition followed the induction phase of acute inflammation defined above. Importantly, between 24 hpi and 48 hpi, neutrophil counts remained above basal levels even as neutrophil ROS production returned to baseline. I hypothesized that this remaining subset of viable neutrophils had shifted towards a pro-resolving phenotype, actively promoting the resolution of inflammation.

4.2.6 Inflammatory neutrophils can modulate the functional responses of macrophages through soluble factors

Recent studies have highlighted the direct contribution of mammalian neutrophils to the resolution of inflammation, based on the internalization apoptotic cells (Esmann et al., 2010; Rieger et al., 2012) and the release lipid mediators (Serhan, 2014; Serhan et al., 2008; Serhan and Savill, 2005). In order to characterize the effect of teleost neutrophils on macrophage functional responses, goldfish neutrophils were stimulated *in vivo* and isolated from the peritoneum at 18 and 48 hpi, as described above. Neutrophils were cultured for 2 h in 0.4 μm transwells in the presence of zymosan or apoptotic cells (AC) to determine if soluble factors could elicit changes in macrophage responses outside of the transwell (Figure 4.6A). I found that when incubated with zymosan, neutrophils isolated 18 hpi induced significant increases in macrophage ROS production, compared to neutrophils isolated at 48 hpi (60% vs. 46% respectively). This suggested that neutrophils from the induction phase of this acute inflammatory response (isolated at 18 hpi) were more suited to activating inflammatory macrophage functions than those from the pro-resolution phase (isolated at 48 hpi). In contrast, when these induction phase neutrophils were incubated with apoptotic cells, they displayed no capacity to reduce

macrophage ROS production below basal levels (Figure 4.6A). This deferred dramatically from pro-resolution neutrophils, which decreased macrophage ROS levels when incubated with AC (28% vs. 38% respectively). This suggested that (1.) neutrophils released soluble factors that modulated macrophage function and that (2.) the types of soluble factors produced are largely dependent on the phenotype exhibited by isolated neutrophils (pro-inflammatory or pro-resolving). In addition, I examined the ability of neutrophils to induce the uptake of apoptotic neutrophils by macrophages. I observed no differences in the ability of macrophages to internalize apoptotic neutrophils following stimulation under these conditions (Figure 4.6B).

Lipid mediators represent key immunoregulatory soluble factors released by neutrophils. Thus, I investigated the potential contributions from leukotriene B_4 (LTB_4), known to be involved in neutrophil recruitment and activation (Sadik et al., 2011), and lipoxin A_4 (LXA_4), an important mediator with pro-resolving function (Levy et al., 2001; Serhan, 2014; Serhan et al., 2008). I found that the secretion of the pro-inflammatory lipid LTB_4 displayed very similar kinetics to those of CXCL8 (Figure 4.7A). A significant increase in LTB_4 (~75 pg per 5 x 10^5 neutrophils) was found between 8 and 12 hpi, followed by an immediate return to basal levels. In contrast, LXA_4 peaked (~300 picograms per 5 x 10^5 neutrophils) during the putative transition point to the pro-resolution phase (24 hpi; Figure 4.7B). A comparison between neutrophil, mononuclear cell and soluble exudate fractions confirmed that neutrophils were the primary producers of LTB_4 and LXA_4 (Figure 4.8). Interestingly, peak LXA_4 release at 24 hpi paralleled that of peak neutrophil apoptosis, supporting this as a transition point towards the resolution of acute inflammation. I next determined if these lipid mediators also induced autocrine

effects on neutrophils. I found that stimulation with LTB_4 promoted increased respiratory burst responses in both naïve neutrophils isolated from the hematopoietic kidney and inflammatory neutrophils isolated from the peritoneal cavity at 18 hpi. Whereas a dose-dependent effect was evident in hematopoietic kidney PMN (Figure 4.7C), peritoneal cavity PMN displayed consistent high levels of ROS production (Figure 4.7D). This is likely associated with the activated status of inflammatory PMN infiltrating the peritoneal compartment. Primary macrophages were then stimulated *in vitro* with LTB_4 or LXA_4 at varying concentrations for 2 h. Although LTB_4 has been shown to activate intracellular killing mechanisms of murine peritoneal macrophages (Wirth and Kierszenbaum, 1985), LTB_4 had little effect on teleost primary kidney macrophage responses (Figure 4.7E). Conversely, LXA_4 tended to decrease macrophage ROS production at all concentrations examined (Figure 4.7E). In addition, stimulation with LXA_4 also resulted in a significant increase in macrophage uptake of apoptotic neutrophils in a dose dependent manner (Figure 4.7F). Macrophages stimulated with the highest concentration of lipoxin A_4 (290 pg) displayed a four-fold increase in apoptotic neutrophil uptake over unstimulated levels (Figure 4.7F).

4.3 Discussion

The acute inflammatory response requires proper coordination of both pro- and anti-inflammatory responses for effective defences against infection, the removal of damaged cells, and the initiation of tissue repair processes. Similar to previous studies in mammals that have examined zymosan-induced inflammation (Medzhitov and Okin, 2012; Yamada et al., 2011), intraperitoneal injection of zymosan into goldfish resulted in

substantial cellular infiltration and marked increases in the generation of antimicrobial products and soluble mediators. Based on this model, 72 h provided an adequate observation period for examination of the induction, regulation, and resolution of acute inflammatory responses (Kolaczkowska et al., 2010).

Neutrophils are essential first-line effector cells of innate immunity; their recruitment into peripheral tissues is central to host defense against invading pathogens. In mammals, neutrophils spend the majority of their lives in bone marrow, where $5x10^{10}$ to $10x10^{10}$ new neutrophils are generated each day (Summers et al., 2010). A large storage pool of mature neutrophils exists here, termed the bone marrow reserve (12–14). Upon receiving the appropriate signals, this pool can be rapidly mobilized to respond to an infection or inflammatory challenge (Boxio et al., 2004; Furze and Rankin, 2008; Rankin, 2010), resulting in a dramatic rise in the number of circulating neutrophils. My results are consistent with those from mammals, where the number of neutrophils within the hematopoietic tissue of goldfish decreased by nearly 70% within 12 hpi. Neutrophil depletion in the hematopoietic compartment led to a rapid rise in peripheral blood neutrophils, with the number of circulating neutrophils increasing 8.4 fold over basal levels. Notably, during this period, there was also a dramatic decrease in proliferation within the hematopoietic compartment. The lack of proliferation, rapid release of neutrophils from the kidney, and increase in peripheral blood neutrophils indicated that a storage pool of neutrophils exists within the hematopoietic tissue of goldfish, similar to mammals. However, mammalian neutrophils represent the largest population of circulating leukocytes during homeostasis. This is not the case in teleost fish, where lymphocytes are the dominant circulating leukocyte (Ramasamy Harikrishnan et al.,

2009). This suggests that teleost fish are far more dependent on the hematopoietic storage pool of neutrophils, which likely represents the majority of mature neutrophils that can traffic to and infiltrate the site of inflammation. In mammals, many of the neutrophils infiltrating the site of inflammation are presumably already in circulation. Their hematopoietic storage pool may then exist as a means to replenish peripheral blood neutrophils following depletion. Consequently, mammalian neutrophils may be able to respond more rapidly to acute injury or pathogen challenge. Importantly, this would also come at the expense of one regulatory safeguard for the release of these potent effector cells from the hematopoietic compartment.

Diverse chemoattractants, such as CXCL8 and LTB_4 (de Oliveira et al., 2013; Kaur and Singh, 2013; Scapini et al., 2005, 2000), can act to recruit neutrophils to a site of inflammation. However, given their destructive potential to host tissues, neutrophil entry must be tightly regulated (Segel et al., 2011; Wright et al., 2010). In zebrafish, increases in CXCL8 expression can be seen within hours of wounding (de Oliveira et al., 2013). Further, CXCL8 is upregulated in response to acute inflammatory stimuli, critical for normal neutrophil recruitment to the wound and resolution of inflammation (de Oliveira et al., 2013). In a similar manner, during induced atopic dermatitis in mice and humans, the influx of neutrophils was largely dependent on the generation of LTB_4 by neutrophils in an autocrine manner (Oyoshi et al., 2012). I show here that both CXL8 expression and LTB_4 production increase rapidly at the site of inflammation between 8 and 12 hpi. This provides two potential contributors to the rapid recruitment of neutrophils to the site of inflammation in teleosts. Further, the narrow window in which CXCL8 expression and LTB_4 production increased are consistent with tight regulation of

this process from the inflammatory site. The majority of LTB_4 was produced by neutrophils at 12 hpi (51 pg per $5x10^5$ cells; Supplemental Figure 2). In contrast, mononuclear cells (lymphocytes, monocytes, and macrophages) were found to produce low levels of LTB_4 at this time point (14 pg per $5x10^5$ cells; Supplemental Figure 2). LTB_4 was shown to increase the production of ROS in both naïve and inflammatory neutrophils. My results highlight the complementary autocrine effect by which lipids can affect teleost neutrophils at and around the site of inflammation.

I found that neutrophil infiltration into the peritoneal cavity peaked directly after the spike in CXCL8 and LTB_4 at 18 hpi, correlating well with previously published data from Chadzinska and colleagues where cellular infiltration peaked between 16 and 24 hpi (Chadzinska et al., 2004). Induction of pro-inflammatory antimicrobial responses correlated well with leukocyte recruitment, as maximal ROS production was observed during peak infiltration (18 hpi). Interestingly, this also correlates with increased expression of pro-inflammatory cytokines within the peritoneal cavity, which are likely involved, at least it part, in activating neutrophils upon arrival. It should be noted that not every cytokine exerts its affect on every cell, and the timing of cytokine production and release are critical to suitable inflammatory activation and modulation. Further, other soluble effectors, including lipid mediators can also impact cytokine production and release (Williams and Shacter, 1997). When compared to a mammalian model, neutrophil influx was found to peak between 4 and 6 hpi in a murine zymosan-induced peritonitis model (Cash et al., 2009; Fujieda et al., 2013; Kolaczkowska et al., 2010), though neutrophil specific responses were not examined. A second murine study showed total leukocyte ROS production peaking at 8 hpi (Rieger et al., 2012). The quicker response

time of mammalian neutrophils may be associated with factors: (1.) neutrophils are the dominant circulating leukocyte in mammals and thus should be able to respond quicker to an infection compared to teleost fish where they only represent approximately 5% of the circulating leukocytes, and (2.) resident peritoneal cells in mice may display an enhanced capacity for recruitment to the inflammatory site in comparison to their teleost counterparts. Importantly, the induction of inflammation was followed by a controlled resolution phase of inflammation, where cell counts, ROS production, and the expression of pro-inflammatory cytokines returned to basal levels between 24 and 72 hpi.

The resolution of inflammation is critical to maintain host health. One important step toward resolution is the induction of programmed cell death in inflammatory cells. Given the potentially destructive nature of neutrophils at an inflammatory site, their half-life is very short, and the removal of apoptotic neutrophils by macrophages is believed to be a crucial component driving the resolution of inflammation (Erwig and Henson, 2007; Haslett et al., 1994). It was previously thought that the internalization of apoptotic cells was an immunologically neutral event; however, there is now evidence indicating the internalization or binding of apoptotic neutrophils by macrophages *in vivo* and *in vitro* promotes the production of anti-inflammatory mediators, notably IL-10 and TGF-β (Fadok et al., 1998; Voll et al., 1997). Among others, the shift in balance between TNF-α and TGF-β further contributes to the 'quenching' of reactive oxygen and nitrogen species (Serinkan et al., 2005). Interestingly, I found neutrophils began entering the apoptotic cascade during the induction of inflammation (0-18 hpi), signifying a gradual switch to pro-resolving responses. Of note, it is difficult to gauge the exact amount of neutrophil apoptosis at the site of inflammation due to confounding issues: (1.) apoptotic neutrophils

are rapidly engulfed by macrophages, and (2.) many apoptotic bodies are lost during isolation due to size and density. As such, I believe that my results are a conservative estimate of when maximal apoptotic neutrophils are likely to be found at the inflammatory site.

The balance between pathogenic and homeostatic signals is crucial for effective pathogen clearance with minimal damage to surrounding host tissues (Medzhitov, 2007). Macrophages are well known to internalize apoptotic cells and subsequently down-regulate their pro-inflammatory responses (Johann et al., 2005; Rieger et al., 2012; Zamboni and Rabinovitch, 2003). In addition, human and murine neutrophils activated *ex vivo* possess the capacity to internalize apoptotic cells, resulting in decreased ROS production (Esmann et al., 2010; Rieger et al., 2012). This establishes mammalian macrophages and neutrophils as active contributors to the resolution of inflammation. We have previously shown this capacity to extend to macrophages, but not neutrophils, in teleost fish (Rieger et al., 2012). Despite our current findings, there is a possibility that there may be specific instances when teleost neutrophils do internalize apoptotic cells. In one case, zebrafish neutrophils were shown to internalize apoptotic macrophages infected with *Mycobacterium marinum* (Yang et al., 2012). However, one would predict significant activation of pattern recognition receptors under these conditions, likely rendering these infected apoptotic cells poor contributors to the resolution of inflammation that is normally exhibited during a classical acute inflammatory process.

My results also indicate that teleost neutrophils are capable of altering their phenotype throughout the acute inflammatory response, contributing to both the induction and resolution of inflammation. Two phases of the acute inflammatory

response in teleost fish became apparent: (1.) an induction phase of acute inflammation from 0-18 hpi, and (2.) a pro-resolving phase from 24-72 hpi (Figure 7). The period between (18-24 hpi) represents a transition point between these two phases, where divergent pro- and anti-inflammatory responses overlap. Neutrophils isolated during the induction phase (18 hpi) were only able to induce ROS production in macrophages. However, neutrophils isolated during the pro-resolving phase (48 hpi) also possess the capacity to down-regulate macrophage ROS production. Although I observed no change in the ability to stimulate an increased uptake of apoptotic cells by macrophages, I recognized this as a possibility under different experimental conditions. I am currently pursuing two potential modifications, 1) neutrophils need to be isolated at a different time point; and 2) the type of macrophage (i.e. inflammatory vs. kidney derived) used for the assay.

We have previously shown that, in the presence of apoptotic cells, teleost neutrophils produce soluble factors that can modulate monocyte and macrophage responses (Rieger et al., 2012). However, these previous experiments were done with neutrophils from the transition point (24 hpi). It has been shown in mammals that lipid mediators are one the primary soluble factors produced by neutrophils. As such, I examined the production of lipid mediators throughout an acute inflammatory response. I found a shift in the production of leukotriene B_4 to lipoxin A_4 in neutrophils during the transition phase (18-24 hpi) of acute inflammation (Figure 6C and Figure 7), further supporting my argument of multi-phenotypic neutrophils. As previously mentioned, LTB_4 secretion parallels that of increased CXCL8 expression within the inflammatory milieu. Current models propose that LTB_4 acts as a secondary chemoattractant important

in initiating the inflammatory process, indicating that teleost neutrophils may be actively involved in recruiting more neutrophils to the inflammatory site. Further, LTB_4 has been implicated in the activation of neutrophils through BLT1 receptor endocytosis, inducing degranulation and increased respiratory burst responses (Dewald and Baggiolini, 1985; Gaudreault et al., 2005). On the other hand, LXA_4 secretion paralleled that of neutrophil apoptosis, peaking 24 hpi. LXA_4 aids in the uptake of apoptotic cells by macrophages, playing an important role in the resolution of inflammation (Godson et al., 2000; Mitchell et al., 2002). Similarly, I found that macrophage internalization of apoptotic neutrophils increased in as more lipoxin was added, suggesting neutrophils appear to participate in the resolution of inflammation by stimulating macrophages with LXA_4 prior to entering apoptosis. LXA_4 also contributed to downregulating ROS production in macrophages and inflammatory neutrophils, suggesting this pro-resolving lipid may play a broader role than previously described. Interestingly, macrophages produce a significant amount of arachidonic acid, one of the necessary precursors to lipid mediator production, highlighting the need for leukocyte collaboration throughout the immune response.

Based on my findings, the induction phase of acute zymosan peritonitis lasts from 0-18 hpi in teleosts (Figure 4.9). It is marked by substantial increases in pro-inflammatory cytokine expression, leukocyte infiltration, and robust antimicrobial responses. At this point a transition period of overlapping divergent pro- and anti-inflammatory responses occurs, priming the environmental milieu for the resolution of inflammation (18-24 hpi; Figure 4.9). During this transition I observed pro-inflammatory cytokine expression return to basal levels, a switch in the secretion of lipid mediators from LTB_4 to LXA_4, and an increase in apoptotic neutrophils. On this note, I hypothesize

the switch in soluble lipid mediators is mediated, at least in part, by the secretion of prostaglandin E_2 (PGE_2) from monocytes, macrophages, and epithelial cells, which I am currently pursuing. The final period of acute peritonitis is pro-resolution period from 24-72 hpi, resulting in a return to homeostasis (Figure 4.9). Interestingly, homeostasis is not an exact state and other events may still be ongoing, such as lymphocyte recruitment and the initiation of adaptive responses. My results provide added insights into the mechanisms by which neutrophils contribute to the induction and regulation of acute inflammatory processes. In addition, the results highlight the involvement of neutrophils in at least the initiation of resolving events, where they play a significant role in lipoxin production and the stimulation of macrophages to take up apoptotic neutrophils.

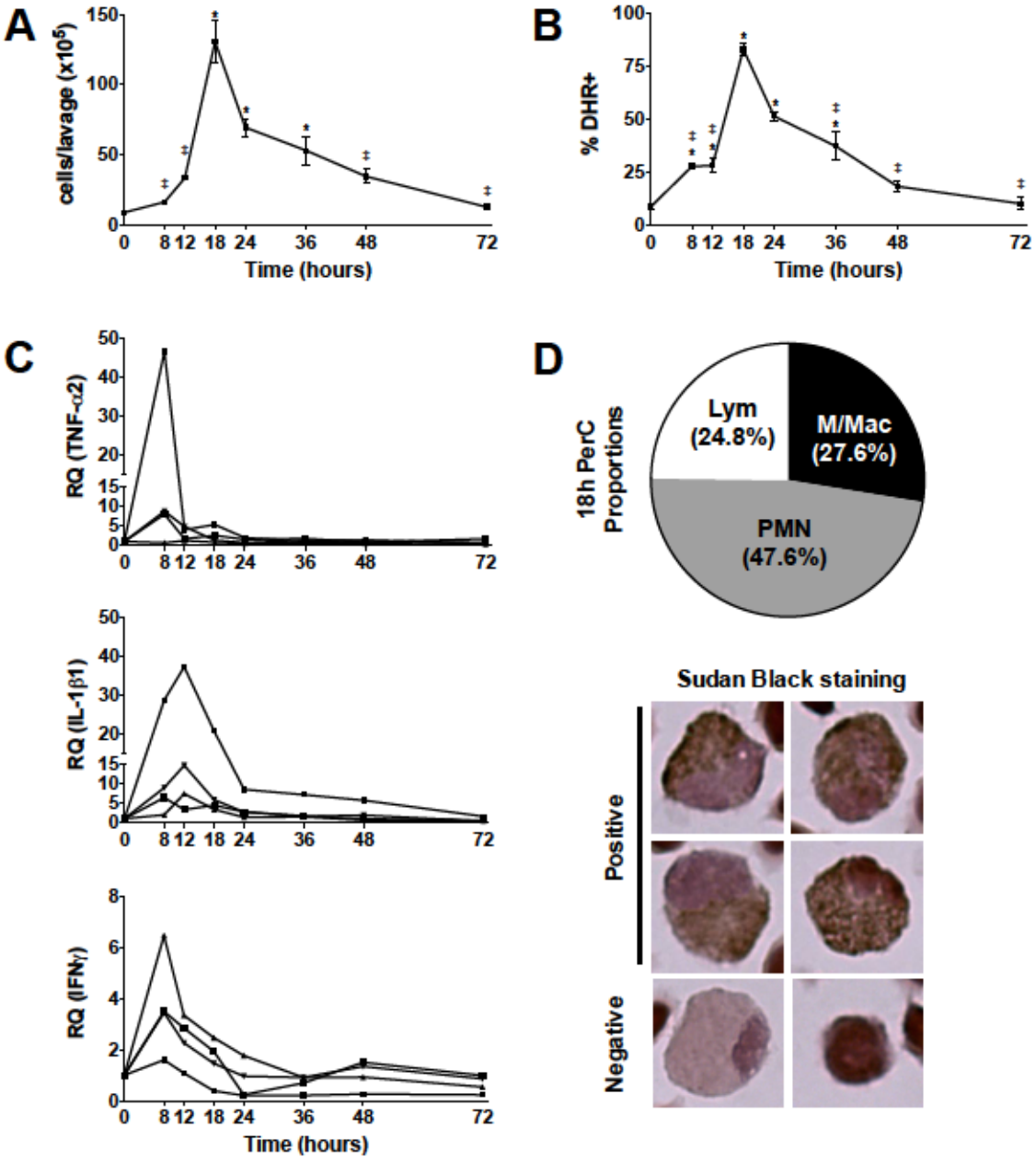
A
cells/lavage (x10^5)
150
100
50
0
0 8 12 18 24 36 48 72
Time (hours)
B
% DHR+
100
75
50
25
0
0 8 12 18 24 36 48 72
Time (hours)
C
RQ (TNF-α2)
RQ (IL-1β1)
RQ (IFNγ)
0 8 12 18 24 36 48 72
Time (hours)
D
18h PerC Proportions
Lym (24.8%)
M/Mac (27.6%)
PMN (47.6%)
Sudan Black staining
Positive
Negative

Figure 4.1. Intraperitoneal administration of zymosan in goldfish induces marked infiltration of leukocytes that is linked to high levels of pro-inflammatory mediators.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. Cells were harvested by peritoneal lavage at 0 h (saline alone), 8, 12, 18, 24, 36, 48, and 72 h, and counted or used for RNA extraction. (A) Cells per lavage at the indicated time points (n=5). (B) Respiratory burst capacity in isolated cells at these time points was determined via DHR staining (n=5). (C) Cytokine levels were measured by Q-PCR. Zymosan induced a general increase in pro-inflammatory cytokine expression levels in the early stages of the time course. Each line represents an individual fish (n=4). (D) Sudan Black staining was used to determine the proportion of neutrophils in the peritoneal exudate at 18 hpi (n=5). Representative images show neutrophils positive for Sudan Black staining, as well as a Sudan Black negative monocyte/macrophage and lymphocyte. All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from 0 hpi, ‡ significantly different from 18 hpi.

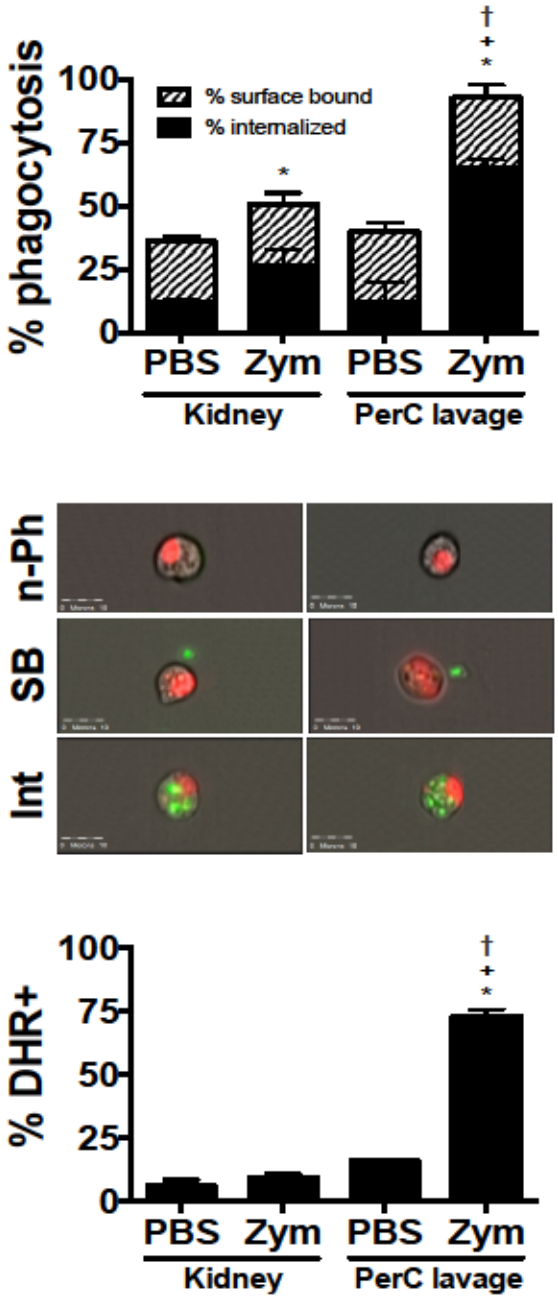
% phagocytosis
100
75
50
25
0
% surface bound
% internalized
*
†
+
*
PBS
Zym
PBS
Zym
Kidney
PerC lavage
n-Ph
SB
Int
% DHR+
100
75
50
25
0
†
+
*
PBS
Zym
PBS
Zym
Kidney
PerC lavage

Figure 4.2. Peritoneal neutrophils display increased phagocytic and respiratory burst capacity in comparison to their hematopoietic counterparts.

Goldfish were injected intraperitoneally with $1xPBS^{-/-}$ or 2.5 mg of zymosan. Neutrophils were isolated from the hematopoietic kidney tissue and peritoneal exudate. (A) Phagocytic capacity of neutrophils. Solid black bars represent internalization, and hatched white bars correspond to surface binding of particles (n=4). Representative images from ImageStream MKII flow cytometer denote non-phagocytic (n-Ph), surface bound (SB), and internalized (Int) events. (B) Respiratory burst capacity of neutrophils (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from PBS injection (kidney neutrophils), + significantly different from zym injection (kidney neutrophils), † significantly different from PBS injection (peritoneal neutrophils).

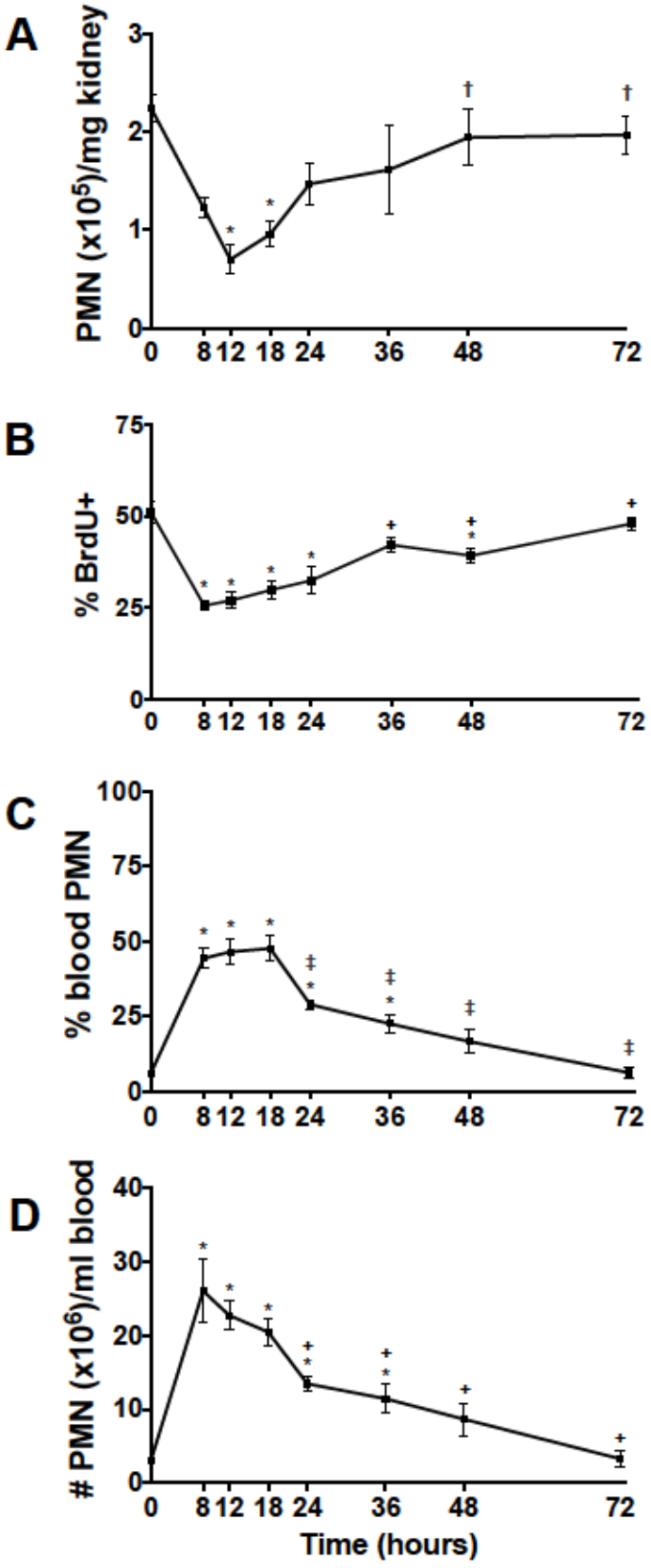
A
PMN (x10^5)/mg kidney
3
2
1
0
0 8 12 18 24 36 48 72
B
% BrdU+
75
50
25
0
0 8 12 18 24 36 48 72
C
% blood PMN
100
75
50
25
0
0 8 12 18 24 36 48 72
D
PMN (x10^6)/ml blood
40
30
20
10
0
0 8 12 18 24 36 48 72
Time (hours)

Figure 4.3. Intraperitoneal administration of zymosan induces an efflux of neutrophils from the hematopoietic tissue into circulation.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan for 0, 8, 12, 18, 24, 36, 48, or 72 h. Prior to kidney isolation, goldfish were injected intraperitoneally with BrdU and incubated for 1 h, to allow incorporation of BrdU into tissues. Kidneys were isolated, and total leukocytes were harvested. Cells were fixed for a minimum of 24 h in 1% formaldehyde, and then stained with anti-BrdU FITC. (A) Prior to harvesting cells, kidneys were weighed. Total kidney leukocytes at each time point were counted using a haemocytometer. To account for varying sizes or fish, counts were divided by kidney weight (in mg) to determine leukocytes / mg tissue (n=4). (B) The percent of proliferating cells within the kidney at each of the indicated times (n=4). (C) Fish were sacrificed, bled at 0, 8, 12, 18, 24, 36, 48, and 72 h, and peripheral blood smears were stained with Sudan Black. Neutrophils were identified as Sudan Black positive, while monocytes and lymphocytes, both stained negative. A minimum of 100 cells was counted to determine the proportion of neutrophils in the peripheral blood (n=4). (D) The number of peripheral blood neutrophils (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from 0 hpi, + significantly different from 8hpi, † significantly different from 12 hpi, and ‡ significantly different from 18 hpi.

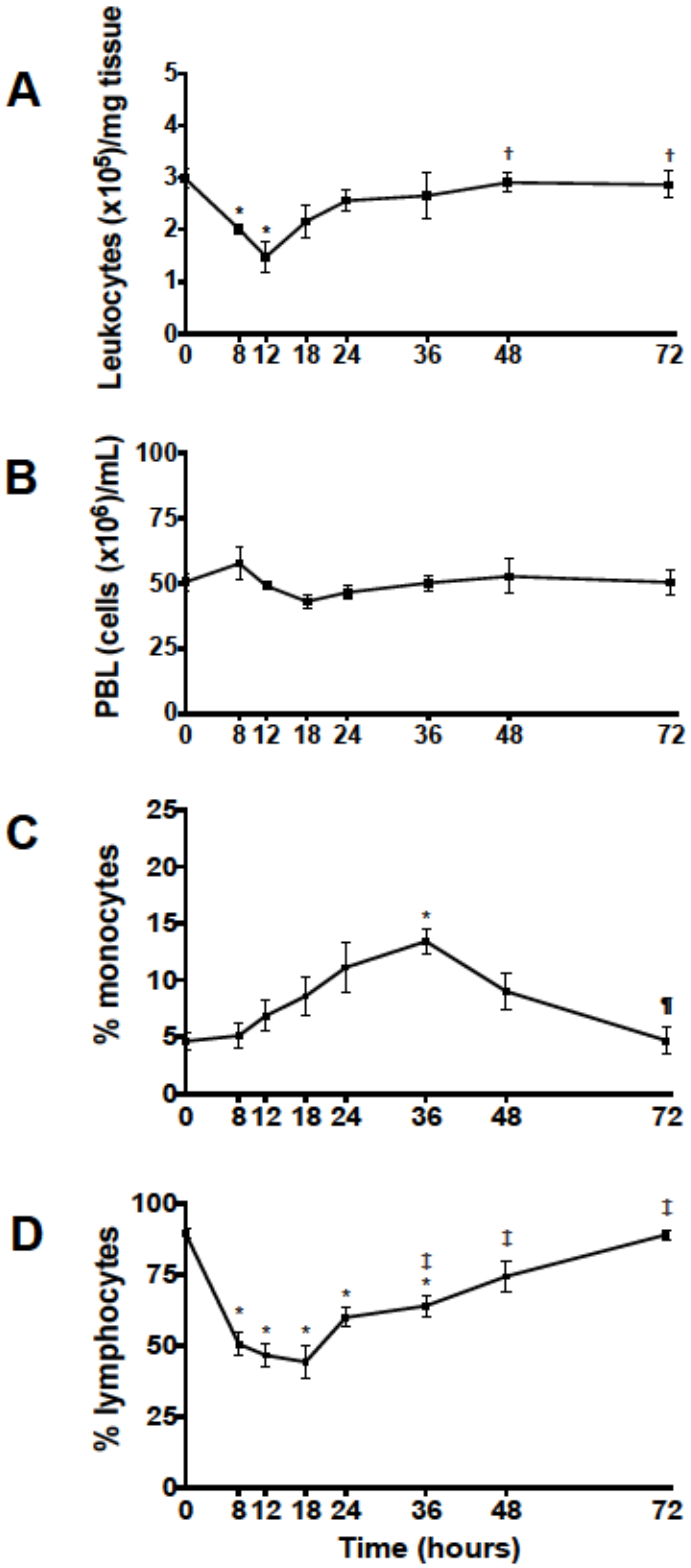
A
Leukocytes (x10⁵)/mg tissue
5
4
3
2
1
0
0 8 12 18 24 36 48 72
B
PBL (cells (x10⁶)/mL)
100
75
50
25
0
0 8 12 18 24 36 48 72
C
% monocytes
25
20
15
10
5
0
0 8 12 18 24 36 48 72
D
% lymphocytes
100
75
50
25
0
0 8 12 18 24 36 48 72
Time (hours)

Figure 4.4. Kinetics of leukocyte release from the kidney and entry into circulation.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. At 0, 8, 12, 18, 24, 36, 48, and 72 h, blood samples were drawn and kidney leukocytes were isolated. (A) Total number of leukocytes per mg of kidney tissue (n=4). (B) Kinetics of total peripheral blood leukocytes (PBL; n=4). (C) Proportion of monocytes in circulation (n=4). (D) Proportion of lymphocytes in circulation (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from 0 hpi, † significantly different from 12 hpi, ‡ significantly different from 18 hpi, ¶ significantly different from 36 hpi.

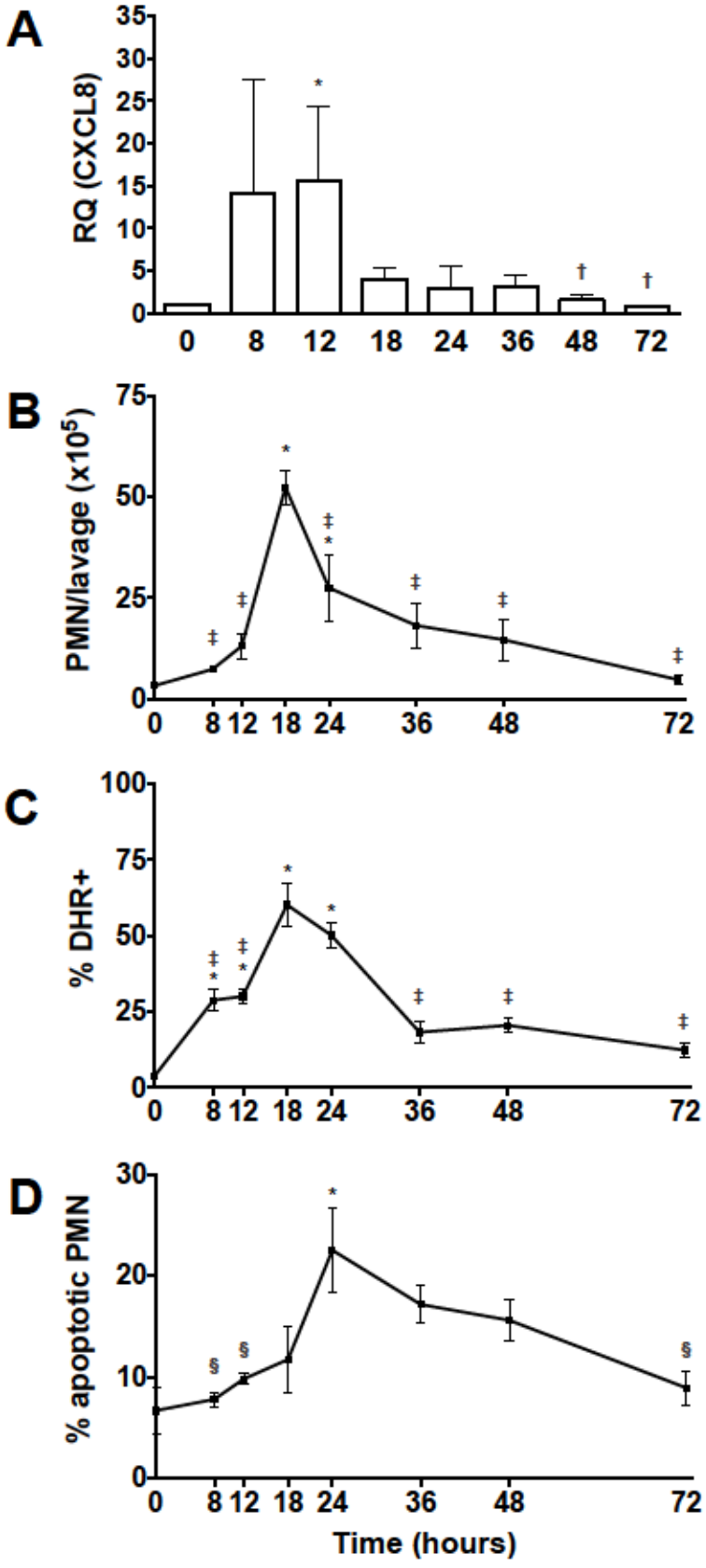
A
RQ (CXCL8)
35
30
25
20
15
10
5
0
0 8 12 18 24 36 48 72
B
PMN/lavage (x10^5)
75
50
25
0
0 8 12 18 24 36 48 72
C
% DHR+
100
75
50
25
0
0 8 12 18 24 36 48 72
D
% apoptotic PMN
30
20
10
0
0 8 12 18 24 36 48 72
Time (hours)

Figure 4.5. Kinetics of CXCL8 expression, neutrophil infiltration, downstream respiratory burst responses and entry into programmed cell death.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. At 0, 8, 12, 18, 24, 36, 48, and 72 h, peritoneal cells were harvested by lavage. (A) CXCL8 levels in total leukocyte population as measured by Q-PCR (n=4). (B) Kinetics of neutrophil infiltration. Neutrophils were isolated using Percoll and counted on a haemocytometer (n=4). (C) Respiratory burst responses of peritoneal neutrophils (n=4). (D) Percentage of apoptotic neutrophils isolated from the peritoneal cavity (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from 0 hpi, † significantly different from 12 hpi, ‡ significantly different from 18 hpi, § significantly different from 24 hpi.

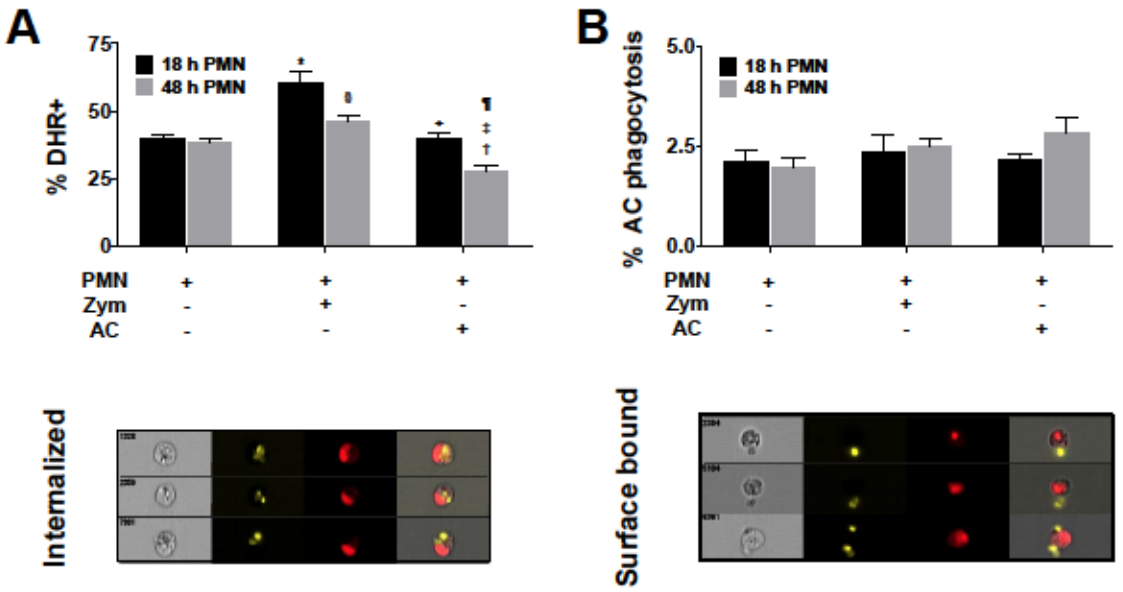

Figure 4.6. Neutrophil soluble mediators affect macrophage functional responses.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. Cells were harvested by peritoneal lavage at 18 or 48 hpi and run over a 51% Percoll gradient to isolate the neutrophils. Neutrophils were added to the upper chamber of a 6-well transwell. Macrophages were seeded in the lower compartment. Neutrophils were incubated with zymosan, apoptotic neutrophils or nothing at a 3:1 (particle: neutrophil) ratio. (A) Macrophage respiratory burst responses following 2 h incubation (n=4). (B) Percent internalization of apoptotic neutrophils by macrophages following 2 h incubation (n=4). Apoptotic neutrophils were generated from neutrophils isolated from the peritoneal cavity 18 hpi. Representative images from ImageStream MKII analyses show internalized or surface bound apoptotic neutrophils. All statistics correspond to a significance of $p<0.05$ using a two-way ANOVA; * significantly different from 18 h PMN alone, + significantly different from 18 h PMN+zym, † significantly different from 48 h PMN alone, ‡ significantly different from 48 h PMN+zym, § significantly different from 18 h PMN+zym, ¶ significantly different from 18 h PMN+AC.

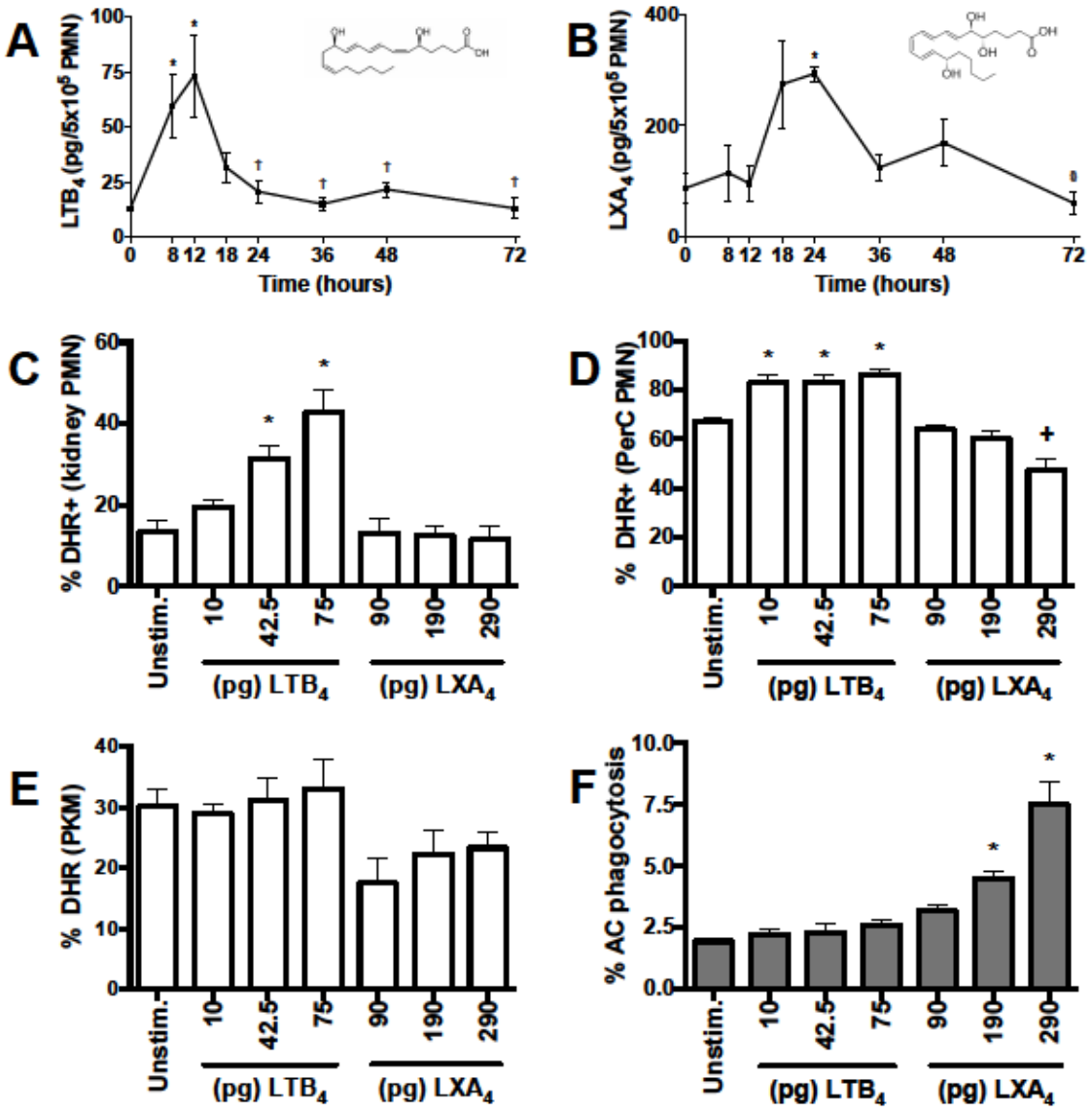
A
LTB4 (pg/5x10^5 PMN)
Time (hours)
B
LXA4 (pg/5x10^5 PMN)
Time (hours)
C
% DHR+ (kidney PMN)
Unstim.
(pg) LTB4
(pg) LXA4
D
% DHR+ (PerC PMN)
Unstim.
(pg) LTB4
(pg) LXA4
E
% DHR (PKM)
Unstim.
(pg) LTB4
(pg) LXA4
F
% AC phagocytosis
Unstim.
(pg) LTB4
(pg) LXA4

Figure 4.7. LTB_4 and LXA_4 affect both macrophage and neutrophil function.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. Cells were harvested by peritoneal lavage at 0, 8, 12, 18, 24, 36, 48 or 72 hpi and run over a 51% Percoll gradient to isolate the neutrophils. PKMs were used on day 7. Kinetics of LTB4 (A) and LXA_4 (B) release in the peritoneal exudate ($n = 3$). Statistics correspond to a significance of $p<0.05$ using a one-way ANOVA; * significantly different from 0 hpi, † significantly different from 12 hpi, § significantly different from 24 hpi. Effect of LTB_4 and LXA_4 on the respiratory burst responses of neutrophils from the hematopoietic kidney (C) and peritoneal cavity (PerC; D). Statistics correspond to a significance of $p<0.05$ using a two-way ANOVA; * significantly different from unstimulated neutrophils, + significantly different from all other concentrations. Effect of LTB_4 and LXA_4 on the capacity of macrophages for respiratory burst responses (E) and internalization of apoptotic neutrophils (F; n=4). All statistics correspond to a significance of $p<0.05$ using a two-way ANOVA; * significantly different from all other concentrations.

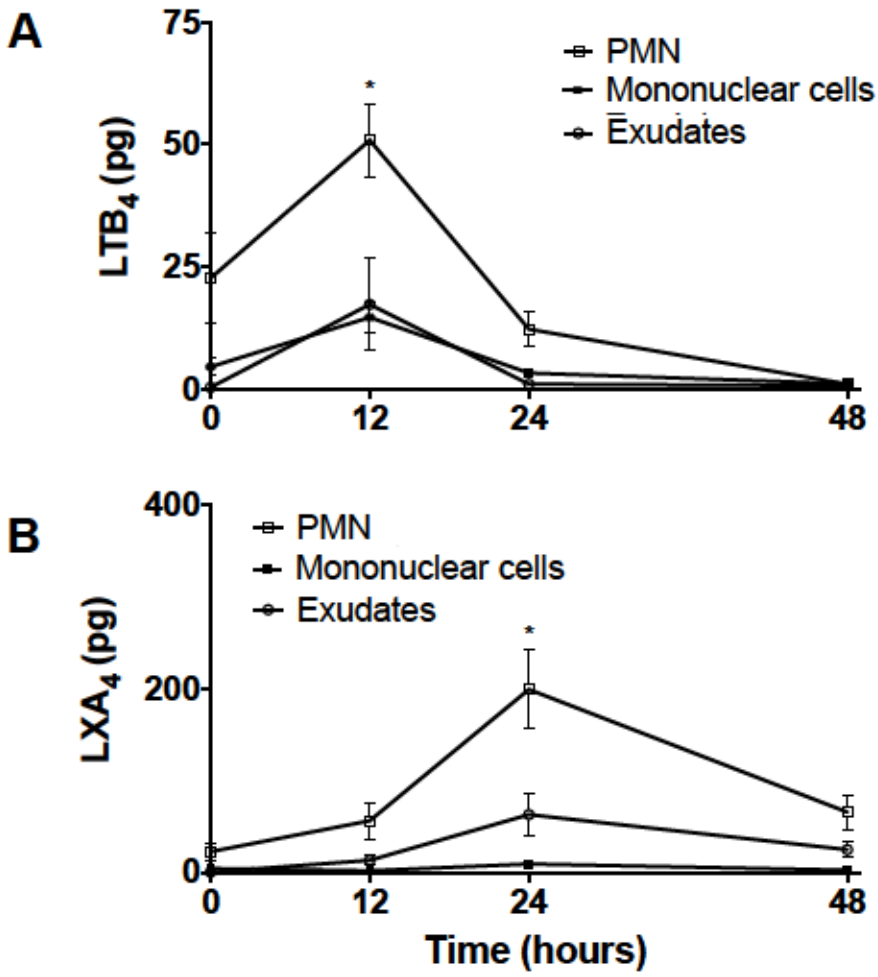

Figure 4.8. Kinetics of LTB_4 and LXA_4 in isolated neutrophils, mononuclear cells, and exudates.

Goldfish were injected intraperitoneally with 2.5 mg of zymosan. At 0, 12, 24, and 48 h, isolated neutrophils, mononuclear cells and exudates were collected. (A) LTB_4 in picograms per $5x10^5$ cells or 50 µL of total peritoneal exudate (n=3). (B) LXA_4 in picograms per $5x10^5$ cells or 50 µL of total peritoneal exudate (n=3). All statistics correspond to a significance of $p<0.05$ using a two-way ANOVA; * significantly different from all other samples/ time points.

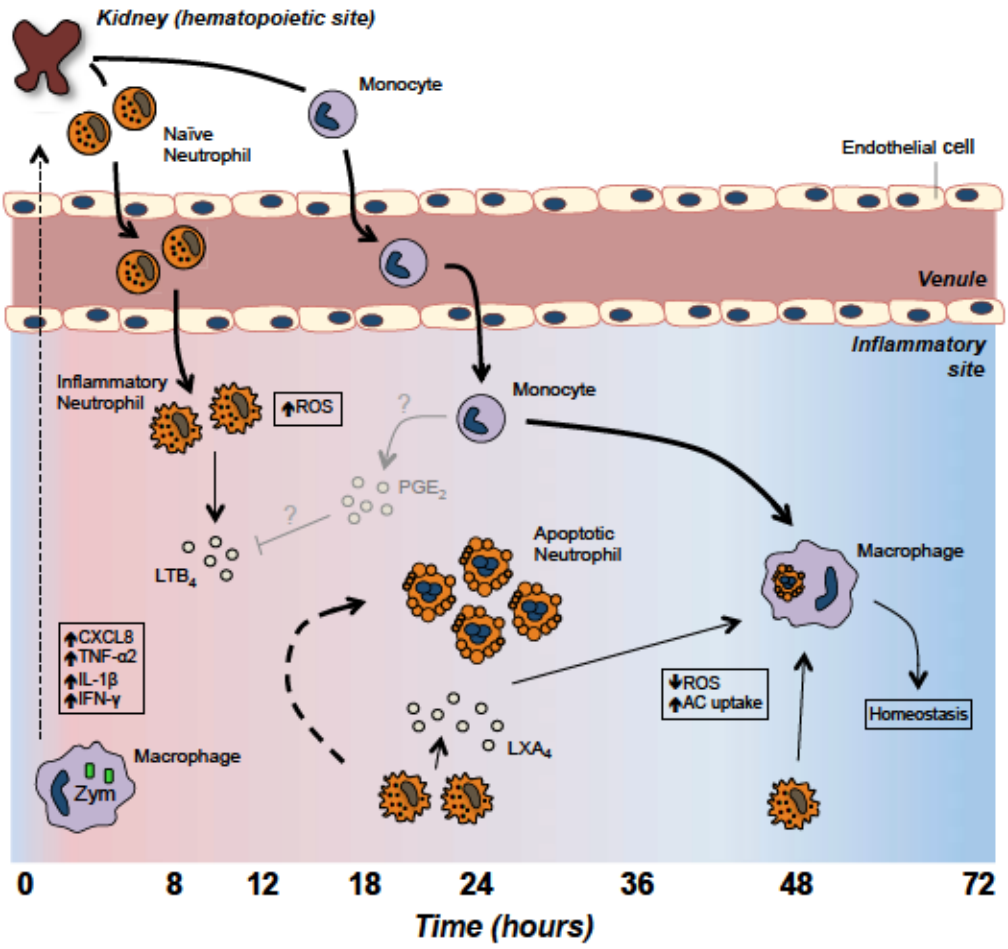

Figure 4.9. Contributions of neutrophils to the induction, regulation, and resolution of the acute inflammatory response in teleost fish.

Pathogen invasion of the host results in the release of chemoattractants and pro-inflammatory cytokines, such as CXCL8, TNF-α, IL-1β, and IFN-γ, inducing the infiltration of neutrophils as well as other leukocytes. Inflammatory neutrophils enter the site of inflammation and are activated by the local environment, resulting in increased ROS production and the release of LTB_4 (pro-inflammatory lipid mediator). As inflammation progresses, a transition from pro-inflammatory to pro-resolution begins to take place (18-24 hpi). Neutrophils switch to the production of LXA_4 and begin to become apoptotic, peaking at 24 hpi. We hypothesize this switch from LTB_4 to LXA_4 is mediated, at least in part, by PGE_2 production (greyed out in model with question marks). The local inflammatory milieu slowly becomes pro-resolving as macrophages are stimulated by LXA_4, decreasing their production of ROS and increasing their uptake of apoptotic neutrophils. This progression eventually leads to the return to homeostasis by 72 hpi.

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Chapter 5. Neutrophils exert protection during *Aeromonas veronii* infections through the clearance of both bacteria and dying macrophages[1]

5.1 Introduction

Aeromonas is one of the oldest known fish pathogens, originally characterized in the 1890s (Janda and Abbott, 2010) and most known for its devastating effects on the fishing industry (both wild and farmed) (Elliott and Shotts, 1980; Monette et al., 2006; Vivekanandhan et al., 2002). Historically, the genus was divided into two major groups. The mesophilic group, which consisted of motile strains that grew well at approximately 35 to 37 °C and were associated with a variety of infections (including in humans). The second group, referred to as the psychrophilic group were non-motile, had optimal growth temperatures between 22 and 25 °C, and were the typically found infecting fish (Janda and Abbott, 2010). This can still serve as a general breakdown in *Aeromonas*

[1] A portion of this chapter has been previously published in:

Havixbeck JJ, Rieger AR, Churchill LJ, and Barreda DR (2017) Neutrophils exert protection in early *Aeromonas veronii* infections through the clearance of both bacteria and dying macrophages. *Fish and Shellfish Immunology 63: 18-30*.

taxonomy, however the use of DNA-DNA hybridization has led to the classification of 18 genetically different species (Janda and Abbott, 2010). However, many of these share biochemical properties.

Herein, I examined *Aeromonas veronii*, a motile, mesophilic *Aeromonad*. It has been characterized as a 'highly virulent pathogen', infecting a range of species from invertebrates to aquatic vertebrates to mammals, including humans. In fish, *Aeromonas* infections are characterized by the presence of fin rot, tail rot, ulcers, and hemorrhagic septicemia- leading to scale shedding, hemorrhages in the gills and anal area, exophthalmia, and abdominal swelling (Bernoth et al., 1997; Hiney et al., 1992; Joseph and Carnahan, 1994; Wiklund and Dalsgaard, 1998). Infections also typically present with a furuncle on the exterior of the fish. However, not all infected fish present with furuncles, but are still capable of shedding bacteria into the environment at high rates (10^5-10^6 CFU/fish/hour) (Noga, 2010).

Skin wounding elicits inflammatory events that lead to rapid infiltration of phagocytes from the circulation to the site of infection (Li et al., 2005; Singer and Clark, 1999). A critical balance exists between the protective mechanisms of phagocytes and the potential for prolonged inflammation. Impaired leukocyte recruitment can lead to chronic inflammatory issues such as impaired wound healing (Miller et al., 2006; Mori et al., 2004), while excessive leukocyte infiltration often leads to chronic wound recurrence (Pierce, 2001). Developing effective models that track the dynamic shift in inflammatory environments resulting from leukocyte availability and recruitment from the hematopoietic tissue to the infection site are important in identifying the mobilization and function of phagocytes during infection.

The role of neutrophils during the inflammatory response against *Aeromonas* is not well understood, as many studies have focused on the interaction of this bacterium with macrophages. It has been shown that many virulent strains are able to infect and resist intracellular killing mechanisms of macrophages (Krzymińska et al., 2009; Lowry et al., 2014; Rosenzweig and Chopra, 2013; Sharp and Secombes, 1993a). Macrophages are capable of removing small numbers of pathogenic *Aeromonas*; however, bacterial cytotoxicity begins to develop when *Aeromonas* outnumbers macrophages, inducing apoptosis (Krzymińska et al., 2009; Sharp and Secombes, 1993b) or pyroptosis (McCoy et al., 2010; Sahoo et al., 2011). In addition, infection leads to the over-production of ROS and NOS by the host, resulting in bacterial-induced apoptosis of epithelial cells from mitochondrial depolarization due to oxidative stress (Krzymińska et al., 2011).

I have previously examined the role of neutrophils during the induction and resolution of inflammation using an intraperitoneal zymosan challenge model (Havixbeck et al., 2015). Interestingly, I found that teleost neutrophils possess the capacity to mediate divergent pro- and anti-inflammatory responses, depending on the environmental inflammatory milieu from which they were isolated (Havixbeck et al., 2015). In addition, neutrophils released copious amounts of lipoxin A_4 (LXA_4), which was found to be a significant contributor to the uptake of apoptotic cells by teleost macrophages (Havixbeck et al., 2015), similar to results previously observed in mammalian studies (Levy et al., 2001; Serhan, 2014; Serhan et al., 2008).

In light of the effects of *Aeromonas* on macrophages and epithelial cells at the site of infection and the newly discovered roles of fish neutrophils in the resolution of inflammation, I hypothesized neutrophils would play a predominant role in the clearance

of this pathogen, as well as the regulation of inflammation associated with infection. Herein, I isolated a wild strain of *Aeromonas veronii* and developed an infection model to examine inflammatory events, with a focus on neutrophil migration, infiltration, and function at the infection site. Following infections, neutrophils exit the hematopoietic kidney and enter into circulation marking a period of neutrophilia 1-2 days post infection (dpi). Shortly thereafter (2-4 dpi), I observed a thickening of leukocytes at the surface of the furuncle, followed by an increase in leukocyte nuclear density within the tissue (7-10 dpi). Neutrophils were capable of migrating toward, internalizing, and killing *A. veronii*. Interestingly, teleost neutrophils were capable of internalizing dying macrophages at high levels. Further, once internalized neutrophils became activated and increased the overall production of reactive oxygen species within the population. Taken together, my results suggest that: 1) neutrophils are capable of detecting specific cellular differences between various types of dying cells, leading to diverse downstream effects following interaction and/or uptake; and 2) not all mechanisms of apoptosis and pyroptosis are identical in their end result- both the method and route of induction are important.

5.2 Results

5.2.1 Bacteria isolated from naturally infected fish were isolated and subsequently used to develop an infection model

Goldfish naturally infected with *Aeromonas* are commonly found within aquatic facilities, with the prevalence increasing during the spring months (spawning season) or during periods of stress (shipping, tank transfer, etc.). Infection generally presents as sores on the body, mouth and oral cavity, or on fins/tail. Sores were classified based on

the level of progress- early stage of infection had a raised, red area; mid stage of infection had a red sore with a white center that had not erupted; late stage of infection had larger sores that had erupted in the center (Figure 5.1A). When goldfish were swabbed, bacteria were only detected from the furuncles, but not from areas without sores (on infected or control fish) (Figure 5.1B).

Bacterial clones were isolated from 6 furuncles (2 body, 1 mouth/oral cavity, 1 eye and 2 fin/tail) and expression of 16s rRNA and *gyrB* were analyzed by PCR (Figure 5.1C). PCR products were purified and sequenced. Sequence alignments identified the isolated bacteria as *Aeromonas veronii* bv *sobria*.

While intraperitoneal injection is a good method to examine the inflammatory response in a controlled environment, it does not recapitulate the true nature of an *Aeromonas* infection (e.g. mechanism of exposure, route of entry of the bacterium into fish). By developing the furuncle infection model I am better able to mimic a fish rubbing up against another fish with a sore. Bacteria from the swabbed area would also be introduced into the water, thus coming into contact with all the fish in the tank. Fish were then harvested at the indicated time points and representative fish are shown in Figure 5.2.

5.2.2 Goldfish with natural or induced *A. veronii* infection have a significant reduction in proliferation within the hematopoietic tissue

Proliferation is crucial to the maintenance of mature leukocytes in the hematopoietic compartment. As a first step in examining the inflammatory response against *A. veronii*, I investigated the effects of infection on the proliferative capacity of

goldfish within the hematopoietic kidney. Interestingly, goldfish that were infected with *A. veronii*, either naturally (mid to late stage of infection; Figure 5.3A) or through an induced infection (Figure 5.3B), showed significant decreases in kidney cell proliferation.

5.2.3 Rapid mobilization of neutrophils from the hematopoietic compartment into circulation results in a period of neutrophilia following infection with *A. veronii*

The mobilization of neutrophils from bone marrow to the inflammatory site has been studied extensively in mammals in both infection and non-infection models (32–35). Several similar studies have also examined the migration of neutrophils in teleost fish (de Oliveira et al., 2013; Deng et al., 2013; van der Aa et al., 2010); little work, however, has been done to dissect the roles of teleost neutrophils in response to a live *A. veronii* infection. Following infection with *A. veronii,* I found that the number of circulating neutrophils increased nearly 10-fold over uninfected levels following infection, resulting in shift of blood neutrophils from ~6% to nearly 50% of circulating leukocytes between 1-2 days post infection (dpi; Figure 5.4B). During the same time period I also observed a significant decrease (5.7-fold) in neutrophils within the hematopoietic compartment (Figure 5.3C). Interestingly, total blood leukocyte counts remained relatively constant throughout the 14 day infection (Figure 5.4A), similar to what I previously observed using a zymosan-induced peritonitis model (Havixbeck et al., 2015). Thus, the increase in circulating neutrophils came largely at the expense of lymphocytes, as peripheral blood lymphocytes decreased by over 50% at 1-2 dpi in order to accommodate the infiltration of neutrophils (Figure 5.4B). Of note, monocytes did not appear to exit the hematopoietic

site (Figure 5.3C) and blood counts remained consistent through all infection time points (Figure 5.4B).

Aeromonas infections have previously been characterized by acute septicemia with accompanying furuncles/ tail or fin rot, and can establish a chronic infection within muscles and organs, especially the kidney (Janda and Abbott, 2010). In light of this I examined the furuncle, blood, and kidney (hematopoietic tissue) of infected fish for the presence of *A. veronii*. Interestingly, the immune response takes over a week to clear *Aeromonas* at the site of infection, as the bacteria could still be detected 10 dpi (Table 5.1). However, with my detection method, I was only able to detect bacteria in the blood 1 dpi, and until 2 dpi in the kidney.

5.2.4 Leukocytes arrive at the furuncle surface, followed by an increase in leukocyte density within the damaged muscle tissue

In mammals, skin wounding triggers an inflammatory cascade resulting in the rapid recruitment of phagocytes from the circulation to the site of infection (Li et al., 2005; Singer and Clark, 1999). Further, the infiltration of leukocytes is key to the elimination and resolution of infection in both mammals and teleost fish (Deng and Huttenlocher, 2012; Kim et al., 2008; Nguyen-Chi et al., 2014; Ozer et al., 2015; Rieger and Barreda, 2011; Well et al., 2007). Furuncle tissue sections were stained with Hematoxylin & Eosin and the leukocyte migration and recruitment, with a focus on neutrophils, was examined. Figure 5.5A shows representative images from each of time point during infection. I found that total leukocyte infiltration into the infection site displayed two prominent peaks with distinct locations within the tissue. The first peak

was found at 2-4 dpi, with a leukocyte layer (solid black arrows) forming outside of the dermis on the surface of the furuncle, increasing approximate 4-fold in thickness over basal levels (Figure 5.5B). The second peak was observed at 10 dpi within the muscle tissue. I found a significant increase (6-fold) in the density of leukocytes (solid white arrow) within the muscle tissue compared with basal levels (Figure 5.5C).

To further dissect the inflammatory environment within the furuncle, I also examined a panel of chemokines (CXCL-8 and CCL-1), pro- (TNF-α2 and IL-1β1) and anti- (IL-10 and TGF-β) inflammatory cytokines. The expression of CXCL-8, an important neutrophil chemokine, peaked early on at 1-2 dpi (Figure 5.5D), correlating well with the mobilization of neutrophils from the hematopoietic kidney into circulation (middle panel Figure 5.3C and Figure 5.4B). Using quantitative PCR, I was able to detect neutrophils within the furuncle though G-CSF receptor expression at all time points during infection (data not shown). However, further analysis is needed to determine the exact movement of neutrophils throughout the infected tissue. Interestingly, I observed no change in CCL-1 expression (Figure 5.5D), one of the chemokines involved in recruiting monocytes. This data was consistent with the lack of monocyte movement throughout the infection model (middle panel Figure 5.3C and Figure 5.4B). The expression of two pro-inflammatory cytokines, TNF-α2 and IL-1β1, underwent modest increases (8-fold, and 3-fold, respectively (Figure 5.5D). In contrast, neither of the anti-inflammatory cytokines, IL-10 and TGF-β, increased significantly during the time points I examined (Figure 5.5D).

5.2.5 Neutrophils migrate toward and rapidly kill *Aeromonas veronii*

In order to examine the chemotactic and pro-inflammatory capacity of neutrophils toward *A. veronii*, I took advantage of various states of neutrophil activation from several different tissue locations. I found that naïve neutrophils isolated from the kidney of goldfish were highly chemotactic toward *A. veronii*. Even at a 1:1 ratio of *Aeromonas*, a two-fold greater number of neutrophils crossed the chemotactic barrier to the well containing bacteria (Figure 5.6A). In addition, the number of neutrophils migrating across the membrane increased in a concentration dependent manner, peaking at approximately 200 cells per field of view in the ratios I examined (Figure 5.6A). Following chemotaxis, neutrophils ideally engulf the bacteria and eliminate the pathogen. In light of this, I isolated peritoneal neutrophils 18 hours post *A. veronii* injection. This time point represents the peak infiltration point of neutrophils following the injection of heat-killed *A. veronii* into the peritoneal cavity (unpublished data), providing me with *in vivo* activated neutrophils. As expected, approximately 66% of neutrophils had internalized *A. veronii*, while roughly 11.4% had bound the pathogen, indicating that 3 out of 4 neutrophils were interacting with the bacteria in some manner (Figure 5.6B). In addition, roughly 75% of these same neutrophils isolated from the peritoneal cavity were positive for intracellular ROS production (Figure 5.6C), a common mechanism by which neutrophils destroy internalized pathogens. Finally, I examined the ability of neutrophils to kill internalized *Aeromonas*. In line with my previous findings, neutrophils were able to eliminate over half of the bacteria within two hours (Figure 5.6D). In combination, these results point to neutrophils as important cells in the defense against *Aeromonas*.

5.2.6 Teleost neutrophils internalize *Aeromonas veronii* induced apoptotic macrophages

Resident tissue macrophages are one of the primary cells responsible for early engagement of pathogenic particles and the release of necessary signals (pro-inflammatory cytokines, chemokines, etc.) that initiate an inflammatory cascade and recruit neutrophils (Davies et al., 2013; Davies and Taylor, 2015; McCarthy, 2015; Medzhitov, 2007; Mogensen, 2009; Takeuchi and Akira, 2010). However, it has been previously noted that *Aeromonas* induces high levels of cell death within macrophage populations (Krzymińska et al., 2009; Rosenzweig and Chopra, 2013). To this end, I examined the ability of *A. veronii* to induce cell death at varying bacteria to cell ratios. I observed significant macrophage cell death at ratios as low as 1:1 (bacteria: cell) (Figure 5.7A). Interestingly, at a 3:1 ratio, nearly all macrophages had been eliminated, consistent with a high level of virulence for this organism. However, when I examined the effect on neutrophils, a ratio of 3:1 induced cell death in roughly 50% of cells, while it took a ratio of 10:1 to eliminate neutrophils entirely (Figure 5.7A).

Previous studies from our lab indicated that teleost neutrophils were incapable of internalizing cycloheximide-induced apoptotic cells (Havixbeck et al., 2015; Rieger et al., 2012). Interestingly, another study found that neutrophils were capable of internalizing dying macrophages that were infected with *Mycobacterium marinum* (Yang et al., 2012). Therefore, I assessed the ability of goldfish neutrophils to internalize dying cells induced through a more natural mechanism- infection with *A. veronii*. I found that, similar to the *Mycobacterium marinum* infected macrophages, goldfish neutrophils were capable of internalizing dying macrophages that had been incubated with *A. veronii* (Figure 5.7B).

In order to ensure that neutrophils were in fact recognizing the dying macrophages, I incubated macrophages rendered apoptotic via cycloheximide with live, heat-killed, or sonicated *A. veronii* and examined phagocytosis. Interestingly, there was only minor surface binding of these apoptotic macrophages (Figure 5.8). This result points to the complexity of cell death mechanisms, leading us to suspect each specific mechanism by which a cell dies is important. From here, I focused my examination on the resulting effect on neutrophils following the uptake of dying macrophages. I observed no changes in neutrophil viability as measured via AnnexinV/PI staining (Figure 5.7C), or mitochondrial membrane potential (Figure 5.7D). However, I did find that following the interaction (binding and/or uptake) with dying macrophages, neutrophils began producing robust levels of reactive oxygen species (Figure 5.7E). This suggests that although neutrophils are internalizing dying cells, they maintain the capacity promote pro-inflammatory functions against *A. veronii*.

5.3 Discussion

Aeromonas veronii is a ubiquitous fresh water pathogen that has been isolated from a variety of hosts, including vertebrates and invertebrates (Austin and Austin, 2007; Deodhar et al., 1991; Graf, 2015, 1999; Horne, 1928; Krzymińska et al., 2009). While this bacteria may have beneficial symbiotic relationships with hosts such as the medicinal leech, *Hirudu medicinalis*, *Aeromonas* is a highly virulent bacterial pathogen linked to diseases in a number of animal species, including humans (Janda and Duffey, 1988; Krzymińska et al., 2011; McCoy et al., 2010; Tomas et al., 2012). For fish, one of the most common acute symptoms during *Aeromonas* infections is the development of severe

tissue damage, known as furunculosis. I observed naturally infected fish over the course of infection, which allowed us to define 3 main stages of acute furunculosis; 1) early stage of infection with the development of a small red sore, 2) mid stage of infection with the eruption of white necrotic or apoptotic tissue exposing the musculature, and 3) late stage of infection with total sore eruption that often results in complete tissue loss at the center of the furuncle. My descriptions were similar to those previously reported in Atlantic salmon, where they defined a peracute stage with a few visible external sores, an acute stage where furuncles may ulcerate releasing a red brown fluid and exposing deep cavities within the musculature, and finally a chronic stage with enormous cavities developing within the furuncle and damage extending beyond the initial point of tissue damage (Munro, 1988).

The progression of infection I observed in the naturally infected fish led us to develop an induced infection to study the immunological responses to *A. veronii* in a controlled environment. This allowed us to control the route of infection and monitor it's course more closely. A patch of scales was removed and the underlying tissue was slightly lacerated, and infected with a swab of *A. veronii*. This created a predictable course of infection, allowing us to examine the mechanisms of immune defense in goldfish.

In both mammals and teleost fish, skin wounding triggers an inflammatory/ immune cascade resulting in the rapid recruitment of phagocytes to the site of infection (Li et al., 2005; Singer and Clark, 1999). In addition, the infiltration of leukocytes is key to the elimination and resolution of infections (Deng and Huttenlocher, 2012; Kim et al., 2008; Nguyen-Chi et al., 2014; Ozer et al., 2015; Rieger and Barreda, 2011; Well et al.,

2007). During infections, neutrophils often represent the first line of effector cells during acute inflammation, where their recruitment into peripheral tissues is central to the immune response and defense against invading pathogens. In both mammals and teleost fish, a large storage pool of neutrophils exists within the hematopoietic tissue (Boxio et al., 2004; Furze and Rankin, 2008; Havixbeck et al., 2015; Rankin, 2010). Upon receiving the necessary signals, this pool is rapidly mobilized to an infection or inflammatory site, resulting in a dramatic increase in the number of circulating neutrophils. Consistent with my previous results following a peritoneal challenge with zymosan (Havixbeck et al., 2015), I observed a rapid efflux of neutrophils, where approximately 80% of neutrophils leave the hematopoietic tissue following infection with *Aeromonas veronii*. The rapid exodus of neutrophils from the hematopoietic compartment lead to a period of severe neutrophilia in the first 48 hours of infection, where I found more than $2.5x10^7$ neutrophils/mL of blood. Interestingly, I observed little to no movement of monocytes out of the hematopoietic tissue, in stark contrast to other inflammatory models where monocyte egress was observed throughout the challenge period. The lack of monocyte egression following infection with *A. veronii* could be due to: 1) the selective recruitment of neutrophils during infection with this bacterium; or 2) adverse effects of *A. veronii* on the monocyte/macrophage population in the kidney. As I have shown here, *A. veronii* induces a high level of cell death in kidney monocyte/ macrophage populations, which may lead to the unavailability of viable monocytes to traffic from the kidney. Interestingly, I have also observed large increases in a negative regulator of monopoiesis, the soluble CSF-1R at the site of infection (unpublished observations). This soluble receptor may be inhibiting CSF-1R signaling in resident

tissue macrophages, and thus lead to a decreased drive for monocyte migration. Finally, recent evidence has shown that mature peritoneal macrophages can rapidly migrate via a non-vascular route into visceral organs to aid in tissue repair (Wang and Kubes, 2016), providing yet another explanation for the lack of monocyte migration. Together, this opens the door for an in depth examination of alternative macrophage proliferation and migration from tissues other than the hematopoietic site during infection from *A. veronii*.

Following an egress of leukocytes from the kidney, I observed increases in leukocyte numbers within the site of infection. Based on cellular morphology and weak Sudan Black staining, the vast majority of leukocytes recruited to the site appear to be neutrophils, though this cannot be fully determined due to a lack of immune cell type specific markers. My examination of two central chemokines responsible for the migration of neutrophils and monocytes, CXCL8 and CCL1 respectively, provided further evidence to support my hypothesis that the neutrophils are dominant cell type recruited to the furuncle. CXCL8 expression within the furuncle showed a robust increase during the early stages of infection. The increase in CXCL8 expression corresponded with the migration of neutrophils from the hematopoietic compartment, into circulation, and into the site of infection. It should be noted that CXCL8 may only play a partial role in neutrophil recruitment, and other chemotactic mediators such as LTB_4, C3a, C5a, and FMLP may also be involved. I observed no increase in CCL1 expression, which parallels with the lack of observed monocyte movement. My data suggests that the cells first migrate to the surface of the wound, exterior to the dermis (stratum compactum). I speculate that the increase of leukocytes at the wound surface during the early stages of infection is to kill and eliminate the invading *A. veronii* within the furuncle. This layer of

leukocytes begins to decrease around 7 dpi, the same time point when few bacteria remain at the infection site. From here, many of the leukocytes appear to migrate into the musculature. These cells may be playing a crucial role in the return to homeostasis and tissue regeneration; however, further work is needed to verify this hypothesis.

The balance between pathogenic and homeostatic signals is crucial for effective pathogen clearance with minimal damage to surrounding host tissues (Medzhitov, 2007). Neutrophils are classically known for their potent pro-inflammatory responses. As shown here, neutrophils were chemotactic toward *A. veronii*, internalized the bacteria, produced robust levels of ROS, and killed the bacterium. However, it should be noted that ROS is used as a marker of a pro-inflammatory killing response, and does not conclusively suggest that ROS is or is not the primary method by which these bacteria are killed. Further analysis should take advantage of commercial anti-oxidants to block reactive oxygen species; followed by an analysis of neutrophilic killing responses toward *A. veronii* to determine the role of ROS in killing. Building upon this by examining other pro-inflammatory antimicrobial killing mechanisms, such as degranulation or NET production, would also aid in painting a larger picture of killing responses that may be involved.

I was also interested in examining what pro-resolving roles, if any, were displayed by neutrophils. I wanted to examine the impact of *A. veronii* on the viability of monocytes and neutrophils. I found that *Aeromonas* had a profound effect on leukocyte viability, especially within the macrophage lineage. Interestingly, previous studies found that *Aeromonas* spp. were capable of inducing both apoptosis (Krzymińska et al., 2009) and pyroptosis (McCoy et al., 2010; Rosenzweig and Chopra, 2013) in macrophages. The

mechanism of cell death is important to the overall outcome of the immune response. Rendering cells apoptotic can ultimately send the host into early resolution, allowing the pathogen to replicate, disseminate, and cause further disease (Erwig and Henson, 2007). Alternatively, the induction of pyroptosis is a pro-inflammatory event, which should allow the host to maintain its pro-inflammatory microenvironment and eliminate the pathogenic threat (Bergsbaken et al., 2009), but can induce more damage to surrounding cells. This places the host and *Aeromonas* at an evolutionary arms race, where it is likely that goldfish macrophages would prefer to elicit a controlled necrotic cell death (pyroptosis) during the early stages of infection, effectively allowing the host to maintain its inflammatory response and clear the infection. However, *A. veronii* would likely prefer to induce a controlled apoptotic cascade, thus leading to decreased pro-inflammatory responses, allowing the bacterium to further infiltrate and disseminate within the host.

Recent evidence has reported that teleost neutrophils were capable of internalizing dying macrophages infected with *Mycobacterium marinum* (Yang et al., 2012). As such, I decided to investigate the capacity of neutrophils to internalize macrophages that had been killed by *Aeromonas*. Much to my surprise, I found that neutrophils were in fact capable of internalizing these dying macrophages. It should be noted that neutrophils were still unable to internalize any other form of induced dying cell, thus suggesting that each specific mechanism by which a cell dies is important, potentially inducing unique cellular features such varying receptors on the cell surface. Another potential explanation is the state of the neutrophil itself. Neutrophils will receive specific extracellular cues from the environment as well as the inflammatory milieu. This may lead to a specific set

of activation steps, which then allow neutrophils to take up apoptotic cells. This represents a unique pathway of further examination, using neutrophils activated under varying conditions both *in vivo* and *in vitro*.

Lastly, I was interested if the internalization of these dying macrophages had any effects on neutrophil viability or downstream responses. Intriguingly, there was no effect on cellular viability, though the neutrophils exhibited healthy ROS responses following internalization. These findings are similar to that of Yang and colleagues who observed the oxidative killing of the *Mycobacterium* infected macrophages. (Yang et al., 2012) Taken together, these results suggest that neutrophils are exhibiting pro-resolving responses by internalizing dying cells, while discerning the exact nature of the dying cell and simultaneously responding in a pro-inflammatory manner. This study provides additional insight into the complicated mechanisms by which neutrophils contribute to the induction and regulation of acute inflammatory responses.

Table 5.1. Mean colony forming units (CFU) of *A. veronii* from the indicated tissues throughout infection

dpi	1	2	4	7	10	14
CFU/furuncle	TNTC	TNTC	TNTC	72.0±9.0	21.3±4.8	TFTC
CFU/blood	1.1±0.6	TFTC	TFTC	TFTC	TFTC	TFTC
CFU/mg kidney	81±9	126±13	TFTC	TFTC	TFTC	TFTC

TNTC- too numerous to count; TFTC- too few too count; dpi- days post infection
Counts represent Mean + SEM; n=6 over a minimum of 2 independent experiments.

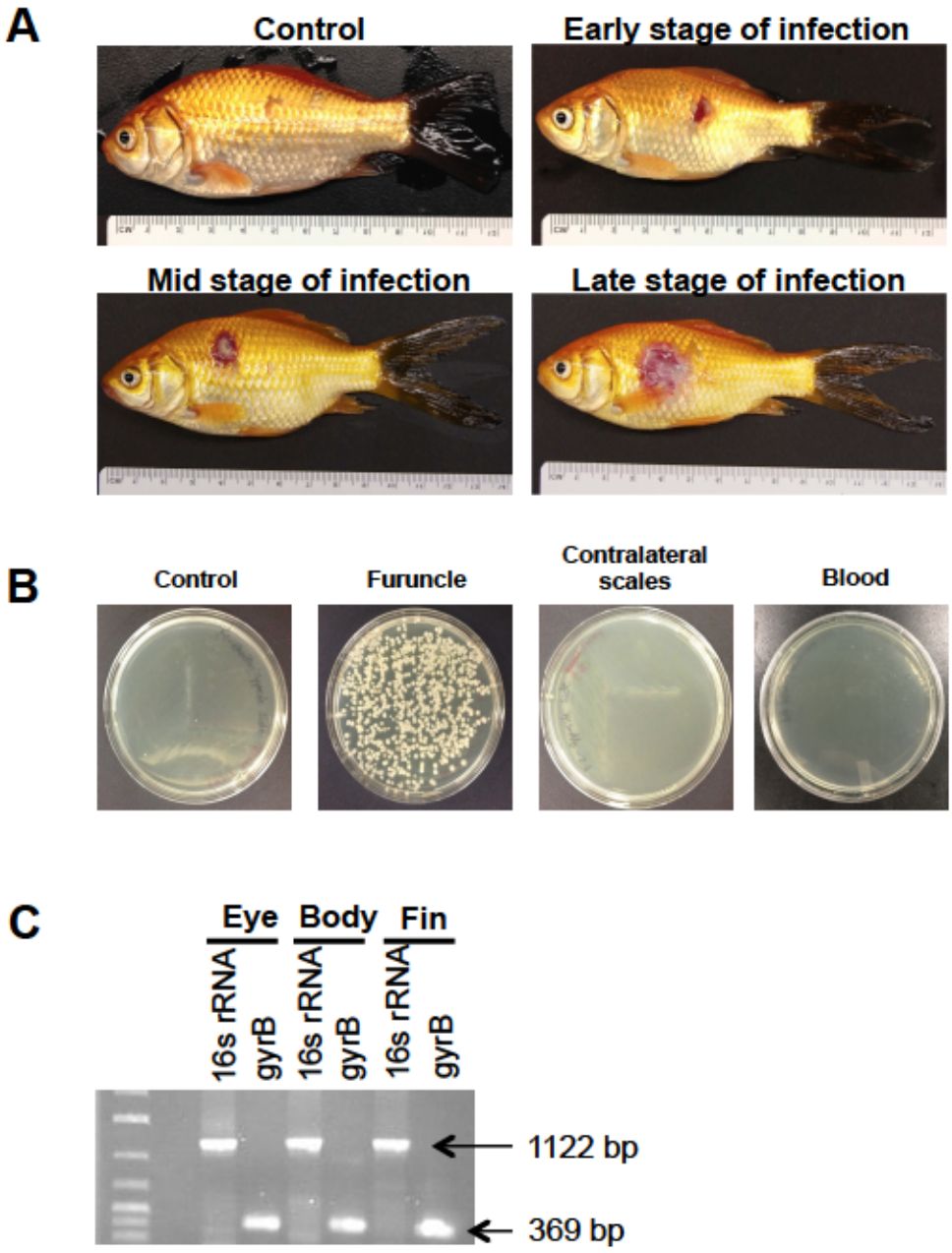


Figure 5.1. Presentation and isolation of a naturally occurring *Aeromonas* infection.

(A) Representative images of goldfish presenting with body sores at various stages of infection. (B) Representative images of plates streaked from swabs of goldfish scales. (C) Goldfish presenting with an *Aeromonas* infection were swabbed and isolated. Bacterial clones were analyzed for expression of 16S rRNA and gyrB. Sequence analysis identified the bacteria to be *Aeromonas veronii* biovar *sobria*.

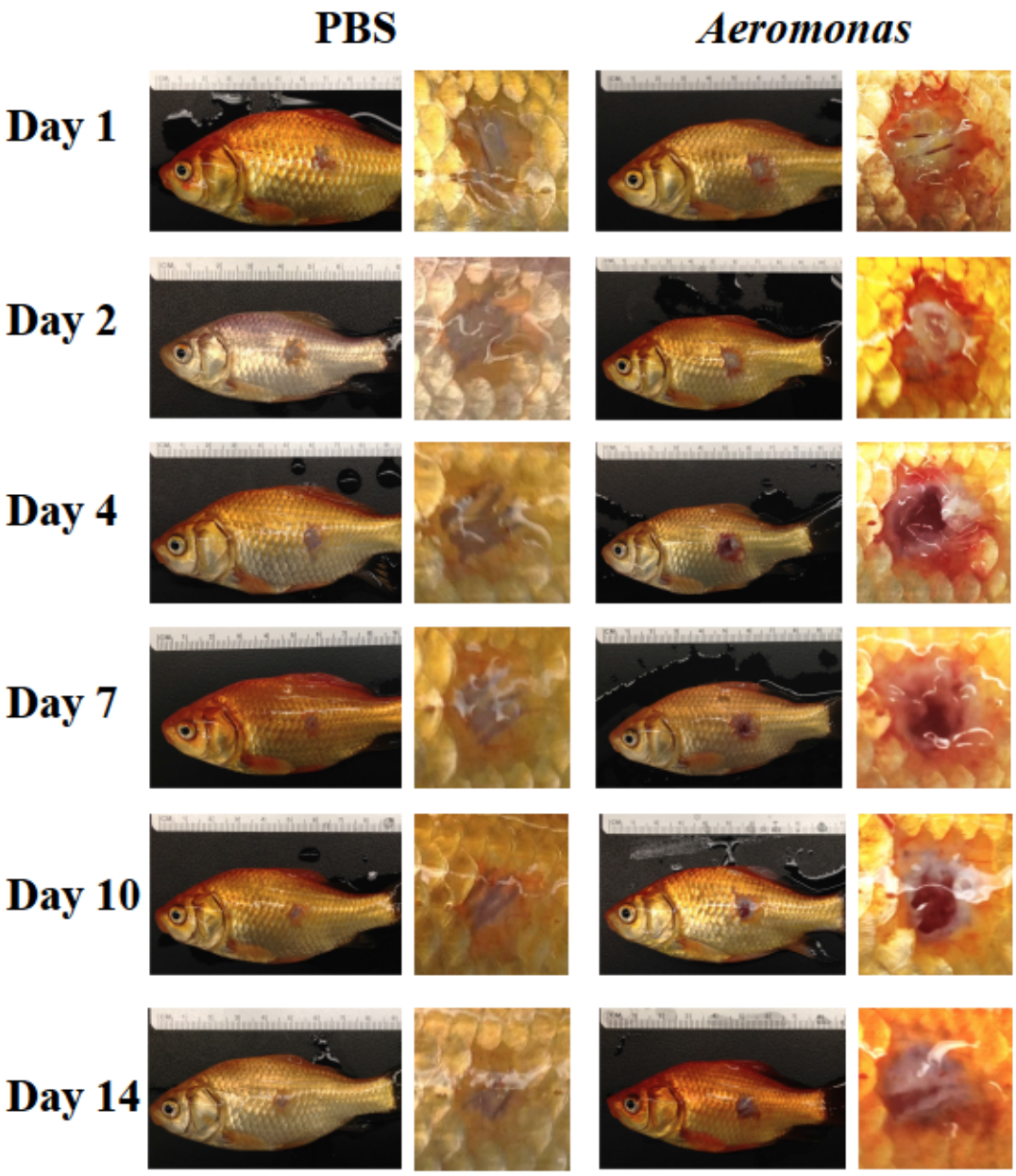
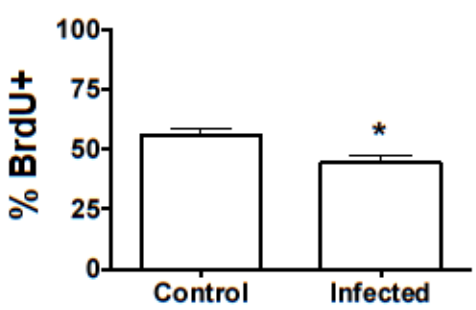
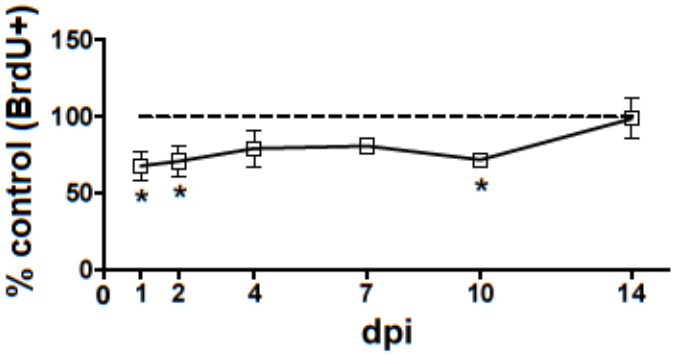
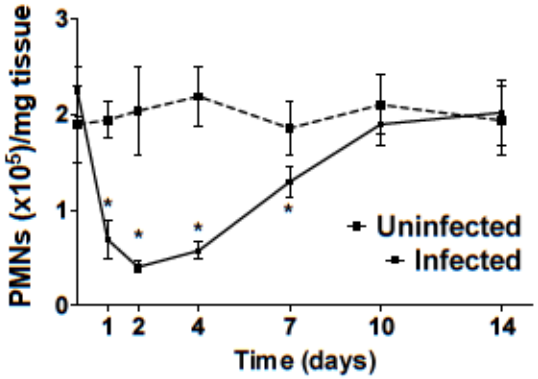


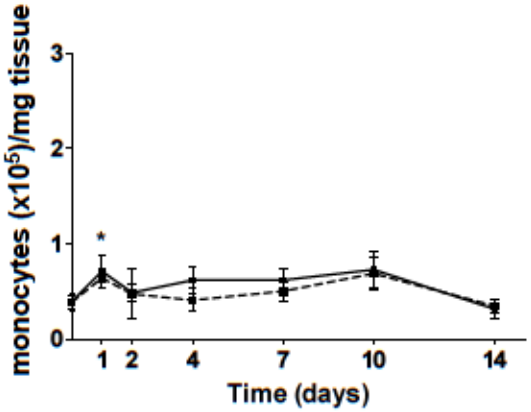
Figure 5.2. Representative model images of goldfish with induced *Aeromonas veronii* infection.

Goldfish were infected with *Aeromonas veronii* by rubbing an exposed area with a swab soaked in a clonal culture. Fish were harvested at the time points indicated.


% BrdU+
100
75
50
25
0
Control
Infected
*


% control (BrdU+)
150
100
50
0
0 1 2 4 7 10 14
dpi
* * *


PMNs (x10[5])/mg tissue
3
2
1
0
1 2 4 7 10 14
Time (days)
Uninfected
Infected


monocytes (x10[5])/mg tissue
3
2
1
0
1 2 4 7 10 14
Time (days)

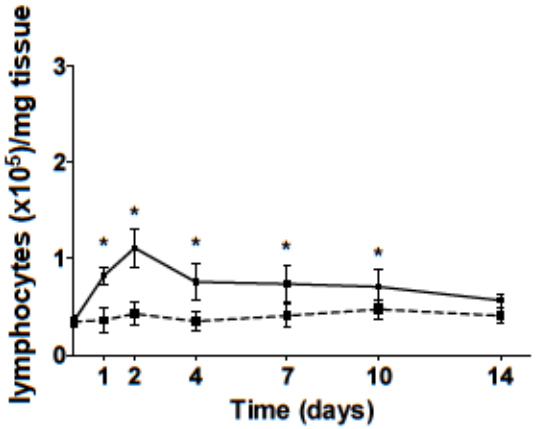

lymphocytes (x10[5])/mg tissue
3
2
1
0
1 2 4 7 10 14
Time (days)

Figure 5.3. Neutrophils rapidly exit the hematopoietic site following infection.

At each of the indicated time points, furuncle tissue was harvested from *Aeromonas veronii*. (A) Prior to kidney harvesting, goldfish were injected i.p. with BrdU and incubated for 1 hour. Kidneys were then harvested from (A) naturally-infected goldfish or (B) goldfish with an induced infection and proliferation was measured based on BrdU incorporation. For naturally infected fish (n=8); for induced infection (n=4); *$p<0.05$ compared to control (Student's t-test for A; Two-way ANOVA for B); error bars indicate SEM. (C) The number of leukocytes ($x10^5$) per mg of tissue. Solid lines represent infected fish. (n=4) Dashed lines represent uninfected controls. Statistics were performed using a two-way ANOVA with a comparison to uninfected controls and the equivalent time point.

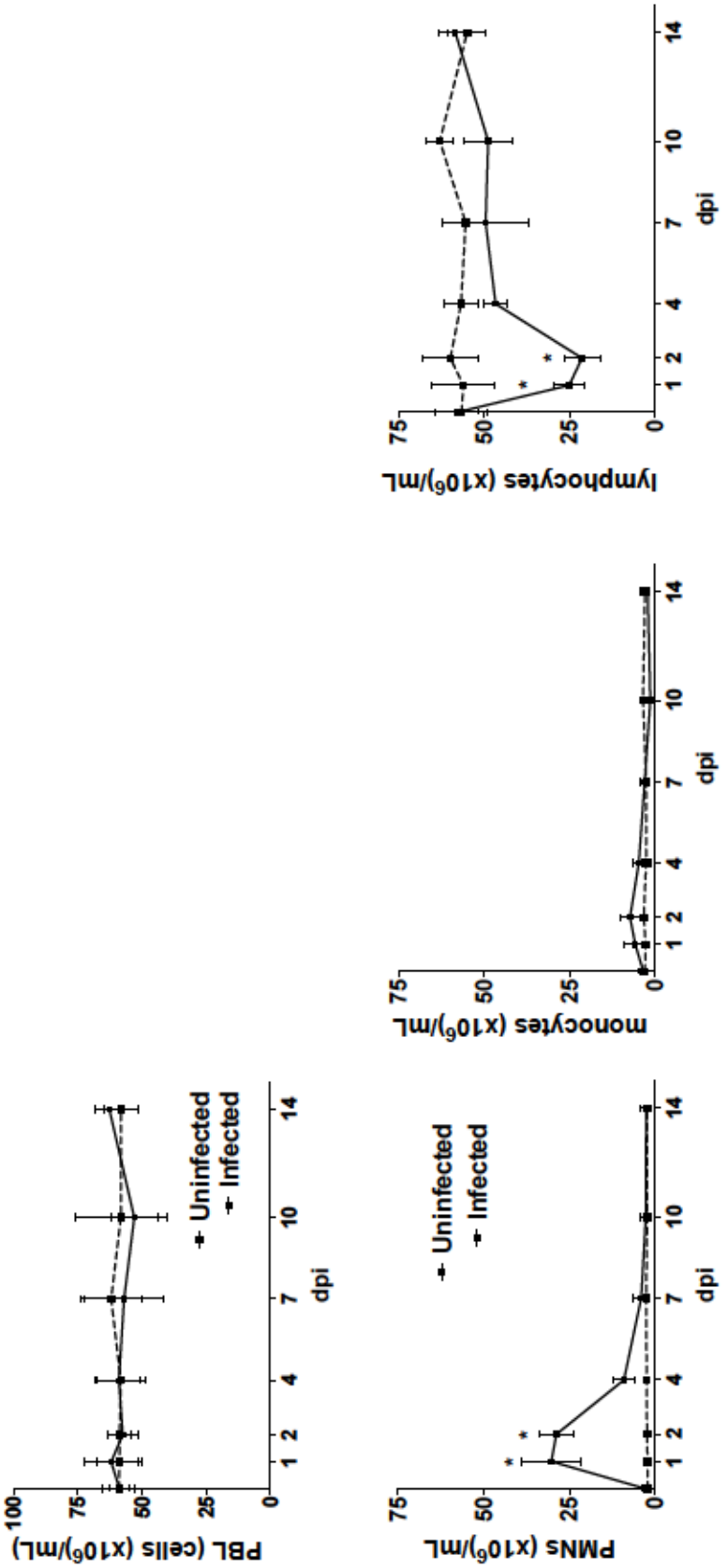

PBL (cells (x10⁶)/mL)
PMNs (x10⁶)/mL
monocytes (x10⁶)/mL
lymphocytes (x10⁶)/mL
Uninfected
Infected
dpi
0
25
50
75
100
1
2
4
7
10
14
*

Figure 5.4. A period of neutrophilia occurs immediately following infection.

Goldfish were infected with *Aeromonas veronii* and bled at the indicated time points. (A) Total number of peripheral blood leukocytes (PBL) per mL of blood (n=4). (B) Breakdown of individual leukocyte subsets and their respective numbers during infection. Solid lines represent infected fish. Dashed lines represent uninfected controls (n=4). All statistics correspond to a significance of $p<0.05$ using a two-way ANOVA with a comparison to uninfected controls at the equivalent time point.

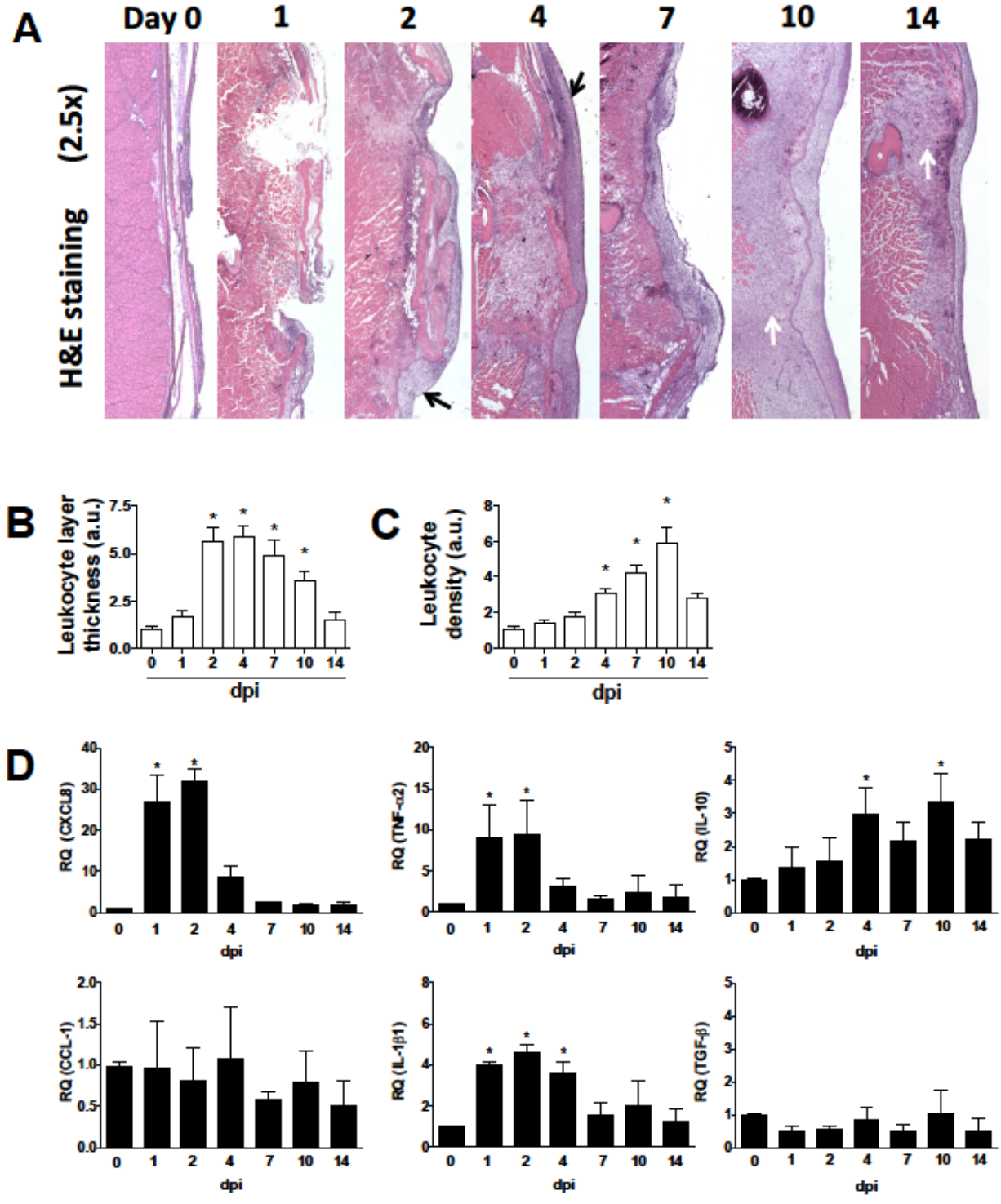

A
Day 0
1
2
4
7
10
14
H&E staining (2.5x)
B
Leukocyte layer thickness (a.u.)
dpi
C
Leukocyte density (a.u.)
dpi
D
RQ (CXCL8)
RQ (TNF-α2)
RQ (IL-10)
RQ (CCL-1)
RQ (IL-1β1)
RQ (TGF-β)
dpi

Figure 5.5. *Aeromonas veronii* induces a robust infiltration of leukocytes into the furuncle.

Goldfish were infected with *Aeromonas veronii* and their furuncles were isolated at the indicated time points. Furuncles were immediately fixed, sectioned, and imaged. (A) Representative images of *A. veronii* furuncle infection (n=4). (B) Using FIJI, the thickness of the leukocyte layer outside of the dermis and muscle tissue was determined (solid black arrows). Ten measurements across the entire furuncle were taken to provide an average thickness for each fish. Images are cropped in order to clearly depict leukocyte layer in question. A.u.- arbitrary units; this refers to the numerical pixel data retrieved when measurements were taken in FIJI (n=4). (C) Leukocyte density (solid white arrows) was also determined using FIJI software. Leukocyte density was determined by color decoupling each layer and analyzing only the Hematoxylin Gill III stain. The same area size was measured at10 locations on each tissue section, and leukocyte density was determined (n=4). (D) At each of the indicated time points, furuncle tissue was harvested from *Aeromonas veronii* and RNA was isolated. Using the cDNA, qPCR was used to assess the expression of chemokines (CXCL8 and CXCL1) and pro-inflammatory (TNF-α2 and IL-1β1) and anti-inflammatory (IL-10 and TGF-β) cytokines (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way ANOVA with a comparison to 0 hpi.

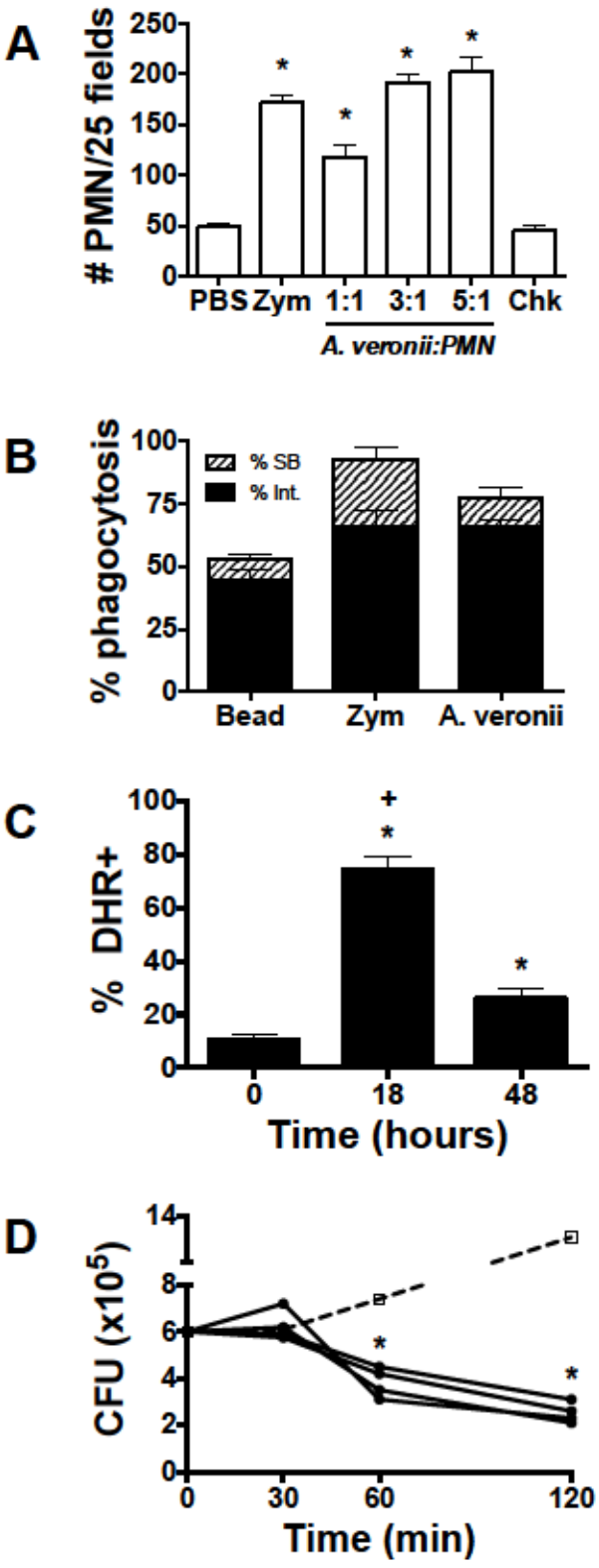

A
PMN/25 fields
250
200
150
100
50
0
PBS Zym 1:1 3:1 5:1 Chk
A. veronii:PMN
B
% phagocytosis
100
75
50
25
0
% SB
% Int.
Bead
Zym
A. veronii
C
% DHR+
100
80
60
40
20
0
+
*
0
18
48
Time (hours)
D
CFU (x10^5)
14
8
6
4
2
0
0
30
60
120
Time (min)

Figure 5.6. Neutrophils exhibit robust inflammatory responses toward *A. veronii*.

(A) Naïve neutrophils were isolated from goldfish hematopoietic kidney and seeded into chemotaxis chambers with increasing amounts of *A. veronii*, beginning with a ration of 1:1 (*A. veronii:* neutrophil) and increasing to a ratio of 5:1. Chk represents a chemokinesis control. Statistics were performed using one-way ANOVA and compared to PBS control (n=4). (B) Goldfish were injected with heat-killed *A. veronii*, and neutrophils were isolated 18 hours post injection, providing *in vivo* activated neutrophils. (B) Phagocytic capacity of activated neutrophils using various targets as a control. Black bars represent % internalized, hatched bars represent % surface bound. (n=4) (C) In addition to isolated neutrophils at 18 hours post injection, neutrophils were also isolated at 48 hours post injection (resolution phase). Respiratory burst capacity in isolated cells at these time points was determined via DHR staining. Statistics were performed using one-way ANOVA and compared to 0 h control (n=4) (D) Activated neutrophils were assessed for their ability to kill *A. veronii ex vivo*. Each black line represents a single replicate. Hatched line represents bacterial only control (n=4) Statistics were performed using one-way ANOVA and compared to 0 min.

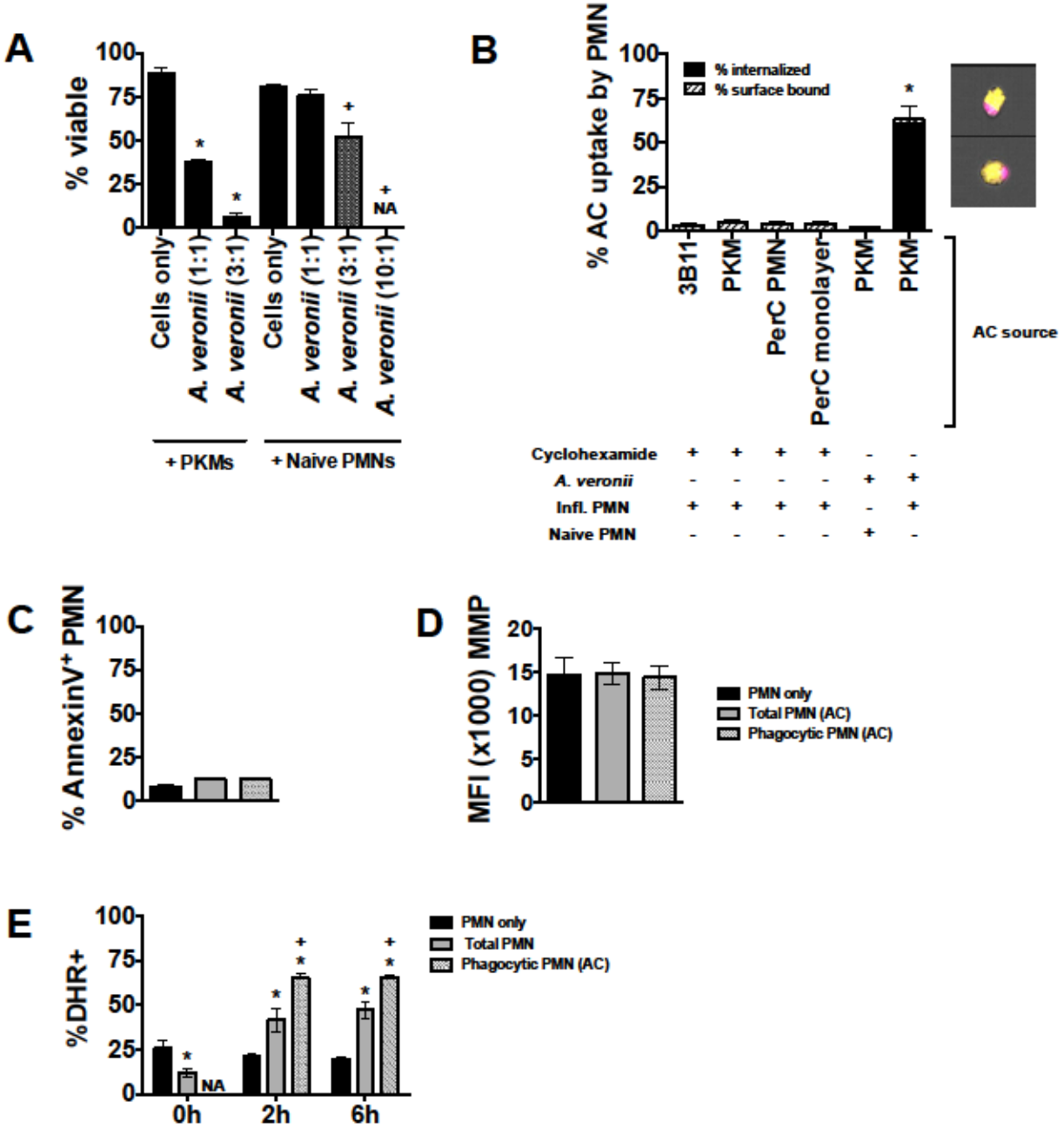

A
% viable
100
75
50
25
0
Cells only
A. veronii (1:1)
A. veronii (3:1)
Cells only
A. veronii (1:1)
A. veronii (3:1)
A. veronii (10:1)
NA
+ PKMs
+ Naive PMNs
B
% AC uptake by PMN
% internalized
% surface bound
3B11
PKM
PerC PMN
PerC monolayer
PKM
PKM
AC source
Cyclohexamide
A. veronii
Infl. PMN
Naive PMN
C
% AnnexinV+ PMN
D
MFI (x1000) MMP
20
15
10
5
0
PMN only
Total PMN (AC)
Phagocytic PMN (AC)
E
%DHR+
NA
0h
2h
6h
PMN only
Total PMN
Phagocytic PMN (AC)

Figure 5.7. Neutrophils internalize dying macrophages and release potent reactive oxygen species.

(A) Primary kidney macrophages and neutrophils were incubated with varying ratios of *A. veronii*: cells and the viability of the leukocytes were subsequently assessed. Grey hatched bar is used to highlight the ratio at which neutrophils become affected by *A. veronii*. Statistics were performed using one-way ANOVA and compared to their respective cell only controls (n=4). (B) Various states of neutrophils (naïve and inflammatory) were examined for their ability to internalize dying cells. Cells were killed with either cyclohexamide or via *A. veronii*. Only one combination resulted in the uptake of dying cells (inflammatory neutrophils took up primary kidney macrophages dying via *A. veronii* infection) (n=6). Following the uptake of dying cells, the viability of neutrophils was examined via AnnexinV/PI+ (n=4; C) and mitochondrial membrane potential (n=4; D) staining. (E) Following the uptake of dying cells, the ability of neutrophils to produce ROS was assessed. Black bars represent PMN only (no addition of dying cells). Grey bars represent total PMN ROS production (phagocytic and non-phagocytic cells). Hatched bars represent only phagocytic PMN (n=4). Statistics were performed using two-way ANOVA within each time point; * is in comparison to PMN only, + is in comparison to total PMN.

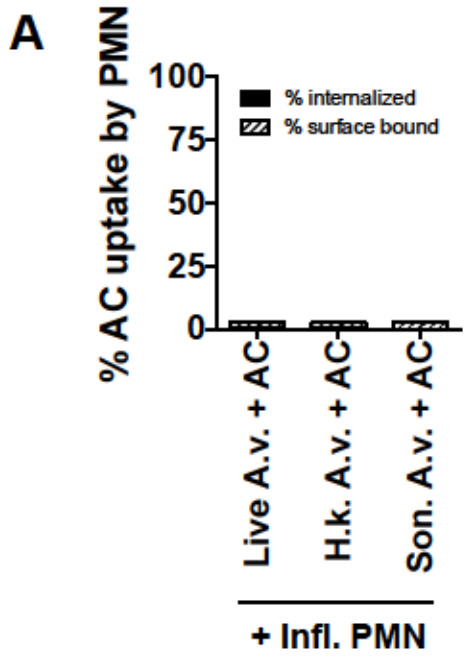


Figure 5.8. *Aeromonas* particles cannot rescue the lack of uptake of dying PKMs by neutrophils.

Primary kidney macrophages were induced to die via cyclohexamide. Following incubation, live, heat-killed, or sonicated *A. veronii* was added to the dead and dying macrophages. In all cases, only minor surface binding was observed (n=4).

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Chapter 6. Teleost neutrophil defense during infection with *Mycobacterium fortuitum*

6.1 Introduction

The role of neutrophils during mycobacteria infections remains highly controversial. Several reports indicate neutrophils are the predominant cell type infected during active tuberculosis (Eum et al., 2010; Francis et al., 2014), a lack of neutrophil killing response (Corleis et al., 2012; Reyes-Ruvalcaba et al., 2008), and a worsening of the infection pathology (Berry et al., 2010; Desvignes and Ernst, 2009; Eruslanov et al., 2005; Nandi and Behar, 2011). Conversely, other studies suggest neutrophils display a prominent killing response during the acute phase of infection (Alemán et al., 2004; Brown et al., 1987; Jones et al., 1990; Kisich et al., 2002; Sugawara et al., 2004). Interestingly, these contrasting results were all observed during infection with *M. tuberculosis*. Moreover, teleost neutrophils have been described as protective, where they internalize infected and dying macrophages at the site of granulomas (Yang et al., 2012). Unfortunately, the vast majority of these experiments are carried out *ex vivo*, examining mammalian neutrophils and *M. tuberculosis*. Perhaps these conflicting reports are due to the inherent variability of mammalian neutrophils isolated from various sources including lungs and blood, differing selective markers, and/or that they are terminally differentiated and short-lived in culture. In light of this, I focused my investigation on teleost neutrophils, which allow for extended culture *ex vivo*, and their interaction with the fish pathogen *Mycobacterium fortuitum*.

Mycobacterium fortuitum is a non-tuberculous mycobacterium (NTM), a grouping that encompasses all mycobacterium external to the *Mycobacterium tuberculosis* complex (Ho et al., 2012). In comparison to other mycobacterial species which may take weeks to grow under laboratory conditions, *M. fortuitum* is fast-growing, with colonies appearing on agar plates within 3 to 4 days. It is capable of infecting a wide range of species, including fish and humans. Fish typically present with lethargy, scale loss, abdominal distension, and skin ulcers, though some fish remain asymptomatic, presenting as long-term carriers of the pathogen. *M. fortuitum* is also known to be zoonotic, leading to skin disease, osteomyelitis, and joint infections in humans. In light of this, it has become an important pathogen for both health and economic purposes (Serra et al., 2007; Sethi et al., 2014; Talaat et al., 1999).

The use of fish hosts to study mycobacterial infections has been used for nearly a century since Dr. Aronson first described the isolation and pathogenesis of *M. marinum* in fish in 1926 (Aronson, 1926). Since then, many more hosts have been used to study *Mycobacterium* including zebrafish, goldfish, and medaka (Broussard and Ennis, 2007; Hodgkinson et al., 2012; Swaim et al., 2006; Talaat et al., 1999, 1998; Yang et al., 2012). For example, Yang and colleagues used the genetically tractable and transparent zebrafish larvae to elucidate the role of neutrophils at and during granuloma development (Yang et al., 2012). Further, several other studies used this same model to identify granulomas as structures that aid in pathogen survival (Davis and Ramakrishnan, 2009; Ramakrishnan, 2013). Although these findings have yet to be corroborated in mammalian models, they signify the importance of using alternative model systems in order to further elucidate the mechanisms of infection and the subsequent host response.

Herein, I focused on the early stages of infection with *M. fortuitum*, during the acute inflammatory response. Using an *in vivo* peritonitis model, I examined the movement and function of leukocytes during this period. One-day post infection (dpi) I observed increased expression levels of CXCL8 and CCL1 in the peritoneal cavity. Consistent with my previous studies in other pathogens, infection with *M. fortuitum* led to the migration of leukocytes from the hematopoietic kidney to the peritoneal cavity, with neutrophils as the predominate infiltrating subset of cells into the initial site of infection. Shortly after the initial release of leukocytes from the hematopoietic kidney, I observed marked increases in the proliferative capacity of kidney leukocytes. This was likely responsible for the increase in kidney neutrophils and monocytes/macrophages I observed from day 2 to 7 post infection. Notably, neutrophils were capable of migrating toward and producing copious amounts of reactive oxygen species (ROS) when stimulated with *M. fortuitum.* However, despite this, when isolated they remained poorly adept in killing *M. fortuitum.* Most interestingly, I found that the total leukocyte population isolated during peak inflammatory periods in the kidney could rapidly eliminate *M. fortuitum*. While all too often we focus in on the responses of single cell subsets, my results highlight the importance of cellular crosstalk during the acute inflammatory process.

6.2 Results

6.2.1 Goldfish rapidly eliminate *M. fortuitum* from the peritoneum but not the kidney

One of the simplest markers of infection is the presence of living pathogens. To this end, I began my study by examining the number of CFU at the initial infection site, the peritoneum, as well as the kidney, a distal location known to become infected in the later phases of mycobacterial infections. I found that goldfish began rapidly clearing *M. fortuitum* from the initial infection site immediately after injection (Figure 6.1). By the first day post infection, nearly 50% of bacteria had been eliminated from the peritoneum. This increases to over 99% by 4 dpi, and complete removal by 7 dpi. Alternatively, in the kidney, the CFU rose steadily to over $9.0x10^3$ CFU/mg at 14 dpi (Figure 1B), after which it declined back to ~$1.0x10^3$ CFU/mg by 28 dpi. Thus, fish appear to eliminate the bacteria rapidly during the acute phase, or the bacteria quickly disseminate from the initial point of infection throughout the host.

6.2.2 Analysis of inflammatory gene expression changes in primary peritoneal leukocytes

The production of soluble inflammatory mediators is vital to the initiation of the acute inflammatory response, where leukocytes use chemokines and cytokines to communicate effectively and mount an appropriate defense against invading pathogens. In order to examine the response of goldfish following exposure to *M. fortuitum*, I measured the changes in gene expression in total leukocytes from the peritoneal cavity. I focused on the chemokines CXCL8 and CCL1, the pro-inflammatory cytokines TNF- α2

and IL-1β1, and the pro-resolving cytokines IL-10 and TGF-β, due to their previously reported importance in mycobacterial infections (Berry et al., 2010; Bohsali et al., 2010; Hodgkinson et al., 2012; Keane et al., 2002; Nandi and Behar, 2011; Pedrosa et al., 2000; Zhang et al., 1995). I observed a 6-fold increase in CXCL8 expression at 1 dpi within the peritoneal cavity (Figure 6.1, top left). CCL1 also exhibited approximately a 5-fold increase in expression at 1dpi (Figure 6.2, bottom left). Interestingly, I observed marked increases in the pro-inflammatory cytokine TNF- α2 (p-value 0.0641; 3.5-fold), but not IL-1β1 (Figure 6.1, middle), suggesting TNF-α2 may play a larger role during infection with *M. fortuitum*. Lastly, we detected peak expression of IL-10 and TGF-β at 7 dpi (Figure 6.2, right), where I observed a 5-fold increase in IL-10 expression (Figure 6.2, top right), and approximately a 2.5-fold increase in TGF-β expression (Figure 6.2, bottom right).

6.2.3 Kinetic analysis of leukocyte migration throughout the course of infection

I previously set out to define the acute inflammatory response using a self-resolving zymosan peritonitis model to better understand the evolutionary origins of phagocyte-driven mechanisms of immunity (Havixbeck et al., 2015). From there, I examined the migration and function of neutrophils during infection with *A. veronii*. Herein, I began by examining the recruitment, migration, and infiltration of leukocytes following injection with the fish pathogen, *M. fortuitum*. Unlike in my previous models, I found that fewer leukocytes exited the hematopoietic tissue following infection (Figure 6.3A), with only 20% fewer cells in the kidney by 2 dpi. Further, the number of primary kidney leukocytes (PKL) actually increased 1.6-fold beyond uninfected levels at 14 dpi

(Figure 6.3A). Following this increase, the number of PKLs returned to near uninfected levels by day 28 of infection. Analyzing this data further, I examined individual cell types, where I observed only neutrophils exiting the hematopoietic tissue within the first 2 dpi (Figure 6.3A), with just under 40% of cells leaving the kidney. Interestingly, all 3 cell types examined- neutrophils, monocytes/macrophages, and lymphocytes, did undergo marked increases in cell numbers between 2 and 14 dpi within the kidney. The discernible increase in cell numbers was especially evident in neutrophils, which increased over 2-fold (Figure 6.3A) and monocyte/macrophages, which increased by nearly 2-fold (Figure 6.3A) during that time.

Continuing down the inflammatory cascade, I assessed the number of peripheral blood leukocytes (PBL) throughout infection. As expected with few exiting leukocytes from the hematopoietic kidney, I found no change in the total number of circulating cells (Figure 6.2B). However, much like other infection models in goldfish, the number of circulating neutrophils did increase significantly (~10-fold) above uninfected controls (Figure 6.2B). The number of circulating monocytes also increased nearly 9-fold between 0 and 1 dpi, from $2.75x10^6$ to $17.5x10^6$ cells/mL (Figure 6.3B). Both increases appear to have come at the cost of circulating lymphocytes, which significantly dropped by over 50% at 1 dpi (Figure 6.3B).

The final step in examining kinetic migration of leukocytes was to assess the number of cells that infiltrated the peritoneal cavity, the initial site of infection. Similar to other models of infection, I found an increase in leukocytes within the inflammatory site, with ~5-fold spike in total peritoneal leukocytes (Figure 6.3C) at 1 dpi. The spike in peritoneal leukocytes was short lived, as the number of cells returned to near basal levels

by 4 dpi (Figure 6.3C). Interestingly, this parallels the removal of *M. fortuitum* from the site. Approximately 50% of infiltrating cells consisted of neutrophils (Figure 6.3C), which corresponded to the period of peak CXCL8 expression (Figure 6.2). However, I also observed increases in both monocytes/macrophages and lymphocytes 1 dpi (Figure 6.3C). Intriguingly, the total number of monocytes/macrophages within the peritoneal cavity fell drastically below that of uninfected control levels at 7 dpi, down to an average of $1.71x10^5$ from $6.0x10^5$ cells/lavage (Figure 6.3C). Moreover, peritoneal lymphocytes were the only cell type in infected fish to exhibit a prolonged increase in numbers (nearly 14 days) when compared to the uninfected control (Figure 6.3C).

6.2.4 Analysis of inflammatory gene expression changes in primary kidney leukocytes

As previously mentioned, chemokines and cytokines form the backbone of soluble inflammatory mediators release during the induction and regulation of inflammation. As infection with *M. fortuitum* progresses it becomes systemic, migrating to other parts of the fish, including the kidney. In order to examine the response of goldfish following the systemic spread of *M. fortuitum*, I also measured the changes in gene expression in isolated leukocytes from the hematopoietic kidney tissue. In the kidney I observed no change in CXCL8 expression throughout the entire time course, indicating something else is responsible for the increase in neutrophils within the hematopoietic site from 2 to 7 dpi (Figure 6.3). However, I did observe a marked increase (~6-fold) in CCL1 at 4 dpi (Figure 6.4, bottom left). Expression then quickly returned to basal levels by 7 dpi. As in the peritoneal cavity, TNF-α2 also underwent an increase in

expression (p-value of 0.0651), peaking at 7 dpi with an approximately 5-fold change (Figure 6.3, top middle), further cementing its role as an important mediator during infection with *M. fortuitum*. Lastly, IL-10 expression peaked (~13-fold) between 7 and 14 dpi (Figure 6.4, top right). Interestingly, the increase in IL-10 expression in the kidney was much higher and more prolonged than that in the peritoneal cavity. It should be noted, that neither IL-1β1 nor TGF-β displayed significant changes in gene expression in the kidney throughout infection (Figure 6.4).

6.2.5 The proliferative capacity of goldfish leukocytes increased following peritoneal challenge with *M. fortuitum*

In mammals, bacterial infection often induces the production of various cytokines and chemokines that can have significant effects on hematopoietic precursor cells in the bone marrow (Chen et al., 2002; Choi et al., 2011; Scumpia et al., 2010; Yáñez et al., 2009). It is only recently that we are beginning to grasp what occurs to hematopoietic precursor cells, including hematopoietic stem cells and their progenitors, during mycobacterial infection. For example, in a chronic murine tuberculosis model, infection results in the expansion of granulocyte-monocyte progenitor cells (Choi et al., 2011). My analysis of leukocyte kinetics determined that I had an increasing number of leukocytes within the hematopoietic kidney tissue (Figure 6.3). Interestingly, I found a marked increase in the proliferative capacity of leukocytes on day 7 and 14 (p-values of 0.0585 and 0.0649 respectively) of infection (Figure 6.5). After 14 dpi proliferation returned to basal levels. The increase in proliferation may indicate one of the methods by which I observed swift increases in the number of neutrophils within the kidney following their

initial exodus.

6.2.6 *Mycobacterium fortuitum* exhibits a bacterial load dependent effect of macrophage and neutrophil viability

It is well known that monocytes and macrophages play an important role during infection with mycobacteria (Behar et al., 2011; Hodgkinson et al., 2012; Iyoda et al., 2014; Schluger and Rom, 1998; Weiss and Schaible, 2015). In addition, a growing body of evidence points toward the importance of early neutrophil responses in controlling mycobacterial infection (Andersson et al., 2014; Hodgkinson et al., 2015; Seiler et al., 2000). However, prior to examining the role of leukocytes during host defense, we were interested in determining whether *M. fortuitum* was capable of impacting the viability of those leukocytes typically responsible for early control during infection. As I increased the ratio of *M. fortuitum* to macrophages, I found fewer and fewer cells remained viable after 24 hours (Figure 6.6A, black bars), hitting a low of approximately 38% viability at a ratio of 10:1 (bacteria: macrophage). Interestingly, the majority of cells died via apoptosis, with significant numbers of apoptotic cells observed at every ratio examined (Figure 6.6B, black bars). The effect of *M. fortuitum* was much less pronounced when incubated with naïve neutrophils. I only observed a significant loss of neutrophil viability at a ratio of 10 bacteria per neutrophil, which left ~50% of neutrophils still viable (Figure 6.6A, hatched bars). In all other ratios examined, neutrophils remained as viable as control cells (~85%, Figure 6.6A). In addition, no significant changes were observed in the number of apoptotic cells (Figure 6.6B).

6.2.7 Neutrophils rapidly migrate in the direction of *Mycobacterium fortuitum*

After determining that neutrophils remained viable at low mycobacterial levels, I was interested in examining their migratory capacity when stimulated by *M. fortuitum*. Using blind well leucite chemotaxis chambers, I found that neutrophils responded in a bacterial load dependent manner. Neutrophils began migrating at the lowest bacterial load examined (1:1; bacteria: neutrophil; Figure 6.7). The number of migrating neutrophils increased significantly (compared to controls) as bacterial load increased, peaking at the highest examined ratio of 10:1 (bacteria: neutrophil; Figure 6.7). I was also curious if the addition of macrophages to the mycobacterial chamber would impact the migration of neutrophils. Interestingly, when macrophages (same number as neutrophils) were incubated with *M. fortuitum* in a 3:1 ratio to neutrophils, I found significantly more neutrophils migrating compared to a 3:1 ratio of *M. fortuitum* to neutrophils alone (Figure 6.7, hatched bar). This lead me to believe that the interaction of macrophages and mycobacteria play an important role in driving the immune response forward.

6.2.8 Peritoneal neutrophils display robust ROS responses but poor killing efficacy

As previously mentioned, neutrophils, monocytes, and macrophages are important mediators in the defense against mycobacterial infection (Andersson et al., 2014; Behar et al., 2011; Hodgkinson et al., 2015, 2012; Iyoda et al., 2014; Schluger and Rom, 1998; Seiler et al., 2000; Weiss and Schaible, 2015). With this in mind, I began by examining the production of reactive oxygen species in peritoneal leukocytes, an important immune mechanism during mycobacterial infection (Yang et al., 2007, 2012). Following infection with *M. fortuitum*, I observed an immediate increase in the ROS production of total

peritoneal leukocytes within the first 2 days of infection (Figure 6.8A, top panel). I then went on to examine an isolated population of neutrophils from the peritoneal cavity, which also proved to produce robust ROS responses during the first 2 days on infection (Figure 6.8A, middle panel). More importantly, I was interested in determining if peritoneal leukocytes were capable of controlling and eliminating *M. fortuitum* at the initial point of infection. In order to determine this, I examined the capacity for total peritoneal leukocytes, or isolated peritoneal neutrophils isolated at 1 dpi to kill *M. fortuitum ex vivo*. Interestingly, when I examined an isolated population of neutrophils, I found them to be inefficient at removing the pathogenic threat within 2 hours (Figure 6.8A, bottom panel). However, I found the total peritoneal leukocyte population to be significantly more capable of killing *M. fortuitum* (Figure 6.8A, bottom panel). Specifically, neutrophils killed ~20% of mycobacteria after 2 hours, whereas, the total peritoneal leukocyte population removed ~50% during that same period (Figure 6.8A, bottom panel).

6.2.9 Kidney leukocytes have a greater proficiency at killing *M. fortuitum*

It is well known that mycobacterial infections in goldfish often become systemic, infecting regions within the host distal to the initial point of infection (Gauthier and Rhodes, 2009; Hodgkinson et al., 2015, 2012, Talaat et al., 1999, 1998). One of the most common places of secondary infection is within the kidney, where mycobacteria accumulates, often forming distinct granules (Hodgkinson et al., 2012; Talaat et al., 1999). In light of this, I also set out to examine the inflammatory responses of leukocytes within this tissue. Similar to my examination of the peritoneal cavity, I began by

examining the production of ROS by the total leukocyte population and an isolated population of neutrophils. I found that both populations exhibited peak ROS production at 14 dpi (Figure 6.8B). Notably, it appears as though neutrophils display a more prolonged increase in ROS production, with ~50% of cells producing ROS from days 7 through 14 post infection (Figure 6.8B, middle panel). Similar to the peritoneal cavity, I isolated total kidney leukocytes during peak ROS production at 14 dpi and assessed their ability to kill *M. fortuitum*. Despite the prolonged increase in ROS production, isolated kidney neutrophils displayed a mediocre capacity to kill *M. fortuitum* (~50% removal; Figure 6.8B, bottom panel). However, when I examined the total leukocyte population, I found a reduction of ~80% of mycobacteria within 2 hours (Figure 6.8B, bottom panel). Thus, my results appear to suggest that in order to exhibit the most efficient responses, all leukocyte subsets must communicate and respond in unison.

6.3 Discussion

A key function of the innate immune system is the detection and destruction of foreign pathogens. Similar to my previous studies that have examined the induction and regulation of the acute inflammation in response to zymosan (Havixbeck et al., 2015) and the fish pathogen, *A. veronii* (Havixbeck et al., 2017.), I set out to further understand the interactions between the acute inflammatory response of goldfish and the rapidly growing fish pathogen, *Mycobacterium fortuitum*. Infection with *M. fortuitum* characteristically causes lethargy, scale loss, abdominal distension, and skin ulcers in fish. Importantly, I also observed an increase in lethargy and abdominal distension during the first 4 days of

infection. It should also be noted that fish can remain asymptomatic, becoming long-term carriers of the pathogen.

Neutrophils are essential effector cells of innate immune response, often considered the first line of defense where their recruitment into peripheral tissues is central to host defense against invading pathogens. I have previously determined that fully mature but functionally naïve teleost neutrophils exist in a hematopoietic storage pool (Havixbeck et al., 2015) awaiting deployment upon receiving the appropriate signals. This was further confirmed when I examined the migration of neutrophils following infection with *A. veronii* (Havixbeck et al., 2017). In this study, I found a similar result, where neutrophils rapidly exited the hematopoietic kidney within the first 2 days of infection. From there, the cells entered into circulation, and then subsequently infiltrated the peritoneal cavity, the initial point of mycobacteria infection. Interestingly, the maximal number of infiltrating neutrophils was ~2.5 fold less when compared to the number of neutrophils recruited following administration of zymosan, although similar numbers were observed when compared 24 hours post injection. In both cases, this correlated with an increase in CXCL8 expression within peritoneal leukocytes, a chemoattractant responsible for recruiting neutrophils to the site of inflammation. Increases in CXCL8 expression has also been observed in injured tail tissue of zebrafish within hours following wounding (de Oliveira et al., 2013). Further, CXCL8 is upregulated in response to foreign inflammatory stimuli, which led to an increase in neutrophil infiltration, highlighting its importance for normal neutrophil recruitment into wounded or infected tissues (de Oliveira et al., 2013). Notably, I also observed an increase in expression of CCL1 within peritoneal leukocytes, a chemokine critical for the

recruitment of monocytes during infection. This increase 1 dpi provides a possible explanation for the increase in peripheral blood monocytes and subsequent peritoneal infiltration. Interestingly, unlike during infection with *A. veronii*, I see a significant migration of monocytes, as well as increased expression of CCL1 throughout infection. However, in both infections (*A. veronii* and *M. fortuitum*) I observed the clearance of pathogens from the initial site of infection, where it was completed within 10 days and 4 days, respectively. Finally, I also found a prolonged increase in the number of lymphocytes within the peritoneal cavity. Typically, lymphocytes have been examined near granulomas where they are known to be involved in long-term defense, however, their presence during the early stage of infection indicate these cells play larger role than previously thought. Collectively, when I consider all stimulants and infections I examined, my findings suggest that teleost fish have developed a level of specialization with numerous methods to respond to the diverse array of pathogens they may encounter. This provides yet another indication of the true complexity of the innate immune response.

In fish, mycobacterial infections are known to become systemic, eventually infecting distal organs within the host, including the kidney, spleen, and liver (Hodgkinson et al., 2012; Talaat et al., 1999, 1998). Herein, I focused on the progression of infection and the acute inflammatory response in the kidney. Interestingly, I found no increase in CXCL8 expression within the tissue, as well as no secondary increase in circulating neutrophils. However, the number of kidney neutrophils continued to increase from days 2 to 7-post infection. This was likely caused by the increase in proliferation observed 2 to 14 dpi. Although, I am unable to definitively identify this increase in

proliferation as the source of the increase in kidney neutrophils, it could be a potentially contributor. Further, it is likely the increase in proliferation also aided in increasing the number of kidney monocytes/ macrophages. Although I did observe an increase in CCL1 expression within the kidney tissue, this did not correlate to an increase in circulating monocytes at that time, suggesting the circulating monocytes were not the source, or I may need to focus on other time points post infection. Of note, during this same period (2-14 dpi), the bacterial load is continuing to increase in the kidney. A likely host response to continued bacterial replication is through an increase in proliferation as a mechanism to expand the number of leukocytes within the kidney. These leukocytes are then prone to encounter *M. fortuitum* themselves, or interact with an already activated leukocyte further inducing the inflammatory cascade. Interestingly, following challenges with zymosan (Havixbeck et al., 2015), or *A. veronii* (Havixbeck et al., 2017), I never observed an increase in proliferation. However, in neither case was there a prolonged bacterial infection within the kidney or any other region within the host. Yet, following infection with *M. fortuitum*, I observed a prolonged bacterial load, and an increase in proliferation throughout infection. This may indicate that like mammals, teleost fish exhibit 'emergency' hematopoiesis, or a demand-driven need for more leukocytes during infection (Panopoulos and Watowich, 2008).

It is well understood that *M. tuberculosis* has a remarkable capacity to survive within the hostile environment of the macrophage (Houben et al., 2006; McDonough et al., 1993; McKinney et al., 2000; Meena and Rajni, 2010; Pieters, 2008; Podinovskaia et al., 2013; Scherr et al., 2009). In addition, this extends to other mycobacterial species including *M. marinum, M. chelonae,* and *M. fortuitum* (Cosma et al., 2006; Hodgkinson

et al., 2012; Stamm et al., 2003; Talaat et al., 1999, 1998; Tobin and Ramakrishnan, 2008). However, we rarely think about the alternative point of view. What is the macrophage trying to do in order to prevent pathogen survival and replication? Several indications point toward the induction of apoptosis in macrophages as an innate defense mechanism against mycobacteria (Behar et al., 2011; Iyoda et al., 2014). The induction of the apoptotic cascade ultimately results in the direct killing of intracellular mycobacteria (Keane et al., 2002; Molloy et al., 1994). I observed a bacterial load dependent effect on macrophage viability, with increasing death of macrophages at every ratio examined. Interestingly, the majority of cell death consisted of cells entering apoptosis. This coincides with the results of Bohsali *et al.,* where non-tuberculosis forming mycobacteria (*M. fortuitum* and *M. smegmatis*) induced greater levels of apoptosis in BALB/c bone marrow derived macrophages and the human monocyte cell line, THP-1 (Bohsali et al., 2010). My observations lend further support to the hypothesis that the strong induction of apoptosis by host macrophages is a major reason for the lack of chronic pathogenicity in rapid-growing mycobacteria. Of note, when I examined the viability of neutrophils following infection with *M. fortuitum*, I didn't find an increase in neutrophil death until the highest ratio. This led us to develop two potential hypotheses, neither of which must be considered mutually exclusive. First, neutrophils may play a larger role in combating mycobacterial infection than previously thought. There is an increasing amount of research pointing toward the importance of neutrophils in combating mycobacterial infections, as Tan and colleagues revealed how macrophages acquire neutrophil granules for the antimicrobial defense against intracellular mycobacteria (Tan et al., 2006). A second potential explanation is that *M. fortuitum* may be preferentially maintaining

neutrophil viability. This hypothesis garners plenty of support as well, where several researchers believe that neutrophils are simply contributing to development of pathology, rather than protecting the host (Eruslanov et al., 2005; Lowe et al., 2012).

The induction of antimicrobial defense mechanisms of leukocytes in response to mycobacteria is slowly being elucidated (Bohsali et al., 2010; Helguera-Repetto et al., 2014; Hodgkinson et al., 2015; Silva et al., 2010; Tan et al., 2006). As mentioned above, it is only recently that a larger focus has been placed on the functional roles of neutrophils (Braian et al., 2013; Eruslanov et al., 2005; Hodgkinson et al., 2015; Martineau et al., 2007; Pedrosa et al., 2000; Yang et al., 2012). To begin my examination, I assessed the ability of neutrophils to migrate toward *M. fortuitum*, where neutrophils migrated in significant numbers at all ratios examined. In addition, Yang and colleagues found that neutrophils were capable of internalizing macrophages infected with *M. marinum* (Yang et al., 2012), thus suggesting macrophages may produce chemotactic factors upon infection. Interestingly, I observed a significant increase in neutrophil migration in the presence of macrophages compared with mycobacteria alone (3:1 ratio of *M. fortuitum*: neutrophils). This suggests to us that when macrophages are undergoing apoptosis in order to prevent further dissemination of *M. fortuitum*, they are also releasing additional chemotactic signals recruiting neutrophils to the site. However, I was ultimately interested in whether or not neutrophils were capable of killing *M. fortuitum*. This led to some unique results. When I isolated neutrophils from the initial site of infection (peritoneal cavity), I found them to produce robust levels of ROS. However, this translated into a poor capacity to kill *M. fortuitum*. Interestingly, when I examined neutrophils from the secondary site of infection (kidney), I found similar data. Thus far,

my results point toward the second hypothesis from above, where neutrophils simply contribute to the infectious pathology, without actually protecting the host. In light of this, I altered my point of view and decided to focus less on a specific cell type and more on the collective host response. The total leukocyte population displayed an increased effectiveness in killing *M. fortuitum,* with the greatest efficacy observed in the kidney. Therefore, it is actually most likely that both hypotheses deserve at least some merit. First, neutrophils are still aiding in the clearance of the pathogen, but must do so with the aid of other cellular subsets, and second, without control significant numbers of neutrophils may also lead to an increase in pathology during infection. Through the development and use of specific cellular markers for fish leukocytes, future studies could focus on specific cellular subsets within the larger group. This would allow for the analysis of unique leukocyte functions while maintaining the necessary cellular crosstalk, an essential requirement during the inflammatory response.

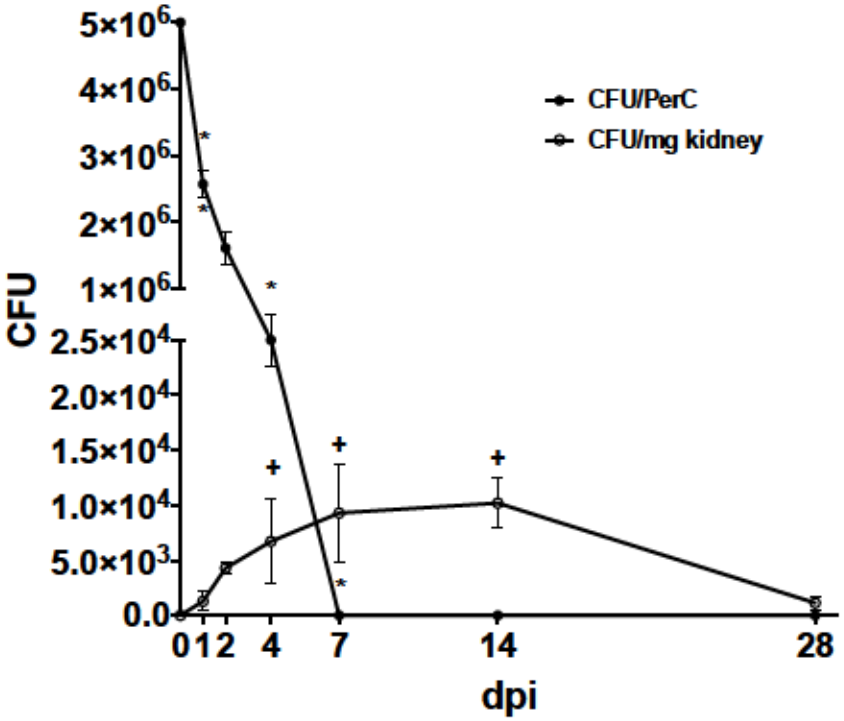

Figure 6.1. Colony forming units (CFU) of *Mycobacterium fortuitum* in the peritoneal cavity and the kidney throughout infection.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At day 0, 1, 2, 4, 7, 14, and 28, fish were terminated and the number of CFUs of *M. fortuitum* was determined by spread plating either peritoneal lavages or macerated kidneys on Middlebrook 7H10 agar plates (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way-ANOVA; * significantly different from Day 0 CFU in peritoneal cavity (PerC), + significantly different from Day 0 CFU/mg kidney.

RQ (CXCL8)
RQ (CCL1)
RQ (TNF-α2)
RQ (IL-1β1)
RQ (IL-10)
RQ (TGF-β)
dpi
0
1
2
4
7
14
28

Figure 6.2. Gene expression changes of chemokines and cytokines in leukocytes isolated from the peritoneal cavity throughout infection.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At day 0, 1, 2, 4, 7, 14, and 28, peritoneal cells were harvested by lavage. Cytokine and chemokine levels were measured by Q-PCR (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way-ANOVA; * significantly different from Day 0.

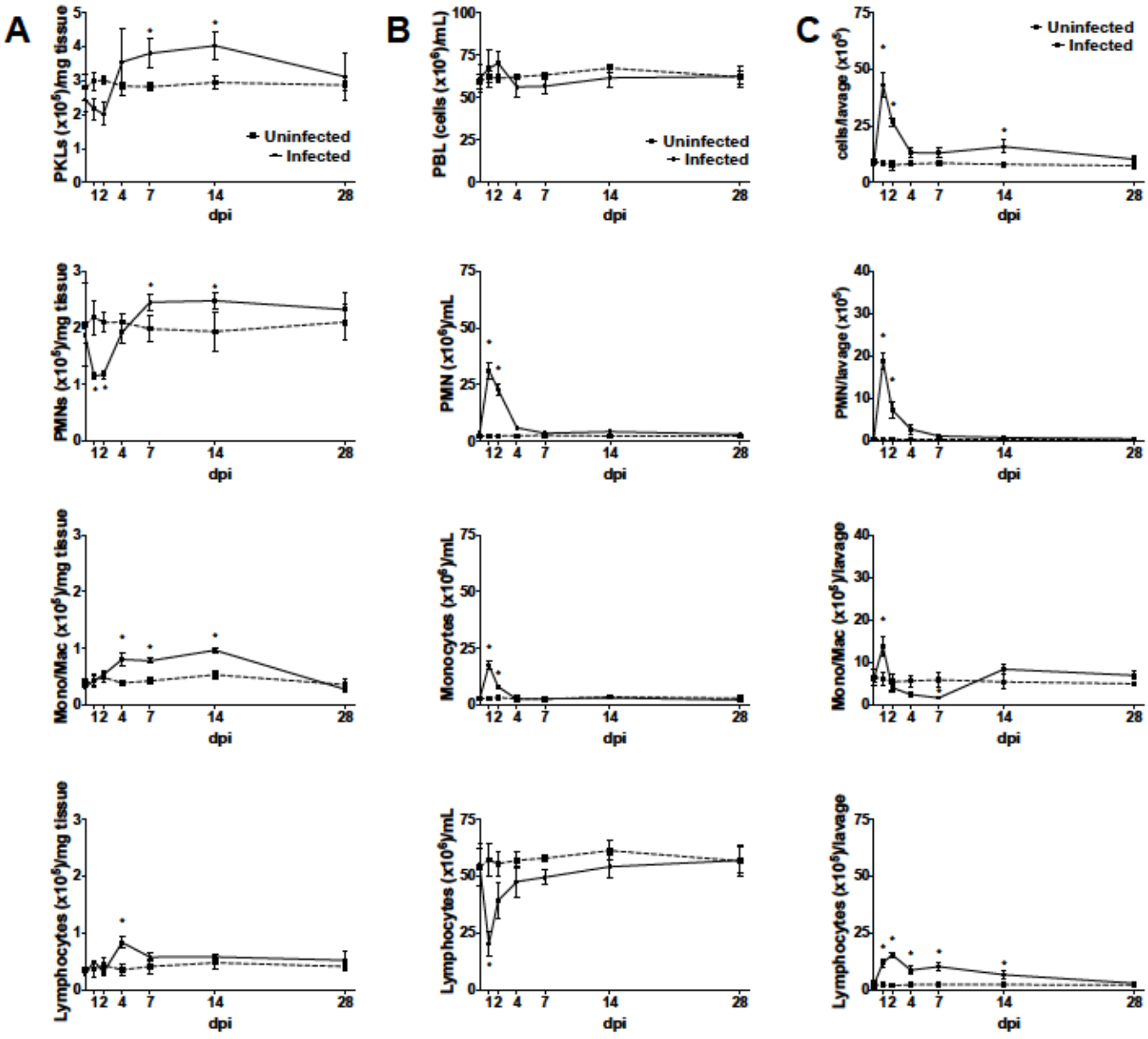
A
B
C
PKLs (x10⁵)/mg tissue
PBL (cells (x10⁶)/mL
cells/lavage (x10⁵)
PMNs (x10⁵)/mg tissue
PMN (x10⁶)/mL
PMN/lavage (x10⁵)
Mono/Mac (x10⁵)/mg tissue
Monocytes (x10⁶)/mL
Mono/Mac (x10⁵)/lavage
Lymphocytes (x10⁵)/mg tissue
Lymphocytes (x10⁶)/mL
Lymphocytes (x10⁵)/lavage
Uninfected
Infected
dpi
12 4 7 14 28

Figure 6.3. Infection with *M. fortuitum* induces the migration of neutrophils from the hematopoietic kidney tissue to the peritoneal cavity.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At day 0, 1, 2, 4, 7, 14, and 28, goldfish were bled via the caudal vein, peritoneal cells were harvested by lavage, and kidneys were removed. (A) Prior to harvesting cells, kidneys were weighed. Total kidney leukocytes at each time point were counted using a haemocytometer. To account for varying sizes or fish, counts were divided by kidney weight (in mg) to determine leukocytes / mg tissue. The total number of leukocytes per milligram of kidney tissue at each designated time point (top panel). The bottom 3 panels represent the total number of individual leukocyte subsets per mg of kidney tissue (n=4). (B) Fish were sacrificed, bled at the indicated time points and peripheral blood smears were stained with Sudan Black. Neutrophils were identified as Sudan Black positive, while monocytes and lymphocytes, both stained negative. The total number of leukocytes per mL of blood at each designated time point (top panel). The bottom 3 panels represent the total number of individual leukocyte subsets per mL of blood (n=4). (C) Kinetics of leukocyte infiltration into the peritoneal cavity. Cytospins were performed, stained with Sudan Black, and counted on a haemocytometer. The total number of leukocytes per lavage at each designated time point (top panel). The bottom 3 panels represent the total number of individual leukocyte subsets per lavage (n=4). All statistics correspond to a significance of $p<0.05$ using a two-way-ANOVA; * significantly different from uninfected controls on the same day of infection. Solid line represents infected fish. Dashed line represents PBS injected controls.

RQ (IL-10)
RQ (TGF-β)
RQ (TNF-α2)
RQ (IL-1β1)
RQ (CXCL8)
RQ (CCL1)
dpi
0
1
2
4
7
14
28
0.0
2.5
5.0
7.5
10.0
5
10
15
20
*
*

Figure 6.4. Gene expression changes of chemokines and cytokines in leukocytes isolated from the kidney throughout infection.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At day 0, 1, 2, 4, 7, 14, and 28, kidneys were removed and flash frozen. Cytokine and chemokine levels were measured by Q-PCR (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way-ANOVA; * significantly different from Day 0.

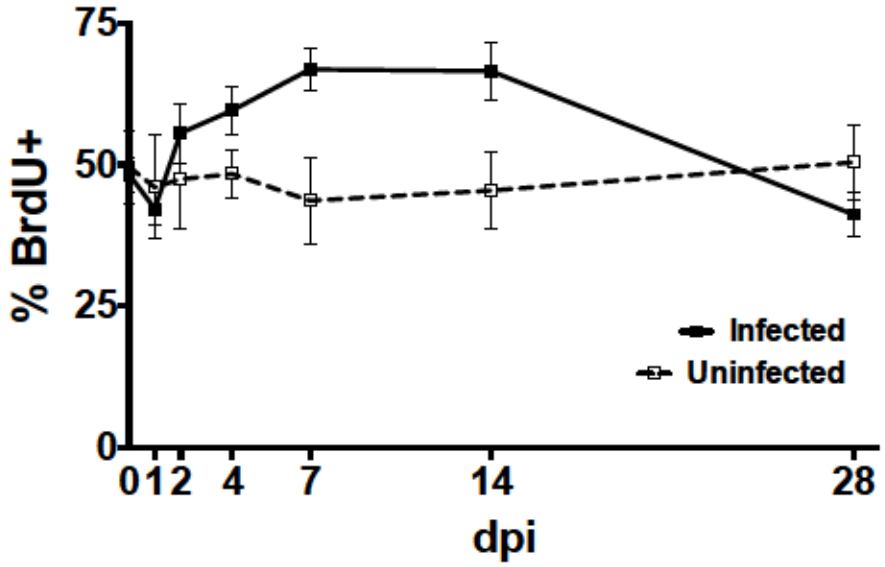

Figure 6.5. Intraperitoneal injection of *M. fortuitum* leads to increased hematopoietic proliferation.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At one hour prior to day 0, 1, 2, 4, 7, 14, and 28, fish were injected intraperitoneally with BrdU and incubated for 1 h, to allow incorporation of BrdU into tissues. Kidneys were isolated, and total leukocytes were harvested. Cells were fixed for a minimum of 24 h in 1% formaldehyde, and then stained with anti-BrdU FITC (n=4). Solid line represents infected fish. Dashed line represents PBS injected controls.

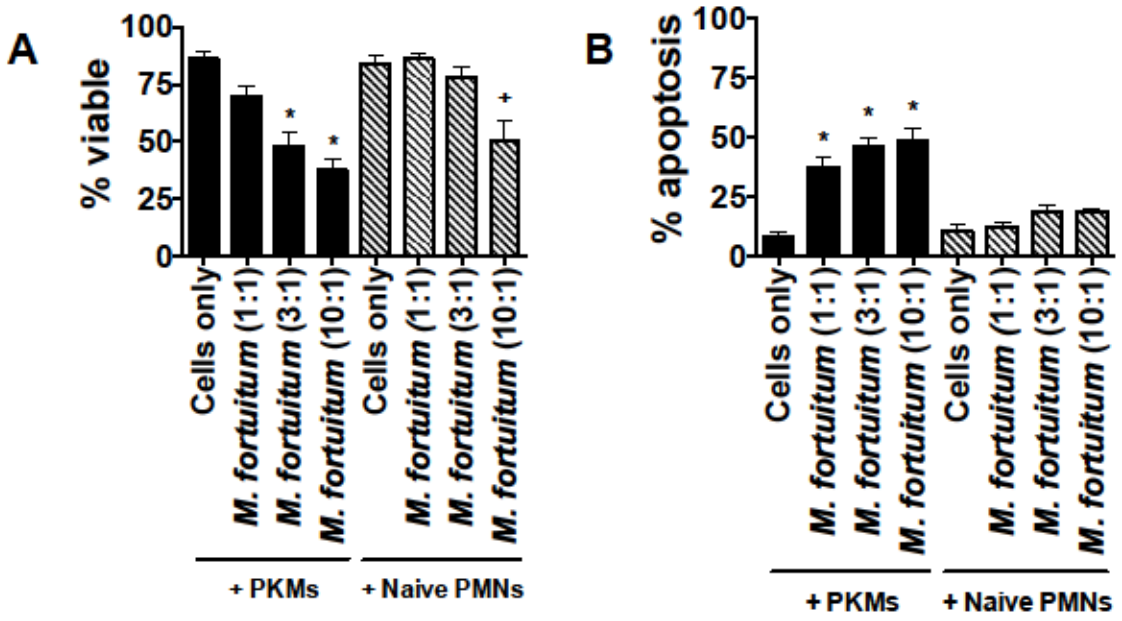

Figure 6.6. Effect of *M. fortuitum* incubation on the viability of primary kidney macrophages and naïve neutrophils *in vitro*.

Primary kidney macrophages (PKM) and naïve neutrophils (isolated from goldfish kidney) were incubated with varying ratios of *M. fortuitum*: cells and the viability of the leukocytes were subsequently assessed using AnnexinV/PI staining. Solid black bars represent primary kidney macrophages, while hatched bars represent naïve neutrophils (n=4). (A) Percent viable cells remaining post incubation. (B) Percent of apoptotic cells which stain positive with AnnexinV. All statistics correspond to a significance of $p<0.05$ using a one-way-ANOVA; * significantly different from PKMs only, + significantly different from neutrophils only.

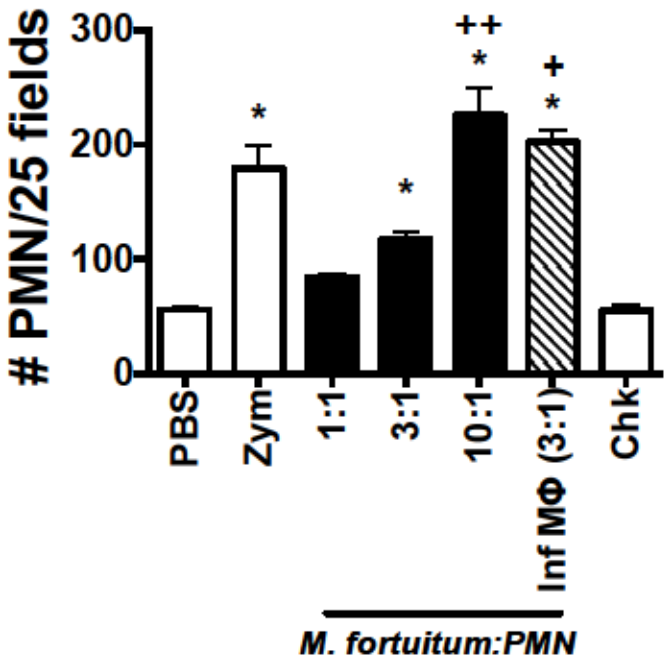

Figure 6.7. Neutrophils rapidly migrate toward *M. fortuitum in vitro*.

Naïve neutrophils were isolated from goldfish hematopoietic kidney and seeded into chemotaxis chambers with increasing amounts of *M. fortuitum*, beginning with a ratio of 1:1 (*M. fortuitum*: neutrophil) and increasing to a ratio of 10:1 (black bars). Chk represents a chemokinesis control. White bars represent positive (zymosan- Zym) and negative controls. The hatched bar represents the addition of PKMs with *M. fortuitum* at a ratio of 3:1 (mycobacteria: macrophage). *M. fortuitum* is also at a ratio of 3:1 to neutrophils (n=4). All statistics correspond to a significance of $p<0.05$ using a one-way-ANOVA; * significantly different from PBS control, + significantly different from 3:1 (*M. fortuitum*: neutrophils) alone, ++ significantly different from 3:1 (*M. fortuitum* alone).

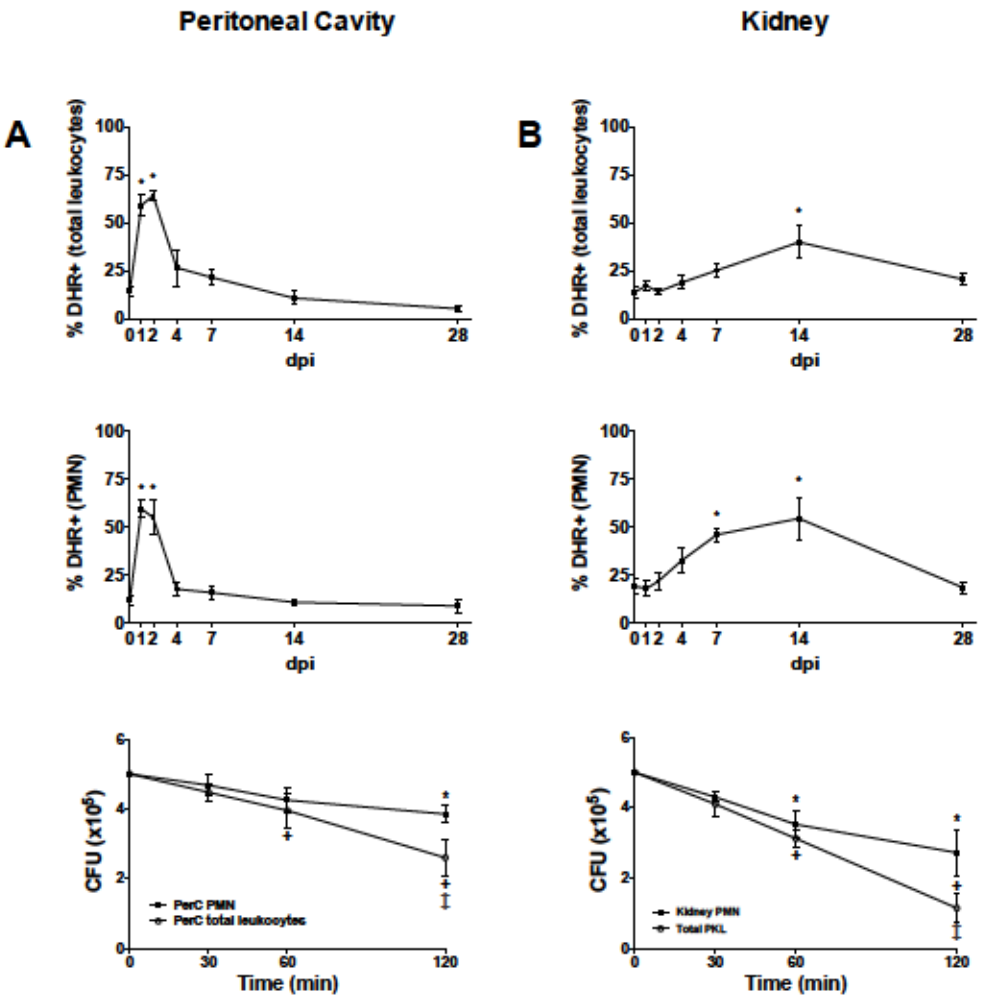

Figure 6.8. Teleost leukocytes mount robust ROS responses and effective killing responses against *M. fortuitum*.

Goldfish were injected intraperitoneally with $10x10^6$ *M. fortuitum*. At day 0, 1, 2, 4, 7, 14, and 28, peritoneal leukocytes were removed by lavage and kidneys dissected. (A) Respiratory burst responses of total peritoneal leukocytes (top panel) and isolated peritoneal neutrophils (middle panel). The bottom panel examines the capacity of total peritoneal leukocytes (open circles) and peritoneal neutrophils (solid circles) isolated 1 dpi to kill *M. fortuitum* (n=4). (B) Respiratory burst responses of total kidney leukocytes (top panel) and isolated kidney neutrophils (middle panel). The bottom panel examines the capacity of total kidney leukocytes (open circles) and kidney neutrophils (solid circles) isolated 14 dpi to kill *M. fortuitum* (n=4). All statistics correspond to a significance of $p<0.05$ using a two-way-ANOVA; * significantly different from isolated neutrophils at 0 minutes, + significantly different from total leukocytes at 0 minutes, ‡ significantly different from isolated neutrophils at 120 minutes.

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Chapter 7. General discussion[1]

7.1 Overview of findings

The acute inflammatory response is a complex biological process initiated following tissue injury or infection. It is composed of an elaborate cascade of both pro- and anti-inflammatory mediators, critical for effective defenses against infection, the removal of damaged cells, and the initiation of tissue repair processes. Central to this process are phagocytes, leukocytes adept in the recognition of pathogens and particles and the induction, regulation, and resolution of inflammatory cascades. In order to execute such a diverse array of functions, phagocytes house an abundance of potent antimicrobial defense mechanisms needed for pathogen clearance, as well as the capacity to produce an array of soluble mediators that direct and shape downstream responses. It should be noted that inflammation is a double-edged sword, where although typically regarded as beneficial to the host, aberrant activation often becomes a detriment. Uncontrolled inflammatory events can lead to tissue damage, chronic inflammation and autoimmune diseases, as well as cancer (Coussens and Werb, 2002; Karin et al., 2006; Matzinger, 2007; Medzhitov and Okin, 2012; Nagy et al., 2007). In light of this, numerous mechanisms have evolved to control the acute inflammatory response, ensuring pathogen clearance and tissue healing, while minimizing deleterious damage to the surrounding tissues.

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[1] A portion of this chapter has been previously published in:

Havixbeck JJ and Barreda DR (2015) Neutrophil development, migration, and function in teleost fish. *Biology* 4(4): 715-734.

My Ph.D. began by focusing on the evolution of functional phagocyte responses and the downstream mechanisms by which they are able to regulate inflammation. I found that the capacity of individual phagocytes to mediate divergent pro- and anti-inflammatory responses has been present for millions of years, extending back to one of earliest vertebrates, *Petromyzon marinus* (sea lamprey; Chapter 3) (Havixbeck et al., 2014). Phagocytes of goldfish and lamprey contributed to both pro-inflammatory and pro-resolving responses *ex vivo* (Chapter 3). A similar dichotomy exists within mammalian phagocytes, where macrophages and neutrophils possess the capacity to balance these two seemingly contradictory responses (Esmann et al., 2010; Rieger et al., 2012). Interestingly, the data presented here appears to suggest a shift from homeostatic/ pro-resolving particles to pro-inflammatory particles as the dominant stimulus (Chapter 3). Goldfish phagocytes displayed a reduced sensitivity to apoptotic cells, translating to an overall greater induction of antimicrobial respiratory burst responses (Chapter 3). However, goldfish phagocytes still remain as central contributors to the resolution phase of inflammation, even though they showcased an improved ability to induce strong antimicrobial inflammatory responses (Chapter 3). These pro-resolving contributions are discussed in greater detail below. Notably, these results would be consistent with the evolution of phagocytic theory, where it began with amitochondrial protoeukaryotes acquiring the proto-mitochondrion (Doolittle, 1998), to unicellular eukaryotes using phagocytosis as a method to 'eat' or acquire endosymbiotic relationships (Jeon, 1995), to present day where vertebrates use phagocytosis as a mechanism for tissue remodeling (Desjardins et al., 2005), immune regulation (Aderem, 2003; Havixbeck et al., 2015;

Rieger et al., 2012), and apoptotic cell removal (Maderna and Godson, 2003; Rieger et al., 2012).

Both mammalian and teleost macrophages are known for their roles in the induction, regulation, and resolution of inflammation. It has previously been shown that they are capable of internalizing both pro-inflammatory/ pathogenic and pro-resolving/ self stimuli, resulting in divergent downstream responses (Rieger et al., 2012; Soehnlein and Lindbom, 2010; Weigert et al., 2009). Subsequently, new evidence in mammals indicated that neutrophils were also capable of internalizing apoptotic cells and inhibiting pro-inflammatory functions, such as respiratory burst and the release of pro-inflammatory cytokines (Esmann et al., 2010). In Chapter 4, I used a self-resolving zymosan peritonitis model to show that teleost neutrophils play a large role in orchestrating the induction, regulation, and resolution of inflammation (Havixbeck et al., 2015). Similar to mammals, I found that teleost neutrophils exist within a large storage pool in the hematopoietic kidney tissue. These neutrophils rapidly exit the kidney, enter into circulation, and infiltrate the inflammatory site when called (Chapter 4). In my *in vivo* analysis of neutrophil function within the peritoneal cavity, I detected various levels of ROS production among neutrophils isolated at different time points. It should be noted, that this data is not without assumptions. Some graphs are presented in line format, which assumes the continued progression of the response from time point to time point. However, there are still likely nuances between time points that may require further scrutiny. Together, this allowed me to identify that neutrophils isolated during the pro-inflammatory stage (0-18 hpi) of inflammation exhibit robust levels of ROS, whereas neutrophils isolated during the pro-resolving phase (24-72 hpi) produced very little,

leading me to hypothesize that mature neutrophils are capable of altering their phenotype throughout the inflammatory response (Chapter 4).

To this point our lab had yet to observe teleost neutrophils internalize apoptotic cells. Unfortunately, this continued when I assessed the ability of neutrophils isolated at multiple points during zymosan-induced inflammation to internalize apoptotic cells. Yet, we previously indicated that teleost neutrophils remained capable of decreasing the respiratory burst responses of macrophages (Rieger et al., 2012). Using a transwell assay, I found that neutrophils isolated at 18 hpi were only capable of increasing the ROS production in macrophages, however, neutrophils isolated during the pro-resolution phase (48 hpi), mediated divergent responses, both increasing and decreasing ROS production in macrophages following stimulation with zymosan or apoptotic cells, respectively (Chapter 4). Notably, a transwell control of apoptotic cells in the absence of neutrophils had no impact on macrophage ROS production. Together, this suggested that 1) neutrophils are releasing soluble factors that modulate macrophage function, and 2) the type(s) of soluble factors produced are heavily dependent on the neutrophil phenotype (pro-inflammatory or pro-resolving). Interestingly, I found no changes in neutrophil gene expression for classical cytokines involved in inflammation (TNFα, IL-1β, IFN-γ, TGF-β, IL-10). Thus, soluble lipid mediators became my target candidates. Specifically leukotriene B_4 (LTB_4), a lipid involved in neutrophil recruitment and activation (Sadik et al., 2011), and lipoxin A_4 (LXA_4), an important mediator with pro-resolving function (Levy et al., 2001; Serhan et al., 2008; Serhan, 2014). When examined, I found the pro-inflammatory lipid mediator, LTB_4, secreted at high concentrations within the first 18 hpi. Similar to mammals, it is likely that LTB_4 is central to continuing the pro-

inflammatory cascade in teleost fish. Interestingly, a switch from the production of LTB_4 to LXA_4 occurs roughly 24 hours after the administration of zymosan. Shortly thereafter, I also observed the peak period at which neutrophils undergo apoptosis. This is indicative of the transition from the pro-inflammatory to the pro-resolving phase of the acute inflammatory response. I found the release of LTB_4 to stimulate the production of reactive oxygen species in macrophages, whereas LXA_4 was primarily responsible for inducing the uptake of apoptotic cells by macrophages. Therefore, although neutrophils were unable to internalize apoptotic cells, they release LXA_4 prior to entering apoptosis, stimulating macrophages to uptake apoptotic cells (Chapter 4).

My results thus far, namely the release of pro-resolving eicosanoids and the capacity to modulate anti-inflammatory functions in other leukocytes led us to believe that neutrophils play a larger role in the resolution of inflammation than previously thought (Chapter 3/4). However, these experiments were conducted following the administration of a pathogen mimic (zymosan) and I was unsure if this was the case during a true pathogenic infection. In order to further examine the role of neutrophils in the resolution of inflammation, I focused on a natural pathogen to goldfish, *Aeromonas veronii*. Using an *Aeromonas* infection model, I found that neutrophils migrated to the site of infection in much the same manner (Chapter 5). In my *ex vivo* experiments, neutrophils exhibited their classical pro-inflammatory phenotype when isolated from the peritoneal cavity of infected fish. They rapidly migrate toward and internalize *A. veronii ex vivo*, where they produce robust levels of ROS in addition to displaying a remarkable ability to kill the pathogen, highlighting their importance in defense (Chapter 5). As mentioned above, neutrophils aid in the induction of inflammatory resolution through the

stimulation of macrophages. However, *Aeromonas* has been shown to induce high levels of cell death within macrophage populations (Krzymińska et al., 2009; Rosenzweig and Chopra, 2013). Similarly, at a 3:1 ratio (*Aeromonas*: macrophages), nearly all macrophages had been eliminated, consistent with a high level of virulence for this organism. In light of this, it led me to question which cells were involved in apoptotic cell uptake. Interestingly, a recent study found that zebrafish neutrophils were capable of internalizing dying macrophages infected with *Mycobacterium marinum* (Yang et al., 2012). I found that, similar to the *Mycobacterium marinum* infected macrophages, goldfish neutrophils were capable of internalizing dying macrophages that had been incubated with *A. veronii* (Chapter 5). Interestingly, following the uptake of dying macrophages, neutrophils increased their production of ROS, remaining pro-inflammatory in nature (Chapter 5). Notably, neutrophils remained unable to internalize any other form of induced dying cell, suggesting that the specific mechanism by which a cell dies is critical to the downstream response. One potential explanation for the selective internalization lies in the expression of surface receptors ('eat me' signals) on the apoptotic cell. The various methods of inducing apoptosis may result in differential expression of surface receptors, which may then be recognized and internalized by the neutrophil. Further, receptor interaction may also signal various downstream responses in the internalizing cell. My results and subsequent hypothesis provide the groundwork for an interesting foray into the mechanisms of cell death, and is thus further discussed in my future directions below.

The primary function of the acute inflammatory response is the immediate and effective clearance of pathogens; where in order to fulfill this function, it requires innate

specificity (eg. innate immune receptors such as TLRs), ensuring the appropriate immune response is mounted. In Chapter 6, I used the fish pathogen *Mycobacterium fortuitum* to further understand the functions of neutrophils within the acute inflammatory response. *M. fortuitum* is another natural pathogen of goldfish, which allows me to investigate and compare the acute inflammatory response, as well as the specific roles of neutrophils in multiple infections. Unlike, *A. veronii, M. fortuitum* contains a secondary component to the acute inflammatory response as it disseminates throughout the host. This allowed me to examine the role of neutrophils beyond the initial site of infection. Much like *A. veronii, M. fortuitum* appeared to have a greater effect on macrophage viability compared to neutrophils. Interestingly, the majority of macrophages underwent apoptosis, a method by which some consider a primary defense mechanism in order to prevent the dissemination of mycobacteria (Bohsali et al., 2010). This may suggest a larger role for neutrophils in the defense against *M. fortuitum*, or that *M. fortuitum* may be preferentially maintaining neutrophil viability. Neutrophils rapidly infiltrate the site of infection, migrating toward mycobacteria, where they produced elevated levels of reactive oxygen species, however, they exhibit poor efficacy in killing *M. fortuitum* at the initial site of infection (peritoneal cavity). Further, their killing responses at a secondary point of infection (kidney) also remained mediocre. Interestingly, the complete population of leukocytes (both in the peritoneal cavity and kidney) killed mycobacterium to a significantly greater extent (Chapter 6), suggesting the need for crosstalk between leukocyte subsets. This has been demonstrated by macrophages, who acquire neutrophil granules for downstream antimicrobial defense against intracellular mycobacteria (Tan et al., 2006).

Overall, my data, as well as the results from several other recent studies (Gómez-Abellán et al., 2015; Jones et al., 2016; Levy et al., 2001; Tobin et al., 2012; Yang et al., 2012) indicate that neutrophils are no longer the simple pro-inflammatory foot soldiers we once described. Neutrophils should now be considered multi-phenotypic regulators of the inflammatory response, where they are central contributors to the induction, regulation, and resolution of inflammation. By understanding their movement and function throughout various immune challenges, we not only gain an understanding of their numerous roles during the acute inflammatory process, we also gain a better appreciation of the complexity in which the host defends itself from foreign invaders.

7.2 Neutrophil-driven inflammatory control

Historically, neutrophils have been viewed as nothing more than foot soldiers, sent to inflammatory sites to kill invading pathogens, while leaving vast amounts of tissue damage in their wake. While in some instances this may be true, in the following sub-sections I discuss how our fundamental understanding of neutrophil functions in disease and inflammation has changed over recent years.

7.2.1 Neutrophils in general inflammation

In humans, neutrophils constitute between ~40-60% of leukocytes in circulation, representing the body's primary line of defense against invading pathogens (Doeing et al., 2003). This number decreases to ~30-40% in swine (Fishbourne et al., 2013), ~15-25% in mice (Doeing et al., 2003), and even further to ~5% in bony fish (Havixbeck et al., 2015; Rey Vázquez and Guerrero, 2007). As we descend into lower vertebrates, the

relative percentage of circulating neutrophils decreases, representing an interesting insight into the evolutionary importance of neutrophils to host defense. Further, despite the increase in peripheral blood neutrophils, the hematopoietic storage pool of mature neutrophils (termed the bone marrow reserve in mammals) remains largely conserved (Boxio et al., 2004; Furze and Rankin, 2008; Rankin, 2010). Therefore lower vertebrates are likely far more dependent on this storage pool of neutrophils. They represent the majority of mature neutrophils available to traffic and infiltrate the inflammatory site, whereas in mammals, the majority of neutrophils are presumably already in circulation. The hematopoietic storage pool in mammals may then exist as a means to immediately replenish peripheral blood neutrophils following depletion. However, the abundance of circulating neutrophils could come at the expense of one regulatory safeguard. Neutrophils in lower vertebrates must pass two important checkpoints for complete activation, 1) exit from the hematopoietic compartment, and 2) entry into the inflammatory site. The first of these steps is largely bypassed in higher vertebrates, likely in favor of a more rapid response to inflammation. Interestingly, this could explain the short life span of circulating neutrophils in humans (5-12 hours) (Basu et al., 2002; Dancey et al., 1976), which may indicate that the continual cycling of peripheral blood neutrophils acts as a method to prevent unwelcome activation. However, more recent findings indicate that human neutrophils may actually subsist within circulation as long 5.4 days (Pillay et al., 2010), suggesting two possible explanations, 1) their method of orally administering deuterium-labeled water also labeled bone-marrow neutrophils, or, alternative mechanisms to prevent aberrant activation are yet to be discovered.

This leads me to take a step back in the inflammatory cascade and examine the differences in hematopoiesis between lower and higher vertebrates during inflammation. Inflammation creates physiological stress on the host, threatening both the quality and duration of life. Successful resolution of inflammation requires a functional immune system to recognize and eliminate the foreign particles without eliciting deleterious tissue damage to the host. Cytokines regulate the development and homeostasis of immune function, and their production typically increases during infection as a method to control the immune response. Among others, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) are the major cytokines involved in regulating the proliferation, differentiation, activation, and survival of neutrophils and their progenitors (Burgess et al., 1977; Lieschke et al., 1994; Panopoulos and Watowich, 2008). The ability of the bone marrow neutrophil pool to respond to the physiological demand during infection is termed 'emergency' granulopoiesis. During infection in mice, G-CSF production can increase over 20-fold, stimulating the proliferation of neutrophilic progenitor cells. In addition, GM-CSF is produced by activated T-lymphocytes (Gasson et al., 1984; Otsuka et al., 1988) and endothelial cells (Broudy et al., 1986), suggesting it also plays an important role during emergency hematopoiesis. In mammals, this response is necessary for a sustained output of circulating neutrophils during infection (Lieschke et al., 1994; Zhan et al., 1998). Interestingly, I found that proliferation of goldfish leukocytes within the hematopoietic kidney tissue actually decreased during peritoneal administration of zymosan, or infection with *A. veronii*. During acute zymosan-induced peritonitis, this may suggest that lower vertebrates lack the ability to initiate 'emergency' granulopoiesis, or simple do not

require it in order to clear the site of inflammation. Thus, diverting their energy to more important mechanisms. The lack of proliferation during infection from *A. veronii* is far more interesting as it is a prolonged infection taking ~10 days to clear the furuncle of bacteria, once again indicating a lack of an 'emergency' response. Alternatively, *A. veronii* may be actively decreasing the proliferative capacity of hematopoietic precursors, including immature neutrophilic precursors.

Neutrophils are typically the first leukocytes recruited to an inflammatory site (Havixbeck et al., 2015), recruited by a host of chemotactic factors, including CXCL8. I used CXCL8 as a marker for the induction of chemotactic molecules throughout my experiments. However, I was unable to directly correlate increases in CXCL8 expression with the recruitment of neutrophils. To build upon this, I would conduct a similar experiment to Yang and colleagues (Yang et al., 2012), by using recombinant CXCL8 injected into the peritoneal cavity. I would expect to see similar results to the aforementioned study, where administration results in the recruitment of neutrophils to the injection site. Similar studies could be conducted for many neutrophil chemotactic mediators, including LTB_4, FMLP, C5a, or C3a. Once at the inflammatory site, neutrophils are capable of eliminating pathogens through multiple complementary mechanisms. Upon activation, neutrophils become powerful killers, utilizing toxic intracellular granules (Flerova and Balabanova, 2013; Meseguer et al., 1994), the production reactive oxygen species (ROS) (Filho, 2007; Katzenback and Belosevic, 2009; Rieger et al., 2012), and deploying neutrophil extracellular traps (NETs) (Palić et al., 2007; Pijanowski et al., 2013). These characteristics are shared between teleost neutrophils and their mammalian counterparts (Borregaard, 2010; Brinkmann et al.,

2004; Brinkmann and Zychlinsky, 2012; Nathan, 2006; Prokopowicz et al., 2012; Winterbourn and Kettle, 2012). This is extensively reviewed in Chapter 1, so will not be covered here. Of note, I used the production of reactive oxygen species, along with cellular migration and infiltration as a marker of pro-inflammatory responses. However, it is important to understand that ROS alone does not highlight the state of an immune response, and in some cases supporting experiments should be conducted. These may include examining neutrophil degranulation and NET production to further highlight the inflammatory state of the cell.

In Chapter 4 I found teleost neutrophils to display multiple phenotypes, one of which implicates a central role for neutrophils in triggering inflammatory resolution. Such mechanisms involved crosstalk pathways during the intimate interactions of neutrophils with other cell types at the site of inflammation. One such example is the production of lipid mediators. Lipid mediators are produced via a variety of methods, often involving multiple cell types. For example, lipoxin A_4 can be formed via platelet-neutrophil interactions, epithelial release of precursor molecules, and aspirin triggered 15-epi-lipoxin. My approach would have allowed for the analysis of the first two methods of production, where precursor products are produced and can be found within the peritoneal exudate, and thus present for lipoxin production by neutrophils during incubation. Naturally, this assumes the necessary precursors were produced and are present within the inflammatory milieu. Interestingly, both LTB_4 and LXA_4 production use arachidonic acid as a precursor, which can be produced by macrophages at the inflammatory site. Again, this highlights the need for cellular collaboration throughout the inflammatory response, whether it is the production of specific mediators, or their

respective precursors. My results indicate that neutrophils release significant amounts of LTB_4 during the pro-inflammatory phase and then switch to releasing LXA_4, which then plays a role in initiating resolution mechanisms (Havixbeck et al., 2015). In mammals, this switch is largely caused by the accumulation of prostaglandin E2 (PGE_2) at the site of inflammation. PGE_2 inhibits the 5-lipoxygenase pathway, preventing further production of LTB_4, while simultaneously increasing the expression of 15-lipoxygenase derived products, including LXA_4 (Levy et al., 2001). Interestingly, PGE_2 is also produced extensively by mammalian neutrophils (Levy et al., 2001), however, it appears as though its impact on LTB_4 production is delayed. Of note, PGE_2 is also produced by monocytes *in vitro* and *in vivo* in response to numerous TLR agonists, as well as IL-1β (Grainger et al., 2013; Zaitseva et al., 2012). Thus, it is conceivable that neutrophils do not produce enough PGE_2 to initiate a class change in lipid mediators on their own. However, the arrival of monocytes at the inflammatory site and their subsequent release of PGE_2 may create an environmental milieu with sufficient levels of PGE_2 to inhibit further production of LTB_4 and begin production of LXA_4. This is further supported by the understanding that monocytes typically arrive at the site of inflammation in the second wave of infiltrating cells (often recruited by neutrophils), thus creating a scenario in which neutrophils must rely, at least in part, on monocytes/ macrophages to display a pro-resolving phenotype. The network between neutrophils and monocytes/macrophages becomes even more complicated as we consider the fact that macrophages are also a primary source of arachidonic acid, an essential precursor to lipid mediator production. Thankfully, the body of research on lipid mediators in bony fish continues to grow. Recent findings indicate that acidophilic granulocytes isolated from the gilthead

seabream are capable of producing prostaglandin D2, a pro-resolving ecosanoid (Gómez-Abellán et al., 2015). Further, PGD_2 went on to inhibit the production of reactive oxygen species and decrease expression levels of IL-1β in these cells (Gómez-Abellán et al., 2015). In addition, leukotriene A4 hydrolase (LTA4H) is essential in balancing pro-inflammatory and anti-inflammatory eicosanoids (Tobin et al., 2012; Tobin and Ramakrishnan, 2013). Importantly, this balance is critical for TNF-α-mediated clearance of mycobacterium in zebrafish and humans (Tobin et al., 2012). A model of lipid mediator release in teleost fish can be found in Figure 7.1. Collectively, this becomes increasingly interesting when we consider the lifespan of neutrophils. The length of time teleost neutrophils are capable of surviving within the inflammatory site is significantly longer than those found in mammals. This allows them to maintain a presence within the inflammatory site, suggesting they may possess unique functions involved in inflammatory resolution. This may include the release of additional mediators, the uptake of apoptotic cells, the modulation and stimulation of other leukocytes, and potentially the initiation of tissue repair mechanisms. Further, teleost neutrophils have been shown to reverse transmigrate out of an inflammatory site *in vivo* in zebrafish (Mathias et al., 2006; Starnes and Huttenlocher, 2012). This may also represent a unique method by which neutrophils contribute to resolution or act as additional antigen presenters. One possible approach would be to create a unique chemical marker specific to teleost neutrophils and combine with *in vivo* imaging. This would create a scenario in which we would not only be able to visualize their migration and infiltration into the inflammatory site, but also the length of time they stay within the inflamed tissue. Additional labelling of other leukocytes would also allow for the examination of the various cellular interactions that

may occur, and the resulting downstream responses. This will begin to identify the crucial interactions necessary for pathogen clearance and the initiation of inflammatory resolution.

Accumulating evidence supports the existence of distinct neutrophil subsets that have diverse roles in infection and inflammation. In both Chapter 4/5, I found neutrophils to exhibit robust pro-inflammatory responses, including the production of reactive oxygen species and the capacity to kill *A. veronii*. Interestingly, I also isolated neutrophils during the pro-resolving phase of zymosan-induced inflammation with characteristics more reminiscent of classical macrophages. These neutrophils, but not those isolated during the pro-inflammatory phase, were capable of mediating divergent responses in macrophages. Moreover, it appeared as though this was mediated through the release of lipid mediators. In Chapter 5, I showed neutrophils were capable of internalizing *A. veronii* infected macrophages. However, these same neutrophils remained unable to internalize any other form of dying cell. This becomes essential when we consider the impact of *A. veronii* on the viability of macrophages, and the lack of monocyte migration I observed throughout infection. These likely place neutrophils in a position where it becomes responsible for both the clearance of *A. veronii* and the dying macrophages. What is truly remarkable is the ability of neutrophils to discern the type of dying cell internalized. Instead of inhibiting pro-inflammatory responses, the normal cue following the uptake of apoptotic cells (Erwig and Henson, 2007a; Fadok et al., 1998; Rieger et al., 2012; Voll et al., 1997), neutrophils increased ROS production following uptake. Interestingly, neutrophils displayed similar characteristics following the uptake of macrophages infected with *M. marinum* (Yang et al., 2012). Taken together, this leads me

to believe that the diversity of neutrophil phenotypes is likely developed through the plasticity of a single neutrophil precursor. However, there is evidence to suggest truly distinct neutrophil lineages may exist. During infection with methicillin-resistant *Staphylococcus aureus* in mice, three distinct types of neutrophils were isolated: 1) pro-inflammatory neutrophils associated with resistance, primarily releasing IL-12 and CCL3; 2) anti-inflammatory neutrophils associated with susceptibility to disease, releasing IL-10 and CCL2; and 3) neutrophils isolated from naïve mice. Of note, it should also be considered that neutrophils could alter their phenotypes throughout infection, and thus do not represent unique lineages. The most compelling evidence for two distinct lineages comes from a study highlighting the selective recruitment of a pre-existing population of circulating neutrophils following avascular transplanted pancreatic islets (Christoffersson et al., 2012). This study demonstrates VEGF-A preferentially recruited a pro-angiogenic subset of circulating neutrophils that deliver large amounts of the effector protein MMP-9, required for islet revascularization and functional integration following transplantation (Christoffersson et al., 2012). These two studies indicate some potential for distinct neutrophilic lineages in mammals, much like the heterogeneity we observe in macrophages throughout the various tissues in mammals (Gordon et al., 2014; Gordon and Taylor, 2005). Collectively, my results point toward the former, where neutrophils alter their phenotypes throughout the inflammatory response. However, this cannot be completely supported without a more comprehensive examination of the repertoire of surface receptors from isolated neutrophils, as well as the soluble molecules released by each subset. This debate becomes rather difficult to tackle to due the likelihood that neutrophil heterogeneity may be partly explained by a differential activation stage or

location. Aged neutrophils or neutrophils that have undergone reverse transmigration also display a unique set of cellular receptors (de Oliveira et al., 2016). Further, novel neutrophil populations have been identified during infection (Pillay et al., 2012), cancer (Fridlender et al., 2009), autoimmunity (Denny et al., 2010), and pregnancy (Ssemaganda et al., 2014). Thus despite our continued identification of various phenotypic differences, it is still not clear whether these are derived from a single precursor or completely separate lineages. Teleost neutrophils isolated from the blood may represent an interesting location in which we may begin to identify phenotypic differences from lineages differences. In Chapter 4, I found that neutrophils isolated from the inflammatory site exhibited a far different phenotype from the naïve neutrophils in the hematopoietic kidney tissue. This was further observed in their respective capacities to internalize *Aeromonas* infected dying macrophages. By examining a location between these two points, we can gain insight into functional differences in activation sate, surface receptor expression, and whether or not they release different soluble mediators.

7.2.2 Neutrophils in host-pathogen interactions

Our increased understanding of neutrophil plasticity has expanded our knowledge of their diverse functions in infection and inflammation as they move between host defense, immune modulation, and tissue damage. Importantly, novel technologies, including intravital microscopy, imagining flow cytometry, and transgenic models have also increased our capacity to study neutrophil-pathogen interactions.

7.2.2.1 Defense against *Aeromonas*

Aeromonas hydrophila has been reported to induce the formation of NETs during infection of carp (Brogden et al., 2012). However, more interesting is the capacity of *A. hydrophila* to degrade NETs via nuclease activity. Neutrophils incubated in the presence or absence of *A. hydrophila* showed no significant differences in cell death, indicating that the bacteria make no attempt at blocking NET formation via the induction of neutrophil death. This suggests *Aeromonas* may actively trigger the release of NETs and their associated products without incurring any real damage, creating a scenario by which neutrophils are only contributing to the pathology of disease. Interestingly, the initiation of a severe inflammatory response and the subsequent induction of substantial tissue damage appears to be a hallmark of *Aeromonas* infections (Munro, 1988; Huizinga et al., 1979; Itoh et al., 1999; Sood and Nerurkar, 2014). In my experiments, induced infection led to the development of furunculosis, a common acute symptom of infection. Further, I found *A. veronii* to induce significant levels of ROS production in neutrophils, however, we know that *Aeromonas* express catalase genes, which protect against reactive oxygen species (Rio et al., 2007). Arguably, this may indicate that secondary mechanisms of antimicrobial defense should be examined. This may include degranulation and the related molecules (defensins, antimicrobial peptides, and lysozyme) or the production of NETs. Alternatively, *Aeromonas* may activate neutrophils to induce significant levels of tissue damage to the host, allowing it to migrate deeper into the tissues, potentially going septic. Some of this may in fact be true, as I was able to isolate *A. veronii* from the kidney of goldfish as early as 1 dpi. Other reports have also suggested that infection can become chronic, with bacteria residing within the muscle and organs for years (Janda and Abbott,

2010). In addition to the initiation of severe tissue damage, *Aeromonas veronii* and *Aeromonas hydrophila* were shown to be poorly internalized by J774 macrophages (Krzymińska, 2008). Further, those bacteria that were internalized were capable of replicating up to three hours post infection (Krzymińska, 2008), indicating they likely possess several immune evasion mechanisms, including catalase (Rio et al., 2007). I as well as several others, have also shown *Aeromonas* to induce apoptosis in macrophages and epithelial cells (Krzymińska et al., 2009, 2011), suggesting *Aeromonas* uses the induction of cell death as a method of immune evasion by eliminating the cells responsible for initiating and orchestrating the acute immune response. This also leads to the question, how are neutrophils recruited to the site of infection if macrophages are so easily killed.

The first method is through the way in which macrophages undergo cell death. Previous studies found that *Aeromonas* spp. were capable of inducing both apoptosis (Krzymińska et al., 2009) and pyroptosis (McCoy et al., 2010; Rosenzweig and Chopra, 2013) in macrophages. This suggests the mechanism of cell death is a critical to understanding the overall outcome of the immune response. Conventional apoptosis can send the host into early resolution, allowing the pathogen to replicate, disseminate, and cause further disease (Erwig and Henson, 2007b). Alternatively, pyroptosis is a pro-inflammatory event, which should allow the host to eliminate the pathogenic threat (Bergsbaken et al., 2009), although, this may come at the cost of inducing more damage to surrounding tissues. It is possible that goldfish macrophages would prefer to undergo pyroptosis during the early stages of infection, allowing the host to maintain a pro-inflammatory state and clear the pathogen. Interestingly, it has previously been suggested

that infected macrophages undergo pyroptosis in order to deprive intracellular pathogens such as *Salmonella*, *Legionella,* and *Mycobacteria* of their immune protected niche (Krysan et al., 2014). Thus it is conceivable that macrophages use the induction of pyroptosis as an immune defense mechanism. Alternatively, *A. veronii* may prefer to induce a controlled apoptotic cascade, leading to decreased pro-inflammatory responses, allowing the bacterium to further infiltrate and disseminate within the host. However, the reverse scenario could be considered just as likely. The induction of apoptosis may be preferred by goldfish macrophages. In this event, they can control cell surface receptor expression, initiating a specific set of downstream responses in the internalizing phagocyte. Conversely, *A. veronii* may wish to induce pyroptosis, spewing inflammatory mediators, further cascading the pro-inflammatory response. This could recruit neutrophils and continue the necessary destruction of host tissues required for its dissemination. This is best summarized in Figure 7.2. Determining which scenario is most likely becomes interesting when I examine my data. I found that *A. veronii* was detectable within the kidney 1 dpi, signifying it had already disseminated throughout the host well before late stage furuncle formation (4-7 dpi). This suggests that *Aeromonas* does not require excessive tissue damage to disseminate. Alternatively, when neutrophils internalized the dying macrophages they remained pro-inflammatory, producing robust amounts of reactive oxygen species, suggesting either form of cell death would continue the induction of inflammation. It should also be noted that the tissue damage associated with furuncle formation might simply be a by-product of pathogen clearance, as I found neutrophils to be highly chemotactic toward *A. veronii*, where they internalized, initiated the production of reactive oxygen species, and most importantly, were capable of killing

the pathogen. In addition, *A. veronii* was still detectable within the furuncle until 10 days post infection.

The second method by which neutrophils may be recruited lies in the virulence factors of *Aeromonas*. Virulence factors as a whole are discussed in Chapter 1 and therefore will not be discussed here. However, one enzyme of importance, enolase, was identified in *A. hydrophila* from clinical sources (Sha et al., 2009). Enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, although it has also been identified for its involvement in microbial diseases where it can bind the bacterial cell surface (Sha et al., 2009; Zimbler et al., 2015), contributing to pathogensis. *A. hydrophila* surface-expressed enolase binds plasminogen, allowing host plasminogen activator to cleave it, forming plasmin. Plasmin is a serine protease capable of inducing leukocyte chemotaxis, cell migration into tissues, and downstream signaling events (Syrovets et al., 2012). Interestingly we can build upon this by examining *Yersinia pestis*. *Y. pestis* produces the Pla protein, which is similar in sequence to the outer membrane proteins OmpT and PgtE (known as omptins) in *E. coli* and *S. enterica*, respectively. This protein is necessary for adherence and the invasive properties needed to cause pneumonic plague (Caulfield and Lathem, 2012; Lathem et al., 2005). A closer examination reveals Pla protein is capable of cleaving plasminogen to plasmin, resulting in the activation of macrophages, and subsequent release of IL-1β and the recruitment of neutrophils (unpublished research). As discussed in Chapter 1, *Aeromonas* produces many outer membrane proteins with enzymatic activity, and some are required for adherence and infection (Torres et al., 2005). Omptin proteins are widespread and expressed in a variety of enterobacteria, thus suggesting that *Aeromonas* may also contain a similar protein.

In Chapter 5, I found teleost neutrophils to be capable of internalizing dying macrophages. This has also been reported in other teleost models (Yang et al., 2012), as well as in mammalian models (Esmann et al., 2010). Interestingly, this leads many to question the capacity of neutrophils to internalize large apoptotic cells. However, it is likely that neutrophils are in fact only internalizing apoptotic bodies or fragments of pyroptotic cells. This 1) may leave larger bodies still present at the site to be internalized by other cells; or 2) larger bodies continue down the apoptotic cascade to smaller bodies, which are then internalized by neutrophils; or 3) neutrophils are capable of internalizing larger dying cells (addressed in future directions). It should be noted that it is highly unlikely that neutrophils are the only cells internalizing dying macrophages at the inflammatory site under all conditions. However, during infection with *A. veronii* where macrophage viability is vastly affected and monocyte recruitment appears to be hampered, they are likely one of the major players in cellular clean up during the early stages of infection. It would be interesting to examine macrophage viability *in vivo* throughout infection using a TUNEL (or equivalent) assay, which would provide us with a truer sense of macrophage numbers immediately following infection.

Based on my results, I hypothesize that *A. veronii* is attempting to quietly invade the host, while the goldfish immune system has evolved several mechanisms to deal with infection that just happen to induce the destruction of tissue as a necessary consequence (Figure 7.2). The fact that *Aeromonas* induces macrophage apoptosis/pyroptosis was likely evolved as an immune evasion strategy needed to continue infection. As goldfish (and other hosts) develop new methods to combat *Aeromonas* infections, the pathogen must also adapt new mechanisms to continue to infect.

7.2.2.2 Defense against mycobacteria

Much of our current understanding of mycobacterial infections comes from the study of *M. tuberculosis*. One-third of the worlds population is infected with tuberculosis, however, active tuberculosis generally presents in only 5-10% of individuals. In humans, disease can range from self-limiting pleuritis to hematogenous dissemination of large numbers of mycobacteria throughout the host in tuberculosis (van Crevel et al., 2002). This range of disease manifestation is often linked to the quality of host defense against the immune evasion strategies of the bacilli. It has long been thought that protection and elimination of *M. tuberculosis* lied in the success or failure of T lymphocyte and macrophage interactions. More recently innate phagocytic cells have been found to play a key role in the initiation and direction of adaptive T-cell immunity. However, much remains unknown about the specific interactions and function of neutrophils during infection. As discussed in Chapter 6, there is a large divide among scientists as to whether neutrophils are beneficial or detrimental during infection. Thankfully, numerous studies using a variety of host models, including humans (Kisich et al., 2002), mice (Eruslanov et al., 2005), rats (Sugawara et al., 2004), and fish (Hodgkinson et al., 2015; Meijer et al., 2008; Meijer, 2015; Swaim et al., 2006; Yang et al., 2012) allow me to tackle this debate.

It is well understood that mycobacteria has a striking ability to survive within macrophages (Cosma et al., 2006; Houben et al., 2006; McDonough et al., 1993; McKinney et al., 2000; Meena and Rajni, 2010; Pieters, 2008; Podinovskaia et al., 2013; Scherr et al., 2009; Stamm et al., 2003; Talaat et al., 1998, 1999; Tobin and Ramakrishnan, 2008). Using the zebrafish model, macrophages have been shown to

migrate into other tissues following internalizing of the pathogen, where they form organized granuloma-like aggregates (Swaim et al., 2006; Tobin and Ramakrishnan, 2008). Further, this migration is specifically induced by the bacteria (Clay et al., 2007). This explains, at least in part, the rapid clearance of *M. fortuitum* from the peritoneal cavity. Once inside, mycobacteria attempt to evade the antimicrobial functions of the macrophage as it slowly multiplies. A complete discussion of the evasion strategies is discussed in Chapter 1 and thus will not be reviewed here. Interestingly, the vast amount of literature suggest adaptive immunity is required for granuloma formation, however, examining the mycobacterial interactions in zebrafish embryos suggests innate immunity is sufficient to induce granuloma formation, thus challenging the accepted dogma. The looming question is how does the host defend and/ or prevent dissemination and infection?

The induction of macrophage apoptosis has been discussed as a potential defense mechanism, preventing the dissemination of the pathogen (Behar et al., 2011; Iyoda et al., 2014). Interestingly, intracellular mycobacteria are ultimately killed (Keane et al., 2002; Molloy et al., 1994), suggesting self-sacrifice as an important immune defense during infection. In Chapter 6, I found a substantial level of apoptosis in macrophages infected with *M. fortuitum,* further supporting the notion that the strong induction of apoptosis by host macrophages is a major reason for defense against chronic pathogenicity (Behar et al., 2011; Bohsali et al., 2010). Interestingly, neutrophils appear to play a significant role at the granuloma. Zebrafish neutrophils are recruited to the granuloma by signals released from infected macrophages (Yang et al., 2012). Some neutrophils then rapidly internalize the dying macrophage and subsequently kill the internalized mycobacteria through

NADPH oxidase-dependent processes (Yang et al., 2012). My data supports these findings, where neutrophils were far more chemotactic toward a mixed culture of macrophages and *M. fortuitum* when compared to *M. fortuitum* alone. This suggests that similar recruitment mechanisms are likely released by *M. fortuitum* infected goldfish macrophages. In Chapter 6, I found significant numbers of neutrophils exiting the hematopoietic kidney tissue, entering circulation, and infiltrating the primary point of infection (peritoneal cavity), suggesting an overall importance in defense. This appeared to be in response, at least in part, to the increased expression of CXCL8 (or IL-8), produced by, peritoneal macrophages as well as other tissue cells. This is by no means novel, and has been shown before in numerous model systems (Juffermans et al., 1999; Zhang et al., 1995). Interestingly, this appears to be under the control of TNF-α and IL-1β, where neutralizing these cytokines results in substantially lower levels of CXCL8 (Zhang et al., 1995). A further examination of TNF-α reveals it's also critical to stimulate the killing of *M. tuberculosis* by human neutrophils (Kisich et al., 2002). This helps explain the increased expression of TNF-α2 within leukocytes at both the peritoneal cavity and the kidney. Interestingly, this same group also found killing to be non-oxidative in nature, suggesting an alternate mechanism by which it is mediated. What is rather surprising is the increased ROS expression I observed from all leukocytes during infection. Further, this was also observed by several other groups (Hodgkinson et al., 2015; Yang et al., 2012), one of which was critical to the killing of *M. marinum* within infected macrophages by zebrafish neutrophils (Yang et al., 2012). Thus, neutrophil mediated killing likely occurs through a multitude of antimicrobial mechanisms. It would be interesting to examine the various downstream antimicrobial mechanisms (ROS,

degranulation, NO, NET formation) possessed by neutrophils on mycobacterium. Although my results indicated that isolated neutrophils were relatively poor at killing mycobacteria, determining the method in which they do have some success will aid in our understanding of the cellular collaboration needed to eliminate the pathogen. Martineau and colleagues found that tuberculosis infection and the capacity to restrict bacterial growth was inversely proportional to the number of circulating neutrophils (Martineau et al., 2007), cementing their importance during innate control of the pathogen. Interestingly, they found the neutrophil peptides cathlecidin LL-37 and lipocalin 2 restricted growth of the organism. Another study revealed how macrophages acquire neutrophil granules for the antimicrobial defense against intracellular mycobacteria (Tan et al., 2006). In addition, human neutrophil peptides, rabbit defensins, and porcine leukocyte protegrin all proved antibacterial against *M. tuberculosis in vitro* (Miyakawa et al., 1996). Collectively, this points toward the importance of neutrophilic granules in innate defense. Unfortunately, all mammalian studies employed the use of commercially available kits to determine neutrophil peptide involvement. When I examined neutrophil degranulation in response to *M. fortuitum*, the results were never conclusive and thus not included in this thesis. However, this presents a unique path for future research of innate defense mechanisms. In addition, as antibiotic resistance among human pathogens increases, the potential use of neutrophil peptides for tuberculosis therapy may come as a solution.

Adaptive immunity also plays a significant role in defense, which has been previously reviewed extensively (Jasenosky et al., 2015; Orme, 2004; Wolf et al., 2008), and therefore will not be discussed in great detail here. However, several studies have

examined the role of lipid mediators during infection with mycobacterium, and their direct impact on the initiation of T cell immunity. Interestingly, *M. tuberculosis* and *M. marinum* promote their survival through the dysregulation of lipid mediators. This began when 5-lipoxygenase knockout mice were found to be more resistant to tuberculosis (Chen et al., 2008). Moving further, several groups demonstrated that by inhibiting PGE_2 production and stimulating lipoxin production, *M. tuberculosis* inhibited apoptosis and prevented the cross-presentation of its antigens by dendritic cells, thus impeding T cell immunity (Dietzold et al., 2015; Divangahi et al., 2010; Tobin and Ramakrishnan, 2013). Moreover, they are not the only pathogens to take advantage of these soluble mediators. Both *Pseudomonas aeruginosa* and *Toxoplasma* secrete enzymes with 15-lipoxygenase activity, allowing them to modulate host-inflammatory cascades by directly producing anti-inflammatory eicosanoids from host-derived intermediates (Bannenberg et al., 2004; Vance et al., 2004). Prostaglandin E2 and D2 function as inflammatory mediators during the induction phase of acute inflammation. Later, through lipid mediator class switching, prostaglandins induce a pro-resolving phenotype in neutrophils by inducing lipoxin generation (Levy et al., 2001). Moreover, although lipoxins function to inhibit neutrophil recruitment and inflammatory function, it can signal for monocyte migration (Chiang et al., 2006). Therefore, mycobacterium could create an environment in which infiltrating neutrophils are stimulated to secrete lipoxins, in turn recruiting monocytes, which ultimately differentiate into macrophages. This allows for further infection, prevention of macrophage apoptosis, and dissemination throughout the host. Simultaneously, neutrophil lipoxin production prevents the cross-presentation of antigens by dendritic cells, preventing the initiation of T cell immunity (Dietzold et al., 2015; Divangahi et al.,

2010; Tobin and Ramakrishnan, 2013). Collectively, as a significant producer of lipid mediators, this highlights the critical nature of neutrophils to pathogen control.

7.3 Future directions

7.3.1 Are all apoptotic cells created equal?

In Chapters 4 and 5, I discussed the potential for complexity in the induction of apoptotic cascades far beyond what we currently understand. In Figure 5.7B, I showed that goldfish neutrophils do in fact possess the capacity to internalize apoptotic cells, however, the method by which these cells undergo cell death is absolutely critical. Apoptotic cells are essential in directing the inflammatory response when internalized, but how this exactly occurs is relatively unknown. For example, research from the Barreda lab has shown that the internalization of cyclohexamide generated apoptotic cells by teleost macrophages leads to the down-regulation of pro-inflammatory responses, namely the production of ROS (Rieger et al., 2012). However, in Chapter 5, I showed that goldfish neutrophils increased ROS production following the uptake of dying macrophages previously infected with *A. veronii*. The uptake of seemingly similar dying cells resulted in completely opposite downstream responses. In light of this, and with several studies suggesting the internalization of dying cells by neutrophils as critical events during inflammation in fish and mammals (Esmann et al., 2010; Prokopowicz et al., 2012; Tobin and Ramakrishnan, 2013), further examination is necessary.

Apoptotic cells have already been shown to express a wide variety of surface receptors (ex. phosphatidyserine and thrombospondin), as well as humoural secretions (ex. fractalkine, LPC and S1P (Hochreiter-Hufford and Ravichandran, 2013)) giving rise

to 'find me' and 'eat me' signals. Thus, I hypothesize the cause for divergent responses is two-fold: 1) the 'find me' signals released by dying cells will vary depending on the method of induction, and 2) cells undergoing cell death exhibit different 'eat me' surface markers contingent on the method of induction, thus presenting itself in a unique manner. Both 'find me' and 'eat me' signals will likely lead to gene expression and functional changes in the internalizing cell.

A secondary, but complimentary line of examination should also investigate the role of neutrophil activation on the uptake of apoptotic cells. The activation state may also represent important criteria for apoptotic cell internalization. Further, it would also be beneficial to isolate the apoptotic bodies based upon size and assess the capacity for uptake of increasingly larger apoptotic bodies. Neutrophils may in fact be capable of internalizing larger particles than we have previously observed. This would open an entirely new line of inquiry into the phagocytic mechanisms used by neutrophils, potentially leading to novel receptor or pathway identification.

7.3.2 Examine crosstalk between macrophages and neutrophils during infection

In Chapters 5 and 6, I examined the role of neutrophils during infection with two prominent aquatic pathogens, *Aeromonas veronii* and *Mycobacterium fortuitum*. Further, both pathogens are also capable of infecting humans to varying degrees of severity. During both infections, I found a significant degree of interplay between neutrophils and macrophages. In Chapter 5, neutrophils were responsible for internalizing dying macrophages that had been previously infected with *Aeromonas*. This leads to two

particular questions. First, what happens to the macrophages once the infection is under control? It should be noted that I observed no increase in CCL1 expression within the furuncle (Figure 5.5), and no migration of monocytes during the early stages of infection (Figure 5.3/5.4). I predict that the remaining macrophages (those that were not killed by *A. veronii*) begin to proliferate within the tissue. This would not be the first time resident tissue macrophages have been observed proliferating *in vivo* during infection. For example, infection with the nematode *Litomosoides sigmodontis* causes macrophages to proliferate in the pleural cavity of mice (Rückerl and Allen, 2014), and the proliferation of tissue macrophages is often seen as a signature of Th2 inflammatory responses (Davies et al., 2013; Davies and Taylor, 2015; Jenkins et al., 2011). This may potentially be examined through the use of peritoneal injected BrdU (which we already know traffics to tissues within 1 hour), followed by tissue sectioning and antibody staining, and subsequent fluorescent microscopy. Following the regeneration of the macrophage population, I predict that neutrophils will play a significant role in driving pro-resolving responses, much like I observed in Chapter 4. This leads to the second question, what role, if any, do neutrophils exhibit in tissue remodeling? Many chronic inflammatory diseases are characterized by a sustained infiltration of neutrophils, including cystic fibrosis and rheumatoid arthritis. The uncontrolled infiltration and activation often leads to severe tissue damage. However, neutrophils have also been shown to produce proteins that promote angiogenesis and stimulate keratinocyte and fibroblast proliferation (Theilgaard-Mönch et al., 2004). In addition, neutrophils have been implicated in repairing cardiac tissue following an acute heart attack, by polarizing macrophages toward a reparative phenotype (Horckmans et al., 2016). Thus, it is possible neutrophils

play a similar role during the tissue repair process following furuncle formation. Figure 7.3 demonstrates how this hypothesis builds upon my current findings.

In Chapter 6, I found isolated neutrophils to be less effective at killing *M. fortuitum*, when compared to the total population of leukocytes. In addition, several studies have reported the interaction of neutrophils and macrophages in defense against mycobacteria, including the uptake of infected macrophages by neutrophils at granulomas (Yang et al., 2012), and the acquisition of neutrophil granules by macrophages for downstream antimicrobial defense against intracellular mycobacteria (Tan et al., 2006). With this in mind, it would be interesting to examine specific periods during the mycobacterial infection following the depletion of either population. One method by which this can be achieved is through depletion by antibodies against a specific cellular marker (ex. Ly6G). I predict that if neutrophils were depleted at 7 dpi, granuloma formation would become severe and a chronic inflammatory state will ensue. The depletion of neutrophils would result in a build up *M. fortuitum* within the tissues during granuloma formation. In addition, infected macrophages would continue to disseminate throughout the host, further spreading the disease.

7.3.3 Do teleost fish possess 'emergency granulopoiesis'?

During my Ph.D., I examined the proliferative capacity of hematopoietic cells following multiple immune challenges. Interestingly, the only time I observed an increase in proliferation was following peritoneal administration of *M. fortuitum*. Unfortunately, I was unable to definitely determine if this increase in total leukocyte proliferation directly led to an increase in granulopoiesis. However, I predict that much like mammals, the

teleost immune response invokes 'emergency granulopoiesis' during sustained bacterial challenges. Further, I believe it would be of more importance to teleost fish due to the low levels of circulating neutrophils. Humans are prepared to send two large waves of infiltrating neutrophils during the inflammatory response, the first being neutrophils in circulation, and the second being neutrophils released from the bone marrow. Due to the fact that teleost fish possess far lower levels of peripheral neutrophils, the ability to initiate emergency granulopoiesis becomes even more important. This becomes even more interesting if the pathogen is capable of altering the hematopoietic response of the host. An interesting approach to examine this question would be to begin with multiple sequential injections of zymosan (controlled pathogen mimic), while monitoring neutrophil migration. This would be paired with an *in vivo* BrdU assay on isolated hematopoietic neutrophils. Together this may provide insight into neutrophil specific proliferation and migration following repeated exposures.

7.3.4 Acute inflammatory response in mice following *A. veronii* challenge

A. veronii has been implicated in numerous infections in mammals (Lowry et al., 2014), including wound and soft-tissue damage. In Chapter 5, I used a furuncle model of infection in goldfish to analyze the acute inflammatory response against *A. veronii*. Using a comparable footpad infection model, we could dig deeper into the mechanisms of the acute inflammatory response against *A. veronii*. This becomes more beneficial as our ability to analyze individual cellular subsets within mixed populations increases due to the availability of numerous labeling reagents. Harnessing state-of-the-art multi-photon

microscopy allows for an *in vivo* examination of neutrophils, their interactions with other leukocytes, as well as their movement within the tissue.

In Chapter 5, I hypothesized that neutrophils were first migrating to the surface of the furuncle in order to clear the intruding pathogen. From there, we speculated that neutrophils migrated deeper into the muscle tissue in order to initiate resolution and tissue repair. I predict that mammalian neutrophils will do just that, substantiating the data I found in goldfish. Mammalian neutrophils will display prominent roles in pathogen clearance, acting as the 'quarterback' of the immune system until macrophages proliferate and outnumber *A. veronii*. On this note, examining this infection in mice would also allow us to determine if mammalian monocyte migration is impacted, similarly to teleost monocytes. If monocytes are recruited during infection in mice, this creates a unique situation by which the teleost immune system responds to *A. veronii* infection, or how *A. veronii* is capable of modulating monocyte recruitment.

Finally, multi-photon microscopy would allow for the visualization of neutrophils interacting with apoptotic macrophages *in vivo*. This would corroborate the *ex vivo* results I observed in goldfish. Further, it may provide additional insights into the mechanism by which uptake is occurring. By labeling with specific markers, we may be able to determine which receptors are expressed on neutrophils and/ or apoptotic macrophages both during and post uptake. Ultimately, examining the response of different species against the same pathogen would allow us to observe the evolution of cellular function and the overall acute inflammatory response during similar infections.

7.4 Relevance

7.4.1 To aquaculture

Commercial overfishing has become an endemic problem throughout our oceans. With dwindling wild fish populations, aquaculture has become a primary alternative to increase the production needed to feed our growing planet. However, this is not without its challenges. One of aquaculture's greatest hurtles is the prevention of infection within fish stocks. Both *Aeromonas* and *Mycobacterium* have been associated with significant outbreaks in farmed fish, often leading to mass deaths. The develop of potential vaccines is already being examined, where recombinant Omp48 from *A. hydrophila* (also found in many other aeromonads) has been shown to induce protective responses in rohu carp (Khushiramani et al., 2012). Thus, furthering our understanding of how these pathogens infect and evade immune responses, as well as the mechanisms of host defense following infection may lead to the development of additional strategies for disease prevention.

7.4.2 To basic biology

Neutrophils are fundamental for host defense and the proper coordination of inflammatory responses. It is well known that neutrophil depletion results in poor induction of inflammatory responses and improper pathogen clearance. However, as we continue to examine their roles throughout inflammation, we are discovering the critical function of neutrophils to the induction of resolution responses and tissue repair mechanisms. Further, by studying neutrophils in lower vertebrates we will gain added information on their mechanisms of inflammatory regulation, and their respective

conservation within higher vertebrates. Further, basic biological research ultimately becomes the foundation on which novel medical therapies can be developed.

7.4.3 To inflammatory diseases

Neutrophils are at the center of numerous chronic inflammatory or autoimmune diseases, including but not limited to cystic fibrosis, rheumatoid arthritis, and systemic lupus erythrematosus. They often play a central role in initiating and perpetuating aberrant immune responses and the subsequent tissue damage during these conditions. Currently, many researches are examining specific neutrophil functions or molecules as therapeutic strategies to help prevent disease progression. For example, blocking neutrophil chemotactic factors such as LTB_4 (BIIL284- antagonist) and CXCL8 (GSK-656933- CXCR2 antagonist) or their respective receptors is one current approach being investigated (Barnes, 2007). Another includes inhibiting adhesion molecules including E-selectin (Bimosiamose) (Barnes, 2007). A third blocks the function of 5-lipoxygenase (Zileuton), preventing the formation of the LTB_4 precursor, LTA_4 (Barnes, 2007). Therefore, continuing to uncover the mechanisms of migration, infiltration, and function of neutrophils across the inflammatory spectrum becomes critical to developing novel therapies to control disease.

7.5 Summary

The primary objective of my Ph.D. was to examine the evolving contribution of neutrophils to the induction, regulation, and resolution of inflammation. I focused on the entire inflammatory cascade, examining the role of hematopoietic neutrophils, the

kinetics of neutrophil migration and subsequent infiltration, and their function throughout various inflammatory responses. Specifically, my research has expanded our current understanding of the plasticity of neutrophils and the importance of these cells to the initiation of resolving events within an inflammatory site. Further, uncovering novel methods of inflammatory control opens the door for additional strategies to prevent and control both aquaculture and human inflammatory diseases.

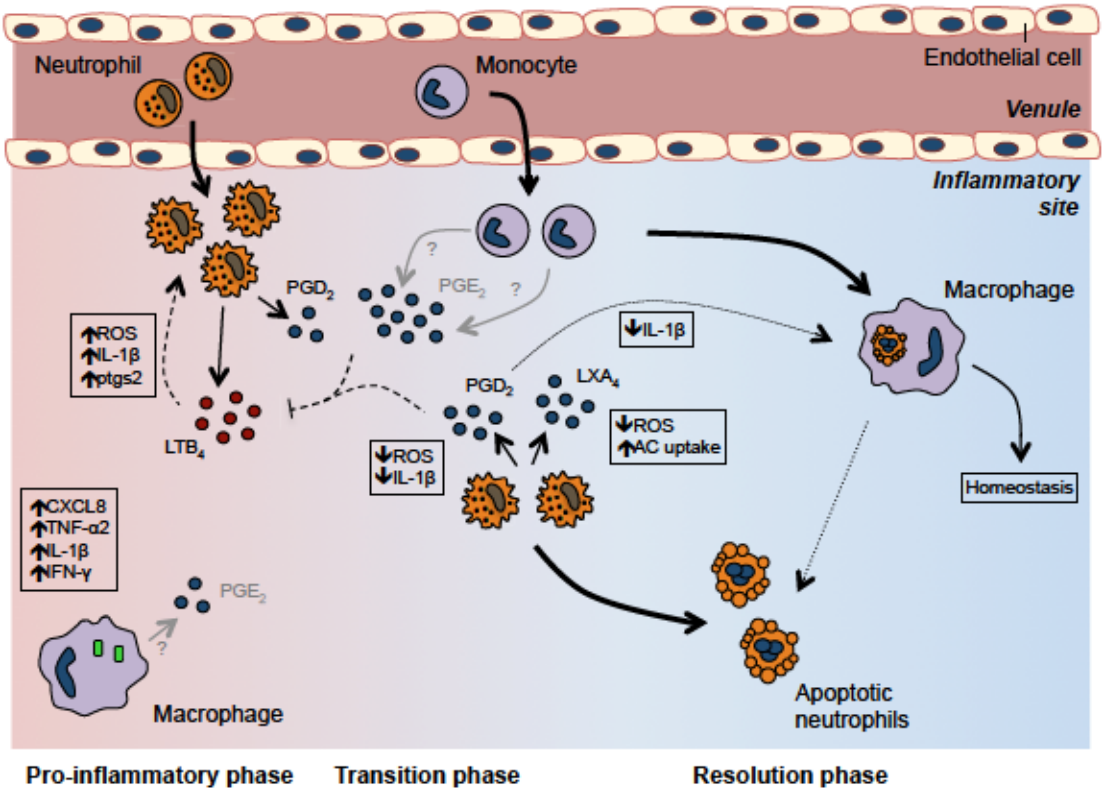

Figure 7.1. Kinetics of lipid mediator release and their downstream effects in teleost fish during the induction and resolution phase of inflammation.

Infiltrating neutrophils release both LTB_4 and PGD_2, with LTB_4 contributing to the activation of both macrophages and neutrophils throughout the induction phase. During the transition phase, PGE_2 (likely from infiltrating monocytes) accumulates, leading to a lipid mediator class switch in neutrophils. Neutrophils continue secreting PGD_2 and begin releasing LXA_4, leading to the inhibition of neutrophil and macrophage pro-inflammatory responses. Further, LXA_4 promotes the uptake of apoptotic neutrophils by macrophages. Solid black lines indicate the transition/ differentiation of cells. The downstream effects (both autocrine and paracrine) of soluble mediators are represented by dashed lines. Dashed lines ending in an arrow indicate activating effects, while dashed lines ending in a perpendicular line represent inhibitory effects.

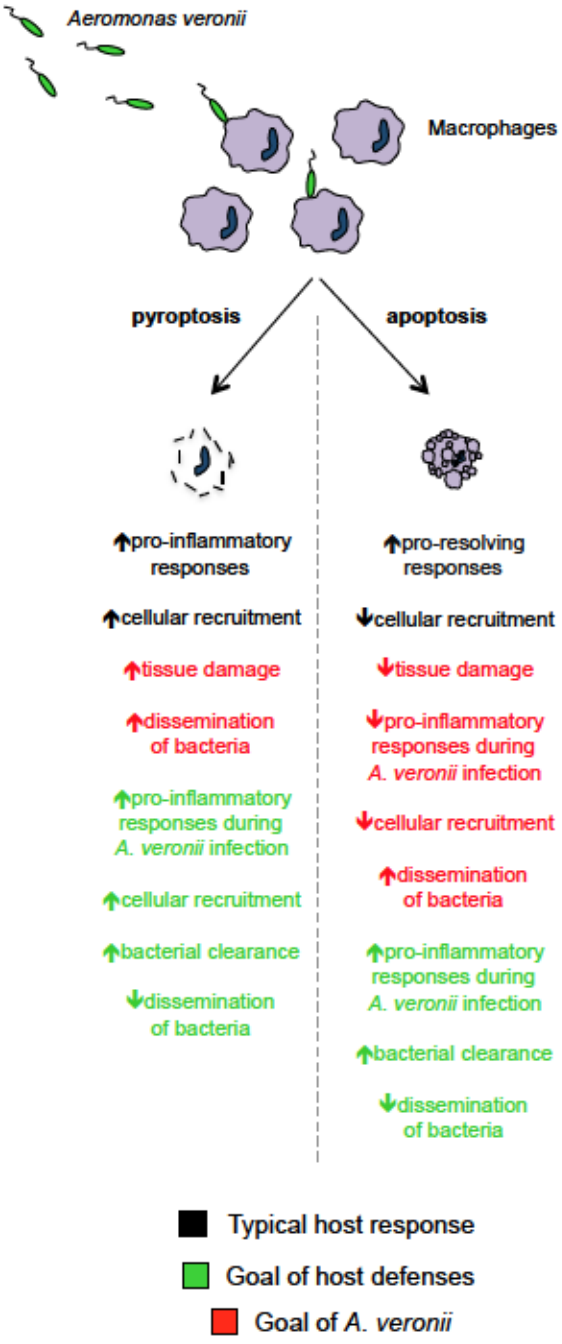
Aeromonas veronii
Macrophages
pyroptosis
apoptosis
↑pro-inflammatory responses
↑cellular recruitment
↑tissue damage
↑dissemination of bacteria
↑pro-inflammatory responses during A. veronii infection
↑cellular recruitment
↑bacterial clearance
↓dissemination of bacteria
↑pro-resolving responses
↓cellular recruitment
↓tissue damage
↓pro-inflammatory responses during A. veronii infection
↓cellular recruitment
↑dissemination of bacteria
↑pro-inflammatory responses during A. veronii infection
↑bacterial clearance
↓dissemination of bacteria
Typical host response
Goal of host defenses
Goal of A. veronii

Figure 7.2. Downstream responses following *Aeromonas* induced pyroptosis or apoptosis in macrophages.

Aeromonas spp. has been shown to induce both pyroptosis and apoptosis in macrophages. This was corroborated by data I found in Chapter 5. This figure summarizes the potential outcomes following either form of cell death. Typical host responses can be found in black. Green text represents the goals/ or known outcomes of the host during the immune response. Red text highlights the goals of *Aeromonas veronii* in order to circumvent host defense, leading to further infection.

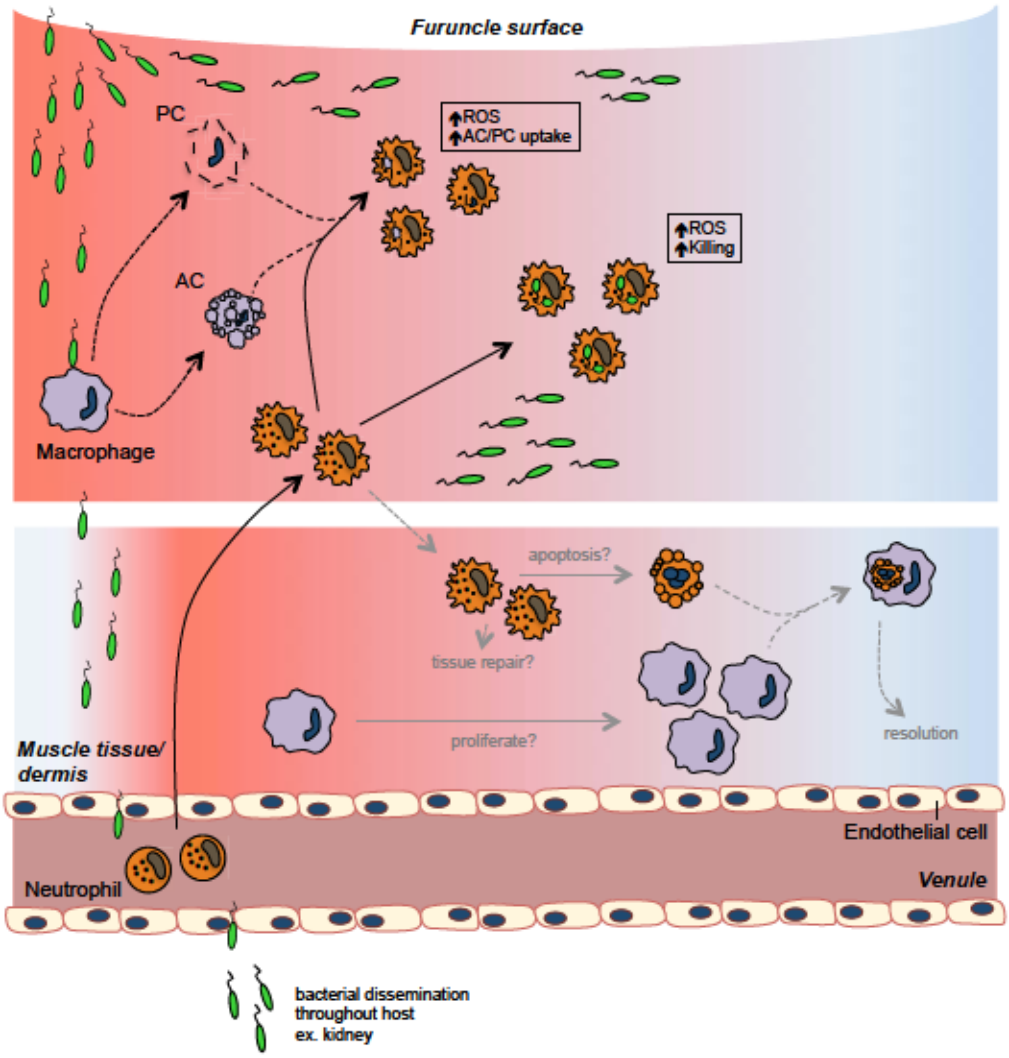

Figure 7.3. Contribution of neutrophils to the defense against *Aeromonas veronii*.

Aeromonas veronii invasion of the host results in the induction of cell death within tissue macrophages. As discussed above, neutrophils are recruited to the infection site where they internalize *A. veronii*, leading to its ultimate destruction. In addition, neutrophils internalize dead or dying macrophage, leading to the induction of pro-inflammatory reactive oxygen species, further propagating the inflammatory response. Solid black lines represent these results. At this point, I hypothesize neutrophils migrate deeper into the muscle tissue/ dermis where they play a role in tissue repair and the resolution of inflammation. Simultaneously, the remaining macrophage population undergoes proliferation expanding the number of tissue macrophages. These macrophages will ultimately internalize the apoptotic neutrophils and continue to regulate tissue repair processes. The hypothesized outcomes are represented by grey lines.

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Appendix I. Multi-parametric analysis of phagocyte antimicrobial responses using imaging flow cytometry[1]

AI.1 Introduction

Phagocytosis is a well conserved cellular mechanism, which serves as a first line of defense against invading pathogens and further contributes to the regulation of downstream effector and regulatory mechanisms of immunity (Underhill and Ozinsky, 2002). Particle internalization marks the successful transition through the threshold of phagocyte activation and promotes the induction of downstream processes. This includes the activation of inflammatory programs (Underhill and Ozinsky, 2002), production of antimicrobial reactive oxygen and nitrogen species (Bogdan et al., 2000; Robinson, 2008), and induction of transcription factor activity resulting in targeted expression of cytokine genes (McDonald and Cassatella, 1997; Scull et al., 2010). Given the heterogeneity among individual phagocyte responses at the inflammatory site, much can

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[1] A portion of this chapter has been previously published in:

Havixbeck JJ, Wong ME, More Bayona JA, and Barreda DR (2015) Multi-parametric analysis of phagocyte antimicrobial responses using imaging flow cytometry. Journal of Immunological Methods 423: 85-92.

be gained from multi-parametric analyses that allow deeper characterization of distinct phenotypes and their contributions to the induction and regulation of inflammation.

Over the years, a number of molecular and cell-based strategies have been applied to the study of phagocytosis (Drevets and Campbell, 1991; Rieger et al., 2010a; Rossi and Lord, 2013; Singboottra et al., 2010). Of these, three common approaches are based on light microscopy, fluorescence microscopy, and flow cytometry techniques. Each allows for the detection of phagocytosis with varying levels of specificity but also displays important limitations that restrict their overall contributions. Microscopy-based approaches (both light and fluorescent) allow the user to directly visualize cells, offering the spatial resolution required to discern particle and/or staining localization. However, microscopy-based assays suffer from time constraints and low event counts, which preclude statistically robust analyses. Flow cytometry-based assays increase the statistical robustness through examination of thousands of cells in a short period of time. In addition, parallel analysis of cellular phenotypes based on surface marker expression may provide increased resolution when mixed populations are analyzed. Flow cytometry, however, lacks the spatial resolution of microscopy-based assays, which prevents the discrimination between bound and internalized particles and the analysis of downstream responses that are not dependent solely on changes in fluorescence level, such as nuclear translocation of NF-κB. The ImageStream multispectral imaging flow cytometry platform offers added resolution by combining the throughput and multi-channel capabilities of flow cytometry with the spatial localization of fluorescent microscopy. Phagocytosis assays are particularly enhanced through the ability to examine cells in the X, Y, and Z plane, which allows the user to analyze cells in all 3 dimensions and therefore distinguish

between surface-bound and internalized particles (Rieger et al., 2010a). Importantly, this platform is also amenable to multi-parametric analyses of cellular responses among distinct subsets in a population. The present study took advantage of these capabilities for *in vitro* evaluation of phagocyte antimicrobial responses against *Aeromonas veronii.* This pathogen has previously been shown to induce apoptosis of epithelial cells through mitochondrial depolarization and oxidative stress (Krzymińska et al., 2011). Previous studies suggest that resident macrophages, are central contributors to the induction and regulation of antimicrobial responses against this pathogen (John et al., 2002)(Sharp and Secombes, 1993). However, the details of this macrophage-*Aeromonas* interaction remain ill defined.

We report here, that both live and heat-killed forms of *Aeromonas veronii* activated murine RAW 264.7 macrophages and induce NF-κB nuclear translocation. Unlike their contribution to epithelial cell apoptosis, *Aeromonas* did not induce significant levels of macrophage apoptosis. However, the number of actively proliferating macrophages decreased upon *A. veronii* challenge. The phagocytic capacity of RAW macrophages internalizing *A. veronii* also increased in a time dependent manner. Interestingly, using a multi-parametric approach, we found that the degree of interaction between these phagocytes and *A. veronii* played a major role on their antimicrobial profile. In short, *A veronii* inhibited respiratory burst responses in macrophages that had phagocytosed the pathogen to a greater extent than those that had simply bound this bacterium, and to a much further degree than those that were not physically associated with *A. veronii*.

AI.2 Materials and Methods

AI.2.1 Cell line

RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum. Cells were incubated at 37 °C/5% CO_2 and passaged every 2-3 days.

AI.2.2 Bacterial growth

Aeromonas veronii was grown overnight in trypticase soy broth to an OD_{600} of 1.0 to 1.3. Heat-killed (Hk) samples were prepared by incubating bacteria at 80 °C for 60 minutes. Live and heat-killed *A. veronii* were then washed twice in $1\times PBS^{-/-}$ and added to cultures at a 5:1 (bacteria: cells) ratio.

AI.2.3 Fluorescent labeling of bacteria

Following heat-killing, *A. veronii* was washed twice and resuspended in $1\times PBS^{-/-}$. Propidium iodide (PI) was added to a final concentration of 4 µg/mL and incubated for 60 minutes in the dark at room temperature with continual rocking. Bacteria were then washed twice with $1\times PBS^{-/-}$ to remove any unbound PI.

AI.2.4 Cell nuclear staining

Cells were stained with a 1:30 dilution of DRAQ5 (Biostatus). Cells were subsequently incubated for a minimum of 2 minutes prior to acquisition.

AI.2.5 NF-κB nuclear translocation

RAW 264.7 cells were incubated with unlabelled heat-killed or live bacteria, for 1 hour at 37 °C/5% CO_2 with complete DMEM media in 5mL polystyrene round bottom tubes (BD Falcon). Following phagocytosis, cells were washed twice with 1×$PBS^{-/-}$ and then fixed with 1% formaldehyde overnight at 4 °C. Cells were then permeabilized with buffer (2 % fetal calf serum supplemented with 0.1% saponin) for 10 minutes at 4 °C before being treated with anti-NF-κB p65 rabbit IgG (Santa Cruz Biotechnology) for 30 minutes at 4 °C, followed by 20 minutes at room temperature. Cells were then washed with permeablization buffer and treated with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) for 20 minutes at room temperature. DRAQ5 stain was added immediately prior to acquisition on an ImageStream MKII (Amnis; EMD Millipore) imaging flow cytometer. At least 10,000 cells were acquired.

AI.2.6 Cell viability

Cell viability was determined as previously described (Rieger et al., 2010b) with minor modifications. RAW 264.7 cells were incubated with live or heat-killed *Aeromonas* for 1, 2, or 4 hours at 37 °C/5% CO_2 with complete DMEM media in 5mL polystyrene round bottom tubes (BD Falcon). Following incubation, cells were washed twice in Annexin V binding buffer (BD Pharmingen) and resuspended in 100 µL Annexin V binding buffer. Annexin V FITC (eBioscience) was added according to manufacturers protocols. Propidium iodide (Sigma) was added to a final concentration of 4 µg/mL. Cells were then incubated for a further 30 minutes and data was acquired on an

ImageStream MKII multi-spectral flow cytometer (Amnis; EMD Millipore). At least 10,000 cells were acquired.

AI.2.7 Cell proliferation

Five-bromo-2'-deoxyuridine (BrdU) was used *in* vitro to determine macrophage proliferation. BrdU (10μM) was allowed to incorporate into RAW macrophages for 1 hour. Cells were fixed in 1% formaldehyde for a minimum of 24 hours in 5 mL polystyrene round bottom tubes (BD Falcon). Cells were then stained according to manufacturers protocols. Briefly, following fixation, cells were washed once in 1x $PBS^{-/-}$ followed by once in flow cytometry buffer (2% FBS in 1x $PBS^{-/-}$). Cells were resuspended in 1 mL of BrdU staining buffer (eBioscience), and incubated at 4 °C for 1 hour. Following incubation, cells were resuspended in 100 μL flow cytometry staining buffer containing DNase I (30μg; Sigma), and incubated at 37 °C for 1 hour. Cells were then washed in flow cytometry buffer to remove DNase I. Following centrifugation, cells were resuspended in 100 μL flow cytometry staining buffer containing anti-BrdU FITC antibody, at incubated at room temperature for 1 hour. Cells were washed twice in flow cytometry staining buffer. Immediately prior to acquisition, a 1:30 dilution of DRAQ5 was added to allow for cell cycle analysis. Analysis of macrophage cell cycle was performed as previously described (Amnis, 2007). Data was acquired on the ImageStream MKII (Amnis; EMD Millipore) and analyzed using IDEAS software. A minimum of $5x10^4$ cells were acquired.

AI.2.8 Phagocytosis and respiratory burst

RAW 264.7 cells were incubated with heat-killed (PI labelled) or live bacteria (Syto9 labelled) for 1 hour at 37 °C/5% CO_2 with complete DMEM media in 5mL polystyrene round bottom tubes (BD Falcon). Following phagocytosis, cells were washed twice with 1×$PBS^{-/-}$ and then fixed with 1% formaldehyde for 30 minutes on ice. Data was acquired on an ImageStream multi-spectral flow cytometer (Amnis; EMD Millipore). At least 10,000 cells were acquired.

To evaluate phagocytosis and the respiratory burst in the same cells, RAW 264.7 macrophages were incubated with PI labelled heat-killed *A. veronii* for 1 hour at 37 °C/5% CO_2 with complete DMEM media in 5mL polystyrene round bottom tubes (BD Falcon). Following phagocytosis, cells were washed twice with 1×$PBS^{-/-}$ then resuspended in 100 µL 1×$PBS^{-/-}$. Dihydrorhodamine (DHR, Molecular Probes) was added to cells at a final concentration of 10 µM and incubated for 5 minutes to allow cells to take up the DHR. Phorbol 12-myristate 13-acetate (PMA; Sigma) was then added at a final concentration of 100 ng/mL. Cells were incubated for a further 30 minutes to allow oxidation of DHR. All samples were properly staggered with respect to timing to accommodate for the transient state of oxidized DHR fluorescence. Data was acquired on an ImageStream MKII (Amnis; EMD Millipore) imaging flow cytometer. Data was analyzed using IDEAS v5.0 software. In short, we first separated phagocytic from non-phagocytic cells (n-Ph). Phagocytic cells were gated further into two populations- cells with surface bound *Aeromonas* (SB) and cells with internalized *Aeromonas* (Int). All three populations (n-Ph, Int, and SB) were then analyzed for DHR fluorescence.

AI.2.9 Flow cytometry acquisition and analysis

Flow cytometry data was acquired on an ImageStream MKII (Amnis) and analyzed using IDEAS software. Cellular debris was defined based on internal complexity, size, and nuclear cell staining, and eliminated from subsequent analyses.

AI.2.10 Statistics

GraphPad Prism software was used to determine the significance between control and experimental groups by paired Student's T-test. Probability level of $p<0.05$ was considered significant.

AI.3 Results

AI.3.1 *Aeromonas veronii* induces NF-κB translocation in RAW 264.7 macrophages

Aeromonas veronii is known to induce inflammatory responses upon infection in a range of evolutionarily-distant species, including arthropods, molluscs, fish, reptiles, and mammals (Janda and Abbott, 2010, 1998). Previous studies suggest that resident macrophages, are central contributors to the induction and regulation of antimicrobial responses against this pathogen (John et al., 2002)(Sharp and Secombes, 1993). However, their early responses against *Aeromonas* remain poorly defined. As a first step in the characterization of these responses, we examined the contribution of *A. veronii* to the induction of early phagocyte inflammatory processes in RAW 264.7 murine macrophages *in vitro*. We found that this pathogen, in both live and heat-killed forms, induced similar levels of NF-κB translocation. In each case, nuclear translocation of NF-κB increased

nearly 7-fold, from approximately 12% in unstimulated macrophages to 80% in stimulated cells (Figure AI.1). Our results indicate that engagement of *Aeromonas veronii*, regardless of its viability state, is sufficient to induce robust activation of this murine cell line.

AI.3.2 RAW 264.7 macrophages maintain viability but decrease proliferation following incubation with *Aeromonas veronii*

Aeromonas is known to contribute to inflammation, in part, through induction of epithelial and immune cell death within 24 to 48 hours of infection (Krzymińska et al., 2011, 2009; McCoy et al., 2010). We were curious to determine whether this process includes a direct effect on macrophage viability. RAW 264.7 murine macrophages were challenged with live or heat killed *A. veronii* for 1, 2, 4, or 24 hours and then stained with PI, AnnexinV FITC, and DRAQ5 (Rieger et al., 2010b). We found that a high level of viability (above 80%) was maintained for the first 4 hours in all macrophages examined regardless of whether live or heat-killed *Aeromonas* were used (Figure AI.2B). However, macrophage viability decreased to below 40% at 24 hours in the presence of either live or heat-killed bacteria (Figure AI.2B). Further, the decrease in viability resulted in a proportional increase in apoptosis (Figure AI.2C). Evaluation of cell morphology and fluorescent staining confirmed classical features of live ($AnnV^-PI^-$), early apoptotic ($AnnV^+PI^-$), late apoptotic/necrotic ($AnnV^+PI^+$), and necrotic ($AnnV^-PI^+$) cells (Figure AI.2D). Given that live and heat-killed forms of *Aeromonas* showed equivalent impact on RAW 264.7 murine macrophage viability and activation, we focused subsequent analyses

on heat-killed *A. veronii* to prevent variability among macrophage responses because of differential bacterial growth and function.

Despite the absence of an impact to macrophage viability, this *A. veronii* challenge was correlated with a decrease in macrophage proliferation. No change was observed in the distribution of macrophages in sub-G0 and G0/1 phase of the cell cycle, but we identified a decrease in the proportion of mitotic (G2/M) macrophages when compared to unstimulated group (3.7% vs 6.6%, respectively; Figure AI.3B). To determine if the decreased proportion of G2/M events translated into decreased proliferation, we examined the impact of *Aeromonas* on the incorporation of BrdU by these macrophages. A significant decrease (~25%) in macrophage proliferation was detected (Figure AI.3B).

AI.3.3 *A. veronii* challenge promotes RAW 264.7 macrophage phagocytosis but inhibits intracellular ROS production

The contributions of phagocytes at an infection site are driven in part by the level of interaction with infiltrating pathogens. Using an imaging flow cytometry-based approach we examined macrophages that interacted directly with *A. veronii* as well as those that had not, to assess the potential for differential contributions from phagocytic and non-phagocytic events (Havixbeck et al., 2014; Rieger et al., 2012, 2010a). Using IDEAS software we separated three distinct subsets of macrophages: (1) non-phagocytic, (2) those with surface bound *A. veronii*, and (3) those that had internalized *Aeromonas*. As expected, we found that the internalization of heat-killed *Aeromonas* increased in a time dependent manner, nearly doubling from 1 to 4 hours (8% vs. 14%, respectively;

Figure AI.4A). However, no change was observed in the degree of *A. veronii* surface binding. Further, the increase in phagocytosis was not coupled with an increase in the phagocytic index (number of internalized particles per phagocytic macrophage; data not shown).

The antimicrobial defence mechanisms in mammalian phagocytes rely on the production of large quantities of reactive oxygen species (ROS), reactive nitrogen species (RNS) and antimicrobial enzymes (i.e. lysozyme) to defend against both intracellular and extracellular pathogens (**Belaaouaj**, 2002; **Campbell et al.**, 1989; **Hyslop et al.**, 1995). To evaluate ROS production among phagocytic and non-phagocytic RAW 264.7 macrophages, these murine cells were challenged with heat-killed PI-labelled *A. veronii* and subsequently stained with dihydrorhodamine (DHR) to measure ROS production. As expected, we found a significant increase in the total number of macrophages producing ROS following a heat-killed *Aeromonas* challenge (27% at all time points compared to 10% in unstimulated macrophages; Figure AI.5A). However, multi-parametric analysis of phagocytosis and ROS production highlighted marked differences among non-phagocytic cells, those that had bound *A. veronii*, and those that had internalized this pathogen. At all time points examined, non-phagocytic cells produced the majority of ROS at 27-29% (Figure AI.5B). In contrast, after 1 h of incubation ROS production decreased to 17% following binding of A. veronii, and further decreased to 3% upon internalization of this gram-negative bacterium (Figure AI.5B). However, a marked increase in the production of ROS from these two subsets was observed in a time dependent manner. After 2 or 4 hours of co-incubation with heat-killed *Aeromonas,* macrophages overcame the initial inhibition, resulting in respiratory burst responses similar to that of stimulated non-

phagocytic cells. However, the potential does exist for differential effects of live versus heat-killed bacteria on phagocytosis and ROS production.

AI.4 Discussion

Phagocytosis is an important, evolutionarily conserved mechanism integral to host defenses against invading microorganisms. Pathogen engagement of the receptor repertoire on the surface of phagocytes and successful transition through the threshold of activation leads to phagocytic uptake, the rapid induction of inflammatory cascades, and the activation of potent antimicrobial mechanisms. In this report, we used an imaging flow cytometry-based multi-parametric approach to assess the differential contributions of phagocyte subsets to antimicrobial ROS production in the face of an *Aeromonas veronii* challenge. Of the *Aeromonas* spp. characterized to date, *Aeromonas veronii* displays virulence in the greatest range of hosts, including humans, where it can cause wound infections, diarrhea, and septicemia (Silver et al., 2011). High levels of endotoxins have previously been shown to induce apoptosis in epithelial cells (Krzymińska et al., 2011), and murine macrophages (Krzymińska et al., 2009). Thus, much interest remains regarding the early responses from resident macrophages to this pathogen.

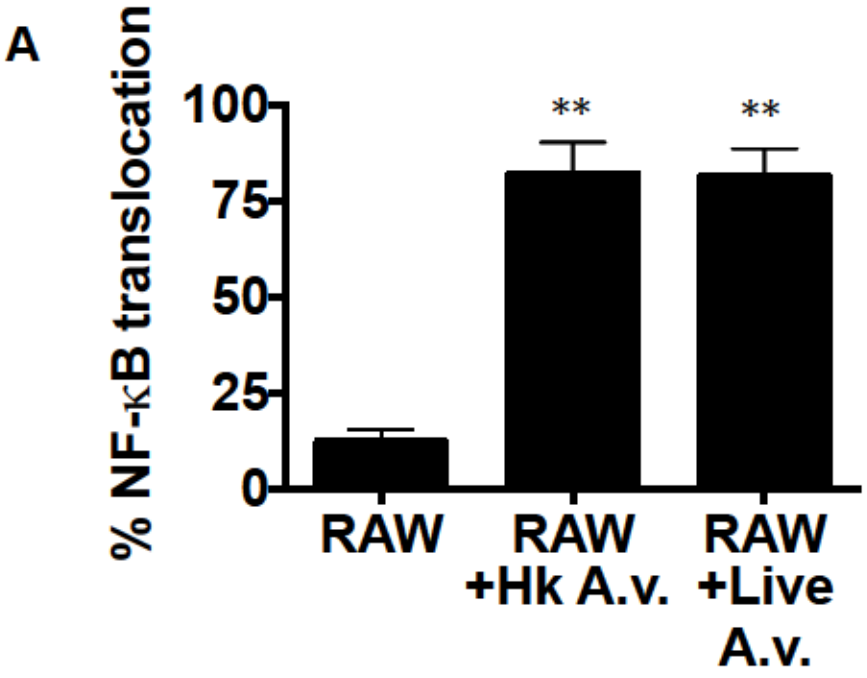
Aeromonas is known to use a number of mechanisms to modulate immune defenses, including the disruption of intracellular signalling (Rosenzweig and Chopra, 2013), the induction of apoptosis (Krzymińska et al., 2011, 2009), and the degradation of reactive oxygen species (Rio et al., 2007). While previous studies have examined time points after an exposure of 24 hours or longer (Krzymińska et al., 2011, 2009), little work has been done to examine phagocyte-*Aeromonas* interactions at earlier time points. We

found that within the first 4 hours following exposure, there was no induction of apoptosis as detected by Annexin V. Phosphatidylserine movement to the outer leaflet of the plasma membrane is one of the initial apoptotic events and is detectable by Annexin V during the first hour of apoptosis (Bratton et al., 1997). Interestingly, though we found no induction of apoptosis at these early time points, we did find a significant change in both cell cycle profiles and proliferation. Based on our results we find that *Aeromonas veronii* has an inhibitory effect on macrophage proliferation as early as one hour after the initial stimulus. In contrast, we identified no significant impact to macrophage viability until after 24 hours on co-incubation *in vitro*. Given the importance recently attributed to local bursts of macrophage proliferation to inflammatory responses against invading pathogens (Davies et al., 2013, 2011), it may be possible that this early inhibition of macrophage proliferation may confer an advantage to this pathogen upon infection.

Multi-parametric analysis of phagocytosis and ROS production highlighted marked differences among non-phagocytic cells, those that had bound *A. veronii*, and those that had internalized the pathogen. The increased interaction of *A. veronii* with phagocytic macrophages led to significant inhibition of ROS production at early time-points (1h). Although differences were still observed after longer incubations (2 and 4 h), RAW 264.7 macrophages appear to overcome the initial inhibition of ROS production. KatA, an antioxidant enzyme catalase has previously been implicated with an improved capacity to foster *Aeromonas veronii* survival under oxidative stress conditions (Rio et al., 2007), and thus may contribute to *Aeromonas* survival and inhibition of ROS production during infection. Furthermore, *A. veronii* has a catalase-independent defensive mechanism against exogenous H_2O_2 during stationary-phase growth (Rio et al., 2007),

which suggests an addition mechanism by which *Aeromonas* is protected from host defenses. While this has not been previously reported for *Aeromonas*, it is a common trait among other intracellular bacteria including *Anaplasma phagocytophilium*, which has been shown to alter the normal processes of human neutrophils by down-regulating ROS production and preventing apoptosis (Borjesson et al., 2005). It will be interesting to determine if this mechanism allows for continued *Aeromonas* survival inside macrophages while fostering continued expansion of host cell death, tissue damage, and inflammation that contribute to subsequent systemic expansion of *Aeromonas* infection beyond the original site of infection.

In this report we used a multi-parametric approach to examine several distinct biological functions concomitantly. Through using this approach we were able to identify previously unreported actions of *Aeromonas* on murine macrophage proliferation, cell cycle, and production of reactive intermediates within the context of macrophage phagocytic responses. The procedures and reagents for each of the assays described in this manuscript provide the potential for integration and detailed multi-parametric analysis of unique subsets. These analyses may provide detailed information amongst mixed cellular populations and provide new insights for comprehensive analysis of the intracellular events that occur following phagocytosis.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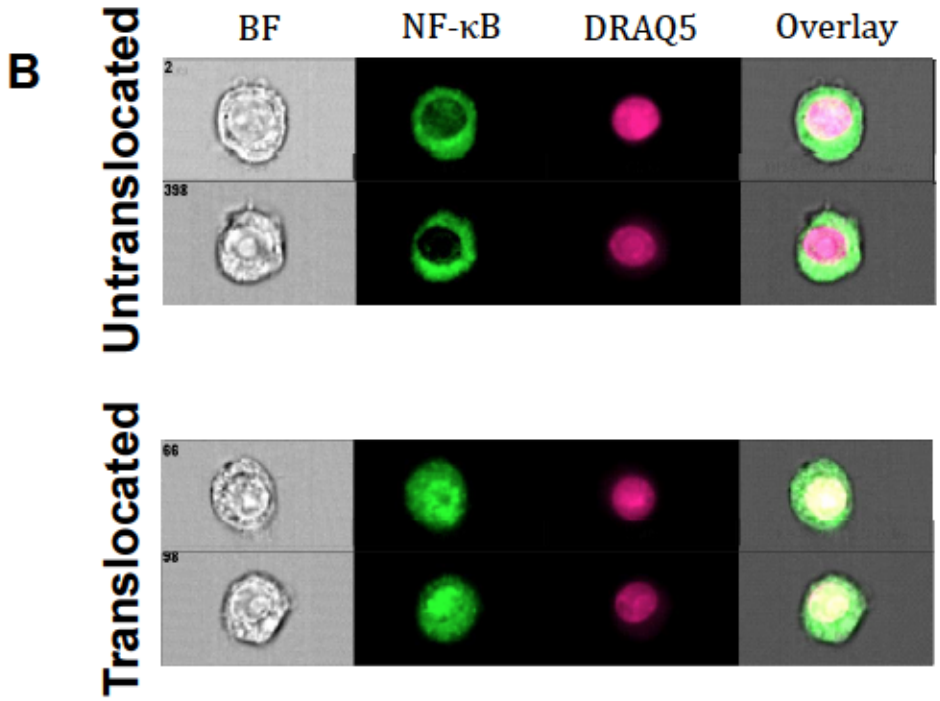
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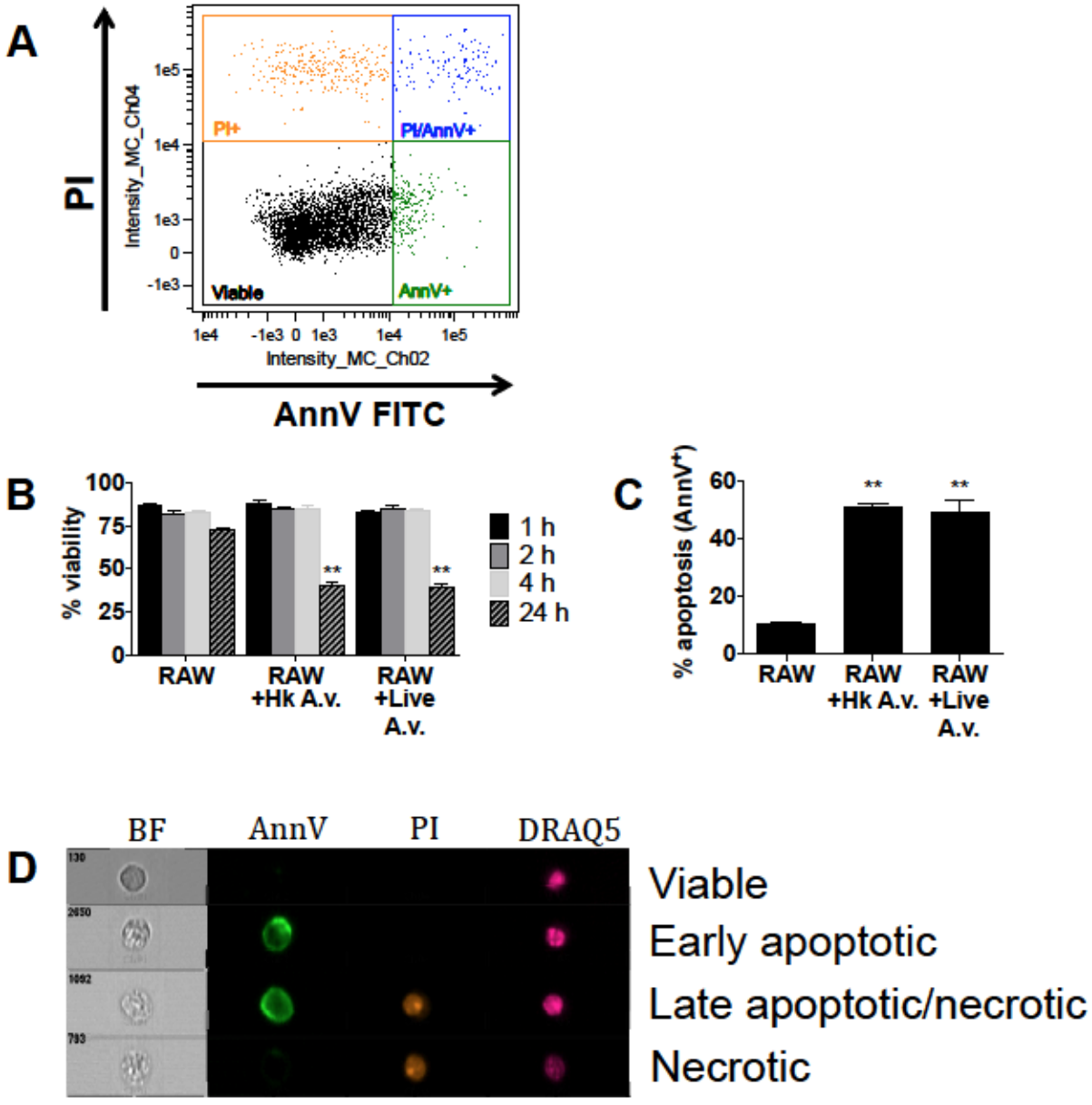
Figure AI.1. *Aeromonas veronii* induces NF-κB translocation in murine macrophages.

RAW 264.7 macrophages were incubated with live or heat-killed *A. veronii* for 1 hour. Cells were fixed, permeabilized, and stained for NF-κB as a marker of cell activation. (A) Translocation of this transcription factor increased in macrophages following incubation with live or heat-killed *Aeromonas*. (B) Representative images highlight macrophages with untranslocated and translocated NF- κB. n=4; **p<0.01; error bars correspond to SEM.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AnnV
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DRAQ5
Viable
Early apoptotic
Late apoptotic/necrotic
Necrotic

Figure AI.2. Effect of *A. veronii* on the viability of murine macrophages.

RAW 264.7 macrophages were incubated with live or heat-killed *A. veronii* for 1, 2, 4, or 24 hours and stained with Annexin V FITC and PI. (A) Dot plot shows viable, early apoptotic, late apoptotic/necrotic and necrotic cell populations ($AnnV^-/PI^-$, , $AnnV^+PI^-$, , $AnnV^-/PI^+$, and $AnnV^+PI^+$, respectively). (B) Percent viability of cells following 1, 2, 4, or 24 hour challenge with either live or heat-killed *Aeromonas*. (C) Percent apoptotic ($AnnV^+$) macrophages at 24 hours. (D) Representative images of each distinct cellular subset. n=4; **p<0.01; error bars correspond to SEM.

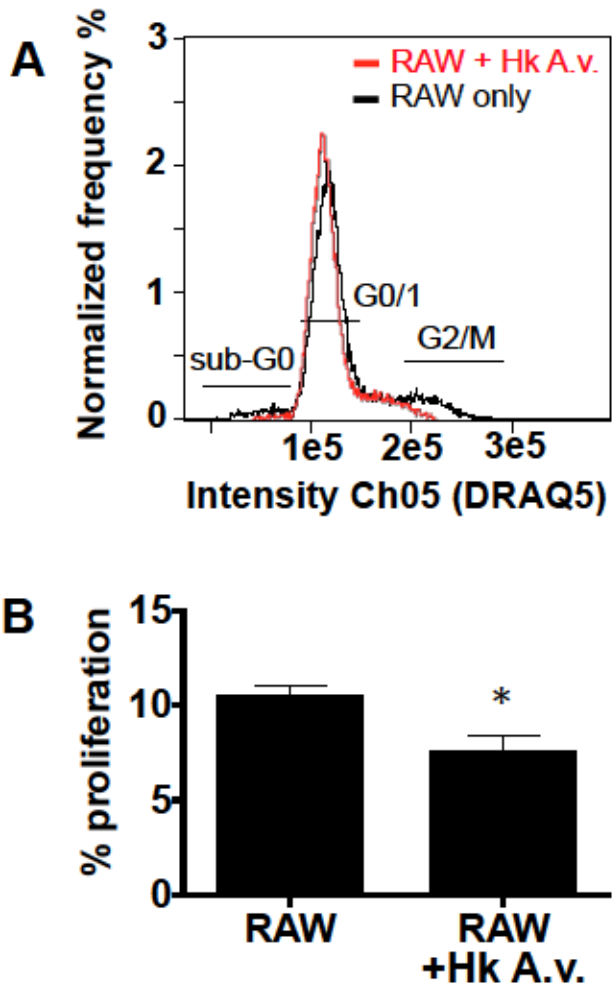

Figure AI.3. *A. veronii* decreases the proliferative capacity of murine macrophages

RAW 264.7 macrophages were incubated with heat-killed *A. veronii* and 10 μM BrdU for 1 hour, fixed with 1% formaldehyde, and stained with an anti-BrdU FITC antibody to identify proliferating cells. DRAQ5 was also added to facilitate cell cycle analysis. (A) Cell cycle overlay of RAW macrophages (black) vs. RAW macrophages challenged with heat-killed *A. veronii* (red). (B) Proportion of proliferating macrophages in unstimulated cultures and those challenged with heat-killed *A. veronii*. n=4; *$p<0.05$; error bars correspond to SEM.

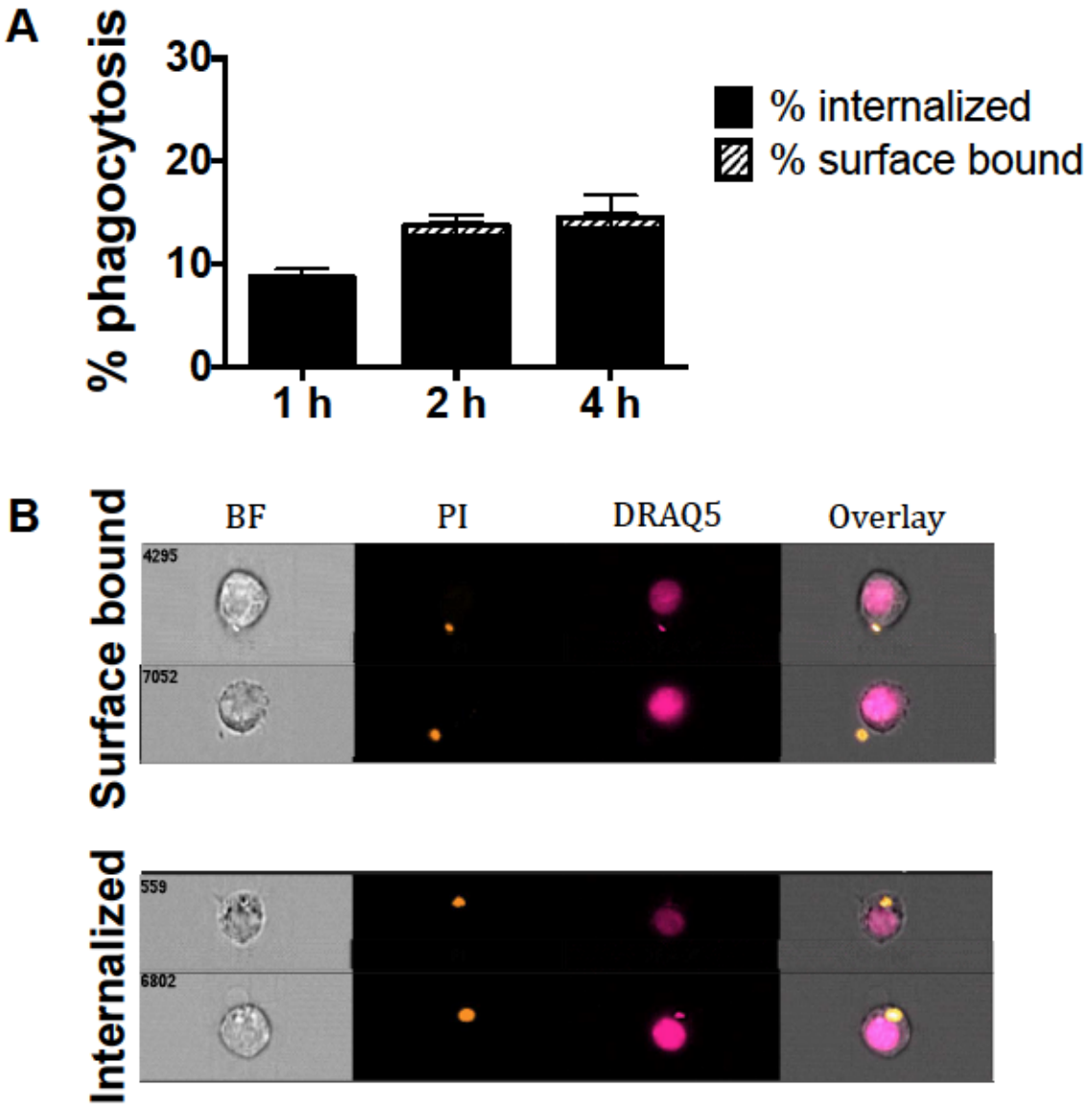

Figure AI.4. Internalization of *Aeromonas* by murine macrophages increases in a time-dependent manner.

RAW 264.7 macrophages were incubated with propidium iodide-labeled heat-killed *A. veronii* for 1, 2 or 4 hours. (A) Proportion of phagocytic macrophages at each time point. Black bars correspond to internalization events, while hatched white bars correspond to surface bound events. (B) Representative images highlight surface bound and internalized *A. veronii*. n=4; error bars correspond to SEM.

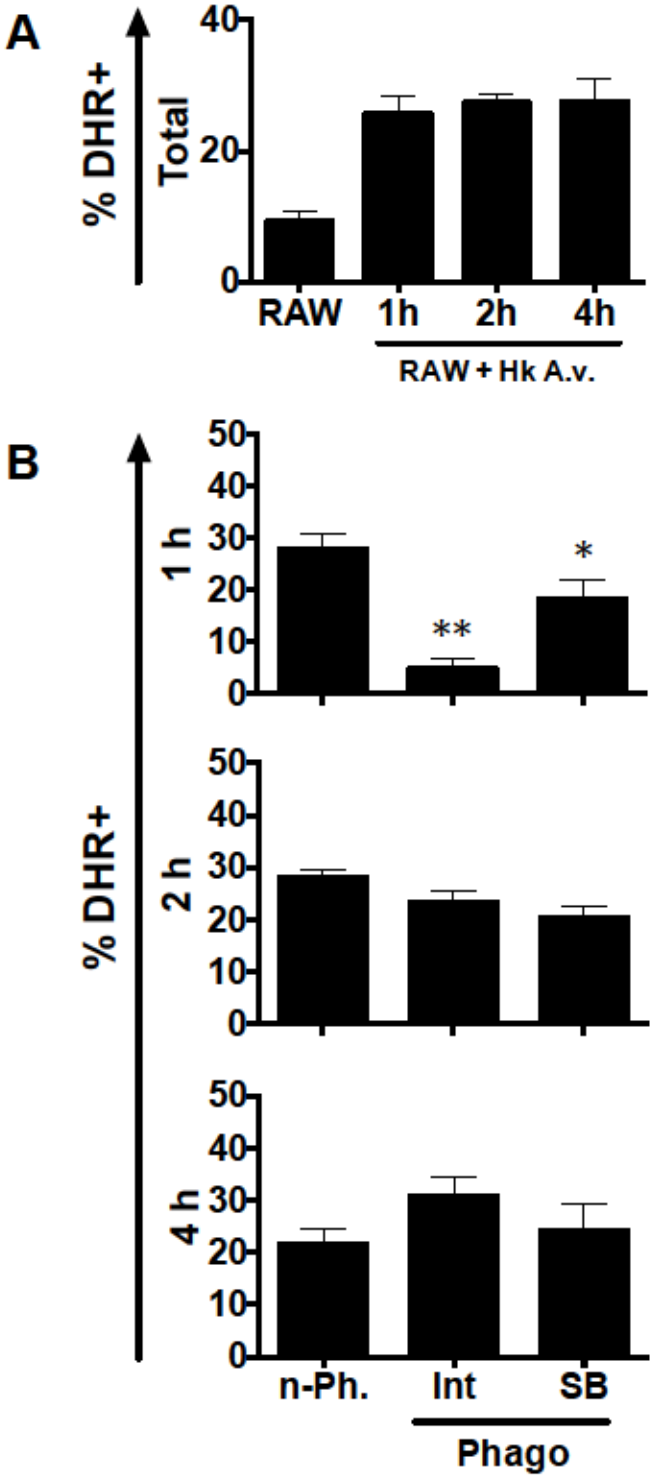
A
% DHR+
Total
40
20
0
RAW
1h
2h
4h
RAW + Hk A.v.
B
% DHR+
1 h
2 h
4 h
50
40
30
20
10
0
**
*
n-Ph.
Int
SB
Phago

Figure AI.5. Murine macrophage respiratory burst killing responses decrease following binding or internalization of *A. veronii*.

(A) RAW 264.7 macrophages were incubated with propidium iodide-labeled heat-killed *A. veronii* for 1, 2 or 4 hours and evaluated for the production of reactive oxygen species using DHR. (B) Evaluation of respiratory burst responses among non-phagocytic macrophages (non-Ph), as well as those that had bound (SB) or internalized (Int) *A. veronii* using DHR. n=4; *$p<0.05$, **$p<0.01$ as compared to n-Ph group; error bars correspond to SEM.

AI.5 References

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